DIVERSITY, DISTRIBUTION AND FUNCTIONAL CHARACTERIZATION OF BACTERIA FROM THE MANGROVE SEDIMENTS OF NORTHERN KERALA, INDIA

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

Under the Faculty of Science

University of Calicut

By

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DECLARATION

I, **THARA PAUL.,** hereby declare that the work embodied in this thesis, **"DIVERSITY, DISTRIBUTION AND FUNCTIONAL CHARACTERIZATION OF BACTERIA FROM THE MANGROVE SEDIMENTS OF NORTHERN KERALA, INDIA"** submitted to the University of Calicut in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology is a bona fide record of the research work carried out by me under the supervision of Dr. Sreedevi N. Kutty., Associate Professor, Department of Zoology, NSS College, Ottapalam and co-guidance of Dr. Sudhikumar A. V., Associate Professor, Department of Zoology, Christ College (Autonomous), Irinjalakuda affiliated to the University of Calicut; Dr. Sebastian. C. D, Professor, Department of Zoology, University of Calicut, Malappuram and Dr. Sarathambal. C., Scientist, ICAR- Indian Institute of spices research, Kozhikode. No part of the thesis has formed the basis for the award of any degree, diploma or similar titles of any university. The contents of the thesis have undergone a plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index was found to be within the permissible limit. I also declare that this thesis is free from AI-generated content.

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This is to certify that **Ms. THARA PAUL** has completed the research work for the entire period prescribed under the PhD ordinance of the University of Calicut. This thesis, **"DIVERSITY, DISTRIBUTION AND FUNCTIONAL CHARACTERIZATION OF BACTERIA FROM THE MANGROVE SEDIMENTS OF NORTHERN KERALA, INDIA"** embodies the results of her investigations conducted during the period in which she worked as a research scholar. I recommend that the thesis be submitted for evaluation for the Doctor of Philosophy in Microbiology degree award from the University of Calicut.

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The Doctoral Committee* has verified the report on plagiarism check with the contents of the thesis, as summarized above and appropriate measures have been taken to ensure originality of the Research accomplished herein.

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** In case of languages like Malayalam, Tamil etc..on which no software is available for plagiarism check, a manual check shall be*

ACKNOWLEGEMENTS

I sincerely thank all those who have supported and guided me throughout the course of this research. I am especially grateful to my research supervisors, ***Dr. Sreedevi. N. Kutty., Dr. Sebastian. C. D., Dr. Sudhikumar. A. V and Dr. Sarathambal. C.*** *for their invaluable guidance, constant encouragement and insightful feedback, which have been instrumental in shaping the direction and depth of my work.*

I express my heartfelt gratitude to ***Rev. Dr. Jolly Andrews CMI****, Principal of Christ College (Autonomous), Irinjalakuda, for his inspiration and support. My sincere thanks go to all the* ***faculty members of the Department of Zoology, Christ College (Autonomous), Irinjalakuda*** *and* ***St. Thomas College Thrissur*** *for their support throughout this journey. I am grateful to* ***University of Calicut zoology department*** *for permitting the use of their laboratory facilities.*

I am indebted to ***Prof. (Dr) Nasser M.****,* ***Prof. (Dr) Pushpalatha E.****, and* ***Dr. Binu Ramachandran****, the former and present subject experts of the University of Calicut, for their constructive suggestions, which significantly contributed to the research.*

I am deeply grateful to all my teachers—from kindergarten to post-graduation—whose dedication and wisdom have helped shape me into the person I am today. I am deeply indebted to ***Dr. Manjusha. K****, for the valuable suggestions and help provided in completing this work. I am grateful to* ***Dr. Abdul Jaleel K. U.*** *and* ***Dr. Jasmin Anas*** *for their technical support.*

This research has greatly benefitted from the collaborative spirit and support of my fellow researchers at Calicut university, Christ College and beyond. Their camaraderie and insights created a peaceful and intellectually stimulating environment. I am thankful to my colleagues in the ***molecular biology lab*** *and* ***CATE Lab****. A few individuals deserve special mention for their support:* ***Dr.Vimala K John, Dr. Chithra K. C****.,* ***Dr. Lakshmi Devi Menon, Dr. Rajeesh P, Dr. Urmila Dr. Vidya. P., Sr. (Dr) Mable rose****,* ***Dr. Sheeja U.M.****,* ***Dr. Priya Bhaskaran****,* ***Dr. Akhilesh V. P.****,* ***Dr. Surya P., Dr. Bindu****,* ***Dr. Sreevidhya P., Dr. Ruksana, Dr. Jisha, Dr. Surya, Dr. Remya****,* ***Dr. Punya****,* ***Dr. Abhijith R. S., Dr. Anoop, Dr. Faisal, Mr. Alok****,* ***Ms. Kiran Kishore****,* ***Ms. Parvathy****,* ***Ms. Alina****,* ***Ms. Aiswarya****,* ***Ms. Anagha****,* ***Ms. Maneeshma****,* ***Ms. Anjana****,* ***Ms. Archana****,* ***Mr. Santhosh, Ms. Silpa K. R.*** and

Mr. Vishnudas E. H. *Their companionship and collaboration have enriched my experience in numerous ways. I sincerely thank the* ***office staffs*** and ***librarians*** *for their invaluable assistance and support in providing access to essential resources that greatly contributed to the success of my research.*

I extend my sincere thanks to the ***"Innovation in Science Pursuit for Inspired Research (INSPIRE)*** **and** ***Kerala State Council for Science, Technology and Environment (KSCSTE)*** *and for providing* ***JRF/SRF financial assistance****, without which this research would not have been possible.*

Most importantly, this thesis would not have come to fruition without the unwavering support and love of my family. I will forever cherish the strength and patience of my beloved husband, ***Mr. Jijo Saju,*** *who stood by me during difficult times, sacrificing his time and enduring my moments of frustration with grace and understanding. My heartfelt thanks also go to my loving parents,* ***Mr. Poulose. P.K.*** *and* ***Mrs. Lilly Poulose****, for their lifelong prayers, encouragement and constant support during this phase. I am equally grateful to my brother,* ***Mr. Kiran Paul,*** *for his affection and encouragement throughout this journey. I would also like to thank my in-laws—* ***Mr. Saju K.U.****,* ***Mrs. Suni Saju, Ms. Jithu Joel, Dr. Joel Kuruvila*** *and* ***Master Moses Joel Kuruvila****—for their unwavering support, especially during the final stages of my work. I cannot forget to acknowledge my dear friend,* ***Ms. Anu*** *and* ***Ms. Gargi*** *whose companionship and emotional support helped lighten the mental burden during challenging times.*

I would like to express my sincere thanks to my ***inmates of hostel Deepti, Carmel*** and ***university*** *for their friendship, encouragements and the joyful moments we shared. I extend my thanks to* ***Fr. Antony Thanickal, Sr. Anie (late), Sr. Leema, Sr. Daisy, Sr. Diya, Sr. Salini, Sr. Sonia and Sr. Lisy*** *for the kind support and assistance throughout my stay.*

Above all, I sincerely thank ***God Almighty*** *for his unwavering guidance, strength and blessings throughout my journey. I am deeply thankful to* ***Nature****—for allowing me to live, for guiding me through this journey, and for providing the inspiration and resources that made this work possible*

Thara Paul

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ABBREVIATIONS

Symbol	Represents
≈	Approximately equal to
MoEFCC	Ministry of Environment, Forest and Climate Change
km^2	Square kilometre
et al	And others
%	Percentage
rRNA	Ribosomal ribonucleic acid
rDNA	Recombinant DNA
PCR	Polymerase chain reaction
CFU	Colony Forming Unit
g	Gram
OD	Optical Density
ml	Millilitre
min	Minute
mg/ml	Milligram per Millilitre
nm	Nanometre
hrs	Hours
LOM	Labile organic matter
ie	That is
cm	Centimetre
N	North
E	East
ºC	Degree Celsius
mg/g	Milligram per gram
ppt	Parts Per Thousand
≤	Less than or equal to
≥	Greater than or equal to
DNA	Deoxyribonucleic acid
bp	Base pair
ATP	Adenosine Triphosphate
mm	Millimetre
µL	Microlitre
w/v	Weight per Volume
v/v	Volume per Volume
Fig.	Figure
SD	Standard Deviation

സംഗ്രഹം

കണ്ടൽക്കാടുകൾ വിവിധങ്ങളായ സൂക്ഷ്മജീവ സമൂഹങ്ങളെ പിന്തുണക്കുന്ന, അത്യന്തം ഉൽപാദനക്ഷമമായ തീരദേശ അതിലോല ആവാസവ്യവസ്ഥകളാണ്. കേരളത്തിലെ പത്തു ജില്ലകളിലും കണ്ടൽക്കാടുകൾ കാണപ്പെടുന്നുവെങ്കിലും സംസ്ഥാനത്തെ ഏറ്റവും വലിയ കണ്ടൽക്കാടുകൾ കാണപ്പെടുന്നത് കണ്ണൂർ ജില്ലയിലാണ്. തൃശൂർ മുതൽ കാസറഗോഡ് വരെയുള്ള ഉത്തര കേരളത്തിൽനിന്നും തെരഞ്ഞെടുത്ത എട്ട് കണ്ടൽപ്രദേശങ്ങളായ കാസറഗോഡ്, എടാട്ട്, പഴയങ്ങാടി, വളപട്ടണം, ഏലത്തൂർ, കടലുണ്ടി, പൊന്നാനി, ചേറ്റുവ എന്നിവിടങ്ങളിൽനിന്നും തുടർച്ചയായ സമയക്രമത്തിനനുസരിച്ചു ശേഖരിച്ച ഉപരിതല ചെളിമണൽ സാമ്പിളുകളിലെ ബാക്ടീരിയകളുടെ വൈവിധ്യം, എൻസൈം ഉൽപാദന ശേഷി, ജൈവികമായ മാലിന്യമുക്തീകരണ കഴിവ്, അവയുടെ വ്യാവസായികാടിസ്ഥാനത്തിലുള്ള ഉപയോഗസാധ്യത എന്നിവയിൽ ആഴത്തിലുള്ള വിലയിരുത്തലാണ് ഈ പഠനത്തിൽ നടത്തിയിരിക്കുന്നത്.

പഠനം നടത്തിയ കണ്ടൽപ്രദേശത്തിലെ ചെളിമണലിന്റെ ഭൗതിക, രാസ ഘടകങ്ങൾ സ്ഥലങ്ങൾക്കനുസരിച്ച് വ്യത്യാസപ്പെട്ടു കാണപ്പെട്ടു. താപനില 21–32°C, pH 6.0–7.0, ലവണാംശം 3–38 ppt, ജൈവവസ്തു 0.2 ± 0.03 മുതൽ 7 ± 0.04% വരെ, കാർബോഹൈഡ്രേറ്റ് 0.01 ± 0.002 മുതൽ 0.25 ± 0.02 mg/g വരെ, പ്രോട്ടീൻ 0.1 ± 0.02 മുതൽ 9.9 ± 0.04 mg/g വരെ, ലിപിഡ് 0.2 ± 0.01 മുതൽ 9.3 ± 0.02 mg/g വരെ, മണൽ 46–97%, സിൽറ്റ് 0.3–55%, പശമണ്ണ് 0.3–35% വരെ എന്നിങ്ങനെ രേഖപ്പെടുത്തി. ബാക്ടീരിയയുടെ സാന്നിധ്യം 6.0 ± 0.03 മുതൽ 8.0 ± 0.04 $\log_{10}$ CFU/10g വരെ കണ്ടെത്തുകയും ജൈവവസ്തു, ലിപിഡ്, പ്രോട്ടീൻ എന്നിവയുമായി അനുകൂല സഹബന്ധം പ്രകടിപ്പിക്കുകയും ചെയ്തു.

ആകെ 17 ജനുസ്സുകളിലായി ഉൾപ്പെടുന്ന 708 ബാക്ടീരിയ ഐസലേറ്റുകൾ തിരിച്ചറിഞ്ഞപ്പോൾ, മെറ്റാജീനോമിക് വിശകലനത്തിലൂടെ 45 ഫൈലകളും 1834 സ്പീഷിസുകളും രേഖപ്പെടുത്തി. ആന്റിബയോട്ടിക് സംശ്ലേഷണം, സീനോബയോട്ടിക് വിഘടനം, സൾഫർ, നൈട്രജൻ, ഇരുമ്പ്, മീഥേൻ തുടങ്ങിയ ബയോജിയോരാസചക്രങ്ങളിലെ പങ്കാളികളായ ബാക്ടീരിയ ജനുസ്സുകളുമായി ബന്ധപ്പെട്ട ജീനുകളും തിരിച്ചറിഞ്ഞു.

എൻസൈം സ്ക്രീനിംഗിൽ ഹൈഡ്രോളിറ്റിക് എൻസൈമുകളായ ലിപേസ് (69%), പ്രോട്ടിയേസ് (55%), അമിലേസ് (48%), സെല്ലുലേസ് (43%), ഡിയോക്സിറൈബോന്യൂക്ലിയേസ് (27%), കെറ്റിനേസ് (3.5%), ലിഗ്നിനേസ് (3%), ലാക്കേസ് (1%) എന്നിവയുടെ മികച്ച പ്രവർത്തനങ്ങൾ രേഖപ്പെടുത്തി. എൻസൈം ഉല്പാദനശേഷി അടിസ്ഥാനമാക്കി തിരഞ്ഞെടുത്ത 35 ഐസലേറ്റുകൾ ഫിർമിക്യൂട്ടീസ്, പ്രോട്ടിയോ ബാക്ടീരിയ എന്നീ ഫൈലങ്ങളിലായിരുന്നു. കോശാന്തര എൻസൈം ഉല്പാദനശേഷി അടിസ്ഥാനമാക്കി ഉയർന്ന ശേഷി കാണിക്കുന്ന സ്ട്രെയിനുകൾ (*എന്റെറോബാക്ടർ ക്ലോയകെ, എന്റെറോ ബാക്ടർ അസ്ബ്യൂറിയേ, സെറേഷ്യ മാർസെസൻസ്, ക്ലെബ്സിയല്ല എയറോജീനസ്*) തിരഞ്ഞെടുത്തു.

വിവിധ pH മൂല്യം, താപനില, ലവണാംശം, ആജിറ്റേഷൻ നിലകളിൽ വളർച്ചാ പരിഷ്കാരം നടത്തി അനുയോജ്യമായ വളർച്ചാ സാഹചര്യങ്ങൾ നിർണ്ണയിച്ചു. രക്തവിഘടനമില്ലാത്തതും സമാന വളർച്ചാ ആവശ്യങ്ങൾ പുലർത്തുന്നതുമായ സ്ട്രെയിനുകൾ ഉപയോഗിച്ച് അഞ്ചു കൺസോർഷിയങ്ങൾ (CS01–CS05) വികസിപ്പിച്ചു. കാണ്ടിറ്റേറ്റീവ് എൻസൈം പരിശോധനയിൽ, ലിപേസ് ഒഴികെ, ഇമൊബിലൈസ്ഡ് കൺസോർഷിയങ്ങളിൽ സ്വതന്ത്ര രൂപങ്ങളേക്കാൾ കൂടുതൽ എൻസൈം പ്രവർത്തനം; അമിലേസ് (373U/ml), ലിഗ്നിനേസ് (270U/ml), സെല്ലുലേസ് (178U/ml), പ്രോട്ടിയേസ് (32U/ml), കെറ്റിനേസ് (24U/ml) എന്നിങ്ങനെ രേഖപ്പെടുത്തി. FST06 സ്ട്രെയിൻ 64U/ml എന്ന ഉയർന്ന ലിപേസ് പ്രവർത്തനം പ്രകടിപ്പിച്ചു. സാനിറ്ററി നാപ്കിനുകളും പോളികോട്ടഡ് കേരിബാഗുകളും ഉപയോഗിച്ചുള്ള ബയോഡീഗ്രഡേഷൻ പഠനങ്ങളിൽ, സ്വതന്ത്ര കൺസോർഷിയങ്ങളിൽ 29%, ഇമൊബിലൈസ്ഡ് രൂപത്തിൽ 8% എന്നിങ്ങനെ വിഘടന ക്ഷമത കാണിച്ചു.

ഈ പഠനം ഉത്തരകേരളത്തിലെ കണ്ടൽ ചെളിമണലുകളിൽ സമ്പന്നമായ ബാക്ടീരിയ വൈവിധ്യവും മെറ്റബോളിക് ശേഷിയും ഉള്ളതായി തെളിയിക്കുന്നു. പരിസ്ഥിതി സംരക്ഷണത്തിലും ബയോറിമീഡിയേഷൻ സാങ്കേതികവിദ്യകളിലും ഇവയുടെ പരിസ്ഥിതിശാസ്ത്ര പ്രാധാന്യവും പ്രായോഗിക സാധ്യതകളും ഈ പഠനം മുന്നോട്ടു വയ്ക്കുന്നു.

പ്രധാന പദങ്ങൾ: കണ്ടൽ ബാക്ടീരിയ, മെറ്റാജീനോമിക്സ്, എൻസൈം പ്രവർത്തനം, കൺസോർഷിയം, ഇമൊബിലൈസേഷൻ, ബയോറിമീഡിയേഷൻ

ABSTRACT

Mangrove ecosystems are productive coastal habitats supporting diverse microbial communities. This study examined the influence of sediment characteristics on bacterial population and diversity. The hydrolytic enzyme potential and bioremediation capacity of the bacterial isolates obtained from the mangrove sediments of eight different stations in northern Kerala, i.e., Kasaragod, Edat, Valapattanam, Pazhayangadi, Kadalundi, Elathur, Ponnani and Chettuva, were determined. Sediment parameters varied across locations, with temperature ranging from 21–32°C, pH 6–7, salinity 3–38 ppt, organic matter 0.2 ± 0.03 to 7 ± 0.04%, carbohydrate 0.01 ± 0.002 to 0.25 ± 0.02 mg/g, protein 0.1 ± 0.02 to 9.9 ± 0.04 mg/g, lipid 0.2 ± 0.01 to 9.3 ± 0.02 mg/g, sand 46 to 97%, silt 0.3 to 55% and clay 0.3 to 35%. Bacterial population ranged from 6 ± 0.03 to 8 ± 0.04 $\log_{10}$ CFU/10 g and correlated with organic matter, lipid and protein content of the sediment. A total of 708 bacterial isolates representing 17 genera were identified, while metagenomic analysis revealed 45 phyla and 1834 species. Genes associated with antibiotic synthesis, degradation of xenobiotic compounds and different bacteria involved in sulphur, nitrogen, iron and methane cycles were identified. Enzyme screening revealed hydrolytic potential for lipase producers (69%), followed by protease (55%), amylase (48%), cellulase (43%), DNase (27%), chitinase (3.5%), ligninase (3%) and laccase (1%). Based on enzyme potential, 35 isolates were identified using molecular methods. Eight strains (*Enterobacter cloacae*, *E. asburiae*, *Serratia marcescens* and *Klebsiella aerogenes*) were selected for consortia development based on the extent of extracellular enzyme production. Growth optimization was done for varying pH, temperature, salinity and agitation. Non haemolytic and compatible strains with similar growth requirements were used to develop five consortia (CS01–CS05). Immobilization of the strains and consortia was done. Quantitative enzyme assays showed enhanced activity in immobilized consortia compared to free forms, except for lipase. The maximum enzyme activity in immobilized consortia was obtained for amylase (373 U/ml), ligninase (270 U/ml), cellulase (178 U/ml), lipase (63 U/ml), protease (32 U/ml), and chitinase (24 U/ml). Strain FST06 exhibited the highest lipase activity (64 U/ml). Biodegradation studies using sanitary napkins and polycoated carry bags demonstrated 29% and 8% degradation by free and immobilized consortia, respectively. The study highlights the rich bacterial diversity and metabolic potential of Kerala's mangrove sediments, emphasizing their ecological importance and potential for application in bioremediation.

Key words: Mangrove bacteria, Metagenomics, Enzyme activity, Consortia, Immobilization, Bioremediation

Chapter 1

GENERAL INTRODUCTION

Chapter - 1

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Mangrove habitat represents a unique and exceptionally productive ecosystems located at the interface of land and sea. Often described as a vital element of blue carbon ecosystems, mangroves are commonly referred to as "blue forests." They are important for coastal protection, conservation of biodiversity and regulation of climate. Mangroves provide a unique habitat for a variety of species and deliver essential goods and services to human beings, including food, timber, fuel and medicine (Carugati et al., 2018). The International Union for Conservation of Nature regards mangrove ecosystems as vital contributors in mitigating climate change and alleviating its adverse impacts on the environment, owing to their remarkable capabilities. These ecosystems possess significant economic value due to the extensive array of ecosystem services they provide (Costanza et al., 1997; Khaleel, 2008). Mangroves represent merely about 0.5% of the global tropical and subtropical coastal regions. But they play a crucial role in contributing terrestrial carbon—the essential element for life and the most vital element on earth, that is transferred to the ocean (Alongi, 2014). Mangrove forests represent unique ecological environments characterized by unusual environmental factors, including salinity, organic matter level, sand nutrient cycling rates, thereby serving as resource-abundant habitats for microorganisms (Alongi, 1996; Clark et al., 1998).

Mangroves represent a global coverage estimated at nearly 150,000 km² (Bunting et al., 2022). India ranks as the third most diverse country (≈ 46 mangrove plant species) for mangrove species and the area covers about 4740 square kilometres (3% of world coverage) (Qian et al., 2024). Mangroves in India are located mainly at the east of the Bay of Bengal (57%), west of the Arabian Sea (31%) and the Andaman and Nicobar Islands (12%) (MoEFCC, 2022). In India, they are mainly present in Kerala, West Bengal, Gujarat, Andhra Pradesh, Maharashtra, Odisha, Tamil Nadu, Goa and Karnataka states (Mitra et al., 2017). India is home to the most extensive biodiversity in mangrove forests worldwide, having 4,107 species, which consist of

23% of plant species and 77% of animal species. Bhitarkanika in Odisha is recognized as the 'Mangrove genetic paradise' of the world (Kathiresan, 2018).

In Kerala, the extent of mangrove forests has significantly decreased from approximately 700 km² to merely 17 km² (Nayana, 2024). The largest mangrove coverage in Kerala is in Kannur district (≈ 8 square kilometres) (Sreelekshmi et al., 2021). Northern section of the Kerala state stores a greater amount of carbon due to its elevated tree density, soil carbon levels, and unique biometric characteristics (Harishma et al., 2020). Kerala is home to 41% of the true mangrove species found in India, although the state comprises only 0.19% of the total mangrove area in the country. 80% of the total mangroves in Kerala is present in Kannur and Kasargod districts, with moderately dense coverage present along the riverbanks in Kasargod, Kottiyur, and Valapattanam, as well as alongside the Pazhayangadi River (Joshy et al., 2021). Other districts that also feature mangroves include Trivandrum, Kollam, Allappuzha, Kottayam, Ernakulam, Thrissur, Kozhikode, and Malappuram.

Globally, mangroves are vanishing in a rapid rate (mainly due to anthropogenic influence), experiencing an annual decline of 1–2%, which is five times the rate of global forest loss. This leads to substantial loss of biodiversity and carbon storage, which also cause climate change (Barathan et al., 2025). Unfortunately, Kerala's mangrove forests face significant threats due to rapid coastal development. Rising land prices and population pressures have driven the conversion of these marshlands for construction, agriculture and aquaculture projects. Inadequate regulations and enforcement have further exacerbated the problem and for the protection Government of India has enacted various regulatory and promotional initiatives. These efforts include raising awareness among private landowners and local communities about the importance of preserving these vital ecosystems. Additionally, responsible tourism initiatives are promoting mangrove tourism, encouraging an increased love for these natural wonders. To guarantee the preservation of current mangroves and expand the area of northern Kerala habitats, the Kannur Kandal Project has been concentrating on acquiring mangrove land, restoring degraded mangrove ecosystems through community and governmental involvement, raising awareness, and conducting research.

The location of mangroves at the intertidal zone of marine and terrestrial habitat makes it the best ecosystem for the growth and development of a vast variety of organisms and helps them to develop special adaptive features. The floral community mainly comprises mangroves and their associates, phytoplanktons and microorganisms such as bacteria, fungi, yeast, algae, etc. The faunal communities include zooplankton, invertebrate epifauna, epibenthos, infauna and meiofauna, insects, spiders, amphibians, reptiles, birds, mammals, fish, crustaceans and molluscs, etc. Mangroves plants belonging to the families Avicenniaceae and Rhizophoraceae are the commonly found ones (Macintosh and Ashton, 2002). According to George et al. (2017), a total of 13 mangrove species have been identified from Kerala (5 families and 8 genera). The bioenergetically significant floral and faunal components of the mangrove ecosystem and also the environmental factors play a crucial role in sustaining its equilibrium and augmenting its biological potential (Harishma et al., 2020).

Mangrove ecosystems host a variety of microorganisms (bacteria, fungi, algae, protozoa, viruses, etc.), where bacteria and fungi contribute about 91% of the total biomass (Zhang et al., 2017). These microbes are found to participate in different biogeochemical cycles, like nitrogen, phosphorus, carbon, sulphur, etc. (Nimnoi and Pongsilp, 2022). The prevalent bacterial groups found in mangroves include sulphate-reducing bacteria, nitrogen-fixing bacteria, phosphate-solubilizing bacteria and methanogenic bacteria. Microbes have the capacity for decomposition and mineralization of leaf litter and transformation of nutrients, help in bioremediation, manage human-generated waste and act as biological control agents for pests affecting plants and animals (Kathiresan and Bingham, 2001).

Microorganisms play an essential role in the mangrove ecosystem, helping in the development and maintenance while also offering biotechnologically valuable products such as enzymes, antibiotics and other bioactive compounds and have potential uses in medicine, agriculture and industry (Thatoi et al., 2013). The intermittent tidal flooding present in the mangrove ecosystem, cause variability in environmental factors (salinity and nutrient availability), which in turn results in special adaptation features for bacteria to survive and constitute a substantial reservoir of biotechnological potential that can be utilized (Sivaramakrishean et al., 2006).

Seasonal variations might have a more significant impact on microbial communities compared to the age of the forest (Ho et al., 2024).

Studying and identifying the bacterial diversity in mangrove habitats helps understand the ecological functions, biotechnological potential and response to environmental changes of these unique ecosystems. Studies of classification, systematics and identification of microbes is interconnected, with identification accuracy being crucial for taxonomic classification (Bisen et al., 2012). Researchers use a combination of culture-based and molecular techniques to identify bacteria from mangrove habitats. Culture-based methods, like culturing bacteria on different media, can help isolate and grow specific bacterial species. Molecular techniques, such as 16S rRNA sequencing, is crucial for identifying bacteria that are difficult to culture and for understanding the overall bacterial diversity in a mangrove ecosystem (Sakhia et al., 2015). Historically, microorganisms have been classified based on morphological, physiological and biochemical traits. However, phenotypic analysis is insufficient for distinguishing bacterial isolates beyond the species level (Bakonyi et al., 2003). Less than 1% of bacteria in natural environments can be cultivated, leaving 99% uncultivable (Stanley, 2002). These results can be achieved through molecular techniques, especially polymerase chain reaction (PCR) (Tasi-Li-Yu and Olson, 1991). PCR offers a faster alternative to traditional culture methods and helps identify hard-to-cultivate bacteria. Universal primers for the 16S rRNA gene have been designed for this purpose. Recent advancements in next-generation sequencing (NGS) have revolutionized microbiology, aiding in the understanding of the microbiome (Do et al., 2022). The study of functional diversity through metagenomics also helps researchers understand the potential range of metabolic and ecological roles that microbial communities in an environment have. It complements taxonomic diversity by revealing the variety of functions, such as nutrient cycling, biodegradation, or the production of bioactive compounds, that are present within a microbial community (Muhammad Riaz et al., 2024). Thus, combining traditional and molecular methods will enhance bacterial identification (Fouad et al., 2002). Accurate identification and classification of microorganisms require selecting the right techniques and understanding their mechanisms and should focus on morphological, physiological, biochemical characteristics and genetic profiles.

The natural ecosystem offers an extensive array of microbial enzyme resources. Enzymes generated by bacteria residing in mangrove ecosystems have diverse applications in multiple industries, such as environmental bioremediation, food processing, textiles and pharmaceuticals. These enzymes are employed for their capacity to decompose pollutants, optimize food processing and enhance processes in the textile and pharmaceutical sectors (Margesin and Schinner, 2001; Sabu, 2003). Bacteria possess a wealth of hydrolytic enzymes, including amylases, nucleases, esterases, lipases, chitinases, ligninases, pectinases, laccases, cellulases, phosphatases and proteases, and they function efficiently in various environments (Mishra et al., 2011).

Various microorganisms produce powerful biocatalysts in the form of enzymes, which differ in their microbial origins, chemical characteristics and operational mechanisms. Typically, microbial enzymes facilitate hydrolysis, oxidation, or reduction reactions. Enzymes serve as biological catalysts that are crucial in all phases of metabolism and biochemical processes. Microorganisms attract considerable interest due to their cost-effective production and potential for genetic enhancement. Microbial enzymes have supplanted numerous enzymes derived from plants and animals (Asad et al., 2011). Bacteria are extensively utilized in the industrial synthesis of enzymes, with their activities evaluated through a range of assay techniques. Researchers are utilizing recent advancements in molecular techniques, such as genomics and metagenomics, to identify novel enzymes from microbial sources (Adrio and Demain, 2005). Investigating the activities of bacterial enzymes yields essential insights into microbial physiology, enhances our understanding of cellular metabolism and bacterial evolution, and elucidates the interactions between bacteria and their hosts. The capacity to analyze enzyme activities in whole cell lysates or crude extracts is crucial for the preliminary identification of specific activities and for acquiring knowledge about enzyme functions in an environment that closely resembles the cellular context (Burns et al., 1998). Enzyme assays evaluate the functionality of enzymes generated by bacteria, typically employing methods such as spectrophotometry, fluorescence, or chromatography. These approaches assess enzyme activity by tracking variations in substrate or product concentration over time, which aids in calculating the rate of enzyme-catalyzed reactions (Yimer and Tilahun, 2018).

Proteases are responsible for proteolysis, which involves the degradation of proteins through the hydrolysis of peptide bonds between amino acids in polypeptide chains (Singh et al., 2016). These enzymes are prevalent and widely found across a variety of sources, including plants, animals and microorganisms. The limitations of plant and animal proteases in meeting global demands have increased interest in microbial proteases (Rani et al., 2017). Bacterial protease enzymes are widely used in industries such as detergents, food processing, leather tanning and pharmaceuticals for their effective protein degradation and stability (Singh and Bajaj, 2017).

Cellulose, a key component of plant cell walls, is broken down by cellulase enzymes into simpler carbohydrates like glucose, making it valuable in biofuel production and food processing (Ferrari et al., 2014). Chitin, a cell wall component of fungi and certain algae, is broken down by the chitinase enzyme (Gonfa et al., 2023). Chitinases are classified into two main types: endochitinases and exochitinases. Chitinase enzymes are used across various industries like agriculture, food, health care, and bioremediation (Meghwanshi et al., 2020). Lignin, being the most abundant renewable source of aromatic polymer in nature, plays a crucial role in carbon recycling through its decomposition. However, its complex and heterogeneous structure renders it chemically resistant to breakdown by most organisms (Yang et al., 2016). Ligninolytic enzymes are used in agricultural, paper, food and textile industries (Fu et al., 2012).

Deoxyribonuclease (DNase) enzyme synthesized by certain bacteria degrades DNA into smaller monomeric units (Mulcahy et al., 2010). Lipases represent a category of enzymes that facilitate the hydrolysis of long-chain triglycerides. Currently, microbial lipases are receiving increasing attention due to the rapid advancements in enzyme technology. They are regarded as the most crucial group of biocatalysts for various industrial applications. The industrial uses of microbial lipases encompass the fat and oleochemical sector, detergent manufacturing, production of biodegradable polymers, food processing, flavor enhancement, medical and pharmaceutical applications, pulp and paper production, biosensor development, waste management, cosmetics and fragrance industries, as well as biodiesel production (Choudhury and Bhunia, 2015). Amylases are enzymes that convert starch into smaller sugar molecules such as maltose, glucose and maltotriose. They are among the most

vital industrial enzymes, utilized in textiles, detergents, the food and paper industries and for ethanol production (Tiwari et al., 2015). Bacterial pectinase breaks down pectin, a polysaccharide found in plant cell walls. This breakdown leads to various beneficial effects in different sectors, including food processing, textile manufacturing and environmental management (Haile et al., 2022). The determination of bacterial enzymes was carried out using two methods, such as qualitative and quantitative (Taillefer et al., 2023). Qualitative tests typically involve visual inspection of clear zones on agar plates following enzyme activity. Quantitative assays commonly employ spectrophotometry to assess the formation of products or the depletion of substrates.

The production of novel and robust enzymes that are industrially significant for biotechnological applications is on the rise. Consequently, it is essential to isolate and identify bacteria from natural sources and to optimize the cultural conditions to achieve maximum enzyme production (Shrestha et al., 2022). The optimization of growth media can significantly affect the growth rate of various microorganisms. Different microorganisms may utilize distinct chemical compounds, such as carbon or nitrogen sources. Environmental isolates might exhibit preferences for varying pH levels or growth temperatures, which can be challenging to anticipate. Furthermore, optimization can also impact the production of enzymes or other metabolites (Singh et al., 2017). Due to the substantial economic implications, achieving optimal fermentation conditions, especially those pertaining to physical and chemical parameters, is a crucial aspect in the development of biological processes (Pereira Duta et al., 2006). Optimizing the conditions for bacterial culture is essential for achieving optimal bacterial growth, yield and the synthesis of targeted metabolites or products. This process requires meticulous adjustments of variables such as temperature, pH, agitation, salinity, nutrient availability and oxygen concentration to establish an environment conducive to bacterial proliferation and the maximum production of desired compounds (Pylak et al., 2021). pH plays a vital role in various physiological processes, including membrane permeability and cell structure and temperature in the production and growth of bioactive metabolites (Bundale et al., 2015). Salinity can trigger stress responses in bacteria, which diminishes the energy available for metabolism and influences enzyme activity (Yao et al., 2023). The agitation of bacterial cultures has a significant effect on enzyme production (Feng et al., 2003).

Optimizing bacterial culture conditions helps to maximize bacterial growth, enzyme production, or the desired outcome in a given application. By carefully adjusting these factors, researchers and industrial practitioners can achieve higher yields, improved efficiency, and more predictable results in various biotechnological processes (Sharma et al., 2021).

Bioremediation refers to the process of utilizing living organisms, mainly microorganisms, to break down environmental pollutants into less harmful substances. Microbial enzymes are essential in bioremediation, utilizing their catalytic properties to break down and convert pollutants into less harmful or innocuous forms (Bhandari et al., 2021). This method is generally preferred over alternatives due to its lower costs and the possibility of complete mineralization. The microorganisms involved may be indigenous or non- native (Vidali, 2001). The efficiency of microbial bioremediation is influenced by several factors, like the type and concentration of pollutants and environmental conditions such as temperature, pH and oxygen levels, as well as the characteristics of the microbial community (Kuppan et al., 2024).

Numerous studies indicate that achieving complete pollutant degradation using a single bacterial strain is challenging. Due to the varying metabolic pathways of different strains, combining bacteria with diverse capabilities into a microbial consortium allows the integration of each strain's strengths, leading to more effective pollutant removal (Fahmy et al., 2025). Microbial consortia significantly outperform single-strain cultures, demonstrating notable effectiveness in pollutant degradation (Bhatt et al., 2021). The co-cultivation of microbial consortia proves to be more efficient than that of individual bacteria, leading to a quicker degradation of pollutants and a substantial improvement in the biodegradation of contaminants present in the soil (Zhang et al., 2021). Bacterial consortia, due to their synergistic interactions among various microorganisms, demonstrate greater efficacy in environmental bioremediation compared to individual microbial or enzyme systems. This superiority arises from their enhanced adaptability and resilience to diverse and complex environmental conditions. Microorganisms within these consortia engage in substance exchange and communication through intricate metabolic regulation networks and signaling molecules, thereby coordinating their collective functions and achieving superior degradation efficiency relative to solitary organisms. Investigations into the

microbial ecology of bacterial consortia are extremely important for understanding their functions and ecological roles in the degradation process, as well as for enhancing their efficacy (Eze et al., 2021). Microbial consortia have been successfully utilized for the removal of organic pollutants from contaminated soils, wastewater and sediments (Chan et al., 2022); inorganic pollutants (Ningthoujam and Pinyakong, 2024); plastic (Salinas et al., 2025); and hydrocarbons (Poddar et al., 2019). In order to establish a successful microbial consortium, it is essential that the bacterial cultures be compatible with one another and devoid of any antagonistic interactions so that they can collectively carry out all necessary metabolic processes for enhanced degradation. For a microbial consortium to function effectively, the individual strains must not only be compatible with each other but also be non-pathogenic. This factor is crucial to prevent any disruption to the overall ecosystem (Hadi et al., 2021).

To improve the effectiveness and durability of bioremediation agents in contaminated environments, it is essential to immobilize the cells. Immobilization refers to the physical or chemical confinement of biocatalysts (including enzymes or whole cells) to a designated area while maintaining certain desired catalytic functions (Karel et al., 1985; Kourkoutas et al., 2004). Recently, immobilized cell systems have gained popularity for applications in various fields such as agriculture, biotechnology, waste management and pharmaceuticals (Zur et al., 2016). This method enhances stability, reusability and catalytic efficiency. A comparison of pollutant degradation over the same duration showed that immobilized microorganisms achieved a removal efficiency exceeding 21% compared to free microbial consortia (Najim et al., 2024). Immobilized microbial cells present a promising strategy in nearly all bioremediation processes due to their superior operational stability, increased biomass loading and improved biodegradation rates than free cells (Xue et al., 2017). Research has indicated that immobilized bacterial cells can effectively eliminate a range of pollutants, such as heavy metals (Podder et al., 2019), polychlorinated biphenyls (Ouyang et al., 2021), dyes (Rodriguez Couto, 2009) and phenols (Krastanov et al., 2013).

Soil pollution is a major global issue due to its limited self-purification and high remediation costs. Plastic and similar materials play a major role in soil pollution due to their prolonged degradation process and their ability to leach toxic substances,

which adversely affects soil health and the integrity of ecosystems. Improper waste disposal, industrial operations and agricultural methods aggravate the accumulation of plastics and microplastics in the soil (Hoang et al., 2024). The incorrect disposal of used sanitary napkins and polycoated paper bags raises numerous environmental issues, such as plastic pollution, contamination of landfills and potential threats to ecosystems and human health. These sanitary napkins and polycoated paper bags, which frequently contain non-biodegradable components like plastic, require hundreds of years to break down, thereby contributing to landfill overflow and the microplastic contamination of both soil and water (Biju, 2023). Therefore, the urgent need is for effective bioremediation.

SIGNIFICANCE OF THE STUDY

Mangrove ecosystems are recognized as hotspots of microbial diversity, hosting numerous unique bacterial species. Although mangrove forests are valued for their ecological significance and the services they provide, certain elements of their habitats, such as the role of bacteria and their ecological functions, have been less investigated. Additionally, studies on the mangrove ecosystem in Kerala, especially in the Northern Kerala mangrove habitat, remains limited. Although mangrove microbial communities play a vital role in the regulation of mangrove ecosystems, they have garnered considerably less attention than microbial communities in other marine and terrestrial environments (Bahram et al., 2018; Thomson et al., 2022). Examining the diversity and functionality of bacteria within an ecosystem aids in understanding the roles various bacteria play in vital processes such as nutrient cycling and decomposition, while also serving as indicators of the ecosystem's health and stability. Furthermore, it provides valuable knowledge about the resilience of ecosystems against disturbances and assists in pinpointing essential microbial species that are vital for sustaining overall ecosystem functionality. The investigation of bacterial consortia represents a growing area of research with numerous applications across fields such as medicine, agriculture and environmental science. These consortia exhibit synergistic effects, rendering them more efficient than single-strain methods for various tasks. The study of bacterial consortia illustrates how various bacterial species from mangrove ecosystems collaborate to accomplish functions that exceed the abilities of individual strains, resulting in enhanced bioremediation and the possibility of novel

biotechnological applications. Examining the functional characterization of bacterial consortia within the mangrove habitat of Northern Kerala is important as it uncovers the microbial diversity and functionality of these distinctive ecosystems, which are essential for biogeochemical processes, nutrient cycling and the overall health of the ecosystem. Gaining knowledge about these microbial communities also provides valuable information about possible biotechnological applications, including bioremediation and the identification of new bioactive compounds.

A database on potential microbial species can provide deeper understanding of the microbial diversity and ecological processes taking place in the area. An annotated library of these species can also be prepared and preserved for further studies. The isolates obtained can be used specifically or in consortia for developing a better way to tackle the waste disposal and management problem. The results of the study can be utilized commercially and for the betterment of existing social problems.

OBJECTIVES OF THE STUDY

1. Isolation of bacteria from the mangrove sediments of North Kerala
2. Classification of the isolates by morpho-biochemical methods
3. To determine the non-culturable bacterial diversity using metagenomics
4. Screening of the bacterial isolates for hydrolytic enzyme production
5. Molecular identification and phylogenetic analysis of the potential isolates
6. To screen the potential isolates for pathogenicity
7. To develop bacterial consortia as bioremediators for recalcitrant waste management
8. To test the efficacy of the consortia by immobilization technology

Chapter 2

INFLUENCE OF SEDIMENT CHARACTERISTICS ON BACTERIAL POPULATION

Chapter - 2

INFLUENCE OF SEDIMENT CHARACTERISTICS ON BACTERIAL POPULATION

2.1 INTRODUCTION

Mangrove habitats consist of specialized salt-tolerant trees and shrubs, prevalent in tropical and subtropical regions. This ecosystem supports a wide variety of flora and fauna, acting as a crucial nursery and refuge for numerous species while also offering coastal protection against erosion and storms, filtering pollutants, improving water quality and sequestering carbon for climate regulation (Mattone and Sheaves, 2024).

Mangrove habitats, characterized by distinctive conditions such as tidal flooding, fluctuating salinity levels, low oxygen (anoxia) in sediments, and rich organic matter are home to a rich diversity of microorganisms (Thatoi et al., 2013). Such challenging environments foster a highly diverse bacterial community that is well-adapted to extreme conditions. Bacteria are essential for nutrient cycling and maintaining ecosystem health, establishing symbiotic relationships with mangrove plants and aiding in the transformation of carbon, nitrogen, and sulphur (Deng et al., 2022).

Ecological factors influence bacterial populations by impacting their community structure, diversity, and functional characteristics. The factors interact with other elements, including soil properties (like particle size) and nutrient availability (such as nitrogen and phosphorus), leading to shifts in bacterial groups that are essential for biogeochemical cycling in these dynamic environments (Fernandes et al., 2022). Examining environmental factors, sediment properties and bacterial diversity within mangrove ecosystems provides insights into their overall health, nutrient cycling, carbon sequestration capabilities, and the identification of microbes that could be utilized in bioremediation and restoration efforts. The present study uncovers the role of these microbes in driving ecosystem processes, affecting the resilience of the system, and highlighting the potential impacts of human activities or pollution on the ecosystem.

2.2 REVIEW OF LITERATURE

2.2.1 Mangrove bacteria

The mangrove ecosystems are exceptionally diverse and sustain a broad range of flora, fauna, and microorganisms, including bacteria, fungus, yeast, and viruses. Mangrove ecosystems because of their significant species richness and distinctive ecological characteristics, helps in climate resilience and providing livelihoods for coastal communities (Ellison, 2008). Bacteria play a vital role in nutrient cycling, organic matter breakdown, and the overall functioning of mangrove ecosystems. Various environmental conditions, including elevated organic matter content, fluctuating salinity, and anaerobic sediments, facilitate the proliferation of specific bacterial species (Gomes et al., 2010).

In a Caribbean mangrove swamp, scientists discovered *Thiomargarita magnific a*, the largest bacteria in the world (Volland et al., 2022). Researchers have extensively isolated nitrogen-fixing bacteria (Munir et al., 2018), sulphate-reducing bacteria (Chen et al., 2018) and cellulose-degrading bacteria among others from mangrove ecosystems (Mhuantong et al., 2015). The bacterial population in mangrove ecosystems can fluctuate according to environmental factors, including sediment composition, water quality, nutrient accessibility, salinity, temperature, the presence of mangrove species, geographical location and microbial interactions (Li et al., 2019).

Bacterial biomass and abundance in mangrove sediments are often superior to those in other oceanic ecosystems (Alongi, 2014). Studies in the Sundarbans mangrove forest of India indicated that bacteria constitute up to 80% of the microbial biomass in mangrove sediments (Alongi et al., 2004). The bacteria reside in mangrove water, sediment and detritus, establishing interactions with plants and animals.

2.2.2 Sediment

Mangrove sediment denotes the material that settles and gathers at the base of mangrove forests, consisting of silt, sand, clay and various organic matter. It provides support for roots, essential nutrients, and a habitat for a wide range of organisms, particularly microbial communities such as bacteria, yeast and fungi (Liu et al., 2024). Numerous studies have highlighted the distinctiveness of mangrove sediments in terms of their microbial composition (Urakawa et al., 1999).

Mangrove sediment is recognized for its substantial bacterial biomass and variety, with estimates varying from 10^6 to 10^9 bacterial cells per gram of sediment (Donato et al., 2012). Factors including sediment texture, organic matter, salinity, nutrient levels and redox conditions affect the bacterial communities in sediment, and the type of sediment has a considerable impact on the composition, abundance and diversity of the microbial populations present within it (Yang et al., 2022). The concentration of bacterial cells in rhizosphere soil can vary from 10^6 to 10^9 cfu/g (Munir et al., 2018).

The growth of mangrove vegetation is heavily influenced by climatic conditions, the physicochemical properties of the sediment and hydrological dynamics. These factors influence the distribution of various mangrove ecotypes and the ecosystem services they provide (Cinco-Castro et al., 2022). Mangrove vegetation supplies organic matter to mangrove ecosystems, comprising leaf litter, woody waste and detritus. This organic substance acts as a substrate for bacterial proliferation and can facilitate bacterial multiplication, decomposition and cycling of organic matter within the environment (Cunha et al., 2012). As noted by Romimohtarto and Juwana (2001) the cycle of organic matter in marine environments mirrors that of organic cycles in freshwater and terrestrial ecosystems. The intricate and variable nature of microbial communities, coupled with local climatic variables, can lead to significant fluctuations in bacterial load in mangrove sediment across different studies and locales (Donato et al., 2012). Bacteria isolated from mangrove sediments have considerable potential across multiple domains, such as environmental remediation, healthcare and agriculture. Their distinctive adaptations to extreme conditions and varied metabolic functions render them important sources of innovative compounds and biotechnological resources (Roy et al., 2002).

2.2.3 Water

The bacterial concentration in mangrove water exhibits regional and temporal variability. Donato et al. (2012) indicated that bacterial concentrations in mangrove water varied from 10^2 to 10^6 colony-forming units (CFU) per millilitre. Tidal flow, freshwater influx, sediment nutrient availability, salinity and organic matter contributions from mangrove plants or neighbouring ecosystems can affect bacterial

load (Araujo et al., 2016). Increased nutrient concentrations, frequently resulting from anthropogenic activity or the influx of freshwater, might result in a heightened bacterial load. Excessive nutrient inputs can lead to detrimental algal blooms and modify the organization of microbial communities (Bhatt and Bharti, 2018).

The salinity of mangrove water is an essential factor in determining bacterial load. Bacterial populations in mangrove ecosystems adjust to diverse salinities, encompassing brackish and hypersaline environments. Salinity variations, either from tide changes or freshwater influx, can influence the composition and abundance of bacterial populations. Bacteria found in mangrove water have helped us understand the variety of microbes, their community structure, metabolic functions and ecological roles in the water of mangrove ecosystems (Donato et al., 2012).

Bacterial diversity and community composition study of mangrove water column in Pernambuco, Northeastern Brazil, revealed a multitude of bacterial species and identified changes in bacterial load and also found that mangrove forests are intrinsically linked to organic material, whether it is transported by the flow of seawater into the mangrove region or derived from the leaf litter of mangroves (Araujo et al., 2016).

2.2.4 Mangrove flora and fauna

Mangrove vegetation mainly comprises specialized trees and shrubs that are tolerant to salt and flourish in intertidal coastal regions. It encompasses various species from different, unrelated taxa that usually thrive between the average sea level and the highest tide levels along tropical and subtropical coastlines globally. Typically, these plants exhibit distinct characteristics with highly specialized morphological and physiological adaptations that enable them to survive in the challenging conditions of marine coastal environments. In India, 43 distinct species of mangroves have been identified (Duke et al., 2023).

Nevertheless, the composition and structure of mangrove vegetation can differ significantly from one area to another or from one region to another, influenced by factors such as soil conditions, rainfall patterns and the inflow of river water into the ocean (Selvam et al. 2004). As per ENVIS, Kerala (2019), the district of Kannur

contains 80% of the total mangrove forest area in Kerala. Furthermore, median data from 1-in-100-year storm events indicate that mangroves can diminish flood depths by 15-20%, with reductions exceeding 70% in certain regions (Spalding and Leal, 2024).

The roots of mangrove plants and their related rhizospheres exhibit significant interactions with bacteria. The bacterial load in mangrove plants can vary according on species, age and health (Ghosh et al., 2020). Bacteria inhabit the roots and establish biofilms, which enhance nutrient absorption and plant development. Microorganisms in mangrove plants are crucial for nutrient cycling and may enhance disease resistance and stress tolerance (Duke et al., 2023).

Alongi and Dixon (1997) examined the microbial communities linked to mangrove plant foliage in North Queensland, Australia, and reported the ability for nutrient cycling. Sessitsch et al. (2001) conducted a long-term fertilizer field experiment to examine the microbial communities linked to the mangrove root systems in the Bhitarkanika mangrove forest, Odisha and found that Bhitarkanika's clay and fine silt make it a better place for mangroves to grow than sandy sediments. Zheng et al. (2019) examined the diversity of endophytic bacteria in the roots of *Kandelia candel*, a common mangrove species in China, and discovered that the invasion of *Spartina alterniflora* can modify the microbial community in the mangrove rhizosphere and influence nutrient metabolism within the mangrove ecosystem. The assembly of microbes associated with roots is influenced by the niche of the compartment and the species of the host, while soil types may also play a role (Sui et al., 2023).

The mangrove ecosystems in India boast an impressive record of biodiversity, possibly the highest of any nation, encompassing a total of 5,746 species. Among these, 4,822 species, which account for 84%, are classified as animals. This diverse group includes nearly all significant taxonomic categories of animals, represented by 21 documented phyla, and constitutes 4.76% of the overall fauna in India (Kathiresan, 2019).

Mangrove ecosystems harbor a varied range of bacteria that form symbiotic interactions with various creatures, providing mutual benefits to both the bacterium and the host. Notable examples are mangrove crabs, such as fiddler crabs (Uca spp.),

which possess specialized structures called gill chambers that harbor specific symbiotic bacteria. These bacteria facilitate the digestion of cellulose and other intricate polysaccharides found in the diet of crustaceans (Shil et al., 2021). Certain marine organisms residing in mangroves possess bioluminescent organs, including specific types of fish, squid and shrimp. These organs harbor light-emitting bioluminescent bacteria, including *Vibrio* spp. Light emission facilitates animal communication, prey attractiveness and defensive strategies (Klose et al., 2010). Fish host diverse microbial ecosystems, comprising both transient and resident bacteria and load in fish can fluctuate according on species, diet and environmental conditions. The gut microbiota of fish can affect digestion, nutrition metabolism and disease resistance (Cunha et al., 2017). The intricate and variable characteristics of microbial communities result in considerable fluctuations in bacterial load both among and within species (Stephens et al., 2016).

2.2.5 Sediment characteristics

The physicochemical characteristics of sediments pertain to factors such as particle size, organic matter content, labile compounds, nutrient concentrations, temperature, salinity and pH levels. It is essential to determine the physicochemical characteristics of mangrove sediment within a habitat for assessing the current ecological condition and equilibrium (Hu et al., 2021).

The consistent and cyclical alterations in climate that align with the seasons are ultimately mirrored in environmental parameters (biotic and abiotic), which subsequently exert a direct or indirect impact on both floral and faunal populations (Choudhury and Panigrahy, 1991). Different types of sediment and their physical features may impact how microbial communities move around in mangrove ecosystems (Kassem et al., 2013; Zhang et al., 2018). The texture of sediment affects oxygen availability, water retention and nutrient availability, which subsequently dictate microbial composition and dispersal. Mangrove forests act as highly effective carbon sinks, and this stable pool of organic carbon, particularly preserved in their sediment's forms part of the "blue carbon" that is efficiently sequestered by the ocean and coastal ecosystems worldwide (Mcleod et al., 2011).

Rainfall, as a significant cyclic phenomenon in tropical regions, induces essential alterations in the hydrological features of coastal marine environments and leads to seasonal variations during the monsoon, post-monsoon and pre-monsoon seasons (Satheeshkumar and Khan, 2009). Salinity levels fluctuate across various ecosystems based on factors such as topography, tidal movements and the influx of freshwater (Paramasivam and Kannan, 2005). The interaction of mangrove bacteria, organic matter/labile organic compounds and sediment properties is critical for nutrient cycling, organic matter decomposition and overall mangrove ecosystem functionality (Lafrance et al., 2015). Satheeshkumar and Khan (2009) conducted a study in the mangroves of Pondicherry, located on the southeast coast of India; and revealed the influence of environmental variables with bacterial load.

Behera et al. (2019) observed how the structure and function of sediment bacterial communities changed over time and space in the intertidal mangrove forest of Bhitarkanika, India and found that seasonality predominantly accounted for the variations in the structural and metabolic patterns of bacteria communities, and they reported that the composition of the bacterial community was primarily influenced by salinity and the organic carbon content of the sediments. Zhang et al. (2018) investigated the characteristics of sediment, including particle size, organic matter content and nutrient concentrations, from the Zhangjiang Estuary in China, and found the growth of microbes in different layers of sediments. McKee and Faulkner (2000) examined the physical and biogeochemical factors affecting the composition of microbial communities in mangrove ecosystems in south Naples, Florida, and found that fine-grained sediments rich in organic matter typically harbour unique microbial communities and exhibit reduced oxygen availability compared to coarser sediments.

Lunau et al. (2005) investigated a coastal ecosystem in the German Wadden Sea characterized by low salinity, analyzing the correlation between particle size and bacterial diversity while also considering the influence of oxygen availability. They discovered that smaller particles, which may reduce oxygen availability, were linked to increased bacterial diversity. Sanders et al. (2012) examined the organic matter content and particle size alterations in various mangrove sediments in response to sea level rise, revealing that particle size influenced oxygen penetration into sediments, subsequently affecting the bacterial community's abundance and composition. A study

by Hossain et al. (2012) with environmental variables and bacterial populations from the Sunderban mangrove habitat showed that smaller particles correlated with increased bacterial diversity. Zhu et al. (2022) investigated the diversity and composition of microorganisms in the Zhanjiang Mangrove Sediments, China, and identified positive association between sediment organic matter and bacterial abundance, suggesting that increased organic matter content facilitates a rise in bacterial populations. Dutta and Subudhi (2015) investigated the structure and composition of bacterial communities in mangrove detritus in Odisha, India, and discovered that sediment organic carbon content significantly influenced bacterial community composition. Li et al. (2019) investigated bacterial populations in mangrove sediments along the southeastern coast of China. The research identified a favourable link between the concentration of organic materials in sediment and bacterial diversity.

The elevated level of organic matter in the sediment is typically linked to a increase in bacterial population. The labile organic matter (LOM), which microorganisms readily utilize, significantly affects bacterial density and biomass. Surface sediments frequently exhibit this relationship due to the greater quantities of LOM (Oni et al., 2015). Organic matter is crucial in facilitating biogeochemical processes and the resulting transformations in near-shore coastal ecosystems. The organic matter present in marine sediments consists of a vast array of macromolecules, enzymatically broken down and used as nutrient and energy sources by microorganisms (Arndt et al., 2013).

Labile compounds are those materials that can be easily transformed, often through biological activity (Strosser, 2010). Carbohydrates represent a crucial part of the organic matter found in all soils, typically making up 5–20% of soil organic matter. Carbohydrates account for 50–70% of the dry weight of the majority of plant tissues, making them the most prevalent materials introduced to the soil through plant residues. Carbohydrates serve as universal components of living organisms, participating in both structural and metabolic functions (Lowe, 1978). Sediment bacteria depend on organic matter for their carbon and energy needs. An increase in organic matter, particularly carbohydrates, offers more accessible resources for bacterial proliferation and activity (Fabiano and Danovaro, 1994).

Among the various components of soil organic matter, proteins constitute the predominant reservoir of organic nitrogen in the soil (Nannipieri and Paul, 2009). Soil protein has been recognized as a reliable indicator of soil health concerning bioavailable nitrogen, as highlighted by recent initiatives of the Soil Health Institute (Hurisso et al., 2018). Proteins, peptides and amino acids serve as crucial nitrogen sources, especially in sediment environments where inorganic nitrogen sources are scarce (Vetter and Deming, 1994).

Lipids are long-chain fatty acids that are released by the sediment microbes, from lipid hydrolysis and are compounds rich in energy (Sousa et al., 2009) and are anticipated to serve as beneficial substrates for microorganisms. Fabiano and Danovaro (1994) reported that bacterial activity, as indicated by the frequency of dividing cells, was significantly correlated with lipid concentration. Bacterial lipids, including fatty acids and phospholipids, are essential indicators of the structure of the bacterial community and its metabolic activities. The availability of oxygen and organic matter content were the main factors that affected lipid degradation (Johns et al., 1977).

Examining the protein, lipid and carbohydrate composition of mangrove habitats, can yield valuable insights into different facets of the ecosystem, such as: 1) Nutrient availability and dynamics- Analyzing their concentrations and proportions aids in comprehending nutrient cycling, trophic interactions and possible food web dynamics within the mangrove ecosystem (Carugati et al., 2018). 2) Effect of disruption- Studies indicate that alterations in mangrove ecosystems, including degradation, can modify the levels of these macronutrients, especially affecting protein and carbohydrate concentrations (Solntsev et al., 2019). 3) Ecosystem functionality and well-being- The relative presence of these nutrients may reflect the general health and operation of the mangrove ecosystem. A discrepancy in nutrient ratios might imply a change in the ecosystem's structure or productivity (Kumar et al., 2019). 4) Sediment organic matter- Lipids and proteins serve as molecular biomarkers that can help trace the origins and transformations of organic matter found in mangrove sediments.

According to the study conducted by Carugati et al. (2018), labile compounds were analyzed in both undisturbed and disturbed mangroves. The findings revealed that the protein fraction of biopolymeric carbon was twice as high in the disturbed

mangrove areas compared to the undisturbed ones. Furthermore, the protein-to-carbohydrate ratio was significantly four times greater in the sediments of disturbed mangroves than in those of the undisturbed mangroves. Lipid content, however, remained relatively consistent across both areas, averaging 9% in undisturbed forests and 8% in disturbed forests, representing their respective proportions of biopolymeric carbon. Investigating these nutrient levels in mangrove ecosystems is crucial for researchers as it enhances their understanding of ecological processes, the potential effects of disturbances, and the overall health and functionality of the ecosystem.

The seasonal and sampling station wise impact of various environmental factors on bacterial populations across different mangrove stations in the northern Kerala mangrove habitat was analyzed in the current study.

2.3 MATERIALS AND METHODS

2.3.1 Study area

The study area is located in the five coastal districts i.e., Thrissur, Malappuram, Kozhikode, Kannur and Kasargod of North Kerala. Eight stations were selected for sediment collection depending on the mangrove forests (Fig. 2.1 and 2.2). The details of sampling stations are given in table 2.1 and 2.2.

2.3.2 Sample collection

Sediment samples were collected from 8 mangrove sites for 2 consecutive years, i.e., first-year sampling during 2020-21 and second-year sampling during 2021-22. The collection was made during the three seasons (monsoon, post-monsoon and pre-monsoon) for a period of 2 years. Approximately 10-20 g of sediment from the surface (0-15 cm) was collected using the hand-corer method and was transferred aseptically into sterile polythene bags for microbial analysis. The samples were collected by removing surface leaf litter and collecting the top sediment. The collected samples were transported to the laboratory in an ice box and processed within 4 hours of collection. A total of five sub-samples were collected from each location and were later pooled, resulting in one composite sample. Temperature, pH and salinity of the sediment were noted. Another fraction of sediment was stored at -20ºC for further analysis (Baker et al., 1977; Gong et al., 2019).

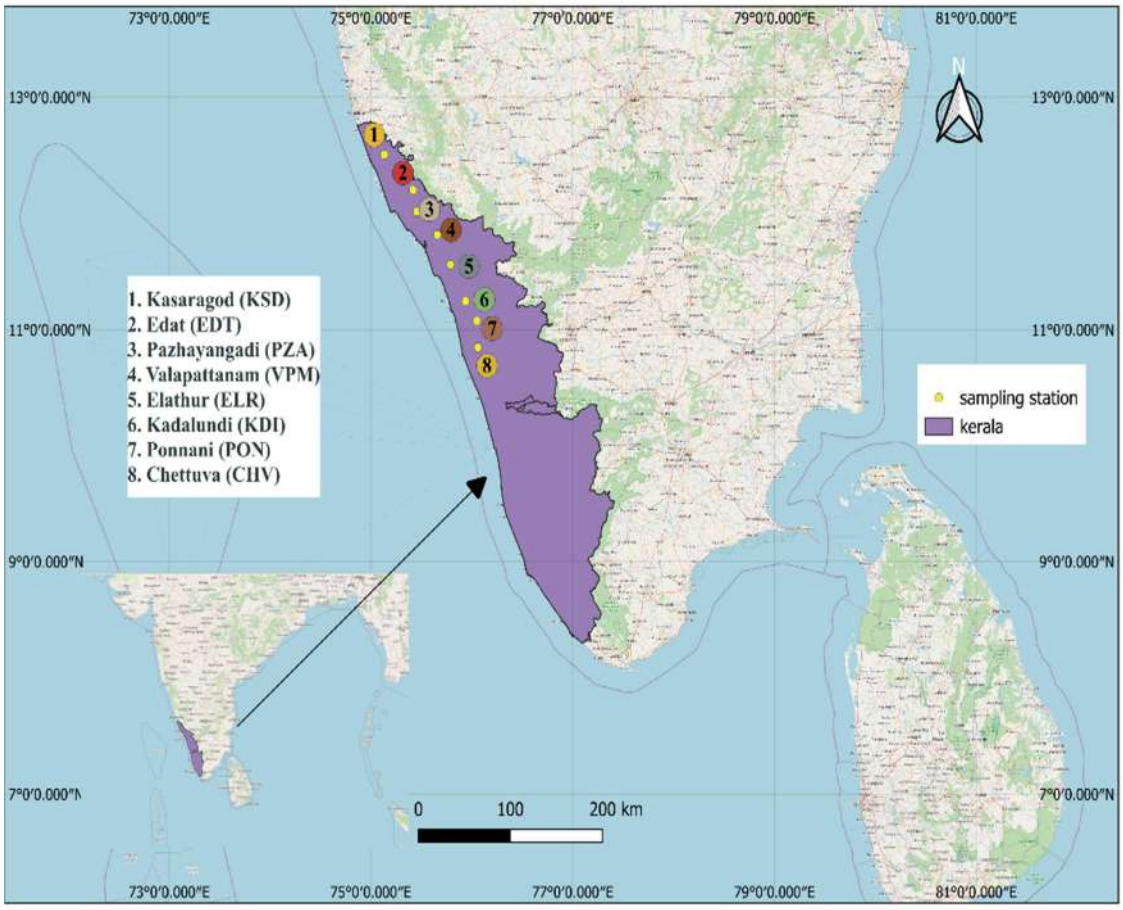


Fig. 2.1 Map showing the sampling stations along North Kerala (developed by using QGIS 3.44.1)

Table 2.1 Details of sampling stations along North Kerala

Sl. No	Sampling station	Abbreviation	District	Latitude	Longitude
1	Kasaragod	KSD	Kasaragod	12°46'87.35"N	74°99'64.67"E
2	Edat	EDT	Kannur	12°09'58.1"N	75°22'34.14"E
3	Pazhayangadi	PZA	Kannur	12°05'31.38"N	75°30'41.77"E
4	Valapattanam	VPM	Kannur	11°58'06.4"N	75°19'34.8"E
5	Elathur	ELR	Kozhikode	11°32'87.5"N	75°75'07.07"E
6	Kadalundi	KDI	Malappuram	11°14'17.1"N	75°83'92.03"E
7	Ponnani	PON	Malappuram	10°78'61"N	75°91'81"E
8	Chettuva	CHV	Thrissur	10°31'21.4"N	76°02'21.4"E

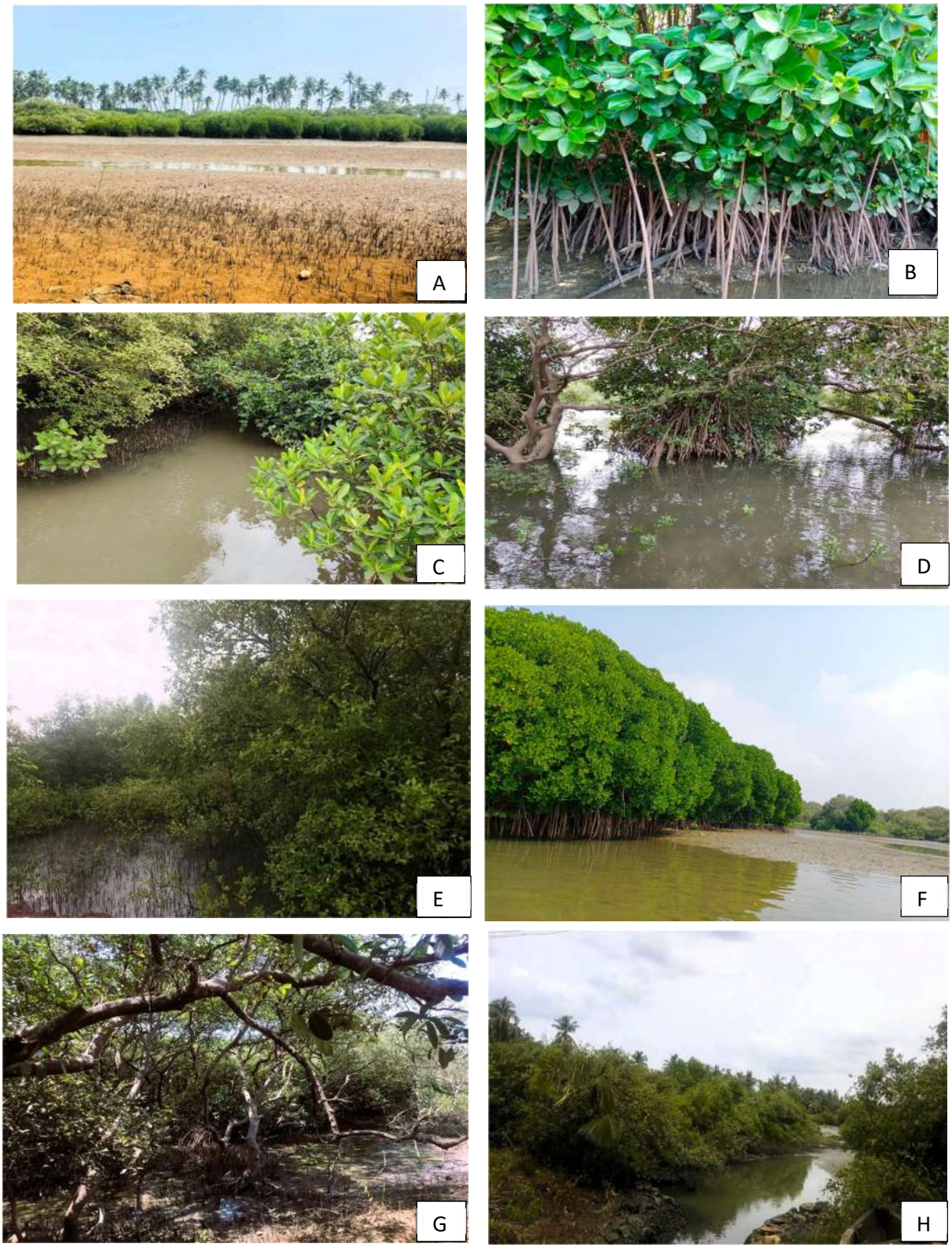


Fig.2.2 **Photographs showing eight sampling stations along Northern Kerala:- A) Kasaragod B) Edat C) Pazhayangadi D) Valapattanam E) Elathur F) Kadalundi G) Ponnani H) Chettuva**

Table 2.2 Characteristics of mangrove sampling stations

Sampling station	Characteristics of the habitat							Reference
	Mangrove forest type	Unique mangrove species present in the area	Anthropogenic influence	River situated	Near to land	Other remarks	Commonly found mangrove species	
Kasaragod	Dense-10-15 type mangrove plant species	*Rhizophora apiculate**	Higher	Chandragiri backwater	Yes	No conservation strategies	*Acanthus ilicifolius, Excoecaria agallocha, Avicennia officinalis, Rhizophora mucronata,* and *Sonneratia caseolaris.*	• Ghajar et al., 2018
Edat	Dense and contain various types of mangrove plant species	*Acanthus ebtacteatus**	Low	Anjarkandy river	No	Highest mangrove covers in Kerala (7.55 km^2, Conservation (Wildlife Trust of India, Kannur Kandal Project), Soil carbon stock of 52.18 t CO_2/ha, 286.88 t CO_2/ha		• Manoj et al., 2024
Pazhayangadi	Not dense	*Acanthus ebtacteatus, Avicennia marina, Rhizophora mucronata, Bruguiera cylindrica**	Higher	Pazhayangadi river	Yes	No conservation strategies		• Rajan and Athira, 2023 • Manoj et al., 2024
Valapattanam	Not dense	*Acanthus ebtacteatus, Rhizophora apiculate**	Higher	Valapattanam river	Yes	No conservation strategies, Carbon stock of 38.59 t CO_2/ha, 212.20 t CO_2/ha		• Rajan and Athira, 2023 • Manoj et al., 2024
Elathur	Dense	*Rhizophora apiculata, Avicennia marina, Rhizophora apiculate**	Higher	Korapuzha river	Yes	Conservation strategies		• Sreelekshmi et al., 2018
Kadalundi	Dense (density- 40-70%)	*Rhizophora apiculata,Acanthus ebtacteatus, Avicennia marina, Acrostichum aureum, and Kandelia candel**	Medium	Kadalundi river	Yes	Conservation strategies, tourist place, includes in Kadalundi-Vallikkunnu Community Reserve		• Vinod et al., 2017
Ponnani	Not dense and thick	*Acanthus ebtacteatus, Bruguiera cylindrica, Rhizophora mucronate**	Higher	Bharathapuzha river	Yes	No conservation strategies, near to fishing harbour and remains open year-round, covers 946.62 hectares		• Sheeja et al., 2020
Chettuva	Dense	*Rhizophora mucronate**	Medium	Chettuva backwater	Yes	Conservation strategies, covers only 5 hectares		• Vinod et al., 2018

2.3.3 Analysis of physico- chemical parameters

Temperature, pH and salinity of the sediment were noted using a portable thermometer, a digital pH meter and a digital salinometer, respectively, at the time of collection.

2.3.3.1 Grain size analysis

Dried sediment samples were used for grain size analysis. Sediment was dried at 60°C for 48 hours. The grain size analysis was done using the Laser Scattering Particle Size Distribution Analyser LA-960, and the percentage of sand, silt and clay were calculated. The results were plotted in a ternary diagram according to Shepard (1954) and Blott and Pye (2012).

2.3.3.2 Organic matter analysis

The sediment samples stored at -20ºC were dried and homogenised for the determination of organic matter. Organic matter was determined according to El-Walkeel and Riley's Wet Oxidation Method (1957) and the results were expressed as percentage organic matter.

2.3.3.3 Estimation of labile compounds

The sediment samples stored at -20ºC were dried and used for the estimation of labile compounds such as carbohydrate, protein and lipid as per standard protocols. Carbohydrate was estimated using phenol sulphuric acid method (Loewus, 1952), protein by Lowry's method (Lowry et al., 1951), and lipid by phosphovanillin method (Knight et al., 1972).

2.3.4 Isolation of sediment bacteria

The isolation of bacteria was performed according to Cappuccino et al. (1983). Sediment samples were pooled and thoroughly mixed. Subsequently, 10 g of the pooled sample was subjected to serial dilutions from 10^{-1} to 10^{-3}. A 100 µl of sample suspension from the dilutions were inoculated and spread on Nutrient agar plates and were incubated at 28± 2°C for 24 hours. The colonies developed were enumerated and colony forming units (CFU) count calculated. Morphologically distinct colonies were picked and purified by the quadrant streaking method and then transferred to Nutrient agar slants for further studies.

Nutrient agar medium		
Peptone	-	5g
Beef extract	-	3g
NaCl	-	5g
Agar	-	20 g
Water	-	1000 ml
pH	-	7

2.3.5 Statistical Analysis

Data analysis was done by using PRIMER7, SPSS, OriginPro 2025 and Graph pad Prism10 (Arkkelin, 2014; Clarke and Gorley, 2015; In and Lee, 2017).

2.4 RESULTS

Mangrove sediments from eight different stations along Northern Kerala were aseptically collected during 2020–21 (first year) and 2021–22 (second year). Bacterial isolation and analysis of sediment physicochemical parameters such as temperature, pH, salinity, organic matter, particle size and labile compounds were done.

2.4.1 Analysis of sediment physicochemical parameters

- **Temperature**

During the study period, sediment temperature at the sampling stations ranged from 21 ± 0.5 to 32 ± 0.4°C. Kasaragod (pre-monsoon season) and Valapattanam (post- monsoon) stations recorded comparatively highest temperature (32 ± 0.4°C). Both Edat (21 ± 0.5°C) and Pazhayangadi (21 ± 0.6°C) stations recorded the lowest temperature. The temperature ranged from 22 ± 1.0 to 30 ± 0.7°C, 21 ± 0.5 to 29 ± 0.1°C, and 22 ± 0.3 to 30 ± 0.6°C during the pre-monsoon, monsoon and post-monsoon seasons, respectively, during the first year of sampling, (Fig. 2.3). The temperature ranged from 26 ± 0.9 to 32 ± 0.5°C, 25 ± 0.4 to 30 ± 0.8°C and 27 ± 0.2 to 31 ± 0.5°C during the pre-monsoon, monsoon and post-monsoon seasons, respectively, during the second year of sampling (Fig. 2.4). No significant station-wise ($p = 0.1$) or season-wise ($p = 0.5$) variations in temperature were observed during the study period.

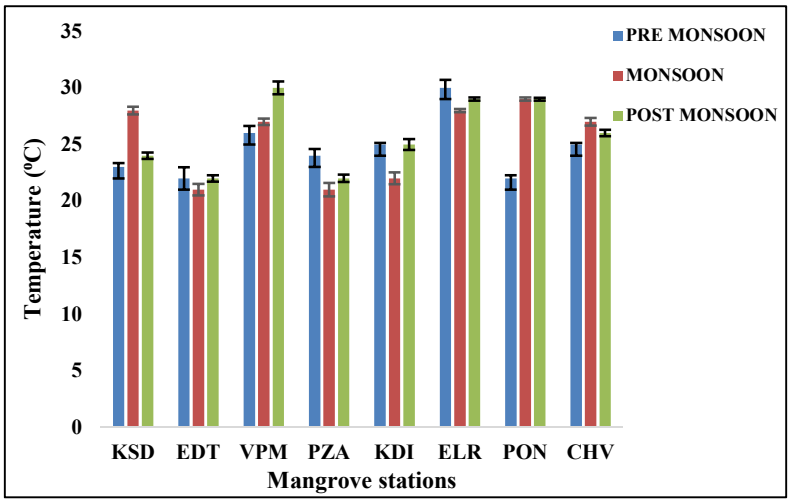


Fig. 2.3 Sediment temperature at sampling stations during the three seasons of study in first year

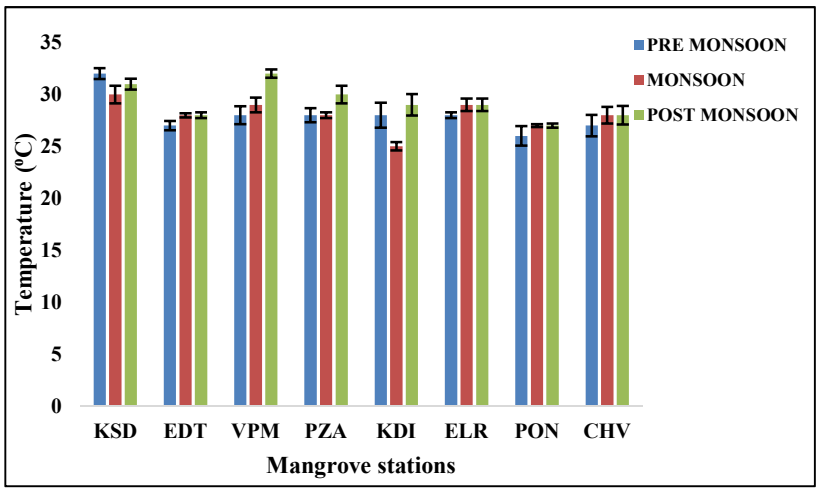


Fig. 2.4 Sediment temperature at sampling stations during the three seasons of study in second year

- **pH**

The observed sediment pH range was 6 ± 0.3 to 7 ± 0.2 during the study period. Valapattanam (7 ± 0.2) and Kadalundi (7 ± 0.1) stations recorded the highest pH during the pre-monsoon season of the first year of sampling. The lowest pH was found at Edat station during the monsoon season in both the first (6 ± 0.3) and second year (6 ± 0.1) of sampling. In the first year of sampling the pH ranged from 6.5 ± 0.4 to 7 ± 0.2, 6 ± 0.3 to 6.9 ± 0.2 and 6.5 ± 0.2 to 6.9 ± 0.3 during the pre-monsoon, monsoon

and post-monsoon seasons, respectively (Fig. 2.5). In the second year of sampling, the pH ranged from 6.2 ± 0.1 to 6.8 ± 0.3, 6.0 ± 0.1 to 6.8 ± 0.2 and 6.3 ± 0.1 to 6.9 ± 0.2 during the pre-monsoon, monsoon and post-monsoon seasons, respectively (Fig. 2.6). Significant station-wise variation in pH was observed ($p = 0.003$), but not during season-wise analysis ($p = 0.09$).

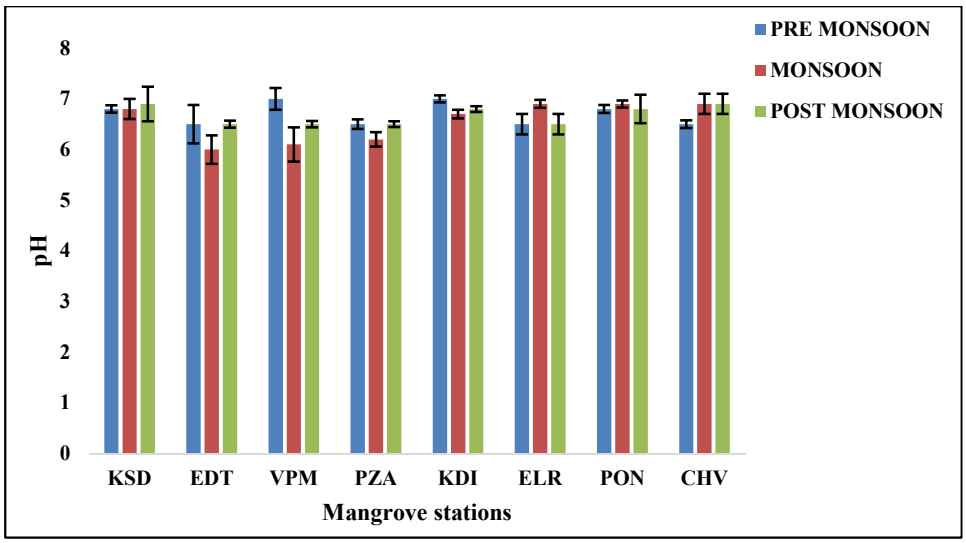


Fig. 2.5 Sediment pH at sampling stations during the three seasons of study in first year

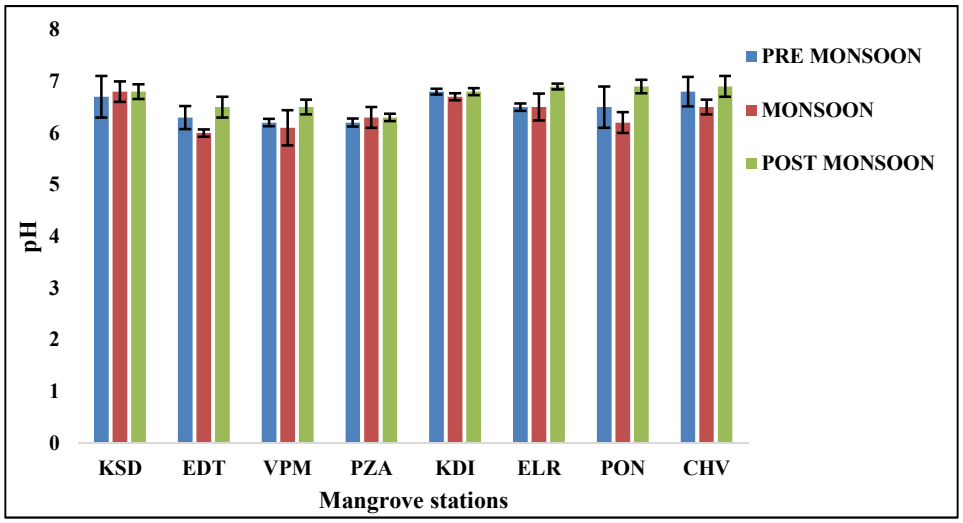


Fig. 2.6 Sediment pH at sampling stations during the three seasons of study in second year

- **Salinity**

During the study period, sediment salinity ranged from 3 ± 0.3 to 38 ± 1.4 ppt in the mangrove stations. In the first year of sampling, salinity ranged from 8 ± 0.9 to 35 ± 1, 4 ± 0.2 to 32 ± 0.8 and 5 ± 0.2 to 37 ± 1.8 ppt during the pre-monsoon, monsoon and post-monsoon seasons, respectively (Fig. 2.7). In the second year sampling period, the range was from 7± 0.3 to 36 ± 0.9, 3 ± 0.3 to 31 ± 2.3 and 5 ± 0.2 to 38 ± 1.4 ppt during the pre-monsoon, monsoon and post-monsoon seasons, respectively (Fig. 2.8). Ponnani station recorded a relatively high salinity of 38 ± 1.4 ppt during the post-monsoon season, while Valapattanam station recorded the lowest salinity of 3 ± 0.3 ppt during the monsoon season. Significant station-wise variation in salinity was observed ($p = 0.001$), but not during season-wise analysis ($p = 0.21$).

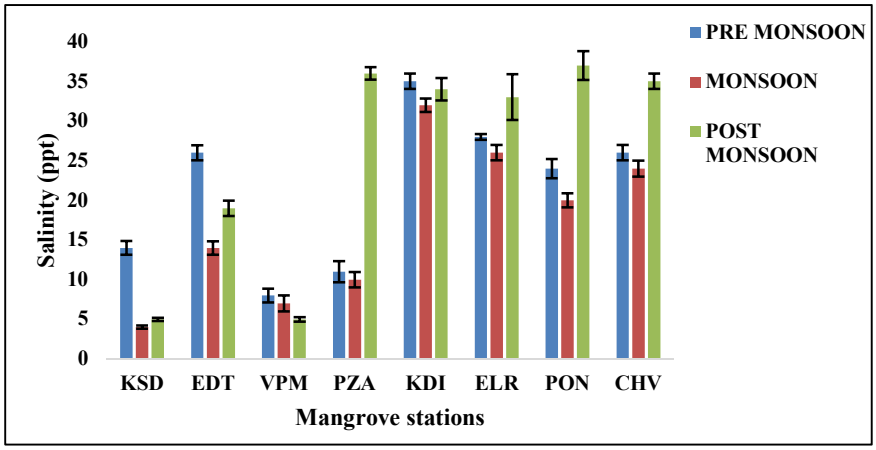


Fig. 2.7 **Salinity at sampling stations during the three seasons in the first year of study**

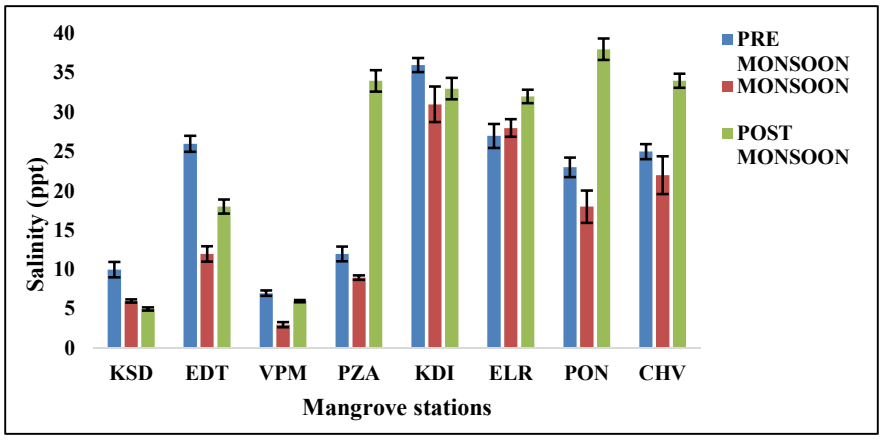


Fig. 2.8 **Salinity at sampling stations during the three seasons in the second year of study**

2.4.2 Sediment texture analysis

The first year of sampling revealed the sediment texture to be sandy in all seasons (Fig. 2.9). However, in the second year during the pre-monsoon season, four stations showed sandy texture, whereas Ponnani, Pazhayangadi, Edat and Kasaragod stations showed clayey sand texture (Fig. 2.10). During the monsoon season of the second year of sampling, all the stations showed sandy texture, except Valapattanam. Valapattanam station constituted a sandy silt sediment texture. Second year of sampling revealed that the sediment texture was mostly sandy during pre-monsoon and monsoon and silty sand during post-monsoon season.

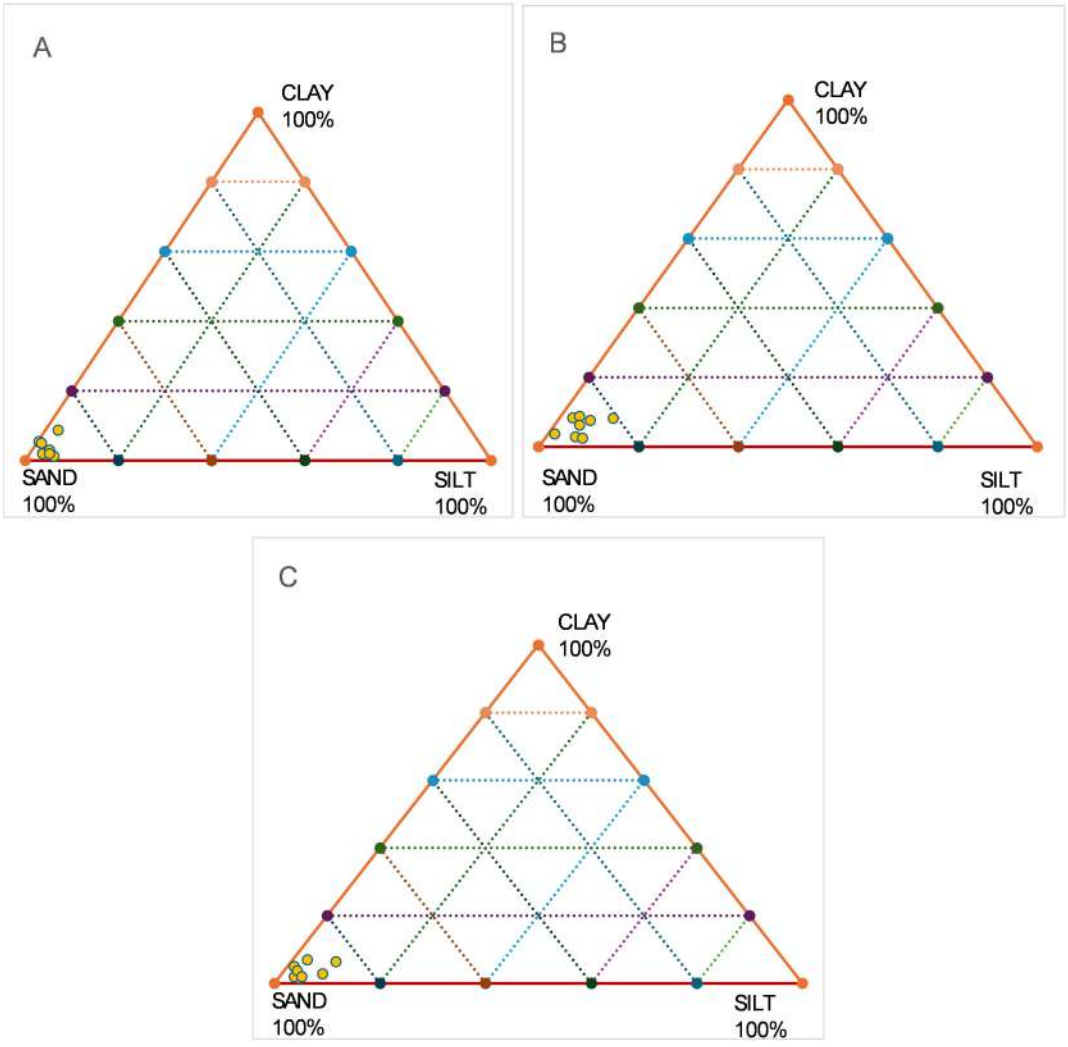


Fig. 2.9 **Sediment texture at sampling stations during the three seasons of first year sampling A) Pre-monsoon B) Monsoon C) Post-monsoon. The yellow dots represent each station.**

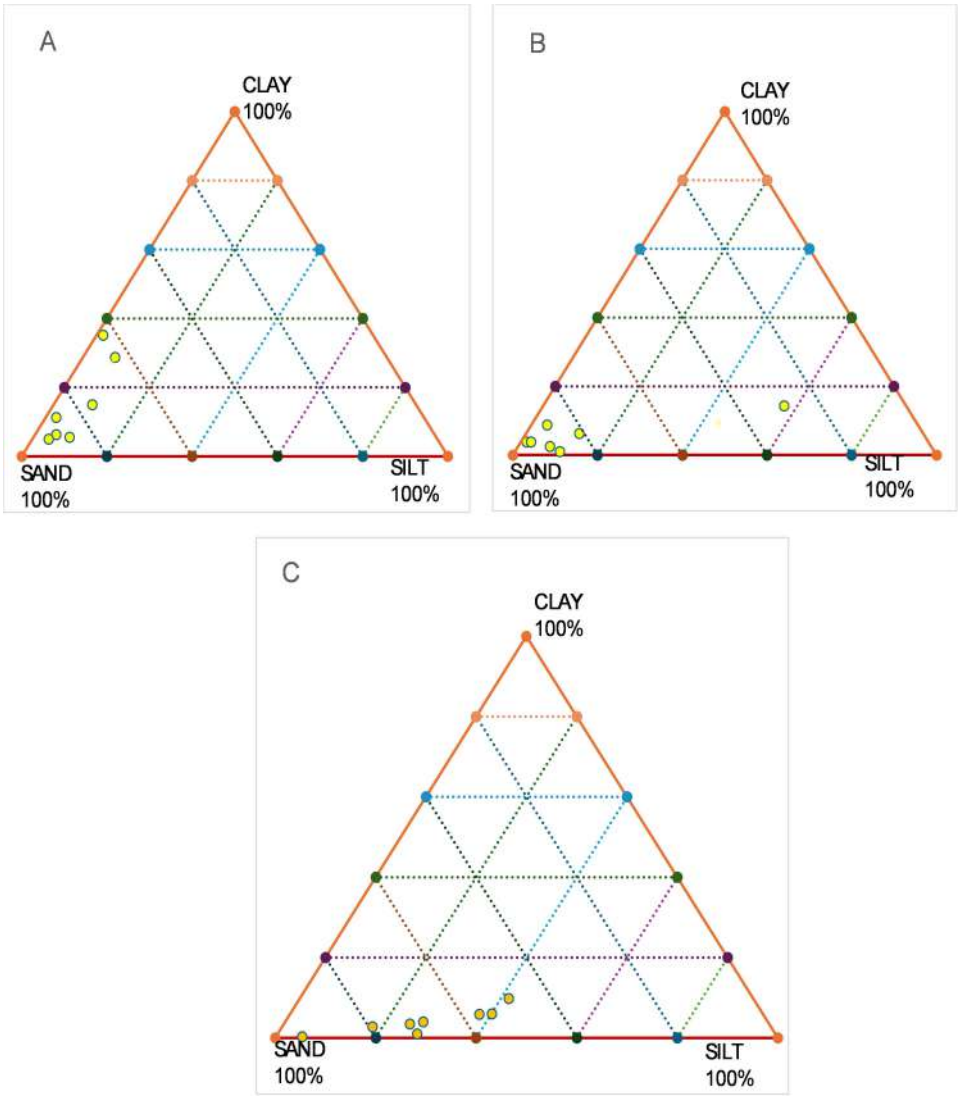


Fig. 2.10 Sediment texture at sampling stations during the three seasons of second year sampling A) Pre-monsoon B) Monsoon C) Post-monsoon. The yellow dots represent each station.

2.4.3 Grain size analysis (sand, silt and clay)

The textural analysis showed varying compositions of sand, silt and clay at different stations during the study period. During the study period, sand content at the sampling stations varied from 46 to 97%, silt from 0.3 to 55%, and clay from 0.3 to 35%.

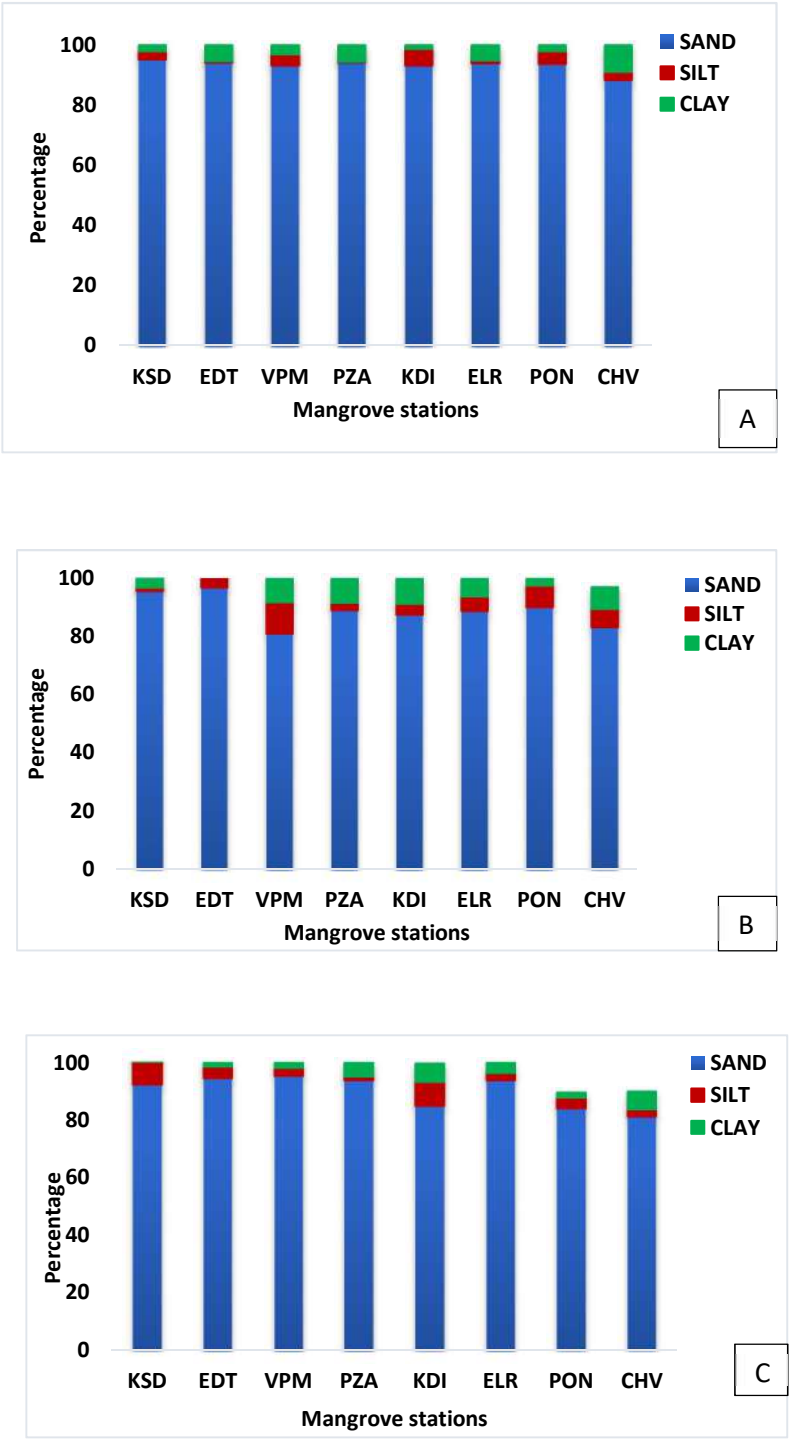


Fig. 2.11 **Percentage of sand, silt and clay at sampling stations during the three seasons of the first-year study. A- Pre- monsoon; B-Monsoon and C- Post-monsoon**

During the first year of sampling, highest sand content recorded was 97% from Edat (monsoon) and lowest was 81% from Valapattanam (monsoon) and Ponnani (post-monsoon) (Fig. 2.11). During the second year sampling, highest sand content recorded was 95% from Kasaragod (monsoon) and Ponnani (post-monsoon) and lowest was 46% from Pazhayangadi (monsoon) (Fig. 2.12).

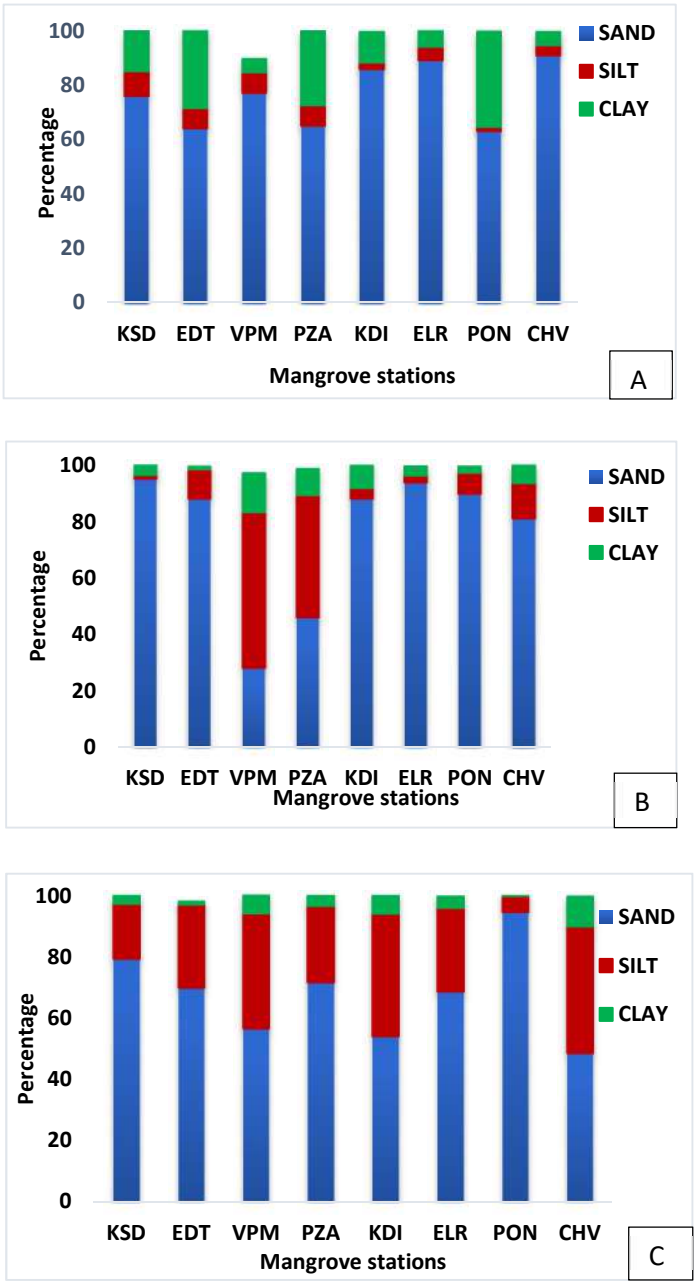


Fig. 2.12 **Percentage of sand, silt and clay at sampling stations during the three seasons of second year study. A- Pre- monsoon; B-Monsoon and C- Post-monsoon**

During first year of sampling, highest silt content recorded was 11% from Valapattanam (monsoon) and lowest was 0.3% from Pazhayangadi (pre-monsoon) (Fig. 2.11). During second year sampling, highest silt content recorded was 55% from Valapattanam (monsoon) and lowest was 1% from Kasaragod (monsoon) (Fig. 2.12).

During first year of sampling, highest clay content recorded was 9% from Kadalundi (monsoon) and Chettuva (pre-monsoon) and lowest was 1% from Kadalundi (pre-monsoon) (Fig. 2.11). During second year sampling, highest clay content recorded was 35% from Ponnani (pre-monsoon) and lowest was 0.3% from Pazhayangadi (monsoon) (Fig. 2.12).

2.4.4 Organic matter

During the study period, organic matter at the sampling stations ranged from 0.2 to 7%. Highest organic matter content in the first year of sampling recorded was 4±0.03% from Edat (monsoon) and Pazhayangadi (monsoon), and lowest was 0.2±0.03% from Chettuva (pre-monsoon) (Fig. 2.13). During the second year of sampling, highest organic content was recorded from Ponnani (7±0.04%) (monsoon) and lowest (0.5±0.02%) from Kasaragod (pre-monsoon) (Fig. 2.13).

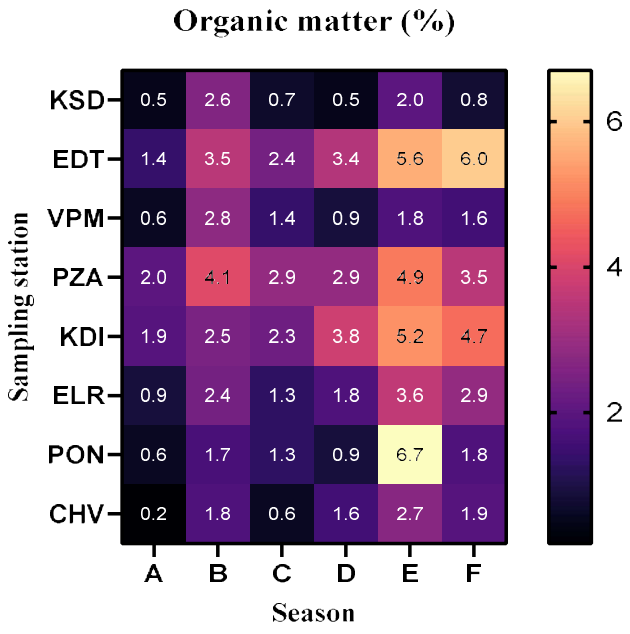


Fig. 2.13 **Percentage of organic matter at sampling stations (A- first year pre-monsoon, B- first year monsoon, C- first year post-monsoon, D- second year pre-monsoon, E-second year monsoon, F-second year post-monsoon)**

2.4.5 Labile compounds

The labile compounds in the sediments consisted of varying compositions of lipid, protein and carbohydrate in different stations during the study period. During the study period, carbohydrate content at the sampling stations varied from 0.01 to 0.25 mg/g. During the first year of sampling, highest carbohydrate content recorded was 0.25± 0.02 mg/g from Kadalundi (pre-monsoon) and lowest was 0.01±0.002 mg/g from Ponnani (pre-monsoon) (Fig. 2.14 I). During the second year of sampling, highest carbohydrate content recorded was 0.14±0.003 mg/g from Edat (post-monsoon) and lowest was 0.02±0.001 mg/g from Pazhayangadi (pre-monsoon), Elathur (pre-monsoon), and Chettuva (pre-monsoon) (Fig. 2.14 I).

Protein content at sampling stations varied from 0.1 to 9.9 mg/g. During the first year of sampling, highest protein content recorded was 9.24±0.03 mg/g from Kadalundi (pre-monsoon) and lowest was 0.07±0.001 mg/g from Elathur (monsoon) (Fig. 2.14 II). Similarly, in the second year too, the highest protein content was recorded from Kadalundi (9.9±0.04 mg/g) during pre-monsoon and lowest was 0.1±0.02 mg/g from Elathur (monsoon) (Fig. 2.14 II).

Lipid content at the sampling stations varied from 0.2 to 9.3 mg/g. During the first year of sampling, highest lipid content recorded was 9.13±0.04 mg/g from Edat (post-monsoon) and lowest was 0.15±0.02 mg/g from Kasaragod (pre-monsoon) (Fig. 2.14 III). During the second year of sampling, highest lipid content recorded was 9.3±0.02 mg/g from Kasaragod (monsoon) and lowest was 0.2±0.01 mg/g from the same station during pre-monsoon (Fig. 2.14 III).

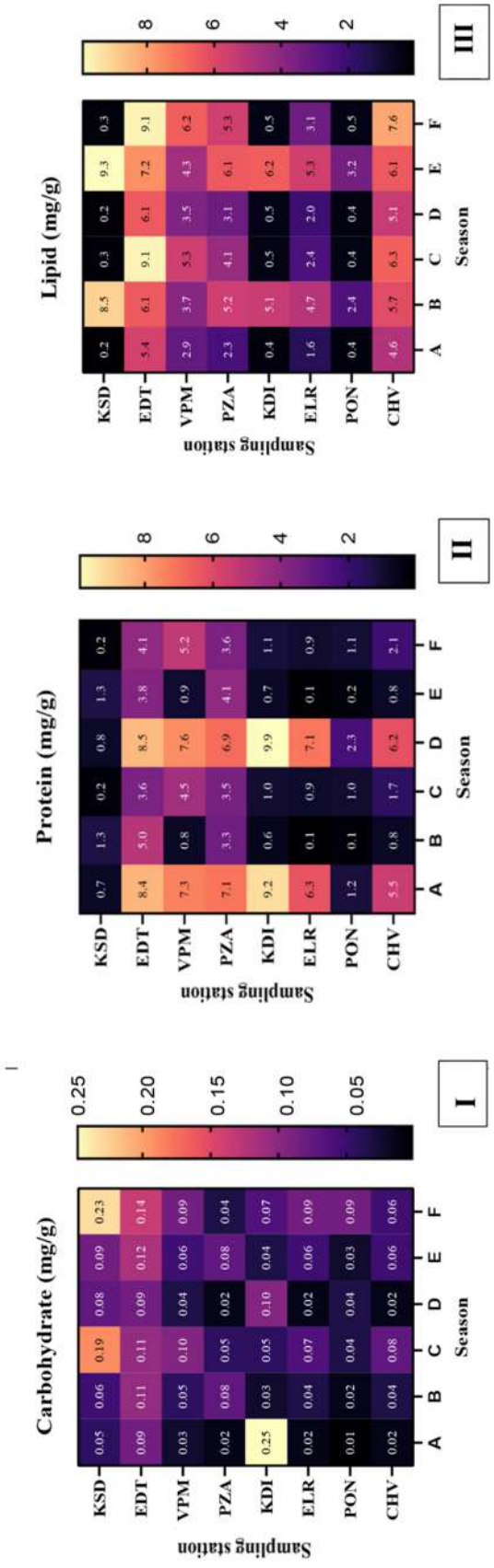


Fig. 2.14 **Concentration of carbohydrate (I), protein (II), and lipid (III) content in sediment samples during the three seasons of second year study (A- first year pre-monsoon, B- first year monsoon, C- first year post-monsoon, D- second year pre-monsoon, E-second year monsoon, F-second year post-monsoon)**

2.4.6 Culturable bacterial population

During the study period, bacterial population at the sampling stations varied from 6 to 8 $\log_{10}$ CFU/ 10g (Fig. 2.15). In the first year of sampling, highest bacterial population recorded was 6.8±0.03 $\log_{10}$ CFU/ 10g from Valapattanam (pre-monsoon) and lowest was 5.5±0.03 $\log_{10}$ CFU/ 10g from Ponnani during pre-monsoon (Fig. 2.16). During second year of sampling, highest counts were recorded from Valapattanam (7.9±0.04 $\log_{10}$ CFU/ 10g) in monsoon and lowest was 5.7±0.02 $\log_{10}$ CFU/ 10g from Valapattanam in post-monsoon (Fig. 2.17). Significant season-wise variation in culturable bacterial population was observed (p = 0.027), but not during station-wise analysis (p = 0.97).

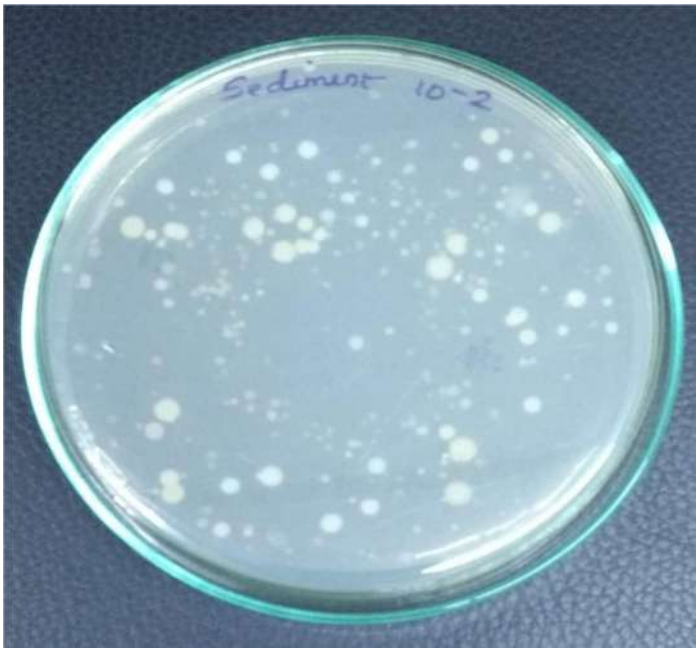


Fig. 2.15 Bacterial colonies on Nutrient agar plates (spread plate technique)

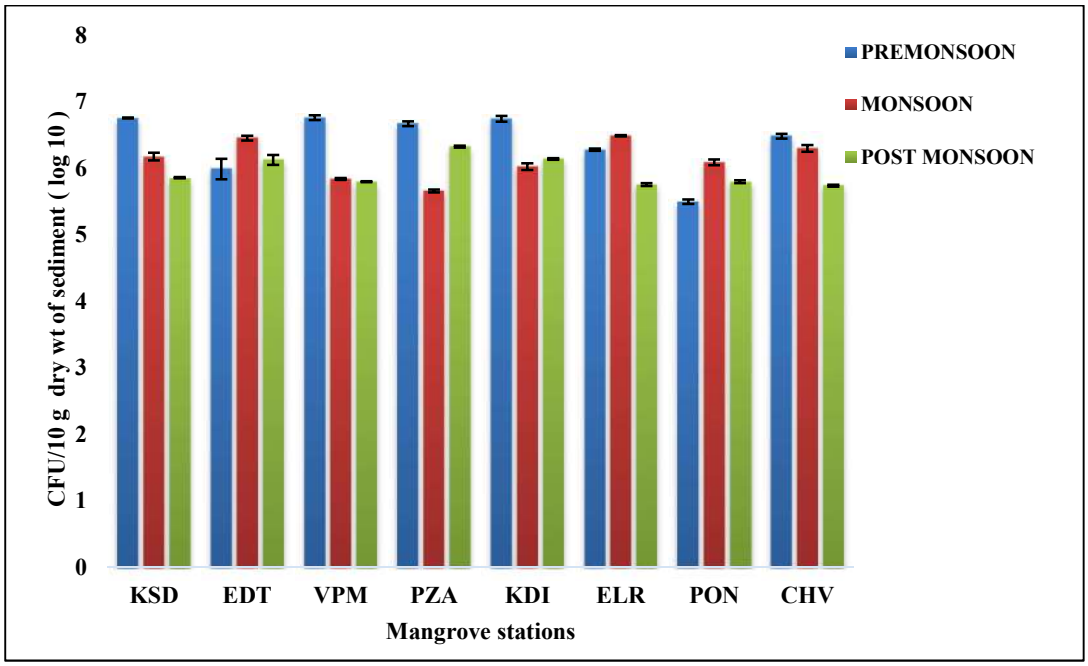


Fig. 2.16 **Culturable bacterial population at sampling stations during the three seasons of the first year of study**

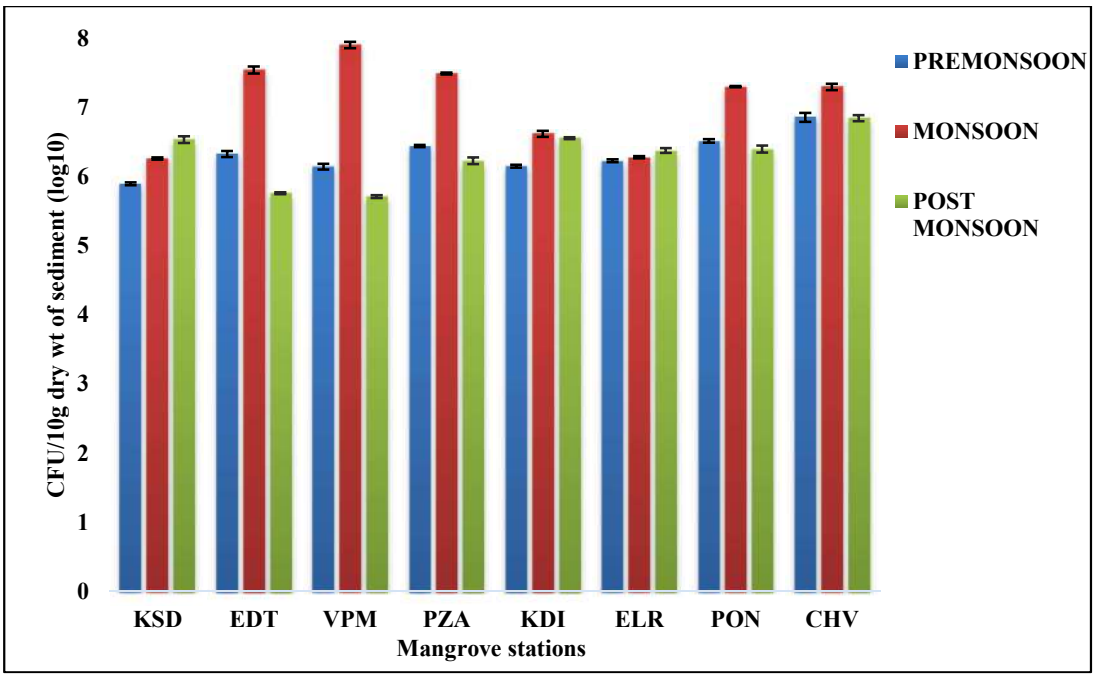


Fig. 2.17 **Culturable bacterial population at sampling stations during the three seasons of second year of study**

2.4.7 Relationship between culturable bacterial population and sediment characteristics- Statistical analysis:

Results of PERMANOVA tests for significant variations in total environmental parameters and bacterial population are depicted in table 2.3. The environmental parameters (Temperature, pH, salinity, labile compounds and grain size) showed significant variation year- wise (P=0.001), season-wise (P=0.001), and sampling site-wise (P=0.001). Significant variation in the bacterial population was found both year-wise (P=0.003), and season-wise (P=0.027), however no variation was observed across sampling sites (P=0.97).

Table 2.3 PERMANOVA tests for significant variations in total environmental parameters and bacterial population in the entire study

Sl.No	Parameters	Year- wise	Season- wise	Sampling station- wise
1.	**Environmental Parameters**	**P= 0.001**	**P= 0.001**	**P= 0.001**
2.	**Bacterial Population**	**P= 0.003**	**P= 0.027**	**P= 0.97**

Results of PERMANOVA tests for significant variations in parameters are presented in table 2.4. This analysis revealed that the organic matter, carbohydrate, protein and lipid showed significant season wise and sampling station wise variation ($p \leq 0.05$). pH and salinity showed significant variation between sampling stations (($p \leq 0.05$), and silt, clay and bacterial population showed significant season wise variation (($p \leq 0.05$).

Table 2.4 PERMANOVA tests for significant variations in environmental parameters (values in shade indicate statistical significance)

Sl. No	Parameters	Season- wise	Sampling station- wise
1	Temperature (ºC)	0.49	0.1
2	pH	0.09	**0.003**
3	Salinity (PPT)	0.21	**0.001**
4	Organic matter (%)	**0.002**	**0.002**
5	Carbohydrate (mg/g)	**0.048**	**0.021**
6	Protein (mg/g)	**0.002**	**0.005**
7	Lipid (mg/g)	**0.004**	**0.002**
8	Sand (%)	0.64	0.47
9	Silt (%)	**0.017**	0.86
10	Clay (%)	**0.029**	0.47
11	Bacterial Population (log_{10} CFU/ 10g)	**0.027**	0.97

- **Non-metric multidimensional scaling (NMDS)**

The multivariate method represents intricate relationships in fewer dimensions. The results of NMDS plot analysis showed that the environmental parameters were most dispersed and scattered irrespective of sampling year and season, indicating that the environmental parameters in Northern Kerala are highly variable (Fig. 2.18).

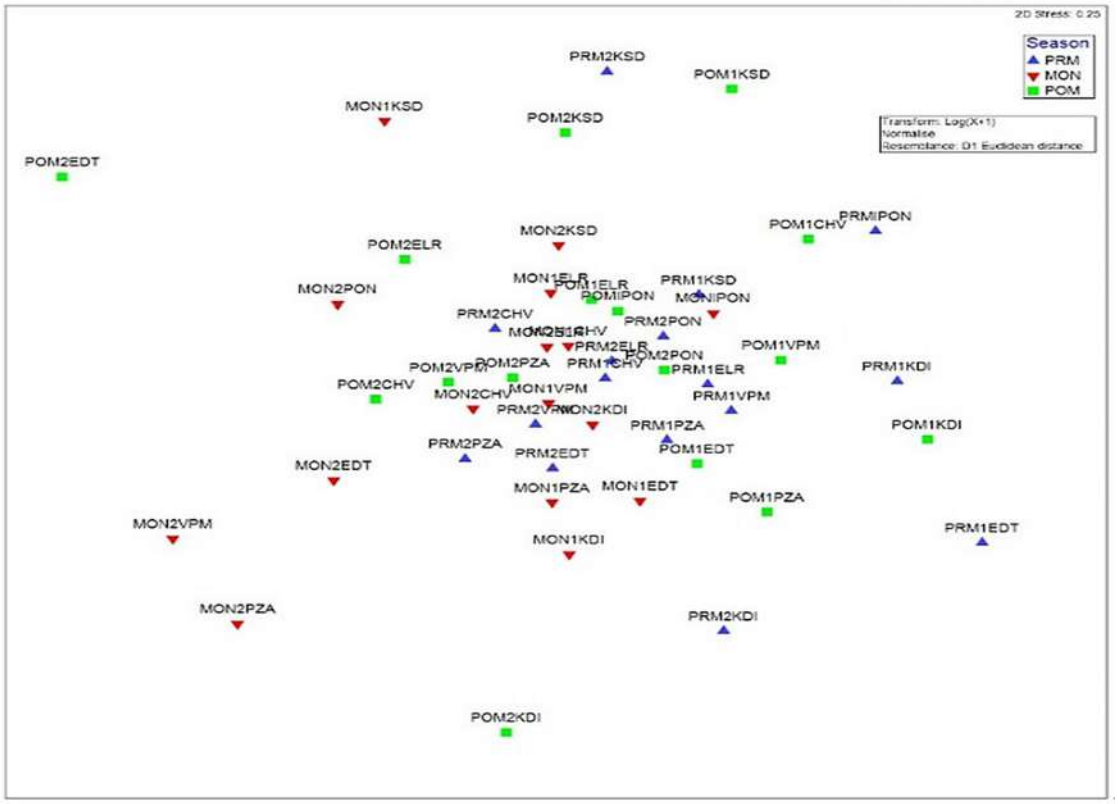


Fig. 2.18 Two-dimensional Non metric MDS plots showing seasonal distribution of environmental variables

- **Correlation analysis**

The correlation analysis between bacterial population and sediment characteristics showed that spearman correlation coefficient was highest with organic matter (r= 0.23) followed by silt (r=0.22), sand (r=-0.13), carbohydrate (r=-0.12), and clay (r= 0.11) (Fig. 2.19). Bacterial population showed positive correlation with organic matter followed by silt, clay, protein, and lipid. The correlation coefficient (r) of sand with silt was -0.70, sand with clay was -0.56, organic matter with lipid was 0.45, silt with temperature was 0.35, sand with temperature was -0.36, organic matter with pH was -0.39, and salinity with pH was 0.33.

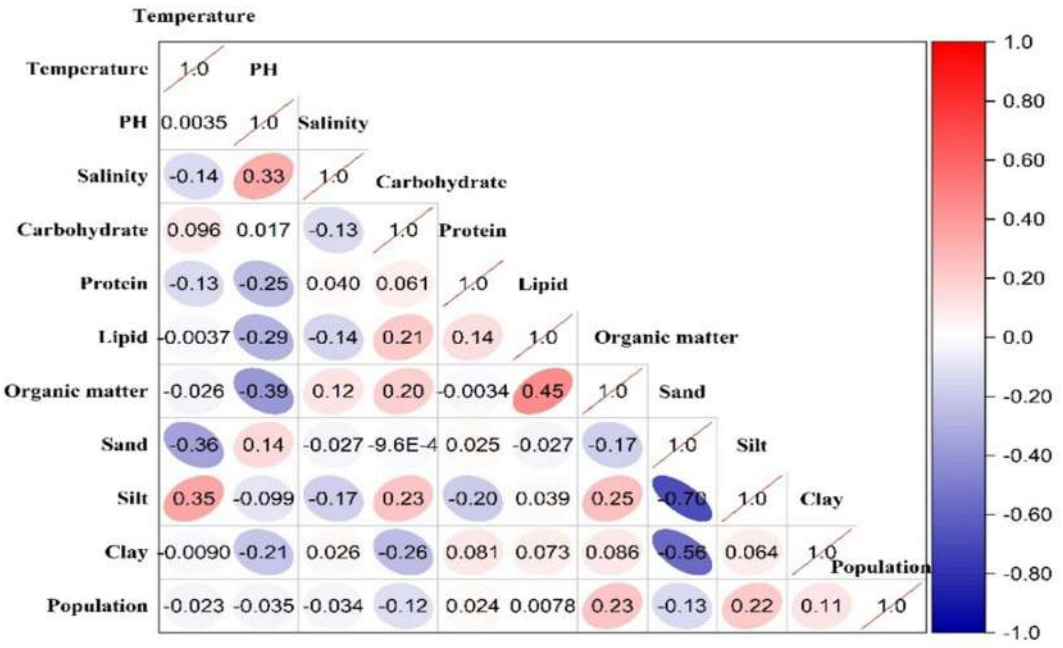


Fig. 2.19 Correlation heat map of environmental variables

- **Principal component analysis**

The environmental factors were visualised using principal component analysis (PCA) (Fig. 2.20; Table 2.5; Appendix I). The PCA axis 1 and 2 explained 24.8% and 16.6% of variation in seasonal distribution, respectively. The factors loading strongly in PC1 were particle size, bacterial population, pH, organic matter content and temperature. Clay content, silt content, organic matter, lipid and protein are closely associated to the bacterial population. The principal component axis, separated the sandy sites with appreciable clay and silt areas, where clay content was closely associated with organic matter and sand content showed close association with salinity.

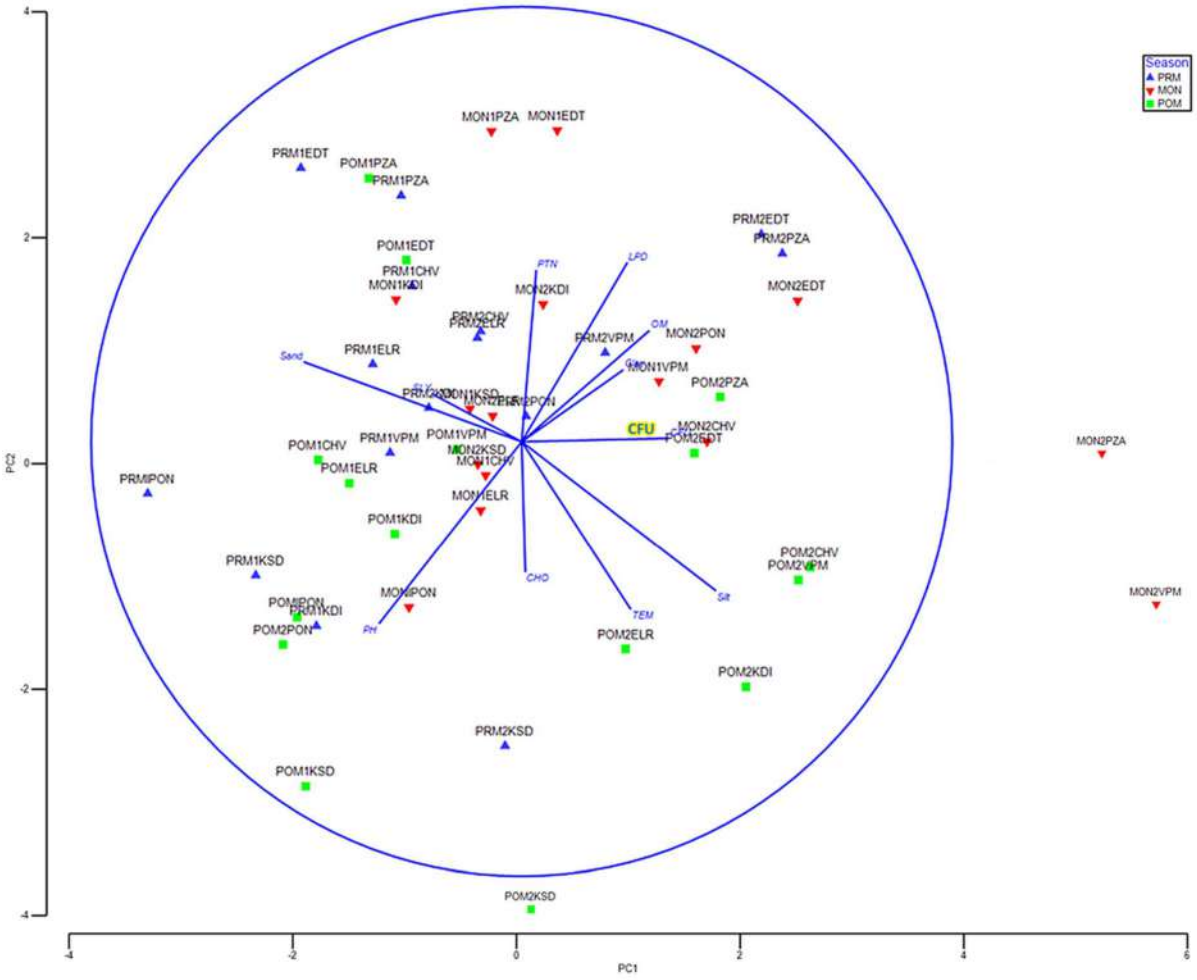


Fig. 2.20 **Principal component analysis (PCA) of environmental parameters in the sampling sites during the pre-monsoon (PRM), monsoon (MON) and post-monsoon (POM) seasons. CFU- Bacterial population; OM- Organic matter; LPD- Lipid; PTN- Protein; SLY- Salinity; CHO- Carbohydrate; TEM- Temperature; 1- First year sampling; 2- Second year sampling.**

Table 2.5 Results of Principal component analysis of environmental factors: eigenvector values

Vector name	Variable	PC1	PC2
TEM	Temperature (°C)	**0.253**	**-0.387**
PH	pH	**-0.331**	**-0.42**
SLY	Salinity (PPT)	-0.208	0.11
CHO	Carbohydrate (mg/g)	0.008	**-0.301**
PTN	Protein (mg/g)	0.034	**0.395**
LPD	Lipid (mg/g)	**0.246**	**0.413**
OM	Organic matter (%)	**0.297**	**0.256**
Sand	Sand (%)	**-0.506**	0.184
Silt	Silt (%)	**0.451**	**-0.344**
Clay	Clay (%)	**0.236**	0.166
CFU	Bacterial population (log_{10} CFU/10g)	**0.342**	0.008

From the current study it was deduced that mangrove habitat in northern Kerala is characterized by bacterial abundance, which is influenced by various environmental parameters and mainly includes clay, organic matter, lipid, protein and silt content of the sediment.

2.5 DISCUSSION

The present study showed that northern Kerala mangrove habitats harbour abundant bacteria, suggesting that they play crucial roles in nutrient cycling and ecosystem functioning. Culturable bacterial population studies have been reported from different mangrove habitats in Kerala which includes, Kadalundi, Kozhikode (Kutty et al., 2020a, Kutty et al., 2020b), Ayiramthengu, Kollam (Varghese et al., 2020), and Kannur (Kutty et al., 2023), In India other studies include, Sundarban, West Bengal (Ghosh et al., 2010), Bhitarkanika, Odisha (Mishra et al., 2012), Manakkudi, Tamil Nadu (Ravikumar et al., 2012), and Goa (Fernandes et al., 2014). Some of the global studies include, Mexico (Gonzalez-Acosta et al., 2006), China (Tam et al., 2002), and Brazil (Dias et al.,2009). These studies revealed a diverse array of bacteria in mangrove environments, suggesting a flourishing ecosystem that plays crucial roles in nutrient cycling, organic matter breakdown, and overall ecosystem vitality. Mangroves, known for their high productivity and biodiversity, depend on microbial communities to

support vital processes such as carbon, nitrogen and sulphur cycling (Ghose et al., 2024).

The current study indicates that the factors responsible for the fluctuations in environmental parameters are probably external or systemic, rather than being specific to the time or location of measurement. Potential explanations may include human influence, pollution, climate change, or significant weather events that affect the environment on a large scale (Srichandan et al., 2019). Bacterial abundance in a mangrove ecosystem can fluctuate between sampling periods due to a variety of factors which includes seasonal changes, shifts in environmental conditions and the natural dynamics of microbial communities.

Unveiling the connection between bacterial populations and sediment characteristics is essential for the management and restoration of important coastal habitats. The interplay between bacterial communities and sediment features in mangrove ecosystems is intricate and layered. Environmental factors affect the distribution of mangrove species, the diversity of microbes, the population of other benthic organisms, and the overall health and stability of the mangrove ecosystem (Haseeba et al., 2025). Microbial communities are influenced by a mix of abiotic and biotic factors. These elements do not function independently; their collective impact dictates the total bacterial abundance and diversity. Bacteria react to a complex interaction of environmental conditions, rather than solely to individual factors. Previous research has also indicated that various factors affect the diversity and composition of bacterial communities in sediments (Wang et al., 2022).

The present study indicates that, clay and silt texture of the soil are significantly linked to the bacterial population, and there is also a strong association between organic matter and clay. As per Lin et al. (2023) and Konstantinou et al. (2023), sediment texture or grain size can significantly influence bacterial population dynamics by affecting factors like pore space, organic matter availability and surface area for attachment. According to Tucker (1999) and Fomina and Skorochod (2020), the high surface area of clay is a key factor in its ability to readily bind organic matter, retain water, and nutrients, making it an important component of fertile soil. The increased organic matter then provides a food source for bacteria, leading to a higher bacterial

load. Silt particles are smaller than sand but larger than clay. As sandy soils have the lowest water retention due to their large particles and pore spaces, this could be the reason for negative correlation (r=-0.13) with bacterial population in the current study. Fine-grained sediments like silt and clay can retain more organic matter and nutrients, creating a favourable environment for microbial growth (Banerjee et al., 2020). In the present study, organic matter concentration shows positive correlation with silt (r=0.2) and clay content (r=0.01) and negative correlation with sand (r=-0.17) supporting the earlier findings.

Bacterial population was found highest during second year sampling as like clay content, suggesting that the clay content positively influenced bacterial numbers. Changes in sediment texture in the ecosystem is influenced by natural tidal actions, sediment deposition, mangrove plants and anthropogenic activities (Siddique et al., 2025). The clay content and bacterial population were seemed to be higher at Edat, Chettuva, Pazhayangadi, Valapattanam, and Ponnani, which may be due to the combination of factors including nutrient availability, sediment composition, salinity, temperature and the presence of specific plants and animals in the particular habitat favourable for bacterial abundance. The sand content showed negative correlation suggesting that higher sand content generally correlates with improved drainage, less salinity and less organic matter retention, while finer sediments are associated with higher moisture retention and salt concentration. The interesting difference observed between the above mentioned and excluded stations (Kadalundi, Kasaragod and Elathur) is that the latter is prevalent in sand composition. Sandy soils tend to have larger particles with wider pore spaces, leading to faster drainage and lower water retention, and lower levels of organic matter, can limit the availability of essential nutrients for microbial activity. This can create a drier environment, which is less favourable for many bacterial species (Vu et al., 2022). High sand content in mangrove sediments is often a result of strong hydrodynamic conditions and sediment sorting during transport (Qi et al., 2025).

Apart from bacterial abundance, sand concentrations were comparatively found higher in mangrove habitats due to factors like the supply of sand from rivers and coastal winds, the physical structure of mangrove roots that trap sediment, and specific site characteristics such as proximity to land or the effects of events like tsunamis that

deposit large amounts of sand (Dewiyanti et al., 2021). During 2020-21 sampling the predominance of sandy sediment may be due to the impact of flood. It's reported that aerobic bacteria are generally more abundant in sandy soils, particularly in the intertidal or supratidal zones, because sandy environments provide greater pore space and thus better oxygen availability for these oxygen-dependent organisms. This contrasts with finer, more compacted sediment that may become anaerobic, creating conditions more suitable for anaerobic bacteria (Richardson et al., 2008).

The pH (r= -0.035), salinity (r= -0.034), and temperature (r= -0.023) showed negative correlation with bacterial population in the current study. In a negative correlation, increase or decrease of these parameters can affect bacterial growth and metabolic activity, creating conditions less suitable for a thriving bacterial community. Different bacterial groups have different pH, temperature, and salinity tolerances, and an imbalanced condition can favour only a few stress-tolerant species, while suppressing the dominance of others (Liu et al., 2024).

According to Fox et al. (2017) classification of organic matter, northern Kerala mangrove habitat has low to moderate level of organic matter. The organic matter content exhibited significant year wise ($p=0.015$), season wise ($p=0.002$) and sampling site wise ($p=0.005$) variation. Variations in mangrove sediments are influenced by factors like rainfall, terrestrial runoff, tidal regimes and organic matter sources (Dan et al., 2021). Station wise variations are mainly due to hydrological regime (location of sampling point), age of mangroves (older mangrove forests -higher autochthonous carbon); younger forests- allochthonous or terrestrial carbon), proximity to source and sediment properties. During monsoon, higher organic matter content is observed due to increased input from terrestrial sources, such as runoff from agricultural fields and river discharges (Ho et al., 2023).

Previous studies reported that higher organic matter content generally leads to increased productivity of the ecosystem with significant carbon storage potential (Prasad et al., 2010). This leads to the accumulation of organic carbon in the sediments, making mangroves important "blue carbon" sinks. Studies showed lower organic matter at high tide and higher content at low tide (Amin et al., 2022). Coarse-grained sediments, like sand, typically have lower organic matter compared to finer-grained

sediments such as clay or silt, which can better trap and preserve organic material (Khan et al., 2012).

Organic matter and clay content influence each other because clay protect organic matter from decomposition through physical entrapment in their pores and chemical binding, while the presence of organic matter can modify clay surface chemistry and aggregation, altering its dissolution rates and the overall microstructure of the soil. This dynamic interaction creates organo-mineral complexes that stabilize soil structure and influence nutrient cycling, water retention and microbial activity (Elert et al., 2015). Organic matter and clay content influence bacterial populations through mechanisms like aggregate formation, nutrient availability, and microhabitat creation. Clay particles stabilize aggregates, which creates diverse microenvironments (different pore sizes, moisture levels) that host distinct bacterial communities. Organic matter provides nutrients and energy for bacteria but also binds with clay, influencing aggregate stability and the decomposition rates of organic material. These interactions create a feedback loop where clay content and organic matter quality shape the bacterial community, which in turn influences the breakdown and stabilization of organic matter (Xia et al., 2020).

The protein (0.1-9.9 mg/g) and lipid content (0.2-9.3 mg/g) was found to be higher than carbohydrate (0.01-0.25 mg/g) in the current study. Proteins are reported as a fundamental factor driving the abundance, activity and diversity of bacteria in mangrove sediments, and performing critical role in the ecosystem's nutrient cycling and overall health (Thatoi et al., 2013). In this study highest protein concentration was observed at Kadalundi during pre-monsoon season and lowest at Elathur during monsoon season. The protein content exhibited significant season-wise ($p=0.002$) and sampling site-wise ($p=0.005$) variation. Variations in mangrove sediment protein content are influenced by factors like organic matter input from terrestrial runoff, tidal flushing, and seasonal rainfall patterns (Nair et al., 2010; Ho et al., 2023). Jayan and Chandramohanakumar (2015) reported that concentration and type of amino acids can vary with depth within the sediment and throughout the seasons. Higher protein content is generally indicative of a eutrophic (nutrient-rich) and less mineralized environment (Satheeshkumar and Khan, 2009). Protein content in mangrove sediments is highest in the pre-monsoon season due to the deposition of organic matter from decaying

mangrove leaves and terrestrial sources, which increases available nutrients and organic carbon during this dry, low-tidal period before heavy rainfall inputs become dominant. Lower tidal inundation and less terrestrial runoff also contribute to the buildup of organic material, including proteins from bacteria and plants, in the sediment (Fernandes et al., 2020). Bacterial abundance showed positive correlation with protein content (r=0.024), and high lipid content is associated with thriving bacterial communities, which are crucial for nutrient cycling and potentially for phytoremediation of pollutants like hydrocarbons, reflecting a healthy and active ecosystem (Semanti et al., 2021).

The lipid concentration showed significant season wise (p=0.004) and site- wise variation (p=0.002), due to varying organic matter input from terrestrial runoff and litterfall, which are higher reported during the monsoon and post-monsoon seasons, leading to increased organic carbon and associated biochemicals, including lipids. Lipid concentration was found highest (9 mg/g) at Kasaragod and Edat. The higher lipid content indicates higher-quality organic matter available to benthic organisms, suggesting good quality energy source. Differences between sampling stations highlight varying influences like tidal flushing and freshwater input, while seasonal changes reflect altered levels of terrestrial input and biological activity (Ho et al., 2023). Lipid content can change with depth (lipids are more abundant in surface sediments than at deeper levels). Bacterial abundance showed positive correlation with lipid content (r=0.01) and also lipid content with organic matter (r= 0.45), suggesting lipids are a significant component of the organic matter in the study area, and that their presence contributes to the overall organic richness leading to bacterial abundance (Semanti et al., 2021).

The carbohydrate concentration showed significant season wise (p=0.048) and site- wise variation (p=0.021), Seasonal and spatial variations in sediment carbohydrate concentrations, especially in tropical systems, indicate the ecosystem's eutrophic condition, the age and source of organic matter, and the presence of labile vs. stable organic carbon, which is influenced by factors like rainfall, light availability, plant species composition, vegetation biomass, riverine input, tidal dynamics, hydrodynamic conditions and microbial activity (Nair et al., 2010). The higher carbohydrate content was reported from Kadalundi during pre- monsoon (0.25 mg/g) and least from Ponnani

(0.01 mg/g) in the present study. Higher carbohydrate content often suggests an accumulation of aged organic matter from increased plant input and higher level during non-monsoon seasons is due to the lower sedimentation from freshwater influx and accumulation of fresh organic matter (Mathew and Gopinath, 2024).

The lowest concentration of carbohydrate among labile compounds in the current study indicates a low proportion of labile organic matter, which suggests that the sediment contains a higher proportion of more recalcitrant (stable) organic matter, potentially reflecting advanced diagenesis or high decomposition rates by microbes (Latt et al., 2001). A negative correlation (r=-0.12) between carbohydrate content and bacterial abundance in the current study is likely due to the process of carbohydrates being rapidly consumed by bacteria, leading to their lower concentration in sediments with higher bacterial activity. The lowest concentration of carbohydrate in mangrove sediment can vary greatly depending on the specific location, sediment type, and disturbance level, but studies suggest that unvegetated areas or areas beneath dead mangrove forests tend to have lower concentrations of organic matter, including carbohydrates, compared to areas with living mangrove stands (Duan et al., 2020).

Protein to carbohydrate (PTN: CHO) and lipid to carbohydrate (LPD: CHO) ratio, is used to analyze the quality and age of organic matter, particularly in aquatic sediments (Thalayappil et al., 2024). In the current study the protein-to- carbohydrate (PTN: CHO) ratio is found to be >1 in all the sampling sites irrespective of year and season, indicating that organic matter was freshly originated and contained more labile organic matter (OM), suggesting a higher quality and more labile food source for benthic organisms compared to aged OM, which typically has a lower protein-to-carbohydrate ratio. Highest ratio was reported from Elathur (PTN: CHO=355:1) and lowest from Kasaragod (PTN: CHO=1:1) and also the ratio was found highest during pre- monsoon in all the sampling stations except in Kasaragod (Monsoon- PTN: CHO=21:1). It may because the pre-monsoon conditions feature less freshwater input, higher marine influence and increased organic matter from planktonic sources, while the onset of the monsoon brings heavy rains, increased freshwater and erosion that washes away some organic matter, leading to lower concentrations. Additionally, pre-monsoon conditions may favour the production and deposition of less degraded, fresher organic matter from benthic and aquatic organisms (Resmi et al., 2016). According to

the study of Carugati et al. (2018) protein fraction of biopolymeric carbon was twice as high in disturbed mangrove ecosystem. The protein-to-carbohydrate ratio was also four times greater in disturbed mangrove sediments.

In the current study the lipid-to- carbohydrate (LPD: CHO) ratio is found to be >1 in all the sampling sites irrespective of year and season, which indicates the better-quality, more nutritionally rich organic matter that is also well-preserved due to a lower input of aged organic matter. This suggests a more labile and less degraded fraction of organic matter, potentially from active biological processes like high microbial abundance, and better conditions for organic matter preservation under anoxic environments (Kumar et al., 2022). Highest ratio was reported from Chettuva (LPD: CHO=255:1) and lowest from Kasaragod (LPD: CHO=1.3:1). Bacterial abundance is positively correlated with protein (r= 0.024) and lipid content (r=0.01) which indicates that organic matter is rich in proteins and lipids, supporting abundance in bacterial population. These organic compounds serve as a food source, driving bacterial growth and increasing overall bacterial biomass and abundance (Danovaro et al., 2000). According to the study of Carugati et al. (2018) lipid content was stable in disturbed and undisturbed mangrove ecosystem.

Bacterial abundance and community structure in northern Kerala mangrove ecosystems are shaped by a complex interplay of various physical, chemical and biological factors, including sediment properties, temperature, salinity, pH, nutrient availability and biological interactions. These factors create diverse ecological niches and influence the metabolic activities and diversity of bacterial communities, making it impossible to attribute their composition to a single element.

Chapter 3

CLASSIFICATION OF SEDIMENT BACTERIA ISOLATED FROM THE MANGROVES OF NORTH KERALA

Chapter - 3

CLASSIFICATION OF SEDIMENT BACTERIA ISOLATED FROM THE MANGROVES OF NORTH KERALA

3.1 INTRODUCTION

Identification of mangrove bacteria helps to understand their crucial role in mangrove ecosystem functions like nutrient cycling and decomposition, to discover new biotechnological resources for industries such as agriculture and pharmaceuticals, and to develop bioremediation strategies for pollution control. Identifying these microbes also helps in monitoring mangrove health and developing conservation methods by understanding how their communities respond to environmental changes.

Traditional methods for classifying isolated sediment bacteria rely on observable characteristics and biochemical tests. Key steps include isolating pure cultures using techniques like the streak-plate method, followed by morphological characterization (shape, size, color, margin of colonies), gram staining and conducting various biochemical tests to determine their metabolic capabilities. This procedure provides a basis for grouping bacteria into broad categories, though molecular methods offer more detailed classification (Christopher and Bruno, 2003).

3.2 REVIEW OF LITERATURE

Mangrove ecosystems are known to harbour diverse and unique bacterial communities. These bacteria play crucial roles in the functioning and ecological dynamics of mangroves. Mangrove habitats exhibit high bacterial diversity due to the complex and dynamic nature of the ecosystems. Mangrove bacteria contribute to important biogeochemical processes such as nutrient cycling, carbon fixation, nitrogen fixation, organic matter degradation and overall ecosystem productivity. They can form symbiotic associations with plants and other organisms. Additionally, bacteria associated with mangrove roots help in nutrient uptake and provide protection against pathogens. They have adapted to the unique environmental conditions of mangrove

ecosystems, including high salinity, fluctuating tidal regimes and oxygen availability and have developed mechanisms to cope with these conditions, such as salt tolerance, production of osmo protectants and specialized metabolic pathways. The type of mangrove species, geographical location and human interference strongly influence the composition of bacteria (Bharti and Grimm, 2021).

To identify bacteria, researchers typically employ a combination of techniques, including phenotypic characterization, biochemical tests, molecular techniques (such as DNA sequencing) and specialized identification databases. These methods help in determining the taxonomic classification and identification of mangrove bacteria based on their unique features and genetic profiles. The field of microbial taxonomy is constantly evolving and new techniques and approaches are continuously being developed. The combination of culture-dependent and independent methods is used in the identification of mangrove bacteria. Isolation of bacteria from samples and culturing them *in- vitro* is involved in the culture-dependent method. Choice of identification method may vary depending on the specific research objectives and available resources.

The isolated bacteria can be identified using various techniques, including morphological observation of bacteria, and can be categorized based on their shape, size and colony characteristics on agar plates. Microscopic observation provides information about morphology, size, arrangement and staining characteristics of the bacterial cells. Staining techniques enhance the visualization and identification of bacteria. Biochemical tests include catalase, oxidase and sugar fermentation, which can help determine the metabolic characteristics of the bacterial isolates.

"Bergey's Manual of Systematic Bacteriology" is a renowned reference work in the field of bacteriology, providing comprehensive information on bacterial taxonomy, classification and identification. The Bergey's Manual of Systematic Bacteriology is published in several volumes, which provide a comprehensive and authoritative resource for bacterial taxonomy and identification, covering a wide range of bacterial species and groups. They provide detailed information on the morphological, physiological, biochemical and genetic characteristics of bacteria. Additional resources, such as molecular techniques and online databases, are often used in

conjunction with Bergey's Manual for more accurate and up-to-date bacterial identification. Rani et al. (2020), Reddy et al. (2019) and Uchida et al. (2014) identified bacterial isolates from different microhabitats using a combination of phenotypic characterization and molecular techniques.

Some examples of commonly used biochemical tests for the identification of bacteria are as follows: The presence of the catalase enzyme, cytochrome oxidase and indole (from tryptophan) is detected using the catalase, oxidase and indole tests, respectively. The methyl red and Voges-Proskauer (MR-VP) test is used to differentiate between mixed acid fermentation and butanediol fermentation pathways. The MR test detects the production of acid, while the VP test detects the production of acetoin (Cappuccino et al., 1983).

Physiological identification of bacteria involves the characterization of their metabolic and growth properties (Abdel-Razek et al., 2019). It provides valuable information about the nutritional requirements, enzymatic activities and physiological capabilities of bacteria. Some common physiological tests used for bacterial identification are as follows: 1) Growth characteristics and observation of colony morphology, growth rate, and pigmentation on different agar media that provide initial clues for identification. 2) Nutritional requirements: Testing the ability of bacteria to grow on different nutrient media or evaluating their ability to utilize specific carbon sources, nitrogen sources, or other nutrients that can help in identification. 3) Enzyme production: Various enzyme assays can be performed to determine the presence or absence of specific enzymatic activities like catalase, oxidase, urease, gelatinase, and lipase tests. 4) Fermentation tests assess the ability of bacteria to ferment different carbohydrates, such as glucose, lactose, or mannitol. These tests can provide important information for bacterial identification. 5) Metabolic pathway assays like the Voges-Proskauer (VP) and Methyl Red (MR) tests help distinguish between different metabolic pathways, such as mixed acid fermentation and butanediol fermentation. 6) Oxygen requirement: Determining the oxygen tolerance of bacteria, whether they are aerobic, anaerobic, or facultative anaerobic, can aid in identification. 7) Antibiotic sensitivity: Assessing the susceptibility of bacteria to a panel of antibiotics can provide information about their antibiotic resistance profile. 8) pH and temperature tolerance:

Evaluating the growth of bacteria at different pH levels and temperatures that can help characterize their physiological adaptability.

The physiological tests are often used in combination with molecular techniques for accurate bacterial identification and gives knowledge about bacterial metabolic capabilities, aiding their classification and identification. Several studies have utilized physiological identification methods to characterize mangrove bacteria. Das and Lyla (2014), Li et al. (2018) and Abdel-Razek et al. (2019) used physiological and biochemical characterization techniques, such as growth characteristics, enzymatic activities, carbon source utilization and antibiotic sensitivity, to gain insights into the physiological diversity and functional capabilities of mangrove bacteria. By combining physiological identification with molecular methods, researchers can obtain a more comprehensive understanding of the microbial communities in mangrove ecosystems.

Biodiversity indices are essential tools for quantifying and comparing the diversity of bacterial communities in mangrove habitats. Several indices can be used for bacterial diversity analysis, each offering different insights into the composition and structure of these microbial communities. The commonly used biodiversity indices for bacterial diversity analysis in mangrove habitats are richness indices, evenness indices, rarefaction and extrapolation, taxonomic diversity, functional diversity, phylogenetic diversity, dominance indices, beta diversity, functional indices, rare biosphere analysis, etc. (Fedor and Zvarikova, 2019). The selection of the appropriate biodiversity indices is based on the research question, data type and study objectives. Often, a combination of indices provides a more comprehensive view of bacterial diversity in mangrove habitats. Additionally, software packages such as QIIME, Mothur and R, along with packages like Vegan, can streamline the calculation and interpretation of these indices. Biodiversity indices provide insights into the overall health and integrity of an ecosystem. A decrease in diversity can indicate stress or degradation, while increased diversity often suggests a healthy ecosystem. Researchers can use biodiversity indices to compare the diversity of species or communities across different habitats, regions, or times. This technique helps to identify areas of high conservation value or regions undergoing significant ecological changes. Biodiversity indices are used to assess the impact of natural or human-induced disturbances, such as habitat destruction, pollution, or climate change, on biological communities. Changes

in diversity can indicate the severity of disturbances. Conservationists use biodiversity indices to monitor the effectiveness of protected areas and conservation efforts. They can track changes in species richness and diversity over time. Biodiversity indices can help identify species that have a disproportionately large impact on an ecosystem's structure and function (Izsak and Papp, 2000).

Studies of bacterial diversity are critical for understanding microbial complexity and its role in various ecosystems and applications. Unveiling bacterial diversity helps us understand its role in ecosystem functioning, nutrient cycling and energy flow. It also helps to assess the impacts of pollution, climate change and land use on microbial communities. Unveiling diversity aids in bioremediation by identifying and engineering bacteria for cleaning up contaminated environments (Horemans et al., 2016). Discovering potential bacteria can lead to the production of biofuels, enzymes, pharmaceuticals and other bioproducts. Monitoring and controlling microbial diversity in food processing and fermentation, investigating how bacterial communities respond to and influence climate change, studying the impact of agricultural practices on soil and plant-associated bacteria, investigating the distribution of bacteria across different geographic regions and their role in biogeochemical cycles, and studying the diversity and evolution of pathogenic bacteria to develop effective treatments and vaccines also aid in unveiling the diversity of microbes in the ecosystem (Fierer, 2017). Understanding bacterial communities and their diversity is fundamental for addressing numerous scientific, environmental and practical challenges.

In the current study, bacteria isolated from the mangrove sediments of Northern Kerala were classified up to the generic level using morpho-biochemical methods.

3.3 MATERIALS AND METHODS

3.3.1 Morphological characterization of isolated bacteria

Morphologically different bacterial colonies were selected by analysing the characteristic shape, size, colour, surface appearance and texture of colonies from each plate and pure cultured. Morphological studies were done by Gram's staining technique and motility checking. Biochemical tests such as the indole test, methyl red

test, Voges- Prauskauer test, citrate utilization test, catalase test, oxidase test, triple sugar iron test and urease test were performed to classify the bacteria (Cappuccino et al., 1983).

3.3.1.1 Colony characteristics: The morphology of the colonies developed on the nutrient agar plate were observed. The colony characteristics, such as colour, configuration (circular, lobate, etc.), margin (irregular, entire, etc.), elevation (flat, convex, raised, umbonate, etc.), opacity and pigmentation, were noted (Cappuccino et al., 1983).

3.3.1.2 Gram staining: Prepared a bacterial smear of suspension on the clean slide with a loopful of culture, air dried and heat fixed. Crystal violet was poured and kept for about 1 minute and rinsed with water. Flooded Gram's iodine for 1 minute and rinsed with water. Then washed with decolourising solution for about 10-20 seconds and rinsed with water. Added safranin for about 1 minute and washed with water. The stained slide was air-dried and observed under oil immersion (100x) using a bright field microscope. Gram-positive bacteria appear purple in colour, while gram-negative ones appear pink in colour. The results obtained were recorded (Cappuccino et al., 1983).

Preparation of Gram's stain reagents

Crystal violet: Dissolved 2.0 g certified crystal violet into 20.0 ml of 95% ethyl alcohol and dissolved 0.8 g ammonium oxalate into 80.0 ml distilled water in separate conical flasks. Mixed the two solutions together and allowed them to stand overnight at room temperature (28°C). Filtered through coarse filter paper before use and stored at room temperature (28°C).

Gram's iodine: Ground 1.0 g iodine (crystalline) and 2.0 g potassium iodide in a mortar and added to 300 ml distilled water. Stored at room temperature (28°C) in an amber coloured bottle.

Decolouriser: Mixed 25 ml of distilled water with 475 ml absolute ethanol and transferred the solution to a screw-cap bottle of 1 litre capacity. Measured 500 ml acetone and added immediately to the alcohol solution. Mixed well and stored at room temperature.

Safranin: Added 2.5 g certified safranin-O to 100 ml 95% ethyl alcohol, and kept as stock. From the stock 10 ml was taken and made up to 100 ml using distilled water and stored at room temperature (28°C).

3.3.1.3 Motility checking- Hanging drop experiment

Held a clean coverslip by its borders and applied Vaseline using a toothpick on its corners. Placed a loopful of broth culture in the middle of the coverslip. Turned the cavity slide upside down on the cover slip and observed the slide under microscope for motility (Cappuccino et al., 1983).

3.3.2 BIOCHEMICAL TESTS

Biochemical tests done for bacterial classification are as follows.

3.3.2.1 Catalase test: Transferred a well-isolated colony to a clean glass slide and added 1 drop of 3% H_2O_2. Observed for immediate bubble formation which was considered a positive result (MacFaddin, 2000).

3.3.2.2 Oxidase test: Using tweezers, placed the oxidase disc on a slide. The well-isolated colony was collected on the edge of a sterile stick, and gently rubbed the colony on the disc. The appearance of a purple colour within 30 seconds was considered a positive result. Colour developed after 30 seconds indicated negative result (Steel, 1961).

3.3.2.3 Indole test: Inoculated the tube of tryptone broth [g/l, Tryptone 10.0 g, Sodium chloride 5.0 g] with a loopful of pure culture and incubated at 37°C for 24 to 48 hours. To test for indole production, added 5 drops of Kovac's reagent directly to the tube. A positive indole test was indicated by the formation of a pink to red colour ("cherry red ring") in the reagent layer on top of the medium within seconds of adding the reagent. If a culture was indole negative, the reagent layer will remain yellow or be slightly cloudy. The colour of the ring was recorded (Cappuccino and Sherman, 2005).

3.3.2.4 Methyl Red test: A tube was taken with MR-VP medium [(pH 6.9) g/l Peptone- 7 g, Glucose- 5 g, Dipotassium phosphate- 5 g] and inoculated with pure culture and incubated at 37°C for 24 hours. After incubation added 5-6 drops of MR

reagent and again incubated for few minutes. Observation of red colour indicated positive result. The colour change was noted (Cappuccino and Sherman, 2005).

3.3.2.5 Voges–Proskauer (VP) test: 18–24-hour pure culture was inoculated to sterilized MR VP medium and incubated at 37°C for 24 hours. After incubation, added 6 drops of Barritt's reagent A (6 g naphthalin in 100 ml of 95 % ethanol) and mixed well to aerate. Then added 2 drops of Barritt's reagent B (16 g KOH in 100 ml distilled water) and mixed well to aerate. Observation of pink-red color at the surface of the tube within 30 min, indicates positive result. The colour change was noted (Cappuccino and Sherman, 2005).

3.3.2.6 Citrate Utilization test: A single isolated colony was taken and streaked on the surface of the Simmon's citrate agar slant and incubated at 28 ± 4°C for overnight. After incubation, intense prussian blue colour in the medium indicates positive result. The colour change of the medium was recorded (Cappuccino and Sherman, 2005).

Simmons citrate agar medium

Sodium citrate	-	1g
Sodium chloride	-	5.0g
Magnesium sulphate	-	0.2g
Mono-ammonium phosphate	-	1g
Dipotassium phosphate	-	1g
Bromothymol blue	-	0.08g
Agar	-	15g
Distilled water	-	1000 ml
pH	-	6.8

3.3.2.7 Urease test: 18 to 24 hour pure culture was streaked on the slant surface of Christensen's urea agar media. Incubated at 28 ± 4°C for overnight. After incubation, observed the slant for colour change. Urease production was indicated by a bright pink (fuchsia) colour on the slant that may extend into the butt. The colour change of the medium was noted (Larson and Kallio, 1954).

Christensen's urea agar medium

Urea	-	20g
NaCl	-	5g
Monopotassium phosphate	-	2 g
Peptone	-	1.0 g
Dextrose	-	1.0 g
Phenol red	-	0.012 g
Agar	-	15.0 g
Distilled water	-	1000 ml
pH	-	6.7

3.3.2.8 Triple Sugar Iron agar: A tube with sterile media was prepared and inoculated the pure culture by stabbing at the butt and then streaking on the slant surface. Incubated at 28 ± 4°C for overnight and observed for the colour change where yellow colour indicated acid production whereas red colour indicated alkali production in media, gas and H_2S production were also noted (Cappuccino and Sherman, 2005).

Triple Sugar Iron Agar (TSI) medium

Yeast extract	-	3 g
Meat extract	-	3 g
Peptone	-	20 g
Sodium chloride	-	5 g
Lactose	-	10 g
Sucrose	-	10 g
Glucose	-	1 g
Sodium thiosulphate	-	0.3 g

Iron (III) citrate	-	0.3 g
Phenol red	-	24 mg
Agar	-	9 g
Distilled water	-	1000 ml
pH	-	7.3

3.3.3 Identification of bacterial genera

The morphological and biochemical results of isolated bacteria were tabulated, grouped and analysed by using Bergey's Manual of Determinative Bacteriology.

3.3.4 Diversity Indices

The diversity indices were calculated using the PAST (4.03 version) software. The data during three seasons were tabulated, and biodiversity indices such as the Shannon-Wiener diversity index, Pielou's Index, Margalef species richness index and dominance index were calculated and plotted. The generated results between the seasons and sampling years were compared (Simpson, 1949; Margalef, 1958; Pielou, 1975; Shannon and Weaver, 1949).

3.4 RESULTS

Morphological and biochemical characteristics of the bacterial isolates obtained during the period 2020-21 (first year) and 2021-22 (second year) were studied. A total of 708 bacterial isolates obtained during the two sampling years were classified based on the morphological and biochemical characteristics.

3.4.1 MORPHO BIOCHEMICAL IDENTIFICATION OF BACTERIA

3.4.1.1 Colony characteristics

After 24 hours of incubation, growth and colony characteristics of all isolates on the nutrient agar plate were noted. 17 different colony characteristics were obtained. Colonies of various sizes were obtained. These colonies exhibited different shapes, including circular, low convex, convex and raised. They also varied in texture, being either smooth or rough, and in appearance, ranging from opaque to translucent, as well

as pigmented and non-pigmented. The pigmented colonies included shades such as white or light yellow, greenish-blue, pale to white, buff-colored, and yellow to orange, among others. Additionally, some colonies were slimy, with edges that were jagged and thinly flared. Other types included slimy or mucoid, smooth, and umbonate colonies. The colonies that were obtained are illustrated in fig. 3.1.

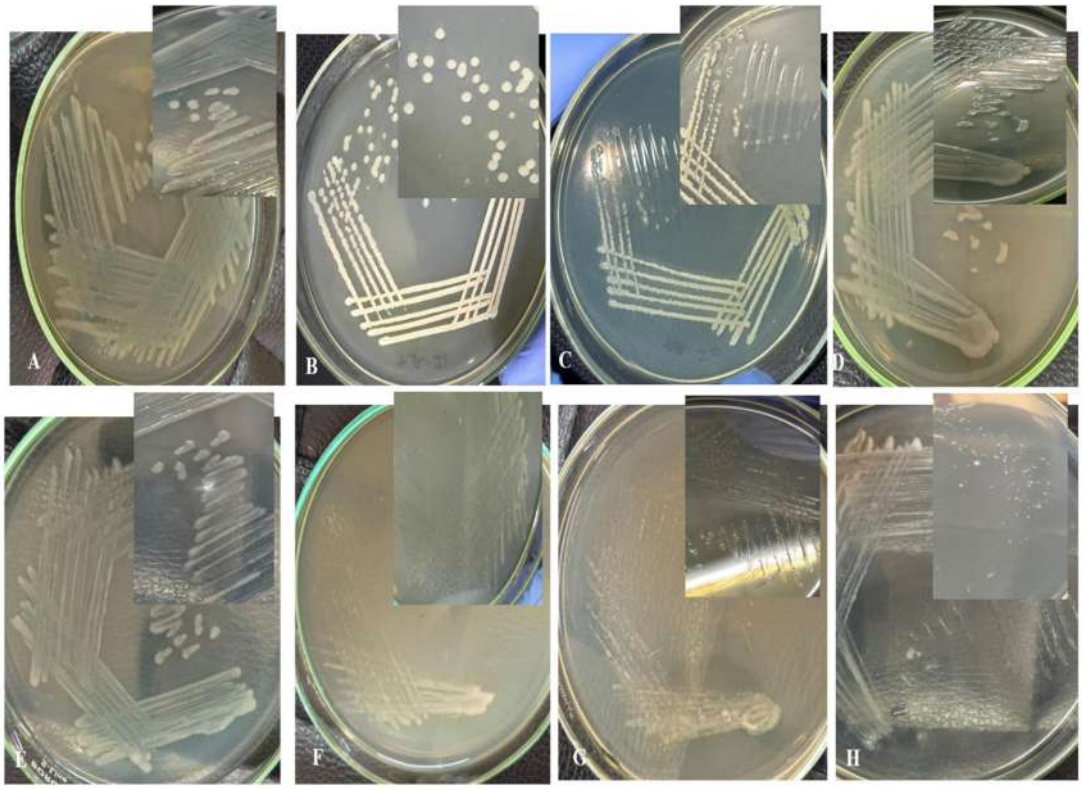


Fig. 3.1 Different bacterial colony morphology on nutrient agar plates

3.4.1.2 Gram staining

The gram staining properties obtained are presented in fig. 3.2. Gram-positive bacteria appeared purple in colour and gram-negative bacteria appeared pink in colour under the microscope. The percentage of gram-positive bacteria was found to be higher than gram-negative bacteria, among the isolates obtained (54% gram-positive bacteria: 46% gram-negative bacteria). The percentage of gram-negative bacteria was found to be higher during first year monsoon, first year post-monsoon and second year pre-monsoon seasons.

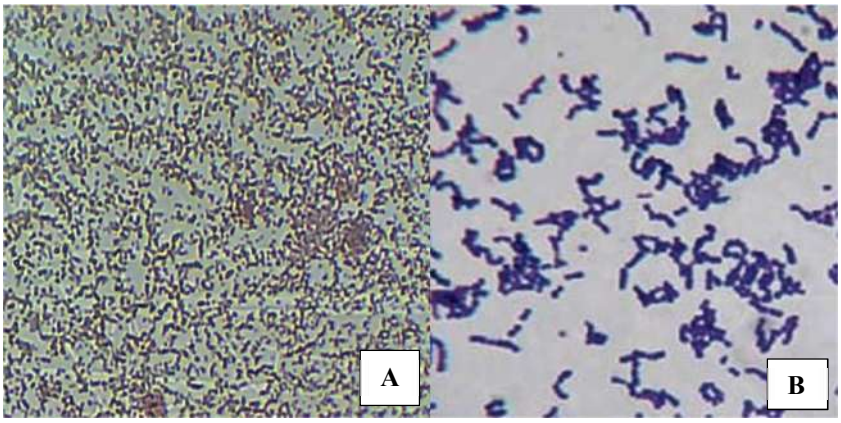


Fig. 3.2 Gram staining A) Gram negative bacteria B) Gram positive bacteria

3.4.1.3 Bacterial motility

The motile characteristics (motile or non-motile) of the bacterial isolates were identified using the hanging drop experiment. Among the isolates obtained, 58% were non-motile and 42% were motile.

3.4.1.4 Biochemical identification of the bacterial isolates

Different biochemical tests were used for the identification of bacterial isolates, which include catalase, oxidase, indole, methyl red (MR), Voges Proskauer (VP), citrate utilization, urease, triple sugar iron agar test (TSI), etc. The results obtained are depicted in fig. 3.3. while the detailed morphological and biochemical characteristics are presented in the appendix II.

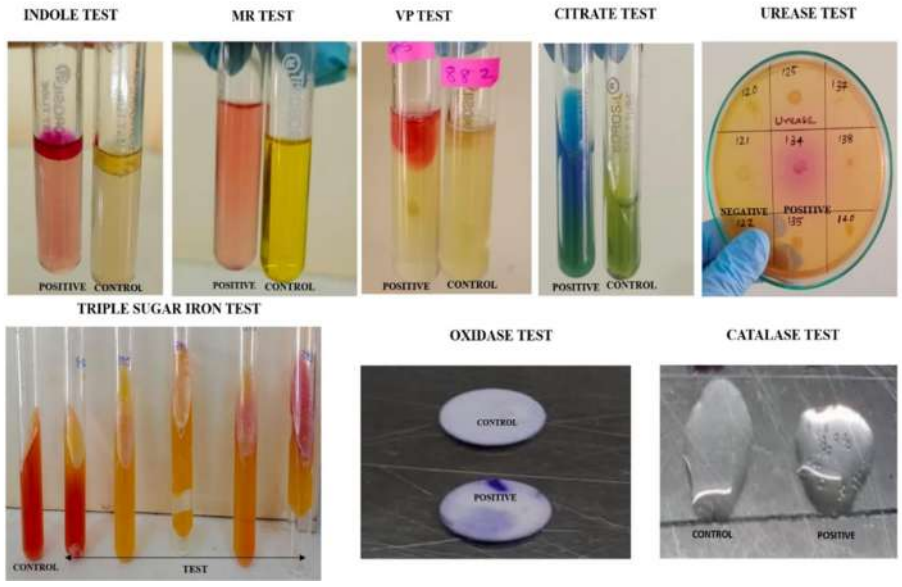


Fig. 3.3 Results of morpho biochemical analysis of the bacterial isolates

3.4.2 Classification of isolated bacteria

By using Bergey's manual of systematic bacteriology, the bacterial isolates were classified up to the generic level. From the results, a total of 17 different bacterial genera were identified from the northern Kerala mangrove sediment, and the percentage of each genus varied according to seasons and mangrove stations. The identified bacterial genera were as follows: Bacillus, Klebsiella, Pseudomonas, Enterococcus, Xanthobacter, Alcaligenes, Enterobacter, Staphylococcus, Vibrio, Aeromonas, Citrobacter, Lactobacillus, Acinetobacter, Serratia, Lysobacter, Proteus and Escherichia. The highest bacterial diversity was observed during the post-monsoon season during both years of sampling (16 genera), followed by the monsoon and pre-monsoon seasons. The genus Bacillus was equally abundant in both sampling years and all seasons. The predominant bacterial genera found in the sediment samples were Bacillus (43%), followed by Pseudomonas (17%), Xanthobacter (7%), Vibrio (6%), Enterococcus (5%), Enterobacter (4%), Staphylococcus (4%), Serratia (4%), and so on.

3.4.3 Season wise diversity analysis

The predominant bacterial genera found in the first year of sampling were Bacillus (41%), followed by Pseudomonas (20%) and Vibrio (7%) (Fig. 3.4). The predominant bacterial genera found in the second year of sampling were Bacillus (45%), followed by Pseudomonas (15%) and Xanthobacter (8%) (Fig. 3.5).

During the first year monsoon sampling (Fig. 3.4 A), 13 genera were isolated, where Bacillus (28%) was predominant, followed by Pseudomonas (23%), Xanthobacter (9%), Staphylococcus (8%), Enterococcus (6%), Alcaligenes (6%), Vibrio (6%), Enterobacter (5%), Aeromonas (3%), Klebsiella (2%), Citrobacter (2%), Lactobacillus (2%) and Acinetobacter (2%). Serratia, Lysobacter, Escherichia and Proteus were not isolated during the entire season.

During the first year post-monsoon sampling (Fig. 3.4 B), 16 genera were isolated; the predominant was Bacillus (30%), followed by Pseudomonas (22%), Vibrio (10%), Xanthobacter (6%), Enterococcus (5%), Enterobacter (5%), Serratia (5%), Aeromonas (3%), Klebsiella (2%), Staphylococcus (2%), Acinetobacter (2%),

Proteus (2%), Alcaligenes (1%), Lactobacillus (1%), Lysobacter (1%) and Escherichia (1%). Citrobacter was not isolated during the season.

During the first year pre-monsoon sampling (Fig. 3.4 C), 10 genera were isolated; Bacillus was predominant (54%), followed by Pseudomonas (18%), Enterococcus (6%), Xanthobacter (5%), Vibrio (5%), Serratia (4%), Lactobacillus (3%), Enterobacter (2%), Acinetobacter (2%) and Lysobacter (2%). Klebsiella, Alcaligenes, Staphylococcus, Aeromonas, Citrobacter, Escherichia and Proteus were not isolated during the season.

During the second year of monsoon sampling (Fig. 3.5 A), 14 genera were isolated; Bacillus was predominant (55%), followed by Pseudomonas (12%), Xanthobacter (9%), Serratia (6%), Lysobacter (5%), Vibrio (4%), Enterococcus (2%), Lactobacillus (2%), Klebsiella (1%), Alcaligenes (1%), Citrobacter (1%), Enterobacter (1%), Staphylococcus (1%) and Aeromonas (1%). Acinetobacter, Escherichia and Proteus were not isolated in the second year monsoon sampling.

During the second year post-monsoon sampling (Fig. 3.5 B), 16 genera were isolated; the predominant one was Bacillus (41%), followed by Pseudomonas (10%), Vibrio (9%), Enterobacter (8%), Staphylococcus (6%), Enterococcus (5%), Serratia (4%), Alcaligenes (3%), Lactobacillus (3%), Xanthobacter (3%), Klebsiella (2%), Acinetobacter (2%), Proteus (2%), Citrobacter (1%), Lysobacter (1%) and Escherichia (1%). Aeromonas was not isolated during the second year post-monsoon sampling.

During the second year of pre-monsoon sampling (Fig. 3.5 C), 13 genera were isolated. Bacillus was predominant (35%), followed by Pseudomonas (22%), Xanthobacter (12%), Enterococcus (8%), Staphylococcus (7%), Enterobacter (4%), Vibrio (4%), Klebsiella (2%), Alcaligenes (2%), Aeromonas (1%), Lactobacillus (1%), Acinetobacter (1%) and Serratia (1%). Citrobacter, Lysobacter, Escherichia and Proteus were not isolated during the season.

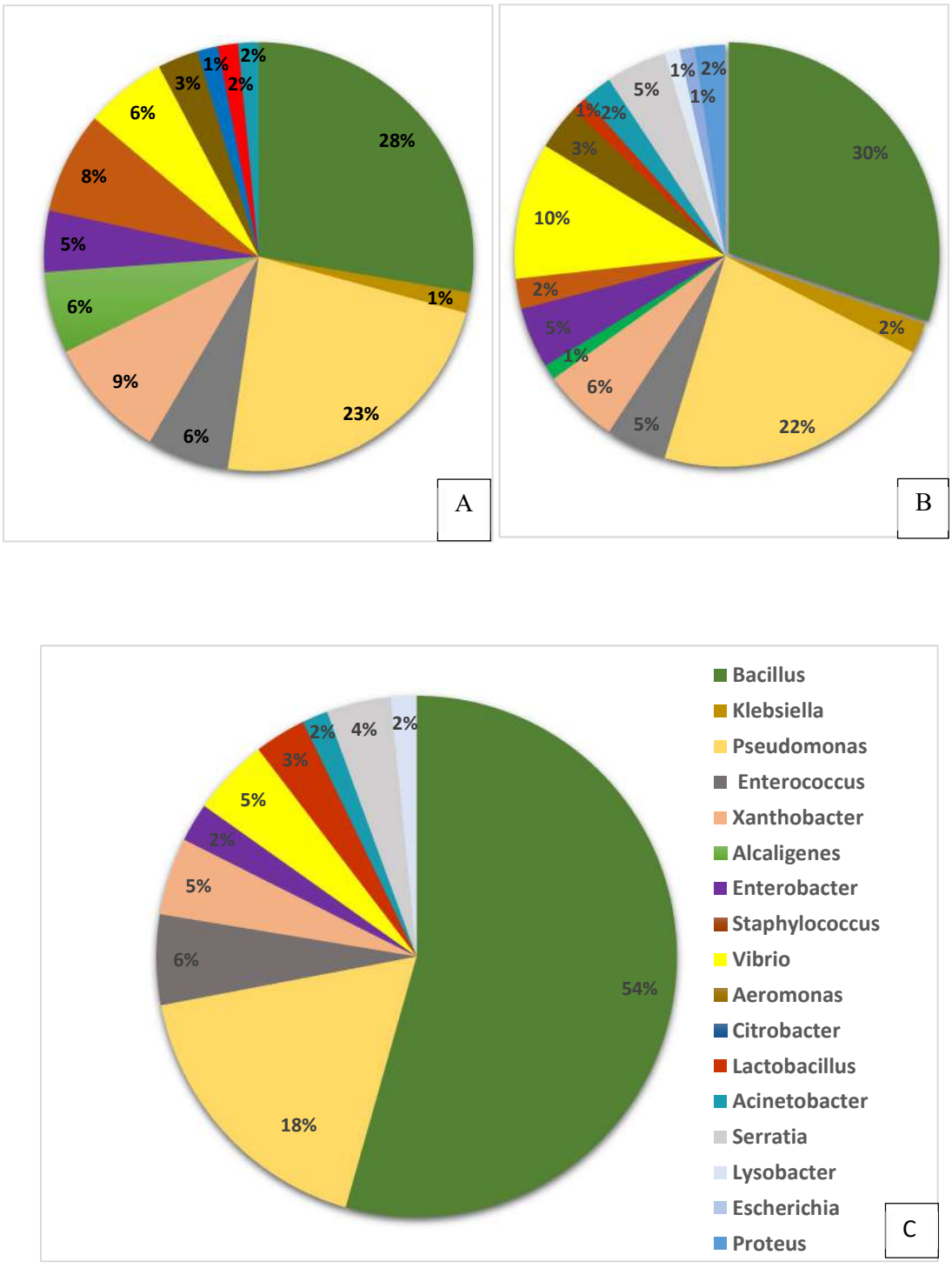


Fig. 3.4 Generic composition of bacteria isolated during the *first year* of study A) Monsoon B) Post-monsoon c) Pre-monsoon

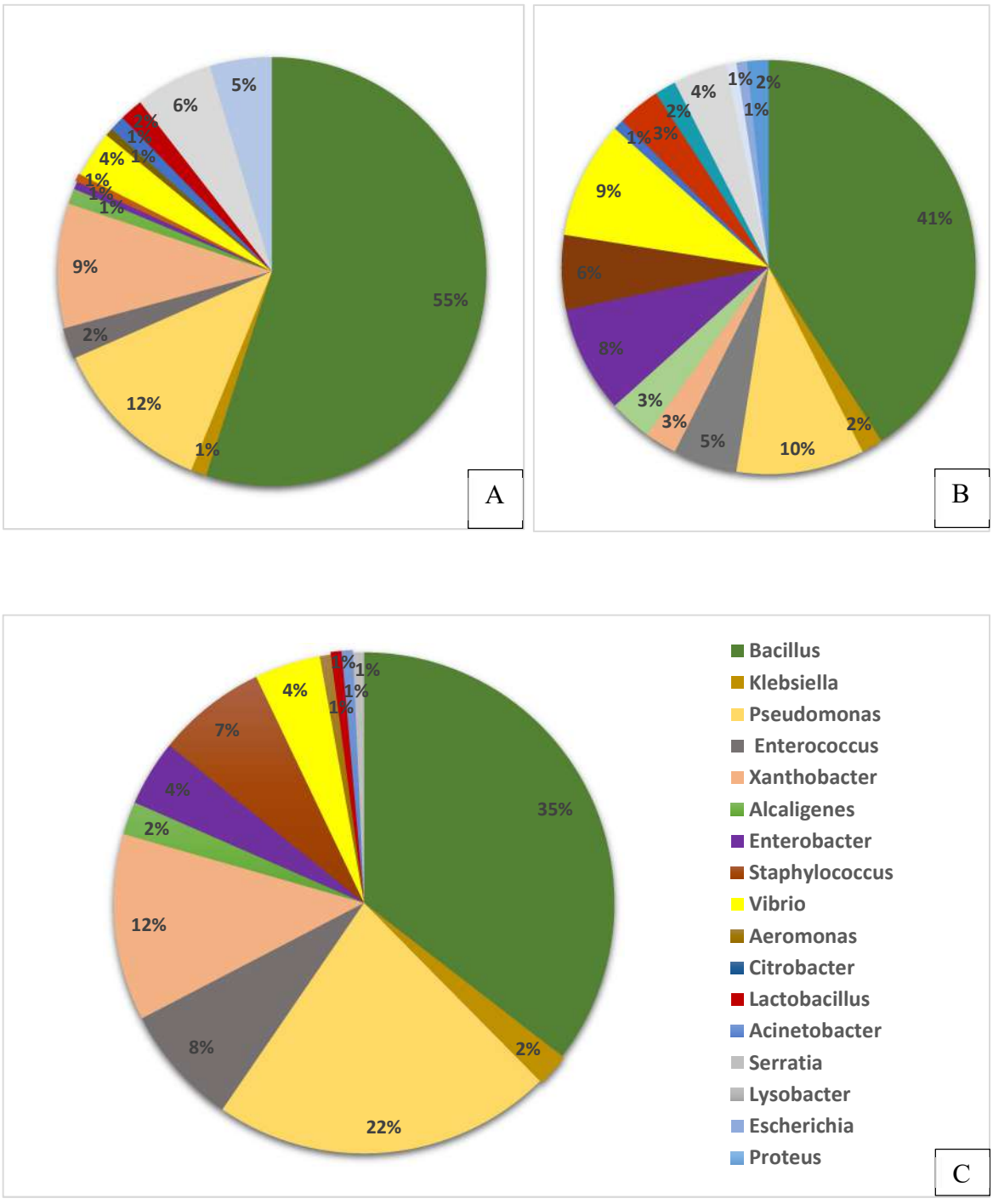


Fig. 3.5 **Generic composition of bacteria isolated during the *second year* of study A) Monsoon B) Post-monsoon c) Pre-monsoon**

3.4.4 Station-wise diversity analysis

Seventeen genera were isolated during both years of sampling. Bacillus and Pseudomonas were obtained from all sampling stations in the first year, and additionally, Xanthobacter was isolated during the second year of sampling. Relative abundance of Bacillus was found highest at Chettuva (56%), followed by Kasaragod (52%) and least at Pazhayangadi (27%). Pseudomonas was found highest at Elathur (28%) and least at Kasaragod (4%). Xanthobacter was found highest at Kasaragod (18%) and least at Ponnani and Chettuva (2%).

The distribution of different bacterial genera during the first year of sampling is presented in table 3.1. Maximum diversity was obtained from Kadalundi (11 genera) and the least from Ponnani and Edat (7 genera). Kasaragod station showed a higher abundance of Bacillus (52%), followed by Staphylococcus (12%) and 9 bacterial genera were obtained. The Edat station showed a higher abundance of Bacillus (38%), followed by Pseudomonas (27%) and 7 bacterial genera were isolated. Valapattanam station showed a higher abundance of Bacillus (35%), followed by Pseudomonas (27%) and 8 bacterial genera were obtained. Pazhayangadi station showed a higher abundance of equal percentages of Bacillus (27%) and Pseudomonas (27%), followed by Enterococcus (15%) and 8 bacterial genera were obtained. Kadalundi station yielded a higher abundance of Bacillus (31%), followed by Pseudomonas (21%) and 11 different bacterial genera. Elathur station showed a higher abundance of Bacillus (44%), followed by Pseudomonas (28%) and 8 bacterial genera were isolated. A higher abundance of Bacillus (47%), followed by an equal abundance of Pseudomonas (17%) and Serratia (17%), was obtained with 7 bacterial genera from Ponnani Station. Chettuva station showed a higher abundance of Bacillus (56%), followed by Pseudomonas (9%) and 10 bacterial genera were isolated. During the first year of sampling, Citrobacter (4%) and Proteus (5%) were obtained only from Kasaragod and Edat, respectively.

Table 3.1 Station-wise percentage of bacterial genera during the first year of sampling

Sl. No.	Genera	KSD	EDT	VPM	PZA	KDI	ELR	PON	CHV
1	Bacillus	52	38	35	27	31	44	47	56
2	Klebsiella	4	0	0	4	3	0	0	0
3	Pseudomonas	0	27	27	27	21	28	7	9
4	Enterococcus	0	0	11	15	5	4	17	3
5	Xanthobacter	8	11	5	4	8	4	3	6
6	Alcaligenes	0	0	0	8	8	0	0	0
7	Enterobacter	8	0	5	0	3	0	0	3
8	Staphylococcus	12	0	0	4	5	6	0	0
9	Vibrio	0	8	11	12	10	2	7	3
10	Aeromonas	0	0	3	0	0	7	0	0
11	Citrobacter	4	0	0	0	0	0	0	0
12	Lactobacillus	4	0	0	0	0	6	0	6
13	Acinetobacter	4	8	0	0	0	0	3	0
14	Serratia	4	3	0	0	0	0	17	6
15	Lysobacter	0	0	3	0	5	0	0	3
16	Escherichia	0	0	0	0	3	0	0	3
17	Proteus	0	5	0	0	0	0	0	0

Table 3.2 shows the distribution of different bacterial genera during the second year of sampling. Among the isolated bacteria, Kasaragod and Pazhayangadi showed higher diversity (11 genera), and Edat and Valapattanam showed the least (7 genera). Kasaragod station showed a higher abundance of Bacillus (40%), followed by Xanthobacter (18%) and 11 different bacterial genera were isolated. A higher abundance of Bacillus (52%), followed by Pseudomonas (16%), was obtained from Edat Station, with 7 different bacterial genera. Valapattanam station showed a higher abundance of Bacillus (45%), followed by Pseudomonas (24%) and 7 bacterial genera were isolated. Pazhayangadi station showed a higher abundance of Bacillus (33%), followed by Pseudomonas (26%), and 11 different bacterial genera were isolated. A higher abundance of Bacillus (38%), followed by Xanthobacter (12%), was obtained from Kadalundi station with 10 different bacterial genera. Elathur station showed higher abundance of Bacillus (48%), followed by equal abundance of Pseudomonas (12%) and Enterococcus (12%) and 10 bacterial genera were isolated. Ponnani station

showed a higher abundance of Bacillus (50%), followed by Enterococcus (19%), and 9 different bacterial genera were obtained. Chettuva station showed a higher abundance of Bacillus (50%), followed by Pseudomonas (20%), and 9 bacterial genera were obtained. During the second year of sampling, Escherichia (2%) and Proteus (5%) were obtained only from Kadalundi and Edat, respectively.

Table 3.2 Station-wise percentage of bacterial genera during the second year of sampling

Sl. No.	Genera	KSD	EDT	VPM	PZA	KDI	ELR	PON	CHV
1	Bacillus	40	52	45	33	38	48	50	50
2	Klebsiella	11	0	0	2	0	0	0	0
3	Pseudomonas	4	16	24	26	8	12	16	20
4	Enterococcus	0	5	0	4	0	12	19	0
5	Xanthobacter	18	14	13	4	12	5	2	2
6	Alcaligenes	4	0	0	4	8	0	0	0
7	Enterobacter	7	0	5	2	5	0	2	10
8	Staphylococcus	5	0	0	15	0	9	0	3
9	Vibrio	5	0	8	4	9	3	5	3
10	Aeromonas	0	0	0	2	2	3	0	0
11	Citrobacter	0	0	0	0	0	2	3	0
12	Lactobacillus	4	5	0	0	0	3	2	2
13	Acinetobacter	2	0	0	0	0	0	0	3
14	Serratia	2	5	3	2	6	3	2	7
15	Lysobacter	0	0	3	0	12	0	0	0
16	Escherichia	0	0	0	0	2	0	0	0
17	Proteus	0	5	0	0	0	0	0	0

3.4.5 Biodiversity indices: The Shannon-Wiener diversity index, Pielou's index, Margalef species richness index and dominance index were calculated from the morpho-biochemical identification of bacterial isolates from northern Kerala mangrove forests. Biodiversity indices of first year and second year sampling are depicted in fig. 3.6 and 3.7, respectively.

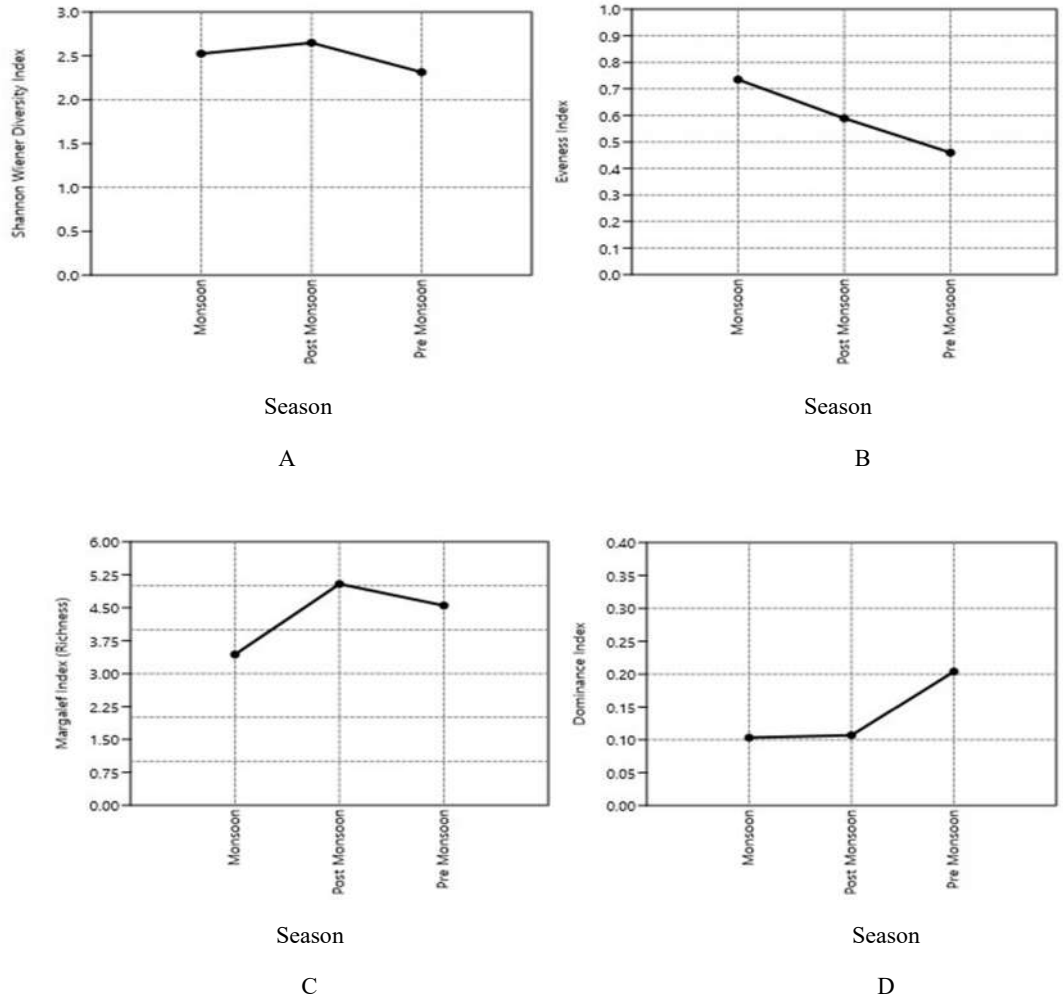


Fig. 3.6 Bodiversity indices of the first year of sampling A) Shannon- Wiener diversity index B) Pielou's Index Pielou's Index C) Margalef species richness index D) Dominance index

The Shannon-Wiener diversity index provided a better analysis of the seasonal variation of bacterial diversity from the northern Kerala mangrove habitat. Shannon diversity was found to be higher during the post-monsoon season in the two years of sampling. The Shannon-Wiener index ranged from 2.3 to 2.6, where the maximum values observed during the first year and second year are 2.6 and 2.5, respectively.

Pielou's evenness index measured the diversity along with species richness. Comparing the pilot index of the two years of sampling, it was possible to see two different patterns. During the first year of sampling, the pilot index was higher during the monsoon season (monsoon > post-monsoon > pre-monsoon), but during the second

year of sampling, more evenness was observed during the pre-monsoon season (pre-monsoon > post-monsoon > monsoon). Pilot index values during the first and second years of sampling were found to be 0.5-0.7 and 0.4-0.6, respectively.

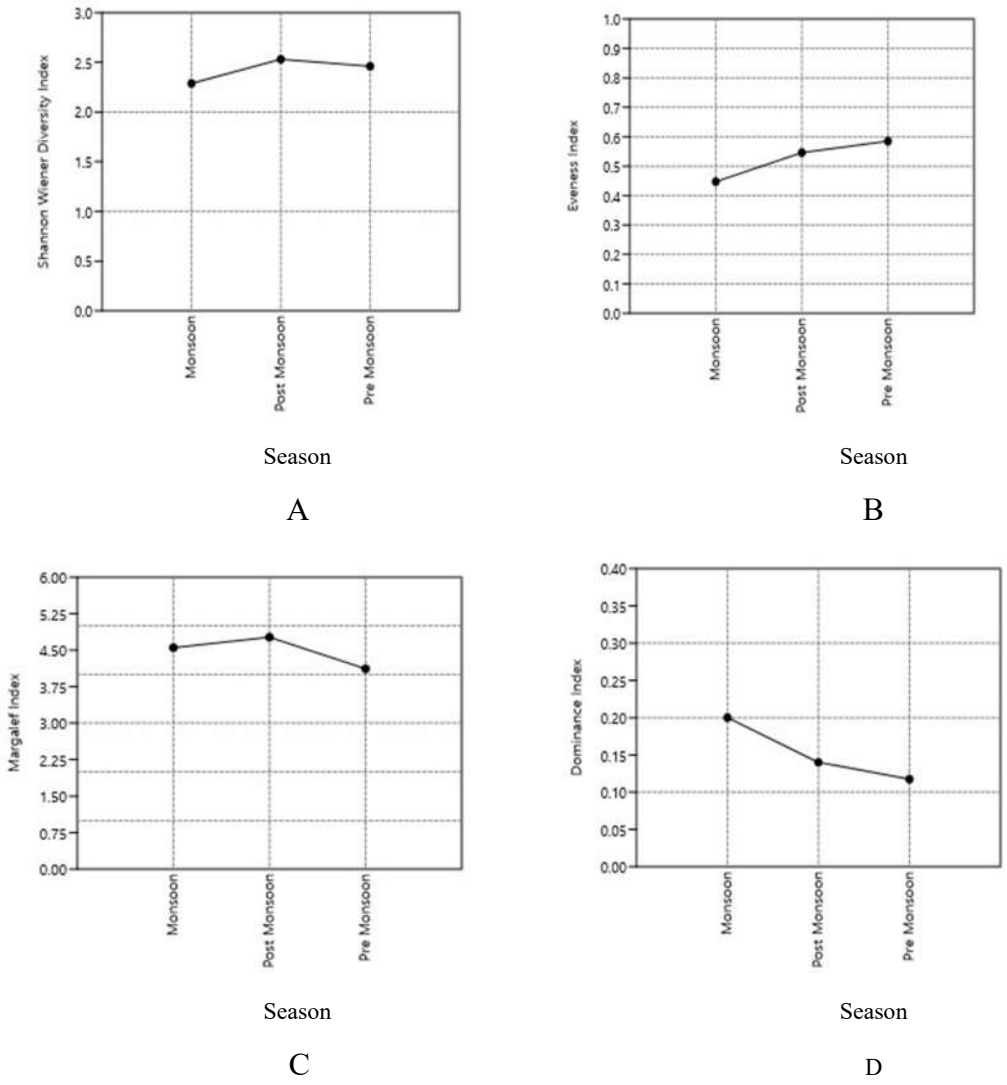


Fig. 3.7 Bodiversity indices of the second year of sampling A) Shannon- Wiener diversity index B) Pielou's Index Pielou's Index C) Margalef species richness index D) Dominance index

The Margalef species richness index indicated the diversity of species in the given dataset. The Margalef index categorized the abundance of the diversity index, plotting a graph with transects from different individual genera. When comparing the two years of sampling, the Margalef index value was observed to be higher during the post-monsoon season. The Margalef index value during the first and second years of

sampling was found to be 3.4-5 and 4.1-4.8, respectively. The order is observed as follows: post-monsoon > pre-monsoon > monsoon during the first year of sampling and post-monsoon > monsoon > pre-monsoon during the second year of sampling.

The dominance index quantified the dominance of one or a few genera in the northern mangrove stations. The dominance indices are negatively correlated with alpha diversity indices (species richness, evenness, diversity, rarity). The range of the dominance index was between 0.1 and 0.2 for both years of sampling. During the first year of sampling, dominance index was higher during the pre-monsoon season. But in the second year, the dominance index was found to be higher during the monsoon season.

3.5 DISCUSSION

Apart from the obvious flora and fauna, mangroves harbour complex bacterial communities. Mangrove microbes significantly contribute to the ecosystem's resilience and productivity. The current study analysed and compared the bacterial diversity of the northern Kerala mangrove habitat using *in vitro* morpho-biochemical identification.

In the current study, 708 bacterial isolates were obtained during a two-year sampling period, and on the basis of morpho-biochemical analysis, 17 different genera were identified: Bacillus, Klebsiella, Pseudomonas, Enterococcus, Xanthobacter, Alcaligenes, Enterobacter, Staphylococcus, Vibrio, Aeromonas, Citrobacter, Lactobacillus, Acinetobacter, Serratia, Lysobacter, Escherichia, and Proteus. Bacillus was the predominant genus identified, followed by Pseudomonas, Xanthobacter, Vibrio, etc. The present study used traditional methods of bacterial identification using morpho-biochemical tests, and the results were analyzed as per Bergey's Manual of Systematic Bacteriology. Studies by MacFaddin (2000), Madigan and Martinko (2005) and Petti et al. (2011) have reported the use of traditional methods for bacterial identification.

The current study observed the highest bacterial diversity during the post-monsoon season. The post-monsoon season often favours bacterial diversity in various ecosystems due to a combination of factors, including increased water availability, nutrient influx and more stable environmental conditions. The post-monsoon period

has significantly increased water availability, rehydrating previously dry areas and creating new ecological niches that support diverse bacterial communities (Schimel and Gulledge, 1998). Rainfall during the monsoon season washes organic matter, nutrients and minerals into ecosystems, enriching the environment, and provides optimal conditions for microbial communities to utilize these nutrients, leading to increased bacterial diversity (D'Onofrio et al., 2010). The post-monsoon season typically features more stable temperature and humidity conditions compared to the monsoon season, which can create a more favourable environment for bacterial growth and diversity (Garcia-Oliva and Camou, 2004). In some ecosystems, the post-monsoon season can result in improved oxygen availability due to reduced waterlogging. Such conditions can benefit aerobic bacteria, allowing them to thrive and contribute to overall bacterial diversity. The monsoon season can subject microorganisms to various stress factors, such as heavy rainfall, waterlogging and temperature fluctuations. The post-monsoon period provides relief from these stresses, allowing bacterial populations to recover and diversify (Mannisto et al., 2007). These may be the reasons for the post-monsoon season to create favourable conditions for bacterial diversity by providing essential resources, stabilizing the environment and reducing stress factors that may limit microbial growth and community development.

Diversity indices such as the Shannon-Wiener diversity index, Pielou's index, Margalef species richness index, and dominance index demonstrated seasonal variations in bacterial diversity within the northern Kerala mangrove ecosystem. The Shannon-Wiener diversity index (2.6) and Margalef species richness index (5) were found to be highest during the post-monsoon season. The Shannon-Wiener diversity index indicates the degree of biological diversity in a given ecosystem by quantifying both species richness and species evenness, with higher values signifying greater diversity (Shannon and Weaver, 1949). A higher Margalef value indicates greater species richness, meaning more different species are present in the sample relative to the number of individual organisms and richness (Margalef, 1958). The highest diversity and richness during the post-monsoon season are due to a combination of increased nutrient availability from organic matter decomposition, elevated organic carbon and nitrates in the water and soil, and the presence of mangrove litter, which supports bacterial activity and nutrient cycling. During the post-monsoon, the

ecosystem is flushed with land-derived organic matter, creating a nutrient-rich environment that supports a wide array of microbial life (Kaliyamoorthy et al., 2025).

Pielou's index was found to be highest during monsoon (first year sampling—0.7) and pre-monsoon seasons (second year sampling—0.6). Pielou's index denotes the evenness of species distribution in a community, with a value of 1 indicating perfect evenness (all species equally abundant) and a value of 0 indicating maximum unevenness (one or a few species dominating) (Pielou, 1975). The dominance index was found to be highest during the pre-monsoon (first year sampling-0.2) and monsoon seasons (second year sampling-0.2) in the current study. The dominance index in an ecosystem denotes the degree to which one or a few species predominate in a community, indicating the proportion of individuals belonging to the most abundant species or species types (Davari et al., 2011). The phenomenon is often due to the influx of freshwater and nutrients from increased rainfall, which supports a wider variety of species and reduces the dominance of any single species. This altered water quality and nutrient availability create more favourable conditions for diverse groups, leading to a more balanced population distribution rather than a few dominant species (Roy et al., 2024).

Bacillus under phylum Firmicutes were found in higher percentages at all mangrove stations. Tong et al. (2019) and Kutty et al. (2023) found Bacillus as the most abundant culturable bacterial genera in mangrove ecosystems. Genus Bacillus, with the most remarkable feature of endospore-formation, belongs to the family Bacillaceae, order Bacillales, and class Bacilli within phylum Firmicutes in domain Bacteria (Logan and De Vos, 2009). Saxena et al. (2020) reported that Bacillus possessing significant genetic and metabolic diversity, is among the most common bacterial genera present in soil, with various species identified across a range of ecological environments, and possesses different ecological functions from nutrient cycling to conferring stress tolerance on plants and producing valuable antibiotics. They offer several benefits to plants, such as nutrient acquisition, growth enhancement through phytohormones and protection against pathogens and abiotic stress.

Enterococcus species are a group of bacteria that can be found in various environments. They are known for their ability to persist and adapt to different

conditions and are often associated with human and animal gastrointestinal tracts and used as an indicator of faecal contamination in water. They can also be found in natural environments. Murugan et al. (2015) have detected Enterococcus species in mangrove sediments and waters. Terrestrial sources, such as runoff and animal waste, can introduce these bacteria into mangrove ecosystems. Laanbroek (2010) found that Enterococcus, like other bacteria, plays a role in nutrient cycling in mangrove sediments. They contribute to the decomposition of organic matter and the release of nutrients, such as nitrogen and phosphorus, into the ecosystem. Some Enterococcus species possess traits that can be harnessed for bioremediation purposes, such as the degradation of organic pollutants.

Xanthobacter species are a group of bacteria known for their nitrogen-fixing capabilities and their presence in various environments, including mangrove habitats. Their presence contributes to the microbial diversity in these ecosystems and can influence nitrogen dynamics, contribute to soil improvement in mangrove ecosystems, enhance soil fertility, and support the growth of mangrove vegetation (Wu et al., 2021). Xanthobacter species can participate in the biodegradation of organic compounds, including hydrocarbons and pollutants, and the degradation of nitrogen-containing pollutants can help mitigate the impact of pollution in mangrove ecosystems, suggesting the use of strains in bioremediation.

Alcaligenes species are more commonly found in soil, water and wastewater environments. Their presence in mangrove habitats would likely be transient and dependent on factors such as organic matter availability and nutrient levels. Some strains of Alcaligenes are known for their ability to degrade various organic pollutants, including hydrocarbons, aromatic compounds and pesticides. In polluted mangrove ecosystems, these bacteria could potentially play a role in bioremediation. Alcaligenes species are used in biotechnological applications, including the production of bioplastics and biofuels. Their metabolic capabilities make them valuable in the development of sustainable technologies (Duan and Hu, 2016).

Pseudomonas are known for their ubiquity in various environments, including mangrove habitats. They are part of the complex microbial communities that thrive in these ecosystems, contributing to nutrient cycling and organic matter decomposition.

Pseudomonas are versatile bacteria, with applications spanning environmental remediation, agriculture and biotechnology. Some Pseudomonas species are well-known for their ability to degrade hydrocarbons and other pollutants. In mangrove habitats affected by oil spills or pollution, these bacteria can be harnessed for bioremediation purposes (Das and Chandran, 2011). Certain Pseudomonas species possess plant growth-promoting traits and can establish beneficial associations with mangrove plants. They contribute to nutrient uptake, stress tolerance and overall plant health. Pseudomonas species can be used as biocontrol agents to protect mangrove plants from pathogens and pests, enhancing the resilience of mangrove ecosystems and have been studied for their role in the production of biodegradable bioplastics, offering an environmental friendly alternative to conventional plastics (Mozejko- Ciesielska and Kiewisz, 2016).

Serratia are a group of gram-negative bacteria that can be found in various environments, including mangrove habitats and are known for their metabolic diversity and ecological significance in nutrient cycling and biodegradation processes. They are often found in the top layers of sediments, where organic matter decomposition is active. Serratia species contribute to nutrient cycling in mangrove ecosystems by depositing organic matter and releasing nutrients like nitrogen and phosphorus (Palit and Das, 2021). Some strains of Serratia are known for their ability to degrade various organic compounds, including hydrocarbons and pollutants. They can play a role in bioremediation efforts in mangrove ecosystems impacted by oil spills and contaminants. Serratia species are used in biotechnological processes for the production of enzymes with industrial applications, including proteases and lipases and some strains have been reported to promote plant growth (Napp et al., 2017).

Klebsiella can form associations with plants, including mangrove species. These interactions may contribute to nutrient cycling and promote plant growth under certain conditions. Certain Klebsiella strains possess the ability to degrade complex organic compounds, including hydrocarbons and pollutants. This potential makes them relevant in bioremediation efforts in mangrove habitats affected by pollution (Zhang et al., 2019).

Prabagaran et al. (2007) found that Vibrio are commonly present in mangrove sediments, waters and associated organisms. They may form associations with mangrove roots, enhancing nitrogen availability for plant growth. They can play a role in the bioremediation of mangrove habitats affected by oil spills or other contaminants. Vibrio species are involved in nutrient cycling processes in mangrove ecosystems. They contribute to the decomposition of organic matter and the release of nutrients, such as carbon and nitrogen, into the ecosystems. In aquaculture practices associated with mangrove ecosystems, selected Vibrio species can be used as probiotics to promote the health of cultured aquatic organisms (Bhatt and Pandey, 2020).

Lysobacter strains have the potential to degrade organic pollutants and hydrocarbons. In polluted mangrove ecosystems, these bacteria could play a role in bioremediation. Lysobacter species are known for producing antibiotics and antimicrobial compounds, and these compounds have the potential to be used in various applications, including the development of novel antibiotics and antimicrobial agents (Huang et al., 2021).

Staphylococcus species are not typically associated with biodegradation in natural ecosystems; however, some strains can contribute to the decomposition of organic matter in environments like wastewater treatment systems. *Staphylococcus aureus*, in particular, is a well-known pathogen in humans and is often the subject of research related to human health and infectious diseases. Studies on antibiotic resistance, virulence factors and the epidemiology of Staphylococcus can have indirect implications for human health in regions near mangrove ecosystems (Tomar and Sangwan, 2013).

Tian et al. (2010) discovered that Enterobacter have been isolated from mangrove sediments and are also known to colonize the roots of mangrove trees, potentially contributing to nutrient cycling and plant health. Some Enterobacter strains can degrade various organic compounds, including hydrocarbons and pollutants. They can play a role in bioremediation efforts in mangrove ecosystems impacted by oil spills or contaminants. Enterobacter species have been associated with plant growth promotion. In mangrove ecosystems, they may contribute to the health and growth of

mangrove vegetation. Biotechnological processes use Enterobacter to produce biofuels, bioplastics and enzymes for industrial applications (Wang et al., 2014).

Lactobacillus species have been sporadically detected in brackish water environments, including estuaries and coastal areas near mangroves. They are often associated with runoff from terrestrial sources. While not commonly associated with mangrove ecosystems, some Lactobacillus strains are known to degrade pollutants in contaminated environments. Lactobacillus species are commonly used in the food and dairy industries for fermentation processes and probiotic production (Liao et al., 2015).

Contamination or runoff from terrestrial sources has occasionally led to the detection of Proteus in aquatic environments, including those adjacent to mangrove forests. Their presence in natural mangrove ecosystems is generally limited. Proteus has been used as a model organism in various areas of biomedical research, including studies on bacterial motility, biofilm formation, and antibiotic resistance (Drzewiecka, 2016).

Transitional zones where freshwater and terrestrial influences intersect with mangrove ecosystems have reported the presence of Acinetobacter. These bacteria are part of the diverse microbial communities in these areas. Acinetobacter are known for their ability to degrade various organic compounds and pollutants, including hydrocarbons and aromatic compounds. They have been studied for their potential use in bioremediation to clean up contaminated environments (Zhang et al., 2014).

Escherichia species can potentially be introduced into aquatic environments near mangrove habitats through various contamination routes, such as agricultural runoff, sewage discharge, or improper waste disposal. Escherichia infections in humans and animals are studied extensively in clinical and medical research to develop diagnostic tools, therapies and vaccines (Jang et al., 2017). Environmental monitoring programs may include the detection of Salmonella and Escherichia as indicators of faecal contamination or potential health risks in water sources near mangroves. *E. coli* is commonly used as an indicator of faecal contamination in water sources. Monitoring *E. coli* levels can provide insights into water quality and potential health risks associated with swimming or recreational activities (Jofre and Blanch, 2010).

Some studies have reported the presence of Aeromonas species in mangrove sediments, where they may contribute to nutrient cycling and organic matter accumulation. They play a role in the biodegradation of organic matter, including plant litter, in mangrove ecosystems (Hossain et al., 2018).

Citrobacter is a gram-negative bacterium commonly found in different environments, including the soil, water, and gastrointestinal tracts of humans and animals. Citrobacter can potentially be introduced into aquatic environments near mangrove habitats through various contamination routes, such as agricultural runoff, sewage discharge, or improper waste disposal. Some Citrobacter strains have been studied for their potential in the degradation of organic pollutants. They can contribute to the cleanup of contaminated environments. Citrobacter, along with other indicator bacteria, may be monitored in water sources near mangroves to assess the impact of human activities on these ecosystems and adjacent water bodies (Wu et al., 2011).

Conventional methods for identifying bacteria are established, cost-effective and provide extensive information about bacterial traits. However, they are time-consuming, labour-intensive, and can be inaccurate, especially for unculturable or slow-growing bacteria. These methods may not always allow for quick and accurate species identification. While molecular methods improve on some of these issues, they also have limitations. Combining traditional and molecular approaches creates a comprehensive strategy for studying environmental bacteria (Sloan et al., 2017). Techniques, such as culturing, microscopic examination, and biochemical identification, have been employed to identify mangrove bacteria at the genus level, indicating Northern Kerala's mangrove habitats are rich in bacterial diversity, with complex and stable microbial communities. Although molecular methods will enhance our comprehension of microbial diversity, traditional techniques continue to provide important insights into the culturable bacteria found in mangroves and further studies are needed to explore the specific functions of these microbial communities.

Chapter 4

DIVERSITY AND FUNCTIONAL ANALYSIS OF MANGROVE BACTERIA USING METAGENOMICS

Chapter - 4

DIVERSITY AND FUNCTIONAL ANALYSIS OF MANGROVE BACTERIA USING METAGENOMICS

4.1 INTRODUCTION

Bacterial identification using metagenomic analysis involves sequencing the genetic material from a sample to identify all bacteria without culturing them, offering a comprehensive view of microbial communities and functional potential. This method, also known as next-generation metagenomic sequencing (NGS), provides rapid and culture-independent detection of organisms, which is crucial for clinical diagnostics and understanding the genetic diversity of microbial environments. Key steps include DNA extraction, sequencing and computational analysis using algorithms to classify bacteria (Zhang et al., 2021).

Metagenomic analysis of bacteria in mangrove habitats provides a comprehensive understanding of their genetic potential and ecological roles, aiding in the discovery of novel bioactive molecules for medicine and biotechnology, identifying genes involved in crucial processes like bioremediation and nutrient cycling for improved environmental management, and establishing reference data for future studies and comparative analyses between different mangrove ecosystems. This approach helps researchers decipher the complex microbial functions, understand how environmental factors influence the communities, and monitor threats such as antibiotic resistance (Das et al., 2025).

4.2 REVIEW OF LITERATURE

Mangrove ecosystems play a crucial role in safeguarding coastlines, facilitating nutrient cycling, and sustaining a variety of marine species, making them complex and dynamic systems. These ecosystems are marked by significant biodiversity, complex food webs, and a remarkable capacity to adjust to changing environmental factors such as salinity and tidal variations. The unique tree species and their root structures create diverse habitats that support a wide array of organisms (Nauta et al., 2023).

Recognizing bacteria in mangrove ecosystems can greatly enhance our comprehension of the functioning of these intricate habitats. Examining the microbial communities, especially bacteria, uncovers their contributions to processes like decomposition and nutrient cycling and their ability to adapt to environmental stressors.

Conventional techniques for identifying bacteria frequently fail to encompass the complete range of bacterial diversity present in intricate ecosystems. Standard methods are recognized for being extremely time-consuming and labor-intensive (such as culture media and biochemical tests). It is estimated that over 99% of microbial populations in nature cannot be cultured using current methods. Therefore, there is a necessity for new culture-independent approaches to investigate the function and diversity of microorganisms in their natural environments. Molecular techniques provide exceptional accuracy and specificity in the identification of bacteria, frequently yielding results significantly quicker than conventional methods (Franco-Duarte et al., 2019).

Gene amplicon sequencing is regarded as the leading method in the past twenty-five years for taxonomical and phylogenetical study of intricate microbiomes that were previously considered difficult to characterize (Bramhachari, 2023). The 16S rRNA gene is used universally and has been reported as a gold standard for bacterial identification. It is the most conserved sequence, ubiquitous, has a housekeeping genetic marker and is a short-length nucleotide sequence (~1542 bp). Hypervariable regions (V5–V6, V3–V4, or V4) are commonly used for sequencing (Bharti and Grimm, 2021).

Metagenomics (next-generation sequencing or high-throughput sequencing) involves the direct genetic analysis of genomes sourced from various environments. It is focused on marker genes and not the whole genome. In such a way, bacteria from direct environmental samples (culture independent) can be sequenced and produce a multimodal phylogenetic analysis (Bharti and Grimm, 2021). Metagenomics offers a comprehensive catalog of all microorganisms found in complex environmental samples, including both unculturable and culturable species, as well as those that are known and unknown. Unlike unimodal phylogenetic analysis, which focuses on the diversity of a single gene such as the 16S rRNA gene, metagenomics organizes the

multimodal genetic makeup of microbial communities, thereby delivering enhanced taxonomic resolution and genomic insights. In recent years, modern next-generation sequencing (NGS) has gradually supplanted traditional Sanger sequencing as the favoured method for metagenomic shotgun sequencing. The 454/Roche and Illumina/Solexa platforms have been widely utilized for the analysis of metagenomic samples sourced from various environments (Riesenfeld et al., 2004).

Functional annotation research in ecology focuses on defining the roles and activities of genes, proteins, or whole organisms within an ecosystem. This understanding is essential for grasping how species engage with their environment and with one another, which in turn affects ecosystem functionality and biodiversity. Such studies are vital for comprehending the impacts of environmental changes, such as climate change, on ecosystems and for supporting conservation initiatives (Laureto et al., 2015). The growing adoption of function-based methods has driven the quest for new metrics designed to assess functional diversity precisely. As a result, categorical classifications of functional traits are being progressively supplanted by continuous multi-trait methodologies. Recently, functional diversity has been acknowledged as a crucial element in sustaining vital ecosystem functions and services.

Nonetheless, metagenomic techniques are demonstrating greater efficacy in uncovering the intricate microbial communities found in mangroves and offer the most comprehensive perspective on the microbial community, encompassing both taxonomy and functional characteristics. This enhances our comprehension of the metabolic, physiological, and ecological functions of environmental microorganisms (Wainwright et al., 2024). Metagenomic research on mangrove ecosystems has been relatively uncommon, particularly in Kerala, India. This study focuses on the metagenomic analysis of the bacterial community within the mangrove habitat of northern Kerala, along with functional annotation.

4.3 MATERIALS AND METHODS

4.3.1 Study site and sample collection

Approximately 250 g of sediment samples were collected during the post-monsoon season, from the surface (0-15 cm deep) removing the surface litter from 8

distinct sampling sites in the mangroves of Northern Kerala habitat which includes; Kasaragod (KSD), Edat (EDT), Valapattanam (VPM), Pazhayangadi (PZA), Elathur (ELR), Kadalundi (KDI), Ponnani (PON), and Chettuva (CHV). Following collection, each soil sample was preserved at −20°C prior to DNA isolation.

4.3.2 DNA extraction and PCR amplification of fragment 16S rRNA

Genomic DNA was extracted from the sediment samples using Power soil DNA isolation kit (MO BIO Laboratories Inc., USA) as per manufacturer's instructions. Afterwards, total DNA was purified and concentrated using DNA Purification kit (Origin, India) following the manufacturer's instruction. The extracted DNA was checked on agarose gel (%) and the quality and amount of extracted DNA was evaluated by Nano-Drop 2000c (Thermo Scientific, Massachusetts, USA).

The extracted DNA was sent to Mediomix Diagnosis and Bioresearch Private Limited, Bengaluru, Karnataka for library preparation using universal bacterial primers. The V3–V4 region of the 16S rRNA gene was amplified using the specific V3 forward primer 341F 5′-CCTACGGGAGGCAGCAG-3′ and the V4 reverse primer 806R 5′-GGACTACHVGGGTATCTAAT-3′ (Muyzer et al., 1993). Polymerase chain reaction (PCR) was performed in 20 µl reaction volume, containing 0.4 µl of Taq DNA polymerase (5 U per µl), 0.8 µl of each primer (5 µM), 2 µl of dNTP (2.5 mM), 4 µl of 10X PCR buffer and $MgCl_2$ (50 mM), and 1 µl (50–100 ng) of genomic DNA and 11 µL sterile distilled water. Thermal conditions for PCR were as follows: Initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds, and 5 minutes of final elongation at 72°C (Sun et al., 2015). The amplified product was utilized for library preparation with the NEBNext Ultra DNA library preparation kit. The quantification and quality assessment of the library were conducted using the Agilent 2200 Tape Station. Using paired-end read sequencing on Illumina HiSeq 2500 platform, the sequencing of 16S rRNA gene amplicon libraries were performed.

4.3.3 Analysis of the sediment microbial community

After sequencing, the quality of paired-end raw reads was checked by FastQC tool (version 0.11.9) (Andersson et al., 2010). Overview of bioinformatic analysis is

presented in fig. 4.1. The high-quality paired-end reads, after primer trimming, were paired and permitted to merge, resulting in the V3–V4 amplicon consensus FASTA sequences. Chimaeras were eliminated, and reads were merged utilizing DADA2 with a minimum overlap of 10 bp and a maximum overlap of 240 bp (Callahan et al., 2016). The selection of operational taxonomic units (OTUs) and the classification of taxonomy were conducted using the pre-processed consensus V3–V4 sequences. The good quality Fasta sequences were used for QIIME program (version 2-2021.11) (Quantitative Insights into Microbial Ecology) (Lawley and Tannock, 2016). Sequence reads were assigned to operational taxonomic units (OTUs) by using a reference-based OTU picking approach with Silva database (Silva 138-99-nb-weighted classifier) (Pruesse et al., 2007).

The QIIME 2 program [Version: 2–2021.11] was employed for the complete downstream analysis includes alpha and beta diversity analysis (Bolyen et al., 2019). Alpha diversity indices i.e., Shannon diversity (Shannon and Weaver, 1949), Pielou's evenness (Pielou, 1975), Simpson index (Simpson, 1949), Chao1 (Faith, 1992), and observed features (Faith, 1992) and Beta Diversity matrices i.e., Jaccard distance and Bray-Curtis distance (Calle, 2019) were estimated using QIIME pipeline. Alpha diversity analysis measures the diversity and complexity within a single sample to assess species richness and evenness and also helps to describe the overall biodiversity. It helps to understand how disturbances affect community structure, and provide a foundational understanding of microbial communities in various environments (Xia and Sun, 2023). Beta diversity analysis is used to compare the similarity or dissimilarity of microbial communities between different samples, helping to understand how environmental factors and host conditions influence microbial composition and function. Common beta diversity metrics include Bray-Curtis dissimilarity, which accounts for microbial abundance, and Jaccard distance, which focuses on the presence or absence of species.

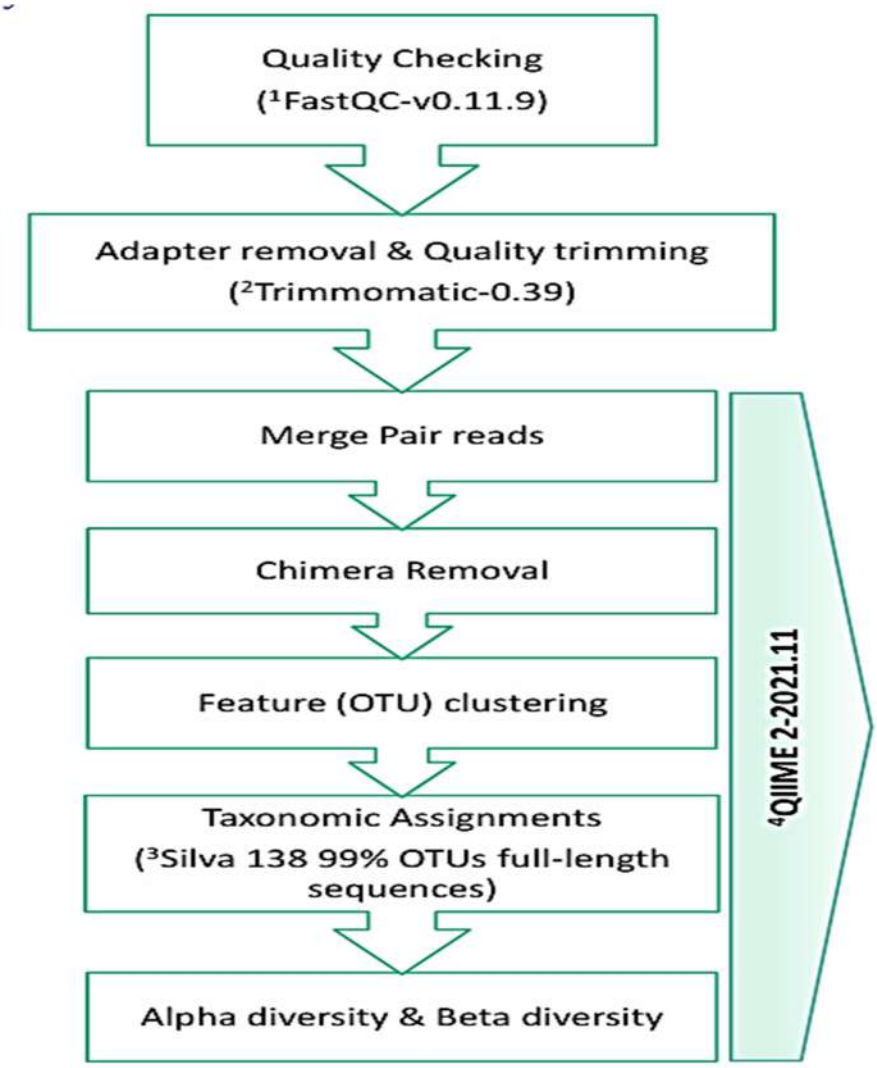


Fig. 4.1 Overview of bioinformatic analysis

4.3.4 Functional annotation

The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; https://github.com/picrust/picrust2; version v2.5.0) software package. The predicted functional characteristics of bacterial community were obtained from the MetaCyc database via PICRUSt2 which predicts the function of bacterial community according to the proportion of marker gene sequences in samples (Douglas et al., 2020). The

Metacyc output was normalized and imported into RStudio (2022.07.2) and STAMP (v2.1.3) for graphical visualization (Coleman et al., 2023).

4.3.5 Data deposition

The metabarcoding data of eight different sediment samples of post-monsoon season, from northern Kerala mangrove habitat were deposited at National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA).

4.4 RESULTS

4.4.1 Amplicon analysis by illumina sequencing

According to the results obtained in the morpho-biochemical analysis, maximum bacterial diversity was observed during the post-monsoon sampling. Hence, the non-culturable diversity analysis was done with the post-monsoon sediment samples. This study was carried out to assess and compare bacterial diversity from eight different mangrove stations along the north Kerala coast, India. A total of 658256 trimmed 16S rRNA V3-V4 sequences were obtained from 8 mangrove sampling stations. Valapattanam had the largest number of OTUs (129668), and the least was observed at Elathur (44701) (Table 4.8).

4.4.2 Taxonomic assignment of the bacteria in mangrove sediments

A total of 129668, 95244, 94638, 86828, 84920, 65080, 57177 and 44701 processed reads were yielded from the samples of Valapattanam, Kadalundi, Chettuva, Kasaragod, Pazhayangadi, Ponnani, Edat and Elathur, respectively. 64% belonged to domain bacteria, 34% belonged to unassigned groups, 0.2% was domain archaea; and 0.1% was domain eukaryota. The percentage of domain bacteria was found highest at Valapattanam (26%), followed by Kadalundi (23%), Kasaragod (17%), Chettuva (10%), Pazhayangadi (9%), Ponnani (7%), Edat (5%), and Elathur (2%). The archaea bacterial percentage was highest at Valapattanam (66%) and lowest at Edat (0.3%).

4.4.2.1 Phylum level analysis

The obtained OTUs could be classified into 45 phyla, where one phylum was unclassified (Fig. 4.2 and table 4.1). The most dominant phylum was Proteobacteria (57%), followed by Bacteroidota (8.5%), Actinobacteriota (8%), Firmicutes (7%),

Chloroflexi (6%), Desulfobacterota (4%), Planctomycetota (2%), Campylobacterota (1.3%), Acidobacteriota (1.2%), Nitrospirota (1%) and others. Bacteria belonging to Proteobacteria, Bacteroidota, Actinobacteriota, Firmicutes, Chloroflexi, Desulfobacterota, Planctomycetota, Campylobacterota, Acidobacteriota, Nitrospirota, Myxococcota (0.7%), Gemmatimonadota (0.6%), Deltaproteobacteria (NB1-j) (0.4%), Deferrimicrobiota (MBNT15) (0.3%); and Verrucomicrobiota (0.2%) were commonly present in all the sampling stations (Fig. 4.3). 0.01% of unclassified phylum was present in the 16S rRNA metagenomic data analysis.

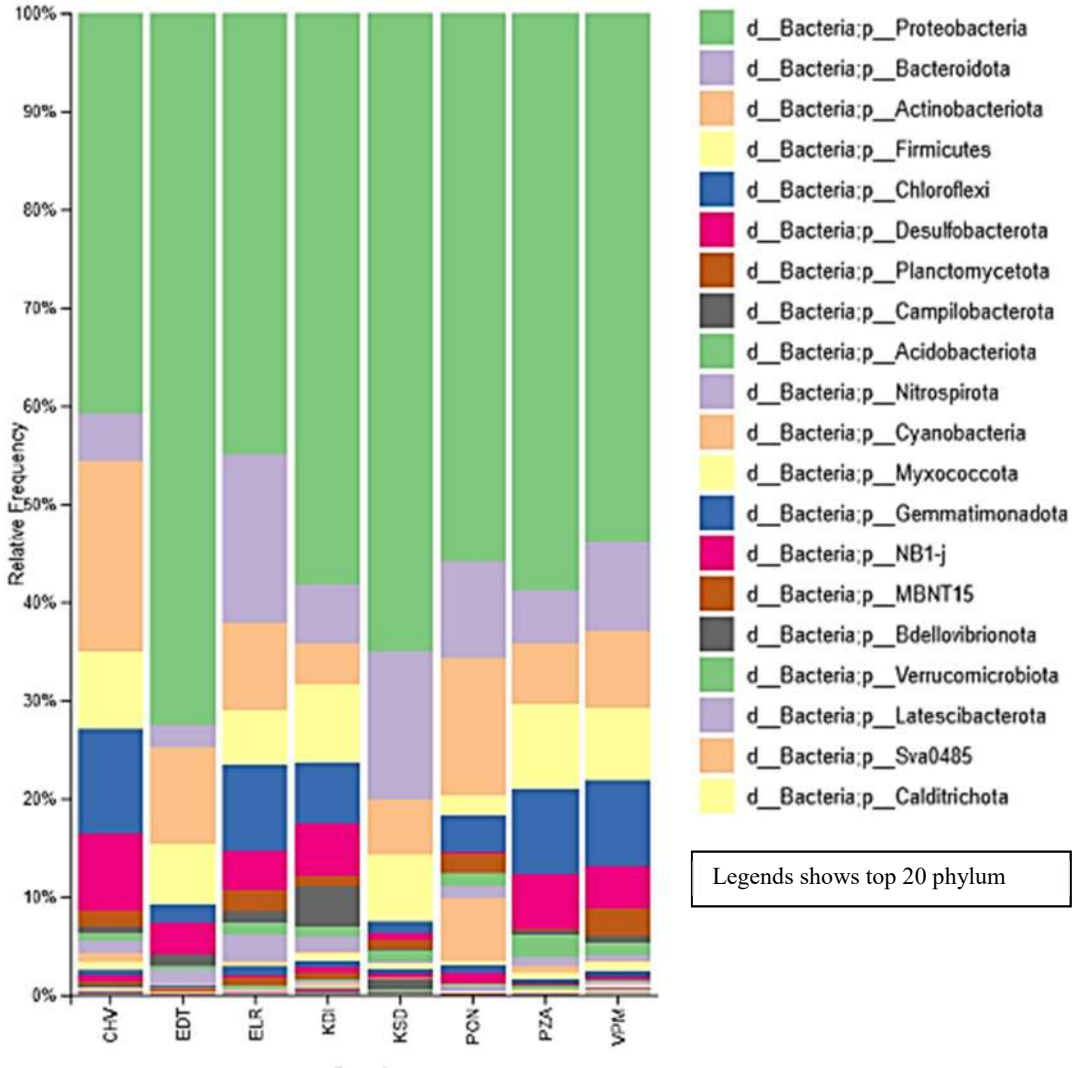


Fig. 4.2 Phylum level classification of bacteria from 8 different stations of Northern Kerala mangrove sediments

Table 4.1 Relative abundance of top 21 bacterial phylum from 8 different stations

SL.NO	PHYLUM	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
1	Proteobacteria	7	1	24	7	6	9	25	20
2	Bacteroidota	6	3	16	9	1	6	28	31
3	Actinobacteriota	24	2	12	13	6	7	25	12
4	Firmicutes	11	1	26.6	2	4	11	26.7	17
5	Chloroflexi	17	2	23	4	1	12	36	4
6	Desulfobacterota	20	2	31	0	4	13	28	3
7	Planctomycetota	11	3	16	10	0	1	50	9
8	Campilobacterota	5	2	71	0	4	3	13	3
9	Acidobacteriota	6	2	23	8	2	17	28	15
10	Nitrospirota	13	5	35	10	7	9	16	6
11	Cyanobacteria	13	1	6	64	0	9	7	1
12	Myxococcota	13	1	26	5	1	10	34	12
13	Gemmatimonadota	10	3	29	10	1	5	25	18
14	Myxococcota (NB1-j)	15	1	32	17	0	3	15	17
15	Deferrimicrobiota (MBNT15	14	5	56	3	7	5	3	7
16	Bdellovibrionota	6	0	4	0	0	2	13	74
17	Verrucomicrobiota	4	3	33	7	1	15	17	19
18	Latescibacterota	8	1	29	6	0	1	50	5
19	Deltaproteobacteria (Sva048	16	1	32	6	0	1	54	5
20	SAR324 clade(Marine Group	16	0	41	2	1	5	34	0
21	Calditrichota	13	1	42	0	6	15	22	0

Fig. 4.3 shows the heat map for bacterial phyla present in mangrove sediments from North Kerala. The heat map reveals a higher frequency of Proteobacteria in all mangrove stations. Proteobacteria, Chloroflexi, Firmicutes and Bacteroidota were found in almost all the mangrove stations.

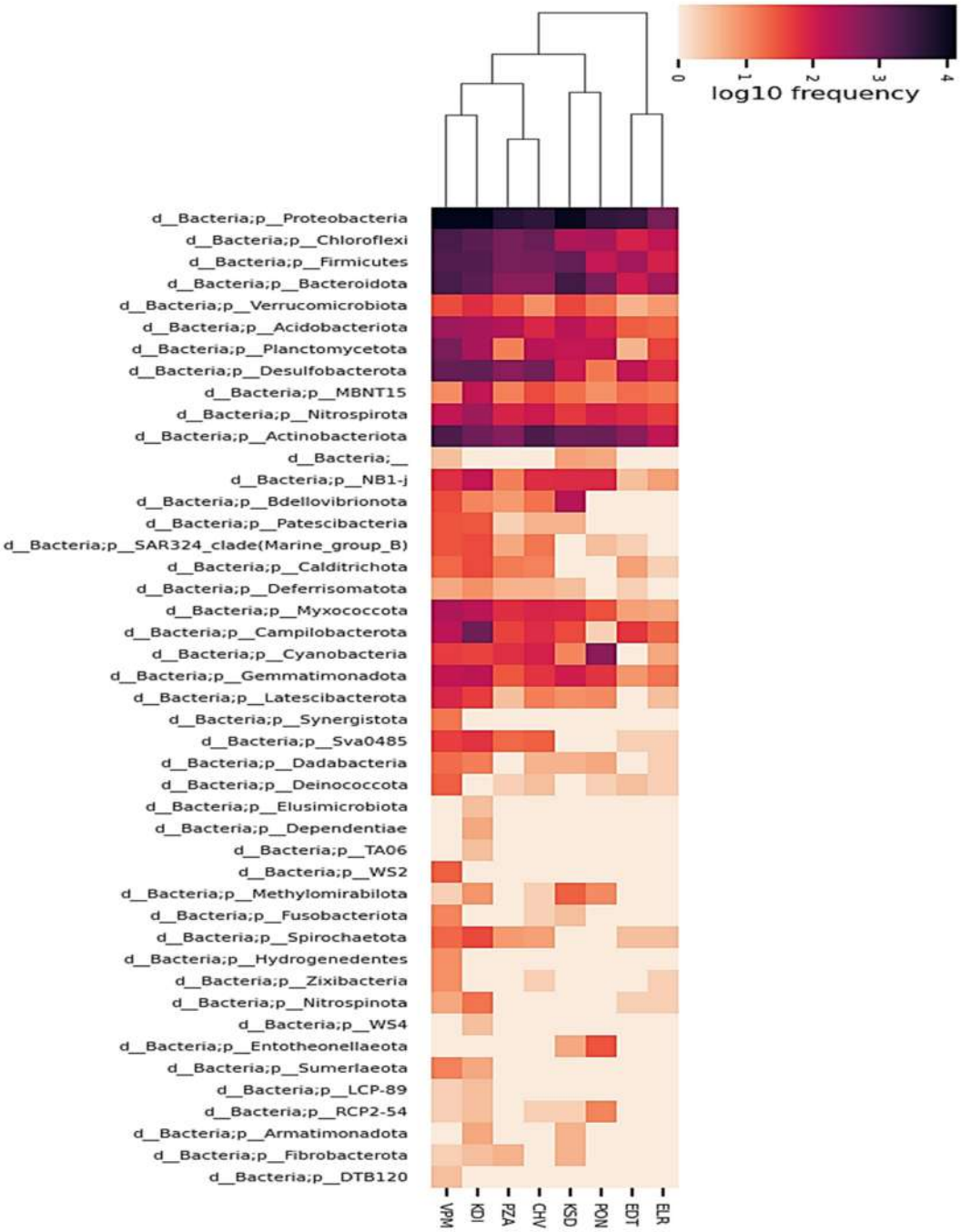


Fig. 4.3 Heat map and cluster analysis of bacterial phyla present in mangrove sediments of Northern Kerala

4.4.2.2 Class level analysis

16S rRNA metagenomic data analysis classifies the total OTUs into 121 classes, including one unclassified (Fig. 4.4 and table 4.2). The predominant class found in the study was Gammaproteobacteria (40.4%), followed by Alphaproteobacteria (16.4%), Bacteroidia (7.6%), Anaerolineae (4.5%), Acidimicrobiia (4.3%), Clostridia (3.6%), Bacilli (3.5%), Actinobacteria (3.0%), Campylobacteria (1.3%), Desulfobulbia (1.3%), Chloroflexia (KD4-96) (1.2%), Planctomycetes (1.0%), Desulfuromonadia (1.0%) and others. In Proteobacteria, 71% belonged to Gammaproteobacteria, 28.8% to Alphaproteobacteria and 0.01% to Zetaproteobacteria. 26.9% of Gammaproteobacteria was found at Valapattanam, followed by Kadalundi (25.6%), Kasaragod (20.9%), Pazhayangadi (7.7%), Edat (7.4%), Chettuva (5.7%), Ponnani (4.7%) and Elathur (1%). Alphaproteobacteria was found highest at Kadalundi (20%) and lowest at Elathur (2.3%). Bacteria belonging to Gammaproteobacteria, Alphaproteobacteria, Bacteroidia, Anaerolineae, Acidimicrobiia, Clostridia, Bacilli, Actinobacteria, Campylobacteria, Planctomycetes, Thermoleophilia, Polyangia, Acidobacteriae, Dehalococcoidia, Phycisphaerae, Nitrospiria, Ignavibacteria, Gemmatimonadetes, Vicinamibacteria, and Verrucomicrobiae were found at all the mangrove stations.

Table 4.2 Relative abundance of top 20 bacterial class from 8 different stations

SL. NO	CLASS	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
1	Gammaproteobacteria	6	1	26	5	7	8	27	21
2	Alphaproteobacteria	11	2	20	13	3	14	19	17
3	Bacteroidia	6	4	17	9	1	6	25	33
4	Anaerolineae	19	2	22	1	2	15	38	1
5	Acidimicrobiia	31	2	12	15	1	2	28	9
6	Clostridia	18	2	31	2	4	18	21	5
7	Bacilli	5	1	22	2	5	4	32	29
8	Actinobacteria	19	2	8	7	14	13	25	12
9	Campylobacteria	5	2	71	0	4	3	13	3
10	Desulfobulbia	19	1	29	0	1	4	46	0
11	KD4-96 (Chloroflexi)	16	3	27.3	13	1	7	27	7
12	Planctomycetes	11	3	17	9	0	1	48	10
13	Desulfuromonadia	21	2	28	0	5	11	21	10
14	Syntrophobacteria	27	3	34	0	3	21	12	0
15	Desulfobacteria	14	1	36	0	6	21	20	1
16	Cyanobacteriia	13	0	5	66	0	8	7	1
17	Thermoleophilia	12	2	19	15	3	9	15	26
18	Thermodesulfovibrionia	16	6	38	0	9	8	21	1
19	Polyangia	14	1	25	3	1	10	38	9
20	Rhodothermia	1	0	2	0	0	2	76	19

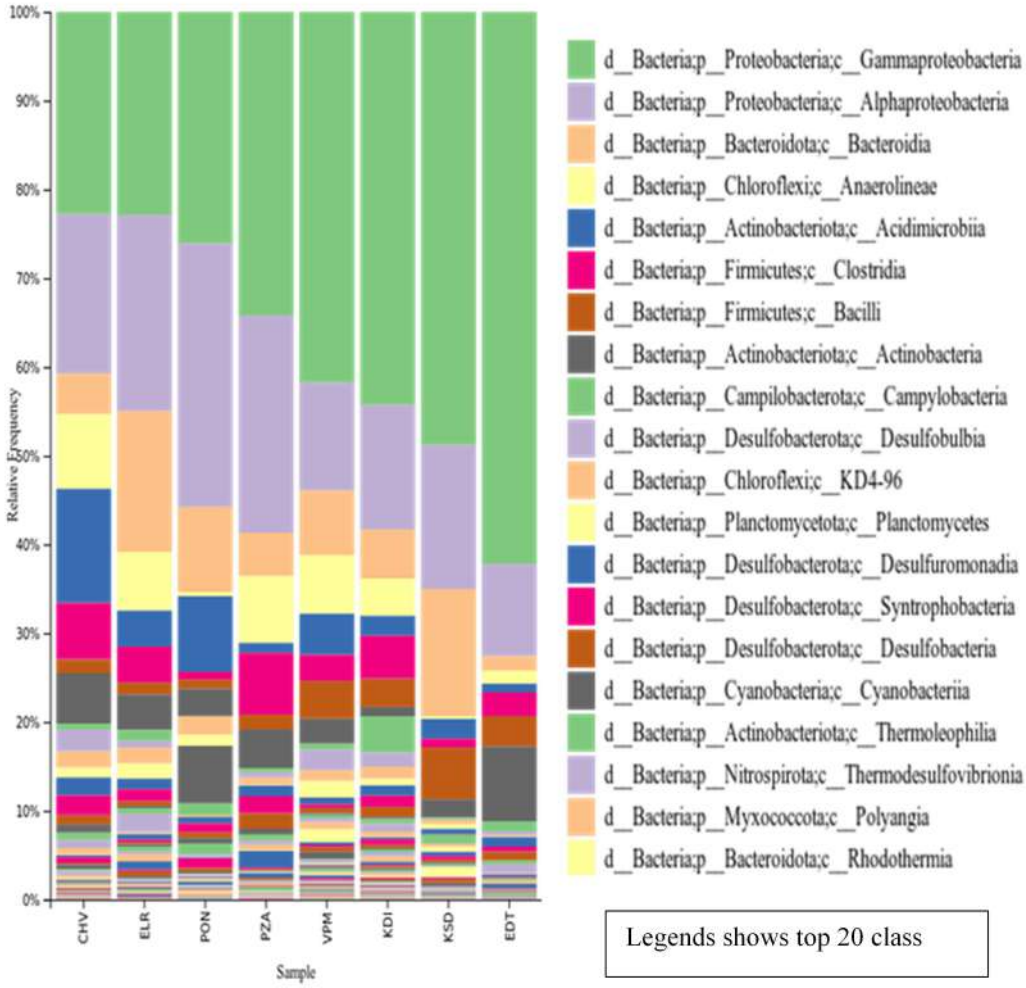


Fig. 4.4 Class level classification of bacteria from 8 different stations of Northern Kerala mangrove sediment

4.4.2.3 Order level analysis

A total of 308 taxonomic orders, including 9 uncultured and 3 unclassified ones, were obtained in the metagenomic analysis (Fig. 4.5 and table 4.3). The most predominant class was Oceanospirillales (11.2%), followed by Alteromonadales (8.9%), Flavobacteriales (5.8%), Rhodobacterales (5.2%), Rhizobiales (4.8%), Vibrionales (4.5%), Anaerolineales (3.3%), Bacillales (2.4%), Thiotrichales (2.3%), Peptostreptococcales-Tissierellales (2.2%), Pseudomonadales (2%), Actinomarinales (2%), Microtrichales (2%), Aeromonadales (1.9%), Sphingomonadales (1.9%), Thiomicrospirales (1.8%), Ectothiorhodospirales (1.5%), Steroidobacterales (1.4%),

Campylobacterales (1.3%), Desulfobulbales (1.3%), Burkholderiales (1.1%), Kiloniellales (1.1%), Cytophagales (1%), Micrococcales (1%) and others. 42% of Oceanospirillales was reported from Kadalundi. The second most abundant order, Alteromonadales (32%), and the third most abundant order, Flavobacteriales (40%), were found maximum at Kasaragod station.

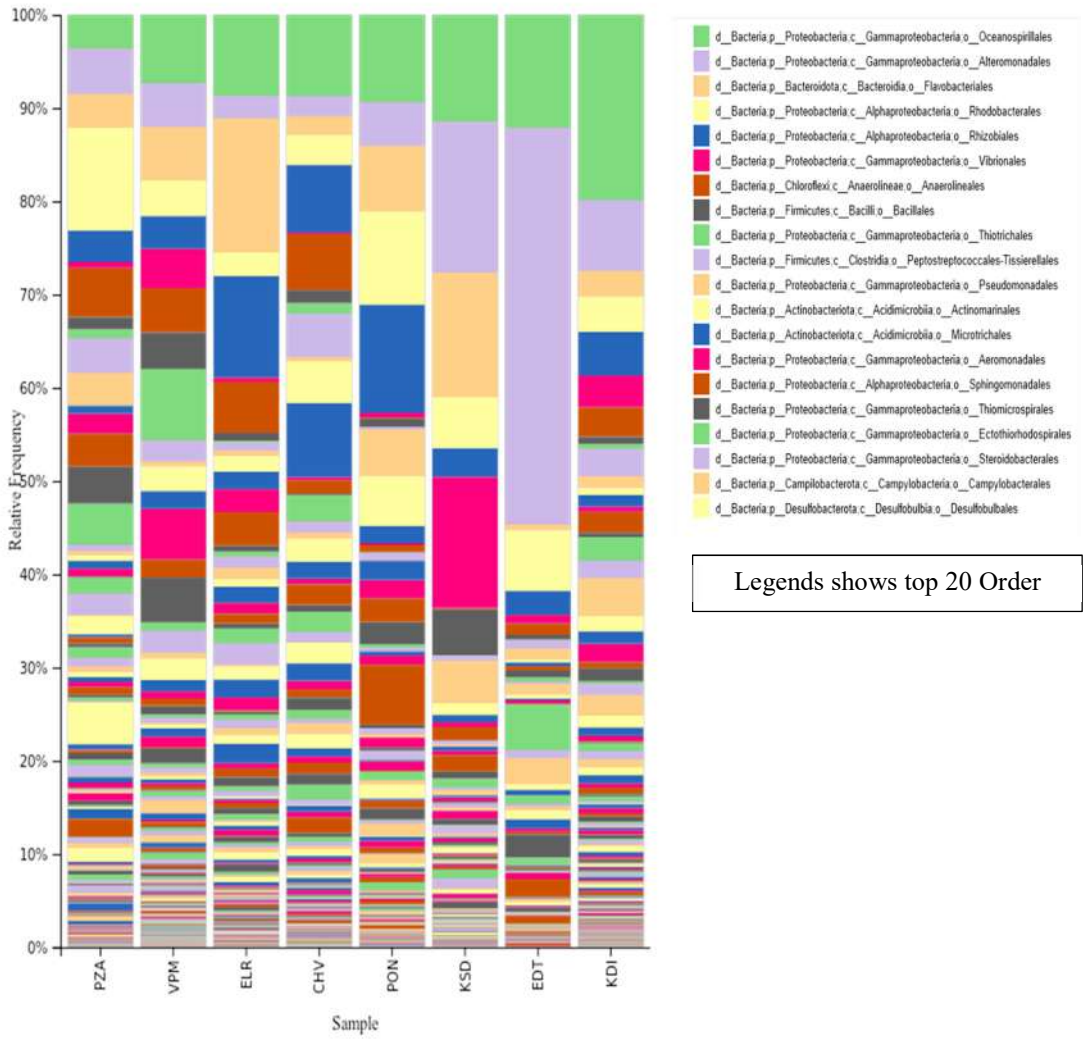


Fig. 4.5 Order level classification of bacteria from 8 different stations of Northern Kerala mangrove sediments

Table 4.3 Relative abundance of top 20 bacterial order from 8 different stations

SL. NO	ORDER	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
1	Oceanospirillales	8	1	42	6	5	3	17	18
2	Alteromonadales	2	0	20	4	23	5	14	32
3	Flavobacteriales	4	4	11	9	1	6	26	40
4	Rhodobacterales	6	1	17	14	6	19.2	19	18
5	Rhizobiales	16	4	23	18	3	6	19	11
6	Vibrionales	0	0	18	1	1	1	24	54
7	Anaerolineales	19	3	23	0	2	15	38	1
8	Bacillales	6	1	8	3	1	5	42	35
9	Thiotrichales	5	0	5	0	0	4	86	0
10	Peptostreptococcales-Tissierellale	21	1	30	1	2	15	26	4
11	Pseudomonadales	2	0	15	18	3	16	7	39
12	Actinomarinales	23	1	9	20	1	0	35	11
13	Microtrichales	41	2	15	7	1	4	25	7
14	Aeromonadales	2	2	7	1	0	10	74	4
15	Sphingomonadales	8	3	28	3	1	17	27	13
16	Thiomicrospirales	1	1	6	0	2	20	71	0
17	Ectothiorhodospirales	18	1	38	0	1	26	15	0
18	Steroidobacterales	9	1	31	5	1	4	44	5
19	Campylobacterales	5	2	71	0	4	3	13	3
20	Desulfobulbales	19	1	29	0	1	4	46	0

4.4.2.4 Family level analysis

A total of 507 families were obtained, of which 22 families belonged to the uncultured group and 4 were classified as unknown (Fig. 4.6 and table 4.4). In the present study, the majority of bacteria belonged to the family Shewanellaceae (5.5%), followed by Alcanivoracaceae (5.5%), Rhodobacteraceae (5.2%), Flavobacteriaceae (5%), Vibrionaceae (4.5%), Anaerolineaceae (3.3%), Marinobacteraceae (2.9%), Thiotrichaceae (2.3%), Saccharospirillaceae (2.3%), an uncultured family under the order Actinomarinales (2%), Aeromonadaceae (1.9%), Sphingomonadaceae (1.9%), Thiomicrospiraceae (1.8%), Planococcaceae (1.5%), Rhizobiaceae (1.5%), Pseudomonadaceae (1.5%), Ilumatobacteraceae (1.4%), Woeseiaceae (1.3%), Halomonadaceae (1.2%), Desulfobulbaceae (1.1%), Kiloniellaceae (1.1%), Sulfurimonadaceae (1%), Thioalkalispiraceae (1%) and others.

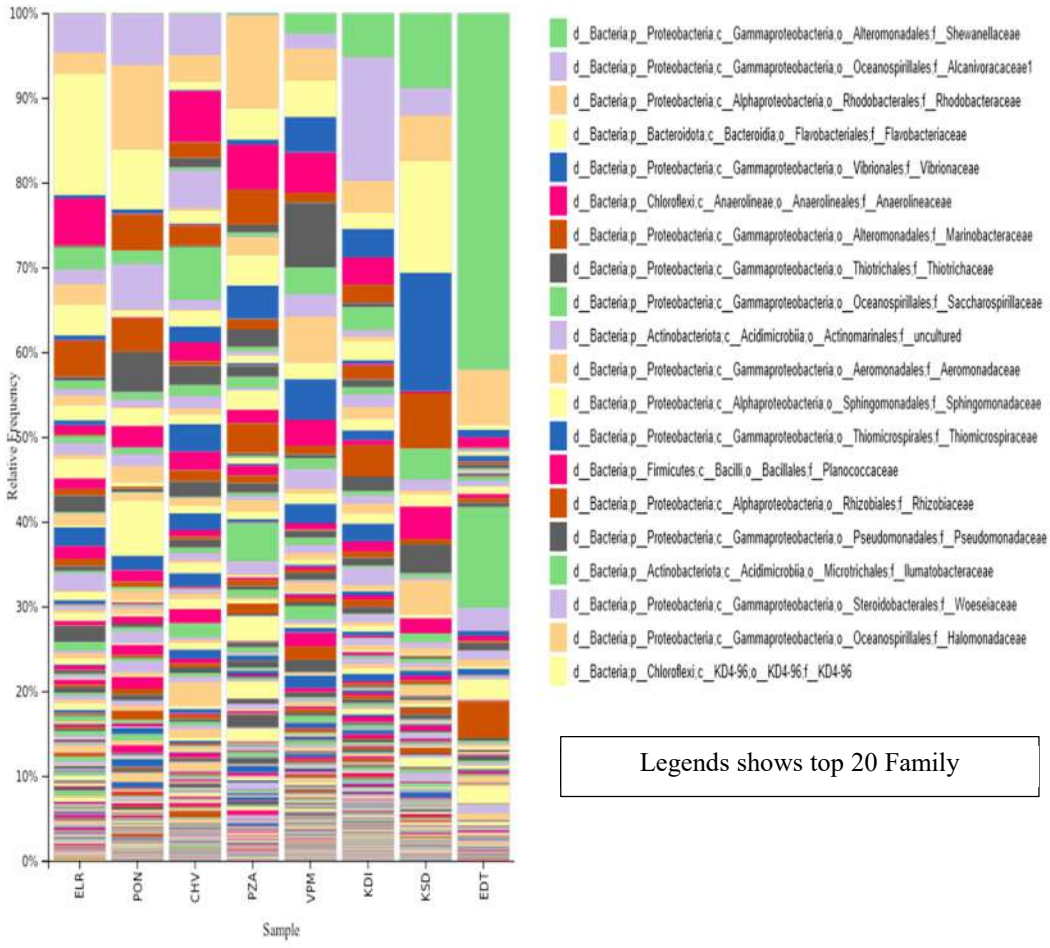


Fig. 4.6 Family level classification of bacteria from 8 different stations of Northern Kerala mangrove sediments

Table 4.4 Relative abundance of top 20 bacterial family from 8 different stations

SL. NO	FAMILY	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
1	Shewanellaceae	0	0	22	0	37	0	12	28
2	Alcanivoracaceae	9	1	63	8	0	0	8	10
3	Rhodobacteraceae	6	1	17	14	6	19.2	19	18
4	Flavobacteriaceae	2	5	9	10	1	7	22	45
5	Vibrionaceae	0	0	18	1	1	1	24	54
6	Anaerolineaceae	19	3	23	0	2	15	38	1
7	Marinobacteraceae	7	0	18	11	0	13	11	40
8	Thiotrichaceae	5	0	5	0	0	4	86	0
9	Saccharospirillaceae	1	2	27	5	0	2	35	27
10	uncultured family (Order A	23	1	9	20	1	0	35	11
11	Aeromonadaceae	2	2	7	1	0	10	74	4
12	Sphingomonadaceae	8	3	28	3	1	17	27	13
13	Thiomicrospiraceae	1	1	6	0	2	20	71	0
14	Planococcaceae	1	0	3	0	0	0	52	44
15	Rhizobiaceae	16	5	25	19	1	7	19	8
16	Pseudomonadaceae	1	0	14	23	2	13	7	40
17	Ilumatobacteraceae	46	1	14	5	1	3	24	6
18	Woeseiaceae	9	1	28	5	1	4	48	5
19	Halomonadaceae	1	2	27	1	0	0	12	57
20	KD4-96 (Phylum Chlorofle	16	3	27.3	13	1	7	27	7

4.4.2.5 Genera level analysis

A total of 1036 genera were found, of which 73 genera were uncultured and 25 unnamed (Fig. 4.7 and table 4.5). The majority of the bacteria belonged to the genus Shewanella (5.5%), followed by Alcanivorax (5.5%), an uncultured genus (3%) under the family Anaerolineaceae, Marinobacter (2.9%), Arenibacter (2.8%), Vibrio (2.7%) and others.

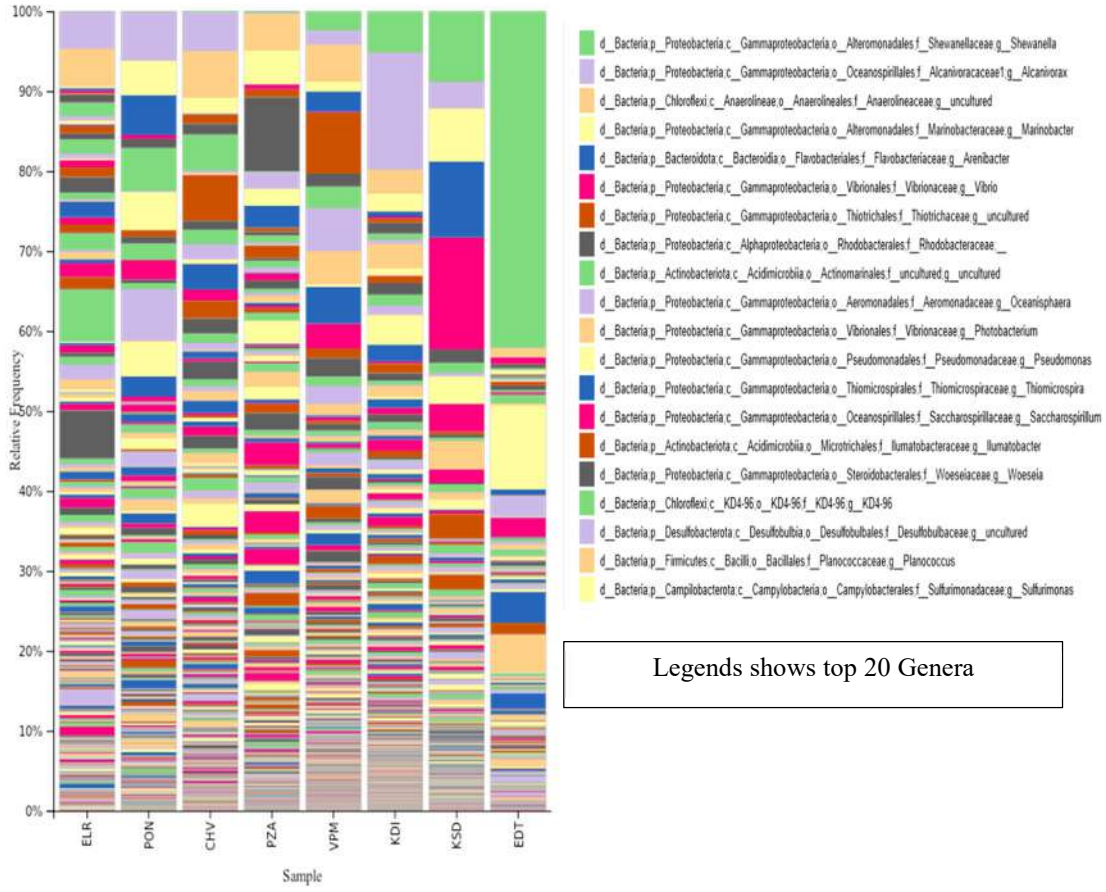


Fig. 4.7 Genus level classification of bacteria from 8 different stations of Northern Kerala mangrove sediments

Table. 4.5 Relative abundance of top 20 bacterial genera from 8 different stations

SL. NO	GENERA	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
1	Shewanella	0	0	22	0	37	0	12	28
2	Alcanivorax	9	1	63	8	0	0	8	10
3	uncultured (Family Anaerolineaceae)	19	3	23	0	2	14	39	1
4	Marinobacter	7	0	18	11	0	13	11	40
5	Arenibacter	0	0	5	13	0	0	23	58
6	Vibrio	0	0	2	1	2	2	1	91
7	Uncultured (Family Thiotrichaceae)	5	0	5	0	0	4	86	0
8	Unnamed (Family Rhodobacteraceae	6	1	15	4	1	40	20	14
9	Uncultured (Order Actinomarinales)	23	1	9	20	1	0	35	11
10	Oceanisphaera	2	0	7	1	0	10	76	4
11	Photobacterium	0	0	40	0	0	0	59	0
12	Pseudomonas	1	0	14	23	2	13	7	40
13	Thiomicrospira	0	0	0	0	0	17	82	0
14	Saccharospirillum	0	0	1	0	0	0	56	42
15	Ilumatobacter	45	1	15	5	0	3	25	5
16	Woeseia	9	1	28	5	1	4	48	5
17	KD4-96 (Phylum Chloroflexi)	16	3	27.3	13	1	7	27	7
18	Uncultured (Family Desulfobulbacea	18	1	24	0	1	2	54	0
19	Planococcus	0	0	3	0	0	0	35	62
20	Sulfurimonas	6	0	90	0	1	1	2	0

4.4.2.8 Species level analysis

A total of 1834 species were obtained in the study, which included 268 unnamed species and 320 uncultured bacteria (Tables 4.6 and 4.7). *Alcanivorax* sp. (3%) was found to be the highest in percentage, followed by an unnamed bacterial species under the genera Arenibacter (2.8%), *Shewanella colwelliana* (2.8%), unnamed species under Vibrio (2.6%), unnamed species under Marinobacter (2.6%), unnamed species under Shewanella (2.4%), and others. The total percentage of bacterial species was found highest at Valapattanam (26%), followed by Kadalundi (23%), Kasaragod (17%), Chettuva (10%), Pazhayangadi (9%), Ponnani (7%), Edat (5%) and Elathur (2%). The identified bacterial species obtained in the metagenomic study is depicted in table 4.7.

Table 4.6 Relative abundance of top 20 bacterial species from 8 different stations

SL.NO	SPECIES	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
1	*Alcanivorax* sp.	13	2	64	7	0	0	1	13
2	Unnamed (Genera Arenibacter)	0	0	5	13	0	0	23	58
3	*Shewanella colwelliana*	0	0	33	0	65	0	1	0
4	Unnamed (Genera Vibrio)	0	0	2	1	1	2	1	92
5	Unnamed (Genera Marinobacter)	7	0	18	11	0	12	10	42
6	Unnamed (Genera Shewanella)	1	0	2	0	7	0	24	64
7	Uncultured (Family Thiotrichaceae)	5	0	5	0	0	4	86	0
8	Uncultured (Family Anaerolineaceae	16	2	22	0	1	17	40	1
9	Unnamed (Family Rhodobacteraceae	6	1	15	4	1	40	20	14
10	Uncultured (Genera Alcanivorax)	3	0	67	11	0	0	12	7
11	Unnamed (Genera Photobacterium)	0	0	40	0	0	0	59	0
12	Unnamed (Genera Oceanisphaera)	2	1	7	1	0	10	76	4
13	Unnamed (Genera Thiomicrospira)	0	0	0	0	0	17	82	0
14	Uncultured (Genera Saccharospirillu	0	0	1	0	0	0	56	42
15	Unnamed (Genera Pseudomonas)	1	0	11	27	0	9	7	45
16	Uncultured (Order Actinomarinales)	24	1	10	19	0	1	38	8
17	Uncultured (Phylum Chloroflexi)	16	2	26	15	1	5	28	7
18	Uncultured (Genera Ilumatobacter)	44	1	14	4	1	4	27	6
19	Unnamed (Genera Planococcus)	0	0	3	0	0	0	37	60
20	Unnamed (Order Actinomarinales)	21	2	5	22	1	0	33	16

Table 4.7 Total relative abundance of bacterial species identified from the metagenomic study

Name of bacterial species	%	Name of bacterial species	%	Name of bacterial species	%	Name of bacterial species	%
Alcanivorax sp.	3	*Citrobacter* sp.	0.01	*Saccharophagus degradans*	0.004	*Litoreibacter arenae*	0.002
Shewanella colwelliana	2.8	*Lactobacillus reuteri*	0.01	*Photobacterium gaetbulicola*	0.004	*Marivita* sp.	0.002
Sulfitobacter sp.	0.6	*Chromatocurvus* sp.	0.01	*Aeromonas veronii*	0.004	*Bacteroides stercorirosoris*	0.002
Planococcus donghaensis	0.4	*Arcomarinus aquaticus*	0.01	*Pseudidiomarina marina*	0.004	*Desulfuromonas thiophila*	0.002
Pseudomonas sp.	0.3	*Sulfuriflexus mobilis*	0.01	*Clostridium pasteurianum*	0.004	*Tepidibacter* sp.	0.002
Halomonas sp.	0.3	*Desulfobacterium* sp.	0.01	*Dyella* sp.	0.004	*Amycolatopsis* sp.	0.002
Shewanella baltica	0.3	*Blastopirellula marina*	0.01	*Idiomarina* sp.	0.004	*Amorphus coralli*	0.002
Achromobacter cholinophagum	0.2	*Rheinheimera* sp.	0.01	*Sediminibacter furfurosus*	0.004	*Altererythrobacter indicus*	0.002
Halothiobacillus neapolitanus	0.2	*Nitratireductor* sp.	0.01	*Xanthomonadaceae bacterium*	0.004	*Streptosporangium violaceochromogenes*	0.002
Arthrobacter sp.	0.2	*Pseudomonas putida*	0.01	*Gelidibacter mesophilus*	0.004	*Simkania negevensis*	0.002
Pseudoalteromonas sp.	0.2	*Rhodococcus hoagii*	0.01	*Sphingobacterial es bacterium*	0.004	*Methylosinus trichosporium*	0.002
Marinobacter sp.	0.2	*Azoarcus* sp.	0.01	*Clostridium cellulovorans*	0.004	*Halobacillus* sp.	0.002
Bacillus sp.	0.1	*Novosphingobium*	0.01	*Sphingomonas*	0.004	*Halodurantibacterium*	0.002

		sp.		sp.		*flavum*	
Acidihalobacter prosperus	0.1	*Ruminiclostridium cellulolyticum*	0.009	*Sphingomicrobium marinum*	0.004	*Lacibacter cauensis*	0.002
Cyclobacterium sp.	0.13	*Lewinella nigricans*	0.009	*Pelobacter venetianus*	0.004	*Nostoc* sp.	0.002
Bizionia paragorgiae	0.12	*Clostridium novyi*	0.009	*Dietzia maris*	0.004	*Geminicoccus roseus*	0.002
Rhodovulum sp.	0.1	*Alcaligenes faecalis*	0.009	*Paraglaciecola hydrolytica*	0.004	*Alicyclobacillus aeris*	0.002
Erythrobacter sp.	0.09	*Mangrovimonas* sp.	0.009	*Labrenzia sp.*	0.003	*Olleya marilimosa*	0.002
Planococcus sp.	0.09	*Nocardioides sp.*	0.008	*Burkholderiales bacterium*	0.003	*Cronobacter dublinensis*	0.002
Bacillus subtilis	0.07	*Staphylococcus hominis*	0.008	*Arcobacter butzleri*	0.003	*Clostridium butyricum*	0.002
Oceanisphaera donghaensis	0.07	*Acinetobacter baumannii*	0.01	*Delftia* sp.	0.003	*Clostridium autoethanogenum*	0.002
Rhodobacteraceae bacterium	0.07	*Paracoccus* sp.	0.008	*Halothiobacillus kellyi*	0.003	*Catelliglobosispora koreensis*	0.002
Bradyrhizobium sp.	0.06	*Porticoccus litoralis*	0.008	*Desulfuromonas* sp.	0.003	*Rhizobiales bacterium*	0.002
Mycobacterium sp.	0.06	*Marinobacterium* sp.	0.008	*Lishizhenia tianjinensis*	0.003	*Geosporobacter ferrireducens*	0.002
Stappia sp.	0.05	*Pontibacter* sp.	0.008	*Rhizobium* sp.	0.003	*Amorphus suaedae*	0.002
Novispirillum itersonii	0.05	*Nitrosomonas* sp.	0.008	*Fulvivirga lutimaris*	0.003	*Thiomicrospira thyasirae*	0.002
Streptomyces sp.	0.05	*Gramella planctonica*	0.008	*Acidibacter ferrireducens*	0.003	*Bacillus decolorationis*	0.002
Kangiella profundi	0.04	*Gaetbulibacter* sp.	0.008	*Lactobacillus ruminis*	0.003	*Paenibacillus* sp.	0.002
Erythrobacter gangjinensis	0.04	*Clostridium* sp.	0.007	*Leucothrix mucor*	0.003	*Staphylococcus epidermidis*	0.002
Mesorhizobium sp.	0.04	*Rhodobium* sp.	0.007	*Microbulbifer marinus*	0.003	*Brevibacillus ginsengisoli*	0.002
Bacillus megaterium	0.04	*Sideroxydans paludicola*	0.007	*Agromyces sp.*	0.003	*Acinetobacter* sp.	0.002
Oceanimonas sp.	0.04	*Thiohalophilus thiocyanatoxydans*	0.007	*Enterococcus faecalis*	0.003	*Myxobacterium* SMH-27-4	0.002
Bacteriovorax sp.	0.03	*Vibrio* sp.	0.006	*Clostridium botulinum*	0.003	*Lactobacillus fermentum*	0.002
Psychrobacter sp.	0.03	*Rhodobacter* sp.	0.006	*Maricaulis maris*	0.003	*Aeromonas salmonicida*	0.002
Marinomonas mangrovi	0.03	*Oceanicola* sp.	0.006	*Algoriphagus sp.*	0.003	*Comamonas terrigena*	0.002
Salinirepens amamiensis	0.03	*Marinomonas sp.*	0.006	*Oleiphilus* sp.	0.003	*Citrobacter freundii*	0.002
Chloroflexi bacterium	0.03	*Lactobacillus delbrueckii*	0.006	*Cereibacter changlensis*	0.003	*Bosea* sp.	0.002
Marinobacter mobilis	0.03	*Stenotrophomonas maltophilia*	0.006	*Muricauda iocasae*	0.003	*Virgibacillus* sp.	0.002
Aeromonas hydrophila	0.03	*Bacillus hwajinpoensis*	0.006	*Thiohalobacter thiocyanaticus*	0.003	*Psychrobacter arenosus*	0.002
Candidatus Tenderia	0.02	*Frankia* sp.	0.006	*Pseudorhodobacter aquimaris*	0.003	*Micromonospora* sp.	0.002
Robertkochia marina	0.02	*Photobacterium* sp.	0.006	*Cohnella sp.*	0.003	*Bacillus circulans*	0.002
Brevundimonas sp.	0.02	*Micavibrio aeruginosavorus*	0.006	*Oceanisphaera marina*	0.003	*Acetobacter lovaniensis*	0.002
Cobetia sp.	0.02	*Gramella jeungdoensis*	0.005	*Estrella lausannensis*	0.003	*Leptolyngbya* sp.	0.002
Polaribacter sp.	0.02	*Ferrimonas gelatinilytica*	0.005	*Phycicoccus endophyticus*	0.002	*Minicystis rosea*	0.002
Comamonas aquatica	0.02	*Oceanicola* sp.	0.005	*Racemicystis persica*	0.002	*Clostridium kluyveri*	0.002
Lutibacter maritimus	0.02	*Bacillus cytotoxicus*	0.005	*Streptococcus hyointestinalis*	0.002	*Formosa* sp.	0.002
Salegentibacter chungangensis	0.02	*Ferrimonas balearica*	0.005	*Fulvimarina pelagi*	0.002	*Pirellula* sp.	0.002
Rhodococcus erythropolis	0.02	*Clostridium acetobutylicum*	0.005	*Staphylococcus sciuri*	0.002		
Sporosarcina sp.	0.02	*Pseudoalteromonas rubra*	0.005	*Ketobacter_alkanivorans*	0.002		

Flavobacteriaceae bacterium	0.02	*Saccharospirillum impatiens*	0.005	*Rhodovastum atsumiense*	0.002	
Arenibacter sp.	0.02	*Thiobacter* sp.	0.005	Yeosuana aromativorans	0.002	
Shewanella sp.	0.02	*Streptococcus saliva rius*	0.005	Prolixibacter bellariivorans	0.002	
Microbaculum marinum	0.02	*Lysinibacillus* sp.	0.005	Oceanisphaera sp.	0.002	
Pirellula sp.	0.02	*Clostridium saccharobutylicum*	0.005	Tumebacillus avium	0.002	
Vibrio natriegens	0.02	*Exiguobacterium* sp.	0.005	Bacillus lentus	0.002	
Muricauda sp.	0.01	*Ilyobacter polytropus*	0.005	Arenibacter nanhaiticus	0.002	
Lutibaculum sp.	0.01	*Acinetobacter radioresistens*	0.005	Clostridium beijerinckii	0.002	
Haliea sp.	0.01	*Rhodospirillaceae bacterium*	0.005	Marinobacter segnicrescens	0.002	
Clostridium perfringens	0.01	*Defluviimonas denitrificans*	0.005	Alcanivorax pacificus	0.002	
Albimonas pacifica	0.01	*Arenibacter latericius*	0.005	Nitrosomonas aestuarii	0.002	
Verrucosispora sp.	0.01	*Lacinutrix* sp.	0.004	Pararhodobacter sp.	0.002	
Nitratireductor basaltis	0.01	*Hanstruepera neustonica*	0.004	Euryhalocaulis caribicus	0.002	
Ilumatobacter fluminis	0.01	*Enhygromyxa* sp.	0.004	Triticum aestivum	0.002	
Thiomicrospira aerophila	0.01	*Marinobacter lipolyticus*	0.004	Altererythrobacter sp.	0.002	
Enterococcus casseliflavus	0.01	*Clostridium oceanicum*	0.004	Carnobacterium funditum	0.002	

4.4.3 Biodiversity indices

The analysis of alpha diversity involved the calculation of Chao 1, observed features, Pielou's evenness, Shannon index, and Simpson index (Table 4.8 A). The trimmed data sequence count was highest at Valapattanam (129668) and lowest at Elathur (44701). Kadalundi (9659) exhibited the highest Chao 1, or species diversity, followed by Valapattanam (7685), Chettuva (6199), Kasaragod (4710), Pazhayangadi (4026), Elathur (3380), Ponnani (3303), and Edat (2043). The observed features were found highest at Kadalundi (9659), followed by Valapattanam (6655), Chettuva (4245), Kasaragod (3670), Pazhayangadi (3280), Ponnani (2546), Elathur (1270) and lowest at Edat (1254). Pielou's evenness, or equity in species abundance, was comparatively higher at Chettuva and Elathur (0.9), followed by Kadalundi and Ponnani (0.88), Valapattanam (0.84), Pazhayangadi (0.82), Kasaragod (0.81) and lesser at Edat (0.74). The Shannon index, i.e., the richness and evenness, was highest at Kadalundi (11.6) and lowest at Edat (7.6). The Simpson index, which shows diversity, was highest at Chettuva, Kadalundi, Ponnani, and Valapattanam (1.00) and lowest at Edat (0.96). Elathur, Kasaragod and Pazhayangadi stations showed 0.99 of the Simpson index.

Beta diversity was represented by the Bray-Curtis distance (Table 4.8 B); the amount of variation in species composition between sampling units was comparatively higher between Ponnani and Edat (0.967) and lowest between Chettuva and Valapattanam (0.788). Jaccard distance (Table 4.8 C), which depicts the difference in microbial composition between two samples based on the presence or absence of species, was comparatively higher between Kadalundi and Edat (0.962) and lowest between Chettuva and Valapattanam (0.758).

Table 4.8 A) Sequence count and alpha diversity analysis of the species population from sediment samples collected from eight mangrove sites along Northern Kerala, India

Features	CHV	EDT	ELR	KDI	KSD	PON	PZA	VPM
Trimmed data sequence count	94638	57177	44701	95244	86828	65080	84920	129668
Chao1	6199	2043	3380	9659	4710	3303	4026	7685
Observed features	4245	1254	1270	9659	3670	2546	3280	6655
Pielou's evenness	0.9	0.74	0.9	0.88	0.81	0.88	0.82	0.84
Shannon index	10.8	7.6	9.3	11.6	9.6	10	9.6	10.7
Simpson index	1	0.96	0.99	1	0.99	1	0.99	1

Table 4.8 B) Beta diversity analysis (Bray-curtis distance) of the species population from sediment samples collected from eight mangrove sites along Northern Kerala

	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
CHV	0							
ELR	0.855	0						
KDI	0.827	0.948	0					
PON	0.853	0.889	0.917	0				
EDT	0.918	0.892	0.934	0.967	0			
PZA	0.897	0.928	0.925	0.964	0.922	0		
VPM	0.788	0.951	0.829	0.917	0.958	0.918	0	
KSD	0.879	0.933	0.924	0.797	0.952	0.952	0.832	0

Table 4.8 C) Beta diversity analysis (Jaccard distance) of the species population from sediment samples collected from eight mangrove sites along Northern Kerala

	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
CHV	**0**							
ELR	**0.874**	**0**						
KDI	**0.890**	**0.955**	**0**					
PON	**0.878**	**0.915**	**0.961**	**0**				
EDT	**0.906**	**0.877**	**0.962**	**0.950**	**0**			
PZA	**0.863**	**0.893**	**0.932**	**0.945**	**0.889**	**0**		
VPM	**0.758**	**0.920**	**0.885**	**0.905**	**0.935**	**0.876**	**0**	
KSD	**0.854**	**0.909**	**0.949**	**0.760**	**0.926**	**0.921**	**0.857**	**0**

4.4.4 Functional annotation of the bacterial community of study area

To formulate the predictions on the functional role of the microbes present in the study site, PICRUSt2 was used. MetaCyc was used for annotations. The genes responsible for the metabolic pathways were revealed in the metagenomic study. Genes responsible for amino acid metabolism (22%) were found predominant, followed by metabolic functions (20%), nucleic acid metabolism (11%), environmental functions (11%), carbohydrate metabolism (8%), cell wall biosynthesis (8%), ATP synthesis (7%), lipid metabolism (6%), metabolism of vitamins (5%) and stress response (1%) (Fig. 4.8).

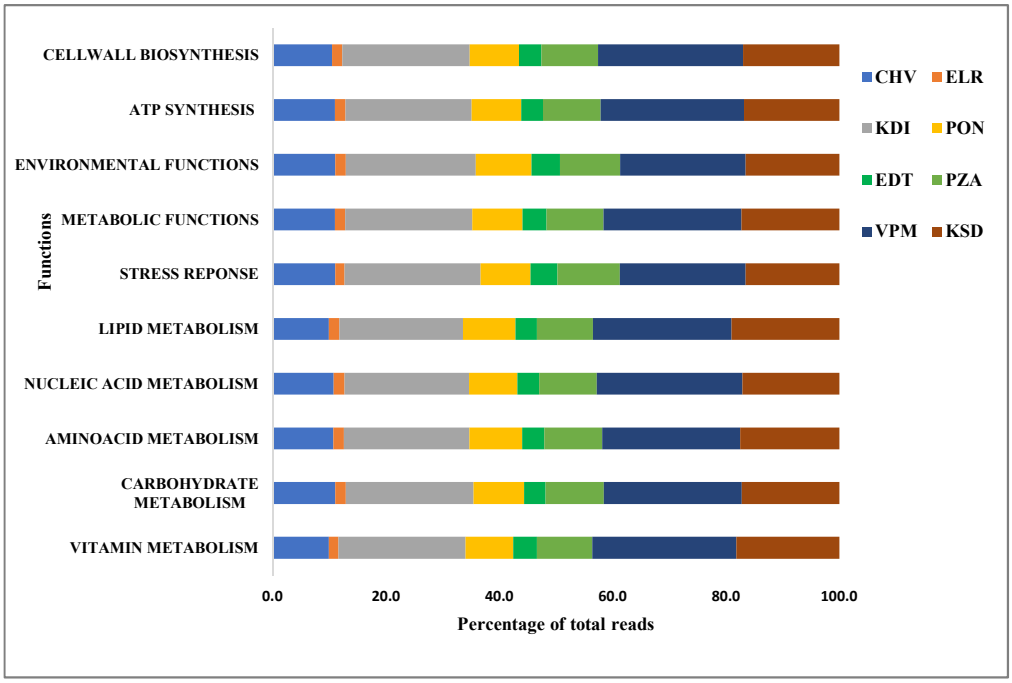


Fig. 4.8 Comparative analysis of pathways from different sampling stations

Comparing the relative abundance of genes in functional pathways, it was found highest at Valapattanam (24%), followed by Kadalundi (22%), Kasaragod (17%), Chettuva (11%), Pazhayangadi (10%), Ponnani (9%), Edat (4%) and Elathur (2%).

4.4.4.1 Biosynthesis pathway

The genes responsible for the biosynthesis of antibiotics, metabolite compounds, etc., annotated in the metagenomic study are shown in Fig. 4.9.

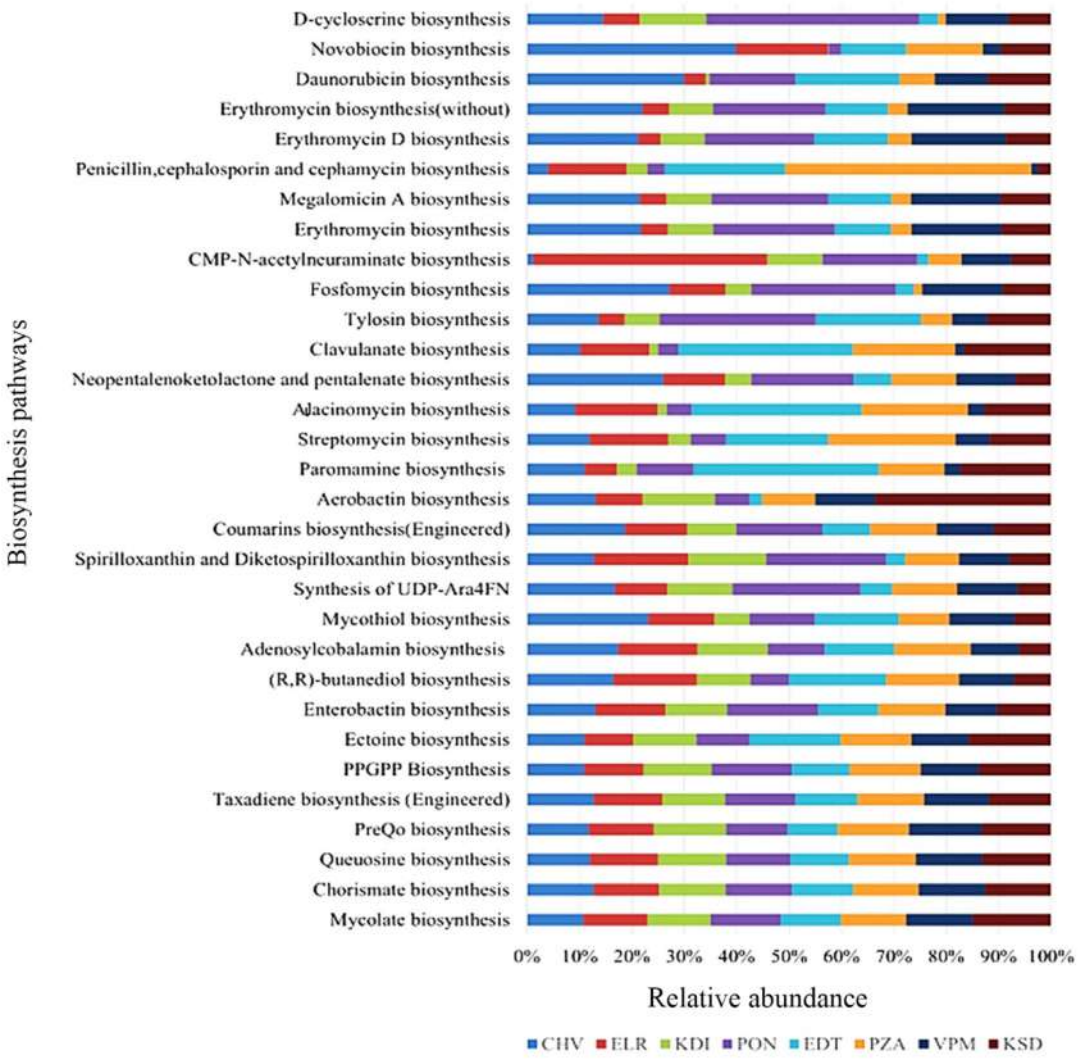


Fig. 4.9 Functional annotation of genes showing the biosynthesis pathway present in the mangrove sediment of North Kerala.

Functional annotation from meta-analysis revealed that certain special compounds are being synthesized in the currently studied mangrove habitat. The genes responsible for the biosynthesis of mycolate (0.7%), chorismate (0.5%), queuosine (0.4%), pre QO (0.4%), taxadiene (0.3%), PPGPP (0.2%), ectoine (0.2%), enterobactin (0.1%), butanediol (0.1%), adenosylcobalamin (0.2%), mycothiol (0.05%), UDP-Ara4FN (0.02%), spirilloxanthin (0.02%), coumarins (0.01%), aerobactin (0.01%) paromamine (0.003%), streptomycin (0.002%), aclacinomycin (0.001%), neopentalenoketo lactone (0.002%), clavulanate (0.0005%), tylosin (0.0005%), fosfomycin (0.0003%), acetylneuraminate (0.0003%), erythromycin (0.0003%),

megalomicin (0.0002%), penicillin (0.0002%), cephalosporin (0.0002%), cephamycin (0.0002%), erythromycin D (0.0001%), daunorubicin (0.0001%), novobiocin (0.0001%), cycloserine (0.0001%) etc. has been obtained in functional annotation.

4.4.4.2 Biodegradation pathway

Fig. 4.10 displays the details of genes responsible for the degradation of certain recalcitrant compounds, annotated in the metagenomic study.

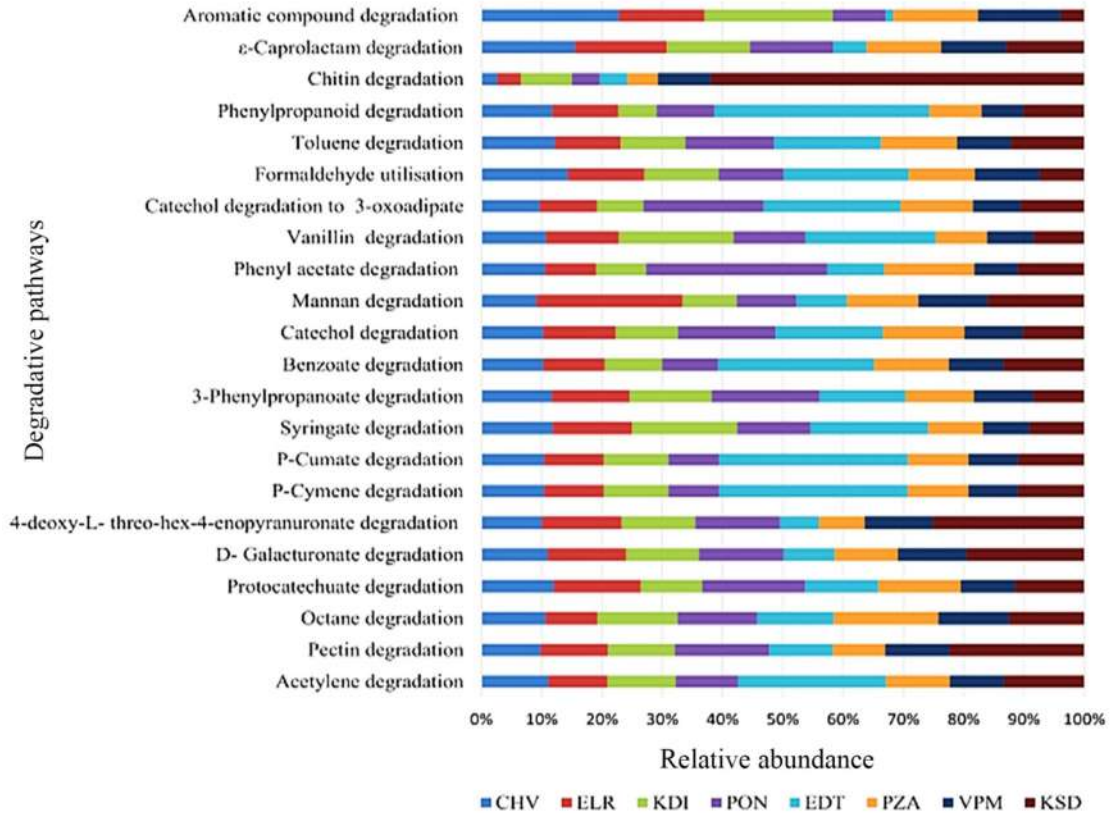


Fig. 4.10 Functional annotation of genes showing the degradative pathway present in the mangrove sediment of North Kerala

Functional annotation from meta-analysis revealed a bacterial pathway responsible for the degradation of several xenobiotic and recalcitrant compounds in the Northern Kerala mangrove habitat. It includes genes responsible for degradation of acetylene (0.26%), pectin (0.25%), octane (0.25%), protocatechuate (0.2%), D-galacturonate (0.2%), 4-deoxy-L-threo-hex-4-enopyrauronate (0.1%), cymene (0.1%), cumate (0.1%), syringate (0.1%), 3-phenyl propanoate (0.1%), benzoate (0.1%), catechol (0.1%), mannan (0.1%), phenyl acetate (0.05%), vanillin (0.05%),

formaldehyde (0.1%), toluene (0.05%), phenyl propanoid (0.03%), chitin (0.02%), ε-caprolactam (0.03%), various aromatic compounds, etc.

4.4.5 Taxonomic assignment of bacterial communities in Sulphur cycle, Nitrogen cycle and methanogenesis

4.4.5.1 Bacteria involved in Sulphur cycle

Bacteria involved in the sulphur cycle, such as sulphur-oxidising bacteria (SOB) and sulphur-reducing bacteria (SRB), were detected from the metagenomic analysis, and the details are shown in fig. 4.11 and table 4.9. In the present study, about twenty different types of bacterial genera were reported as SOBs under nine orders, and details are shown in fig. 4.11A. The SOB belongs to the following orders Acidiferrobacterales, Halothiobacillales, Kapabacteriales, Chromatiales, Campylobacterales, Rhodobacterales, Thiomicrospirales, Ectothiorhodospirales, and Burkholderiales. Thiomicrospira, which belonged to order Thiomicrospirales (27%), was the most abundant SOB, followed by Sulfurimonas under order Campylobacterales (18%), Sulfitobacter under order Rhodobacterales (12%), and Thioalkalispira-Sulfurivermis under order Ectothiorhodospirales (11%). Diversity of SOB was found highest at Valapattanam (all bacteria except those in order Burkholderiales) and Kadalundi (all bacteria except those in order Halothiobacillales) and least at Edat (orders Thiomicrospirales, Rhodobacterales and Ectothiorhodospirales), Ponnani and Kasaragod (orders Acidiferrobacterales, Kapabacteriales and Rhodobacterales). SOB belonging to the orders Acidiferrobacterales, Kapabacteriales, Rhodobacterales, Thiomicrospirales and Ectothiorhodospirales were found from six sampling stations.

The sulphate-reducing bacteria (SRB) belong to the orders Thermodesulfovibrionales, Gammaproteobacteria Incertae Sedis, Chromatiales, Desulfobaccales, Desulfatiglandales, Desulfotomaculales, Peptostreptococcales - Tissierellales, Desulfobacterales, Desulfobulbia, Desulfovibrionales, Desulfomonilales and Desulfuromonadia. The study classified about twenty different types of bacterial genera as SRB under twelve orders (Fig. 4.11 B). Almost all kinds of SRB were found at Valapattanam (except the order Peptostreptococcales-Tissierellales), followed by Kadalundi (except the orders Desulfotomaculales, Thermodesulfovibrionales and

Gammaproteobacteria Incertae Sedis). SRB, under the order Desulfobulbales and Desulfuromonadia, was found at seven sampling stations except Ponnani. Desulfatiglans under order Desulfatiglandales (28%) were the most abundant SRB, followed by Desulfopila under order Desulfobulbales (23%) and Desulfuromonas under order Desulfuromonadia (14%).

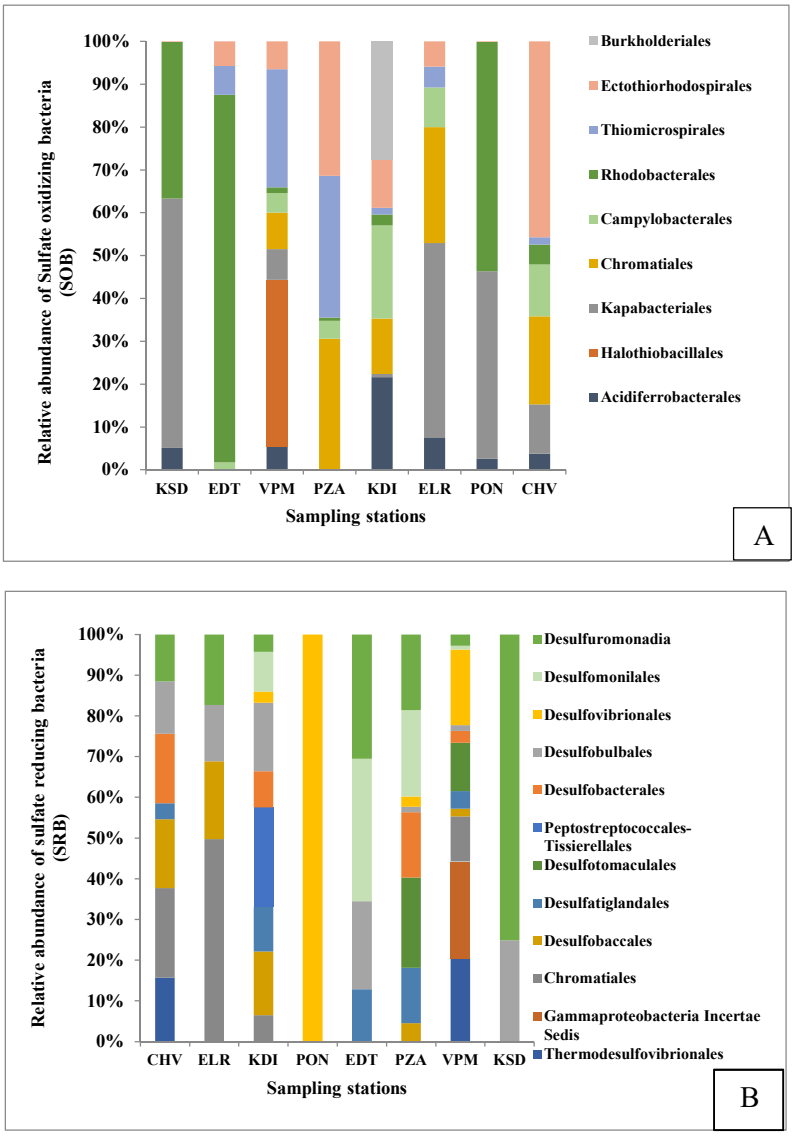


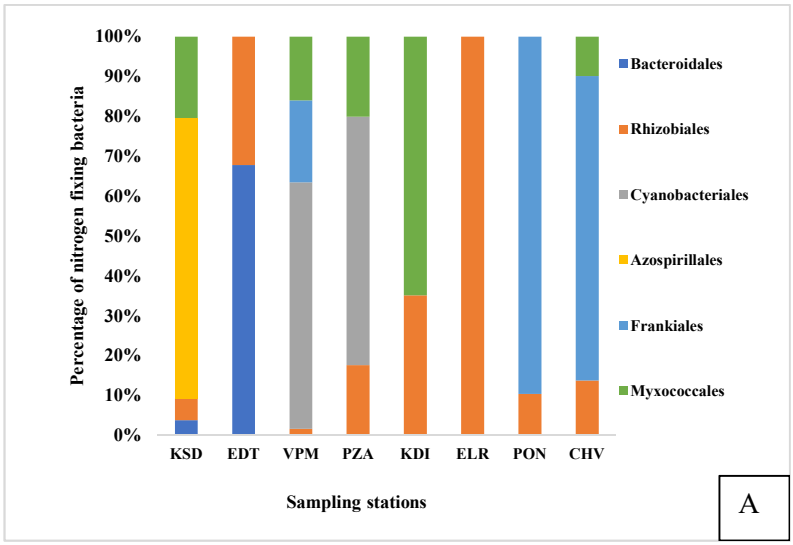
Fig. 4.11 Relative abundance of bacteria involved in sulphur cycle at sampling stations. A: Sulphate oxidizing bacteria; B: Sulphate reducing bacteria

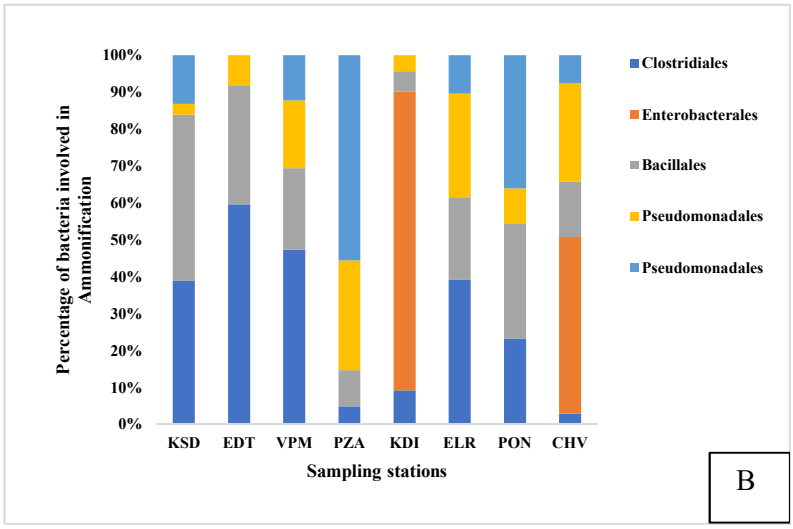
Table 4.9 Abundance of sulphur oxidizing and reducing bacterial genera from different sampling stations

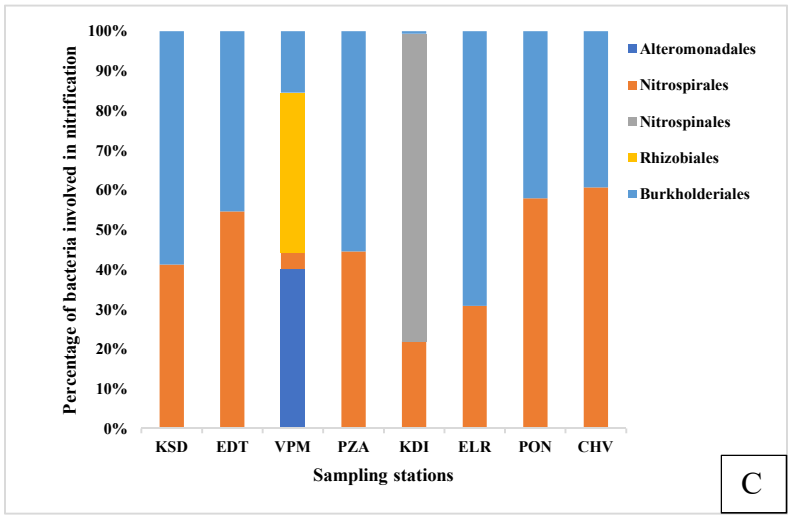
SULPHUR OXIDIZING BACTERIA										
Order	Family	Genera	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
Acidiferrobacterales	Acidiferrobacteraceae	Sulfurifustis	2	1	78	2	0	0	14	3
Halothiobacillales	Thioalkalibacteraceae	Guyparkeria	0	0	0	0	0	0	100	0
Kapabacteriales	Kapabacteriales	Kapabacteriales	5	5	3	29	0	0	18	39
Chromatiales	Chromatiaceae	Candidatus Thiobios	10	3	52	0	0	21	14	0
		Thiohalobacter	0	0	0	0	0	0	100	0
Campylobacterales	Sulfurovaceae	Sulfurovum	5	4	35	0	0	11	45	0
	Sulfurimonadaceae	Sulfurimonas	6	0	90	0	1	1	2	0
		Sulfuricurvum	0	0	0	0	0	0	100	0
Rhodobacterales	Rhodobacteraceae	Thioclava	1	0	2	1	92	1	1	2
		Sulfitobacter	1	0	12	50	0	0	3	34
		Ruegeria	35	0	10	0	0	5	50	0
Thiomicrospirales	Thiomicrospiraceae	Thiomicrorhabdus	5	3	38	0	11	37	6	0
		Thiomicrospira	0	0	0	0	0	17	82	0
		Thioalkalimicrobium	0	0	0	0	0	0	100	0
		Hydrogenovibrio	0	0	0	0	0	100	0	0
Ectothiorhodospirales	Ectothiorhodospiraceae	Thiogranum	12	2	38	0	6	39	2	0
	Thioalkalispiraceae	Thiohalophilus	1	0	50	0	1	5	43	0
		Thioalkalispira-Sulfurivermi	40	0	36	0	0	16	8	0
Burkholderiales	Sulfuricellaceae	Sulfuriferula	0	0	100	0	0	0	0	0
	Hydrogenophilaceae	Sulfuritortus	0	0	100	0	0	0	0	0
SULPHUR REDUCING BACTERIA										
Order	Family	Genera	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
Thermodesulfovibrionale	Thermodesulfovibrionace	Thermodesulfovibrio	14	0	0	0	0	0	86	0
Gammaproteobacteria I	Unknown Family	Sulfuriflexus	0	0	0	0	0	0	100	0
Chromatiales	Sedimenticolaceae	Sedimenticola	20	7	27	0	0	0	47	0
Desulfobaccales	Desulfobaccaceae	Desulfobacca	15	3	64	0	0	10	8	0
Desulfatiglandales	Desulfatiglandaceae	Desulfatiglans	4	0	45	0	3	31	18	0
Desulfotomaculales	Desulfotomaculales	Desulfurispora	0	0	0	0	0	50	50	0
Peptostreptococcales-Ti	Dethiosulfatibacteraceae	Dethiosulfatibacter	0	0	100	0	0	0	0	0
Desulfobacterales	Desulfobacteraceae	Desulfobacterium	36	0	64	0	0	0	0	0
		Desulfobacter	0	0	0	0	0	0	100	0
	Desulfosarcinaceae	Desulfatitalea	0	0	29	0	0	64	7	0
		Desulfatirhabdium	0	0	100	0	0	0	0	0
		Desulfosarcina	18	0	29	0	0	43	11	0
Desulfobulbales	Desulfobulbaceae	Desulfobulbus	0	0	38	0	6	0	38	19
		Desulforhopalus	0	0	0	0	100	0	0	0
		Desulfobacterium_catecholic	0	0	0	0	0	0	100	0
		Desulfopila	13	2	75	0	3	4	1	1
Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	0	0	50	25	0	25	0	0
		Desulfomicrobium	0	0	0	0	0	0	100	0
Desulfomonilales	Desulfomonilaceae	Desulfomonile	0	0	40	0	8	48	4	0
Desulfuromonadia	Desulfuromonadaceae	Desulfuromonas	10	2	17	0	7	42	12	9

4.4.5.2 Bacteria involved in Nitrogen cycle

Metagenomic analysis from mangrove stations revealed the presence of several bacterial genera involved in nitrogen fixation, ammonification, nitrification and denitrification. The details are depicted in fig. 4.12 and table 4.10.


Percentage of nitrogen fixing bacteria
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
KSD
EDT
VPM
PZA
KDI
ELR
PON
CHV
Sampling stations
Bacteroidales
Rhizobiales
Cyanobacteriales
Azospirillales
Frankiales
Myxococcales
A


Percentage of bacteria involved in Ammonification
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
KSD
EDT
VPM
PZA
KDI
ELR
PON
CHV
Sampling stations
Clostridiales
Enterobacterales
Bacillales
Pseudomonadales
Pseudomonadales
B


Percentage of bacteria involved in nitrification
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
KSD
EDT
VPM
PZA
KDI
ELR
PON
CHV
Sampling stations
Alteromonadales
Nitrospirales
Nitrospinales
Rhizobiales
Burkholderiales
C

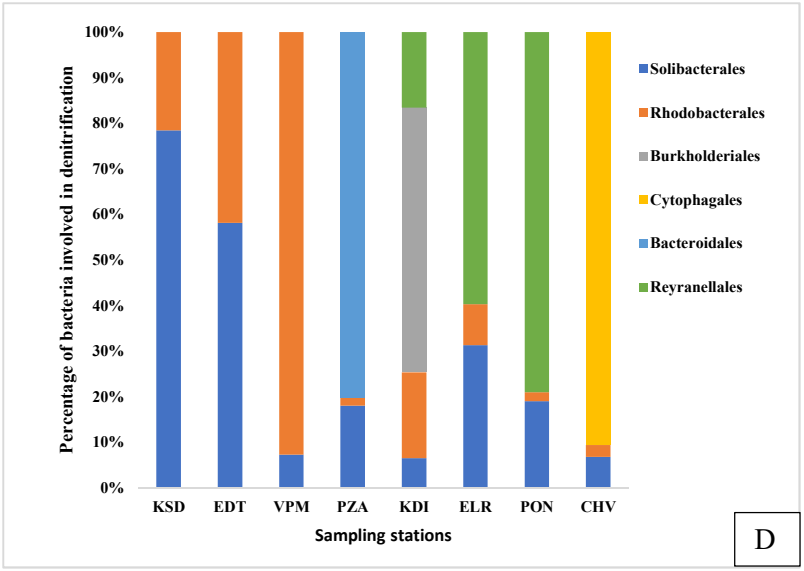


Fig. 4.12 Relative abundance of bacteria involved in nitrogen cycle at sampling stations; A: Nitrogen fixing bacteria; B: Ammonifying bacteria; C: Nitrifying bacteria; D: Denitrifying bacteria

About ten different bacterial genera under six orders were reported to have the ability of nitrogen fixation (Fig. 4.12 A). They belonged to the orders Bacteroidales, Rhizobiales, Cyanobacteriales, Azospirillales, Frankiales and Myxococcales. Nitrogen-fixing bacteria under the order Rhizobiales were found at seven sampling stations except for Elathur, followed by the order Myxococcales, which was found at five sampling stations except for Edat, Elathur, and Ponnani. The most abundant nitrogen-fixing bacteria obtained was Mesorhizobium (32%), followed by Bradyrhizobium (23%) and Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium (14%) and the least were Ochrobactrum, Nostoc PCC-7107 and Azospirillum (1%).

Five different bacterial genera under four orders were found that have the ability for ammonification (Fig. 4.12 B). The order includes Clostridiales, Enterobacterales, Bacillales, and Pseudomonadales. Pseudomonas, Bacillus, and Clostridium were present at all mangrove stations.

Table 4.10 Abundance of bacteria involved in nitrogen cycle from different sampling stations

NITROGEN FIXING BACTERIA										
Order	Family	Genera	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
Bacteroidales	Prolixibacteraceae	Mangrovibacterium	0	0	0	0	95	0	0	5
Rhizobiales	Rhizobiaceae	Ensifer	0	28	50	11	0	6	0	6
		Allorhizobium-Neorhizo	5	42	9	0	0	30	9	5
		Mesorhizobium	6	0	7	7	1	66	5	8
		Ochrobactrum	0	0	0	0	0	100	0	0
	Xanthobacteraceae	Bradyrhizobium	4	4	29	23	8	8	7	16
Cyanobacteriales	Nostocaceae	Nostoc PCC-7107	0	0	0	0	0	50	50	0
Azospirillales	Azospirillaceae	Azospirillum	0	0	0	0	0	0	0	100
Frankiales	Frankiaceae	Frankia	50	0	0	33	0	0	17	0
Myxococcales	Anaeromyxobacteracea	Anaeromyxobacter	6	0	35	0	0	16	13	29
AMMONIFYING BACTERIA										
Order	Family	Genera	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
Clostridiales	Clostridiaceae	Clostridium	14	3	37	4	3	18	17	4
Enterobacterales	Morganellaceae	Proteus	0	0	0	0	29	0	0	71
Bacillales	Bacillaceae	Bacillus	16	2	17	8	2	10	23	22
Pseudomonadales	Pseudomonadaceae	Pseudomonas	1	0	14	23	2	13	7	40
	Streptomycetaceae	Streptomyces	5	0	9	43	0	5	26	11
NITRIFYING BACTERIA										
Order	Family	Genera	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
Burkholderiales	Gallionellaceae	Candidatus Nitrotoga	0	8	17	0	0	75	0	0
	Nitrosomonadaceae	Nitrosomonas	22	2	40	4	1	0	25	6
	Rhodocyclaceae	Denitromonas	33	0	67	0	0	0	0	0
Alteromonadales	Idiomarinaceae	Idiomarina	0	0	0	0	0	0	100	0
Nitrospirales	Nitrospiraceae	Nitrospira	8	3	28	30	3	3	10	14
Nitrospinales	Nitrospinaceae	Nitrospina	0	0	100	0	0	0	0	0
Rhizobiales	Thermopetrobacterace	Thermopetrobacter	0	0	0	0	0	0	100	0
DENITRIFYING BACTERIA										
Order	Family	Genera	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
Solibacterales	Solibacteraceae	Candidatus Solibacter	8	8	11	14	5	23	4	29
Rhodobacterales	Rhodobacteraceae	Paracoccus	3	2	32	1	4	2	47	8
Burkholderiales	Rhodocyclaceae	Denitratisoma	0	0	100	0	0	0	0	0
Cytophagales	Cyclobacteriaceae	Marivirga	100	0	0	0	0	0	0	0
Bacteroidales	Prolixibacteraceae	Prolixibacter	0	0	0	0	0	100	0	0
Reyranellales	Reyranellaceae	Reyranella	0	14	29	57	0	0	0	0

Seven different bacterial genera under five orders were found in the sampling stations with the ability for nitrification (Fig. 4.12 C). The taxonomic order includes Burkholderiales, Alteromonadales, Nitrospirales, Nitrospinales and Rhizobiales. Nitrifying bacteria under the genera Nitrospirales and Burkholderiales were present at all sampling stations.

Six different bacterial genera belonging to six orders were reported from the sampling stations, which have the ability for denitrification (Fig. 4.12 D). Denitrifying bacteria belong to the orders Solibacterales, Rhodobacterales, Burkholderiales, Cytophagales, Bacteroidales and Reyranellales. Paracoccus (59%) was the most abundant denitrifying bacteria under Rhodobacterales, and the least abundant was Denitratisoma (1%) under Burkholderiales.

4.4.5.3 Methanotrophs and iron redox cycling bacteria

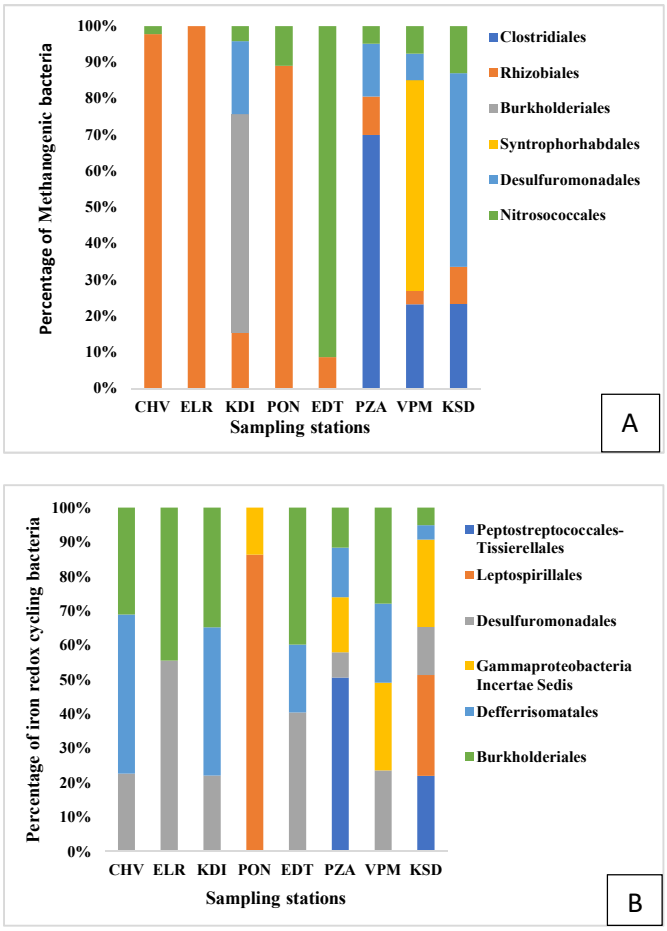


Fig. 4.13 Relative abundance of bacteria involved in methanogenesis (A) and iron redox cycling bacteria (B) at sampling stations.

Eleven different bacterial genera under six orders were reported from the sampling stations that have the ability of methanogenesis (Fig. 4.13 A and table 4.11). Methanotrophs belong to the orders Clostridiales, Rhizobiales, Burkholderiales, Syntrophorhabdales, Desulfuromonadales, and Nitrosococcales. Methyloceanibacter under order Rhizobiales (64%) was the most abundant methanotroph, followed by Methylophaga under order Nitrosococcales (23%), and the least abundant were Lutispora, Roseiarcus and Methylibium (0.3%).

Table 4.11 Abundance of methanotrophs and iron redox cycling bacteria from different sampling stations

METHANOTROPHS										
Order	Family	Genera	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
Clostridia	Gracilibacteraceae	Lutispora	0	0	0	0	0	100	0	0
	Hungateiclostridiaceae	Acetivibrio	0	0	0	0	0	0	67	33
Rhizobiales	Beijerinckiaceae	Roseiarcus	0	0	0	0	0	100	0	0
		Methylosinus	0	0	0	0	0	0	50	50
		Methylobacterium-Methylorubrum	6	0	53	0	0	18	6	18
		Methylocystis	13	0	52	0	17	0	13	4
	Methyloligellaceae	Methyloceanibacter	33	6	23	13	6	6	6	8
Burkholderiales	Comamonadaceae	Methylibium	0	0	100	0	0	0	0	0
Syntrophorhabdales	Syntrophorhabdaceae	Syntrophorhabdus	0	0	0	0	0	0	100	0
Desulfuromonadia	Desulfuromonadaceae	Desulfuromonadaceae	0	0	33	0	0	8	13	46
Nitrosococcales	Methylophagaceae	Methylophaga	1	0	7	1	64	3	13	11
IRON REDOX CYCLING BACTERIA										
Order	Family	Genera	CHV	ELR	KDI	PON	EDT	PZA	VPM	KSD
Peptostreptococcales-Tis	Thermotaleaceae	Geosporobacter	0	0	0	0	0	50	0	50
Leptospirillales	Leptospirillaceae	Leptospirillum	0	0	0	33	0	0	0	67
Gammaproteobacteria In	Unknown Family	Acidibacter	0	0	0	5	0	16	21	58
Defferrisomatales	Defferrisomataceae	Deferrisoma	14	0	38	0	5	14	19	10
Desulfuromonadales	Geopsychrobacteraceae	Desulfuromusa	6	6	26	0	13	10	26	13
	Geoalkalibacteraceae	Geoalkalibacter	10	0	0	0	0	0	0	90
Burkholderiales	Gallionellaceae	Sideroxydans	0	6	0	0	0	83	11	0
		Gallionella	0	0	41	0	7	52	0	0

Eight different bacterial genera under seven orders were reported in the present study as iron redox cycling bacteria, carrying out reduction and oxidation of iron (Fig. 4.13 B and table 4.11). The bacterial orders include Peptostreptococcales-Tissierellales, Leptospirillales, Desulfuromonadales, Gammaproteobacteria Incertae Sedis, Defferrisomatales and Burkholderiales. Desulfuromusa under the order Desulfuromonadales (23%) were the most abundant iron-reducing bacteria, followed by Gallionella under the order Burkholderiales (20%), and the least abundant were Burkholderiales under Peptostreptococcales-Tissierellales (1%).

4.4.6 Data deposition

The metabarcoding data of eight different sediment samples from northern Kerala mangrove habitat are available at National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number PRJNA1166867.

The present study has shown that the mangrove habitat in northern Kerala is abundant in bacterial diversity, with each type playing significant functional roles. The prevalence of each bacterial community is confined to specific mangrove stations,

indicating that their abundance is linked to environmental factors, nutrient availability and stress conditions.

4.5 DISCUSSION

The current study analysed and compared the bacterial diversity of the northern Kerala mangrove habitat using metagenomics. So far, the only metagenomic studies reported from northern Kerala are by Imchen et al. (2017) and Kutty et al. (2023), which are confined to the mangroves of Kannur. The present research uncovers an extensive variety of bacterial species and their distribution among eight distinct mangrove habitats, as well as the complex interactions between microbial communities and their surroundings.

Bacteria are the predominant organisms, playing a crucial role in the mangrove ecosystem's functioning, including organic matter decomposition, nutrient cycling and carbon storage. The relative abundance of bacteria was found to be highest at Valapattanam (26%), followed by Kadalundi (23%), Kasaragod (17%), Chettuva (10%), Pazhayangadi (9%), Ponnani (7%), Edat (5%), and Elathur (2%). The distinctions in bacterial abundance and community structure across various locations are due to environmental factors. These factors, which differ by geography, encompass temperature, soil pH, nutrient availability, and even the existence of specific pollutants. Additionally, the types of organisms present, such as plants or animals, along with human activities, can also affect bacterial abundance (Nathan et al., 2020).

Diversity indices provided a comprehensive view of microbial community structure and how it varies across different sampling stations. Differences in alpha diversity between mangrove stations are due to varying environmental conditions and anthropogenic impacts, such as pollution, land-use changes, nutrient influx and altered salinity (Muwawa et al., 2024).

Chao 1 (9659) and observed features (9659) were found highest at Kadalundi and lowest at Edat (2043 and 1254, respectively). The Chao1 index estimates the species richness (the total number of different species present) in a microbial community (Faith, 1992) and suggests that Kadalundi possesses higher species richness. The number of "observed features" indicates the number of unique microbial

taxa detected in the given sample. This metric serves as a direct measure of the species richness within that specific microbial community. Kadalundi possesses a greater variety of different types of microbes, while a lower number at Edat indicates a less diverse community. The ecosystem with higher species richness and unique taxa is due to higher habitat heterogeneity, offering a greater variety of microhabitats, resources and niches for different species to specialize and thrive (De la Torriente et al., 2020).

Pielou's evenness (0.9) was found to be highest at Chettuva and Elathur and lowest at Edat (0.76) in the current study. Pielou's evenness indicates how equally distributed the microbial abundances are within a community, with a high value signifying a balanced distribution where no single species dominates and a low value suggesting some species are far more abundant than others (Pielou, 1975). The Chettuva and Elathur habitats show higher Pielou's evenness because their species exhibit more equal abundances, meaning there isn't one dominant species and many rare ones. This occurs in diverse or less stable environments with abundant resources, allowing multiple competing species with different traits to coexist, or in highly productive and resource-rich areas.

The Shannon index was found to be highest at Kadalundi (11.6) and lowest at Edat (7.6). The Shannon index indicates both the number of different microbial taxa (richness) and the evenness of their distribution within a sample, reflecting overall community diversity (Shannon, 1948). Kadalundi with greater diversity suggests that there are more unique species and their relative abundance is more uniform.

The Simpson index was found to be highest at Chettuva, Kadalundi, Ponnani and Valapattanam (1) and lowest at Edat (0.96). The Simpson Index indicates the probability that two randomly selected individuals from a community belong to the same taxa, with higher index values signifying lower diversity and greater dominance by a few abundant taxa. A smaller Simpson index suggests higher diversity and more even distribution of taxa within a sample (Simpson, 1949), suggesting that the habitat showing the higher Simpson index likely has a smaller variety of species compared to other habitats, with a few species being much more numerous than others.

In beta diversity analysis using Bray-Curtis distance, higher distance was found between Ponnani and Edat (0.967) and the lowest between Chettuva and Valapattanam

(0.788). The Bray-Curtis distance quantifies the difference in microbial community composition and abundance between two samples. Therefore, lower Bray-Curtis distances signify greater similarity between the communities, and higher distances indicate greater dissimilarity (Calle, 2019). A higher Jaccard distance was found between Kadalundi and Edat (0.962), indicating a lower similarity, and the lowest between Valapattanam and Chettuva (0.758), suggesting the highest similarity. Jaccard distance indicates the dissimilarity in the presence or absence of microbial taxa between two samples, measuring the difference in community composition. A Jaccard distance of 0 means the two samples share the exact same taxa, while a distance of 1 means they have no taxa in common.

Differences in Bray-Curtis and Jaccard distances between mangrove stations are due to varying environmental conditions and anthropogenic influences, which lead to differences in species composition and abundance, and they indicate distinct ecological characteristics, community structure, and potential stress levels at each site. The Bray-Curtis index specifically reflects both species presence/absence and their relative abundance, while the Jaccard index focuses solely on presence/absence, meaning Bray-Curtis will be more sensitive to changes in the dominant species (Calle, 2019).

Using metagenomic analysis, a total of 45 phyla, 121 classes, 308 orders, 507 families, 1036 genera and 1834 species were obtained in the current study. The most dominant phylum reported was Proteobacteria (57%), followed by Bacteroidota (8.5%), Actinobacteriota (8%), and Firmicutes (7%). The heat map revealed that all the mangrove stations had a predominant presence of the phyla Proteobacteria, Actinobacteriota, Firmicutes, Bacteroidota, and Chloroflexi.

Phylum Proteobacteria shows the highest prevalence and abundance in all the sampling stations, suggesting that it is a dominant and consistent member of the Northern Kerala mangrove microbiome. Earlier studies support these findings, with a predominant presence of this phylum being recorded inside Kerala (Chithira et al., 2021; Kutty et al., 2023). Outside Kerala, the studies of mangroves in Brazil (Alzubaidy et al., 2016), Bangladesh (India) (Basak et al., 2016), Goa (India) (Haldar and Nazareth, 2018), China (He et al., 2025), and Malaysia (Lai et al., 2025) have

consistently reported the dominance of Proteobacteria, suggesting a universal dominance of Proteobacteria in mangrove microbiomes. Mendes and Tsai (2014) demonstrated a consistent prevalence of Proteobacteria in Brazilian mangroves, regardless of soil depth. Proteobacteria comprises a large and diverse group of bacteria with gram-negative cell walls. Previous research has indicated that this phylum includes various types of bacteria, such as nitrogen-fixing, ammonia-oxidizing, cellulose-decomposing, sulfate-reducing, nitrifying, denitrifying and ammonia-oxidizing. Members of this phylum exhibit remarkable metabolic diversity, encompassing chemoautotrophic, chemolithotrophic, chemoorganotrophic, and phototrophic types, which account for the majority of bacteria recognized for their medical, industrial, and agricultural importance. The majority of phylum members are either facultative or obligate anaerobes, comprising over 460 genera and more than 2000 species distributed across five classes: Alpha, Beta, Gamma, Delta, and Epsilon (Marin, 2023).

The members of Proteobacteria exhibit metabolic diversity and are present in a broad spectrum of ecosystems because they possess complex groups of genes that are accountable for stress resistance, thereby improving their adaptive abilities and survival, which are the characteristics of dominant groups. The physiological adaptations include the ability of sulphur reduction, which helps in utilizing them as energy sources, switching to anaerobic respiration, using alternative electron acceptors (e.g., nitrate, sulphate); denitrification capability, production of various exopolymers, versatile metabolic pathways, allowing them to utilize various carbon sources; production of antioxidant enzymes; mitigating oxidative stress; and heavy metal resistance, which protects them from harsh environmental conditions in mangrove ecosystem (Puthusseri et al., 2021).

In the present study, the relative abundance of Proteobacteria was found highest at Valapattanam (25%), followed by Kadalundi (24%) and Kasaragod (20%). The presence of Proteobacteria in soil typically signifies a high degree of nutrient cycling, especially concerning carbon, nitrogen and sulphur. The variations in soil Proteobacteria are primarily linked to the nutrient status of soil. Similarly, various studies have noted that the quantity of soil Proteobacteria was notably affected by the physiochemical characteristics of the soil (Khan et al., 2021).

The second most prevalent phylum in the current study, Bacteroidota represents a versatile group of bacteria occupying distinct ecological niches. Bacteroides is a significant bacterial phyla, which corroborates the mangrove data generated globally (Kutty et al., 2023; Alsharif et al., 2024). In the current study, the relative abundance of Bacteroidota was found to be highest at Kasaragod (31%), followed by Valapattanam (28%) and Kadalundi (16%), and the least was observed at Edat (1%). The occurrence of Bacteroidota in soil typically signifies a healthy soil ecosystem. Their prevalence can serve as a measure of soil quality and its responsiveness to agricultural methods (Das et al., 2025).

The third most prevalent phylum identified in this study was Actinobacteriota, which consists of gram-positive bacteria characterized by a high guanine plus cytosine (G + C) content in their DNA and is a significant part of the bacterial diversity, which corroborates mangrove data generated globally (Chithira et al., 2021; Kutty et al., 2023; Ghose et al., 2024). Members of the phylum actinobacteria are diverse and widely distributed in terrestrial and aquatic ecosystems. In the current study, the relative abundance of the phylum Actinobacteriota was highest at Valapattanam (25%), followed by Chettuva (24%) and Ponnani (13%). The presence of Actinobacteria in soil generally indicates a healthy and active soil ecosystem. The prevalence of Actinobacteriota in mangrove sediments is shaped by a complex interaction of both abiotic and biotic elements. Abiotic elements include salinity, the composition of sediments (notably organic matter and metal content), and the availability of nutrients. Biotic elements involve interactions with other microorganisms as well as the presence of particular mangrove species and their root systems (Lai et al., 2025).

The fourth most dominant phylum in the current study was Firmicutes, which is a ubiquitous phylum of gram-positive bacteria, characterized by a low GC content in their DNA (Younas, 2024). Previous studies showed a predominance of Firmicutes in the mangrove stations of Kannur (Kerala) (Kutty et al., 2023), Panangad (Kerala) (John et al., 2024), and China (Tong et al., 2019). In the current study, relative abundance of Firmicutes was highest at Valapattanam (27%), followed by Kadalundi (26.6%) and Kasaragod (17%). The presence of Firmicutes in soil generally indicates active decomposition of organic matter, potential bioremediation of pollutants, and involvement in nutrient cycling. Gupta et al. (2018) reported an abundance of

Proteobacteria and a striking decrease in Firmicutes. The prevalence of Firmicutes in mangrove sediments is affected by several factors, including sediment characteristics (such as salinity, pH, and nutrient levels), tidal patterns, depth, and biological factors like the presence of mangrove trees and other organisms (Liu et al., 2024).

The current study interestingly observed that certain bacterial phyla are restricted to specific mangrove habitats, such as DTB120, Hydrogenedentes, Synergistota, and WS2 at Valapattanam and WS4, TA06, Elusimicrobiota, Dependentiae at Kadalundi. Phyla Synergistota, DTB120, WS2, WS4, Elusimicrobiota and Dependentiae have not been reported previously from mangrove habitat. DTB120 is an uncharacterized phylum showing nitrate reduction and iron oxidation capabilities and has been reported from hydrothermal vents (McAllister et al., 2021). Hydrogenedentes is a candidate phylum and is involved in sulphur, nitrogen, and carbon cycling, and it is adapted to survive in the dark, anoxic, subsurface regions, as reported by Momper et al. (2018) and it has been reported previously from mangrove ecosystems located in China (Lin et al., 2019). Synergistota are anaerobic bacteria that thrive in various anoxic environments and perform anaerobic digestion, carbohydrate and protein breakdown, impacting biogas production and waste treatment and have been isolated from wastewater sludge. WS2 is a candidate phylum, possesses novel metabolic pathways, potentially having the ability to degrade complex organic compounds or participate in nutrient cycling, and is detected in various environments, including soil, aquatic systems, and possibly in association with plants or animals. This widespread distribution implies that these microorganisms play major roles in diverse ecosystems, though their specific ecological functions remain to be fully elucidated (Pedrinho et al., 2024).

The phylum Elusimicrobiota (formerly known as "Termite Group 1") plays significant roles in various ecosystems, particularly as symbionts in the guts of termites and other insects. They are also found in diverse environments like soils, marine sediments and sewage sludge. Elusimicrobiota are involved in the breakdown of complex carbohydrates, such as cellulose and xylan, and some strains can fix nitrogen (Mies et al., 2025). The phylum Dependentiae is a group of bacteria characterized by their obligate intracellular lifestyle and close association with protists. They are part of the "Microbial Dark Matter," a vast collection of bacteria not yet

cultivated, and are found in diverse environments like freshwater sediments and soil (Weisse et al., 2023). WS4, or Wurtsmith 4, is a candidate phylum found in various environments, including soil and aquatic ecosystems, and they are believed to play roles in the decomposition of organic matter and the cycling of nutrients, although their exact metabolic capabilities and ecological niches are not fully understood (Albers et al., 2018). Bacteria of candidate phylum TA06 generally inhabit anoxic sediments in hydrothermal vents, cold seeps, and methane hydrate-bearing subseafloor sediments worldwide and perform a role in carbohydrate metabolism and the production of hydrogen (Huang et al., 2019). TA 06 was reported from a pristine mangrove habitat by Torres et al. (2019) and the least described.

Phylum Proteobacteria, Bacteroidota, Actinobacteriota, Firmicutes, Chloroflexi, Desulfobacterota, Planctomycetota, Campilobacterota, Acidobacteriota, Nitrospirota, Myxococcota, Gemmatimonadota, NB1-j, MBNT15 and Verrucomicrobiota were reported from all the sampling stations. The phylum Desulfobacterota plays a vital role in the cycling of sulphur and carbon in various anaerobic environments. They are known for their sulphate-reducing capabilities and are also involved in other processes like mercury methylation and detoxification, iron reduction, and extracellular electron transfer (Murphy et al., 2021). Planctomycetota plays significant roles in various ecosystems by contributing to carbon and nitrogen cycles, particularly through anaerobic ammonium oxidation and the degradation of organic matter (Wang et al., 2024). Campilobacterota are key players in sulphur cycling, acting as primary producers through chemolithoautotrophy, and also participate in symbiotic relationships with various organisms (Wang et al., 2023). Acidobacteriota contribute to biogeochemical cycles, degrade organic matter, and potentially influence plant growth (Kalam et al., 2020).

Nitrospira plays a vital role in the nitrogen cycle (nitrification) (Edwards et al., 2013). Myxococcota plays a significant ecological role as microbial predators and producers of bioactive compounds and helps regulate microbial community structures and nutrient cycling (Zou et al., 2024). Gemmatimonadota plays diverse roles in various ecosystems, including soil, polar regions, wastewater treatment, marine environments and extreme environments. They are known for their involvement in biogeochemical cycling of carbon, nitrogen and sulphur, as well as their potential for

producing secondary metabolites (Mujakic et al., 2022). NB1-J lineage (within Deltaproteobacteria) is involved in nutrient cycling (Waite et al., 2020). The MBNT15 (Deferrimicrobiota) plays a role in complex organic matter breakdown, aerobic respiration, iron reduction and reducing nitrogen and sulphur compounds (Begmatov et al., 2022). The existence of these ecologically significant bacteria in mangrove ecosystems offers valuable information regarding the health, productivity, and resilience of these distinctive environments. These bacteria are essential for nutrient cycling, carbon sequestration, and sustaining the overall equilibrium of the mangrove ecosystem, indicating that the mangrove habitat is a vibrant and healthy ecosystem.

In the current study, Class Gammaproteobacteria (40%) was the most abundant. followed by Alphaproteobacteria (16%). Gammaproteobacteria is a class of gram-negative bacteria within the phylum Proteobacteria, characterized by a diverse range of genera (~250) (Sharma et al., 2022). Predominance of Gammaproteobacteria in mangrove sediment was found in previous studies inside Kerala which includes Kannur, Kerala (Imchen et al., 2017; Kutty et al., 2023); Puthuvype, Kerala (Nathan et al., 2020); and outside Kerala which includes, China (Jiang et al., 2013); Saudi Arabia (Ullah et al., 2017); Goa, India (Haldar and Nazareth, 2018); and Sundarban, India (Ghosh and Mitra, 2025). Earlier research suggests that members of Gammaproteobacteria perform a critical role in nitrogen oxidative pathways, the carbon cycle, organic matter degradation, and sulphur oxidation (existing either freely or as chemosynthetic symbionts) (Li et al., 2012). In the current study, relative abundance of Gammaproteobacteria was highest at Valapattanam (27%), followed by Kadalundi (26%) and Kasaragod (17%). Gammaproteobacteria are identified as potential health indicators of ecosystems and reported to be dominant in uncontaminated mangrove sediment. Gammaproteobacteria are the core bacteria with the highest colonization potential in the guts of soil invertebrates, and it was identified that they were the best indicators of soil pollution. Gammaproteobacteria also closely correlated with the abundance of antibiotic resistance genes (Zhang et al., 2021).

In the current study, the order Oceanospirillales (11%) was the most abundant. Oceanospirillales (11%) is an order of gram-negative bacteria within the Gamma-proteobacteria, comprising strictly aerobic marine obligate hydrocarbonoclastic bacteria that utilize hydrocarbons as their primary carbon source (Garrity et al., 2005).

Predominance of Oceanospirillales was reported from mangrove habitats of Sundarban, India (Ghosh and Bhadury, 2018), and Goa, India (Haldar and Nazareth, 2018). In the current study, relative abundance was found to be highest at Kadalundi (42%), followed by Kasaragod (18%) and Valapattanam (17%). Members of this bacterial order play a significant role in mangrove ecosystems, primarily through their involvement in nutrient cycling and pollutant biodegradation (crude oil and other organic pollutants), and are seen abundantly in hydrocarbon-contaminated mangrove sediment (Naether et al., 2013).

In the current study the family Shewanellaceae (5.48%) was the most predominant, followed by Alcanivoracaceae (5.45%). The family Shewanellaceae (5.48%) within the order Alteromonadales are gram-negative, facultatively anaerobic bacteria widely distributed in nature; they are found in marine environments, marine organisms, deep sea, iced fish and proteinaceous foods (Alsharif et al., 2024). It presently consists of three genera, Shewanella, Parashewanella and Psychrobium, the latter being represented by a single species. Since the second half of the 1990s, the number of novel species in the Shewanellaceae has steadily increased, suggesting that the diversity of this family has only started to emerge. Earlier works at mangrove habitats reported the presence of Shewanellaceae from mangroves in Kerala, India (Imchen et al., 2017); Goa, India (Ghosh et al., 2022); and the Red Sea (Alsharif et al., 2024). The majority of Shewanellaceae species are psychrophilic and halophilic, capable of thriving at 4°C. They produce polyunsaturated fatty acids in the phospholipids of their cytoplasmic membranes, and certain members exhibit distinctive metabolic traits. Consequently, these bacteria are considered for use in microbial fuel cells due to their capacity to generate electricity (Satomi, 2014). In the current study, abundance was found maximum at Edat (37%), followed by Kasaragod (28%) and Kadalundi (22%). Shewanellaceae members are reported to play a role in organic matter cycling, biogeochemical cycling, biomineralization and oil-polluted mangrove habitats, and their abundance can be influenced by urbanization and other environmental factors.

The predominant genus found in the current study was Shewanella (5.47%), followed by Alcanivorax (5.45%). Genus Shewanella is ubiquitous in natural environments, occurring mainly in marine and freshwater environments, composing a

diverse group of facultative anaerobic bacteria, and comprising of about 70 species of bacteria with versatile capabilities (Ikeda et al., 2021). The distinct feature of many Shewanellae is the ability to utilize a wide array of final electron acceptors (such as oxygen, nitrate, sulphur compounds, metals, and organics) in the absence of oxygen, and this property allows them to survive in diverse habitats.

Earlier studies reported the presence of Shewanella from mangrove habitats at Kannur, Kerala (Imchen et al., 2017; Kutty et al., 2023); Qatar (Farhat et al., 2023); China (Liu et al., 2024); Red Sea (Alsharif et al., 2024); and Goa, India (Ghosh et al., 2022). Shewanella exhibit a wide range of physiological diversity, enabling them to thrive in extreme and harsh environments. Due to their diverse metabolic functions, Shewanellae are important in carbon cycling, iron reduction, biomineralization, bioremediation of contaminated environments, and are used in microbial fuel cells (Farhat et al., 2023). In the present study, abundance of Shewanella was highest at Edat (37%), followed by Kasaragod (28%) and Kadalundi (22%). The prevalence of Shewanella in mangrove ecosystems is shaped by a mix of physical, chemical and biological elements. These factors include salinity, nutrient levels, tidal changes, sediment properties and the availability of organic matter. Furthermore, anthropogenic factors such as pollution and changes to the habitat can greatly affect Shewanella populations (Ellison, 2021).

The second most abundant genus in the study, Alcanivorax, are well-known hydrocarbon degraders such as benzene, chlorobenzene and toluene, particularly under saline conditions, demonstrating potential for bioremediation of hydrocarbon-contaminated saline water. They have been reported to be present in several hydrothermal plumes (Dede et al., 2023). Earlier studies reported the presence of Alcanivorax from mangrove habitats in Brazil (Brito et al., 2006), the Red Sea (Alsharif et al., 2024), and Qatar (Siddique et al., 2024), mainly suffering from oil pollution. In the present study, the genus Alcanivorax was found to be most abundant at Kadalundi (63%), followed by Kasaragod (10%), and least abundant at Edat (0.02%); this is also the first report of Alcanivorax from Kerala, India. Alcanivorax plays important roles not only in the marine hydrocarbon degradation but also biodegradation and transformation of synthetic plastic waste. Abundance of

Alcanivorax bacteria can be an indicator of alkane contamination in marine environments (Dong et al., 2024).

In the current study the predominant and identified bacterial species were Alcanivorax sp. and Shewanella colwelliana. The abundant species in the present study, Alcanivorax sp., is an important member of the hydrocarbonoclastic group, which belongs to the class Gammaproteobacteria and is known for its ability to degrade hydrocarbons (sole source of carbon and energy) in marine habitats and is useful in bioremediation and biofuel production. Alcanivorax sp. are reported to be psychrotolerant and biosurfactant producers, capable of producing alkane monooxygenases, and they possess cytochrome P450 genes that enable them to withstand harsh conditions. Additionally, they are regarded as degraders of polystyrene and acetaldehyde (Cappello et al., 2022). Studies regarding the occurrence and ecology of Alcanivorax sp. from mangrove habitat are rare, but they have been reported from mangrove habitat from Brazil (Santos et al., 2011), the Red Sea (Alsharif et al., 2024), and Qatar (Siddique et al., 2024). The present study is the first report of Alcanivorax sp. from Kerala, India. The abundance of species was found to be high at Kadalundi (64%), followed by Chettuva (13%) and Kasaragod (13%).

In the present study the second most abundant bacterial species is *Shewanella colwelliana,* which is a facultative anaerobic, mesophilic, H_2S-producing, surface-adhering, psychrotrophic (4°C to 30°C), gram-negative marine bacterium under the family Shewanellaceae, naturally found in coastal environments and has been isolated from various sources, including seawater, sediment, and marine animals, and is noted for its production of pigments like melanin (Fuqua and Weiner, 1993). The present study reported a high abundance of *Shewanella colwelliana* from Edat (65%), followed by Kadalundi (33%). Members were shown to have a range of metabolic capabilities, including the ability to reduce iron and manganese oxides, production of an exopolysaccharide (adhesive under aqueous conditions) and bioactive compounds (antibiotics and antimicrobial peptides), degradation of pollutants (heavy metals and polycyclic aromatic hydrocarbons), and important reservoirs for antibiotic resistance genes (ARGs) (Sher et al., 2025). S. colwelliana is a versatile and biotechnologically important bacterium that has a range of applications in fields such as bioremediation, bioactive compound production and marine biotechnology.

The bacterial communities identified in this study are reported to have significant roles in ecosystems, with the bacteria commonly found in a specific environment often carrying out vital ecological functions. These functions include decomposition, nutrient cycling, and the maintenance of ecosystem balance. The presence and quantity of particular bacteria, along with the roles they fulfill, can reflect the characteristics of the habitat they occupy.

In the functional annotation, genes associated with metabolism of biomolecules were found predominant (68%), followed by other metabolic functions (20%), environmental functions (11%), and stress response (1%), indicating a versatile and biochemically active microbiome. Earlier reports from various mangrove habitats from Kannur, Kerala (Imchen et al., 2017; Kutty et al., 2023); Sundarban, India (Ghosh et al., 2022); and Brazil (de Carvalho et al., 2024) support the present study. Dominance of bacterial genes related to the synthesis and degradation of biomolecules in mangrove ecosystems suggests a significant degree of microbial activity and nutrient cycling. These bacteria are vital for breaking down organic matter, releasing key nutrients, and enhancing the overall health and stability of the mangrove environment.

Considering the biomolecule metabolism, amino acid metabolism was found to be predominant (22%), followed by nucleic acid metabolism (11%). Both the synthesis and degradation pathways of amino acids were found to be high at Valapattanam, followed by Kadalundi and Kasaragod in the present study. Mangrove sediments can be considered large reservoirs of amino acids (Gaye et al., 2024). Jayan and Chandramohanakumar (2015) found that amino acids in Kerala's mangrove sediments are key indicators of organic matter diagenesis, varying with depth and season. The presence of specific amino acids is influenced by protein breakdown by bacteria, highlighting the importance of decomposition in the presence of specific compounds.

The genes for polyamine biosynthesis (Norspermidine), mycothiol biosynthesis, and methylglycoxal degradation play a crucial role in stress response by influencing various cellular processes and contributing to stress tolerance. Norspermidine acts as a signaling molecule, influencing biofilm development (Wotanis et al., 2017). Mycothiol maintains redox balance and protection against oxidative stress (Newton et al., 2008). Methylglyoxal (MGO) pathways act as a detoxification system (by-product of glycolysis) (Ferguson et al., 1998).

The current study annotated the genes responsible for different antibiotic production pathways, including cycloserine, novobiocin, daunorubin, erythromycin, penicillin, cephalosporin, cephamycin, megalomicin, fosfomycin, tylosin, aclacinomycin, streptomycin, and paromamine. Earlier studies reported genes responsible for the production of antibiotics from mangrove habitats in India (Ghosh et al., 2022) and Indonesia (Setyati et al., 2021). Antibiotics are antagonistic compounds, and the competitive environment within mangroves, where bacteria constantly battle for resources, drives the production of these bioactive compounds. Mangrove habitat with high production of antibiotic-producing bacteria is due to the unique and often stressful environmental conditions. This makes them a promising source for discovering novel antibiotics and understanding microbial interactions (Ghosh et al., 2022).

In the functional annotation analysis, genes responsible for synthesis of some important compounds was found, which includes, mycolate (contribute to the bacterial resistance to antibiotics), chorismate (synthesis of various essential aromatic compounds), queuosine (biofilm formation and bacterial virulence), PreQo (crucial for growth and survival), taxadiene (bioactive molecule), PPGPP (regulating the stringent response), ectoine (protects from harsh environments), enterobactin (siderophore), butanediol (stress response signalling molecule), adenosylcobalamin (a form of vitamin B12), mycothiol (cellular protectant against oxidative stress), clavulanate (inhibit bacterial β-lactamases), and acetylneuraminate (influence bacterial interactions with hosts and other microbes) (Abdallah et al., 2019; Kadam et al., 2024).

The presence of bacterial stress response and defence genes in the mangrove habitat indicates that the bacteria present are facing challenging or stressful environmental conditions. These genes are activated to help the bacteria survive, adapt, and potentially thrive in those conditions.

In the current study, genes responsible for several degradative pathways, including xenobiotics (includes protocatechate, catechol, phenyl acetate, phenyl propanoate, cymene, cumate, hydroxy phenylacetate, vanillin, aminophenol, mandelate, cinnamate, nicotinate, coumarate, hydroxy acetophenone, etc.) and recalcitrant compounds (includes atrazine, biphenyl, benzoyl CoA, chlorosalicylate,

nitrobenzoate, toluene, etc.), were identified. Recalcitrant compounds are substances that are highly resistant to degradation, particularly by microbial activity. They persist in the environment for extended periods, posing challenges for environmental remediation. Xenobiotics and recalcitrant compounds enter mangrove ecosystems from various sources, including industrial and urban areas. These sources include wastewater discharge, industrial effluents, stormwater runoff, and leaching from agricultural lands. Additionally, activities like shipping and fishing in coastal areas can also introduce contaminants (Balasundaram et al., 2022).

The genes for degradation of several recalcitrant compounds such as toluene, 2-nitrobenzoate, chlorosalicylate, benzoyl CoA, biphenyl, naphthalene and atrazine were identified in the current study, which suggests the bacterial potential for bioremediation of pollutants in mangrove environments. The release of various xenobiotic and recalcitrant organic pollutants in the environment from various anthropogenic sources has caused adverse effects on mangrove habitat.

Toluene, a common environmental pollutant, can be degraded by various bacteria under both aerobic and anaerobic conditions. Many bacteria, including Acinetobacter junii, Thauera sp., and Pseudomonas sp., have been identified as capable of utilizing toluene as a carbon and energy source, and studies report the presence of toluene degraders from mangrove habitats. Anthropogenically, toluene can enter the environment through industrial activities, including the leather processing, mining, thermal power plants, and metal-based engineering sectors, often carried by rivers into mangrove ecosystems (Mahanty et al., 2024).

Nitrobenzoates (NBs), including 2-nitrobenzoate (2NB), are used in the synthesis of chemicals, pharmaceuticals and dyes. These compounds are toxic to living beings because of their genotoxicity, mutagenicity, and hematologic toxicity (Ju and Parales, 2010). Earlier studies report the presence of toluene degraders from mangrove habitat (Ramos-Mendoza et al., 2024). Chlorosalicylates are often found in industrial wastewater and sewage effluents. Excessive levels of chlorosalicylate can lead to soil acidification, reduce nutrient availability, and potentially harm soil microbes and plant growth (Nikodem et al., 2003).

Benzoyl CoA, a universal biomarker for anaerobic degradation of aromatic compounds. The anaerobic benzoyl-CoA pathway is ubiquitous in the environment and plays a key role in anaerobic aromatic transformations and carbon cycling. Earlier studies have reported the presence of benzoyl CoA degraders from mangrove habitats (Porter and Young, 2014).

Naphthalene soil is a hazardous polycyclic aromatic hydrocarbon (PAH) compound that is being released into the environment, especially following the age of industrialization. Ability to degrade naphthalene indicates the capability of a microorganism to carry out bioremediation involving more complex compounds. Earlier studies have reported the presence of naphthalene degraders from mangrove habitats (Hamid et al., 2025).

Atrazine is a herbicide identified in soil and water due to its long half-life, moderate persistence, high mobility, and solubility that can be influenced by many factors. The accumulation of atrazine in marine sediments leads to persistent contamination, which may damage the succeeding submerged plants and create potential threats to the environment. Earlier studies have reported the presence of atrazine degraders from mangrove habitat (Soni et al., 2025).

The present study reported the presence of a hydrocarbon degradation pathway (octane). Octane, a linear hydrocarbon and component of gasoline and crude oil. Hydrocarbon-degrading bacteria play a crucial role in bioremediation, particularly in cleaning up oil spills. Pseudomonas sp. is reported to have the capability of octane degradation (Duque et al., 2022).

Genes responsible for the degradation of some important compounds were identified in the present study, which includes caprolactam, chitin, pectin, xylose, phenylpropanoid, formaldehyde, catechol, vanillin, phenyl acetate, mannan, 3-phenyl propanoate, syringate, cumate, cymene, 4- deoxy-L- threo-hex-4-enopyranuronate, and acetylene. Caprolactam, a monomer of nylon 6, can contaminate soil and water, posing risks to aquatic life and potentially impacting human health. Bacteria capable of degrading caprolactam, such as those from the genera Arthrobacter, Achromobacter, and Brevibacterium, play a crucial role in bioremediation, breaking down caprolactam into less harmful compounds (Baxi, 2013). Formaldehyde-degrading bacteria and

acetylene-degrading bacteria (acetylenotrophs) convert compounds into less harmful substances, contributing to natural detoxification processes in various environments (Ruan et al., 2024). Cumate, phenyl acetate, and 3-phenylpropionate are chemical compounds often found in polluted environments (Aziz et al., 2024).

The presence of various bacterial pathways for degrading pollutants in mangrove ecosystems highlights significant microbial activity and adaptability, emphasizing their crucial role in nutrient cycling and bioremediation. These pathways, which target hydrocarbons, xenobiotics and plant-derived compounds, demonstrate the ability of mangrove bacteria to break down diverse organic matter and pollutants, thus improving ecosystem health and resilience.

In the current study bacteria that play important roles in sulphur cycle, nitrogen cycle, iron cycle and methanogenesis were found, highlighting the significant contribution of microbial communities in maintaining the health and function of these vital coastal ecosystems.

The microbial-driven sulphur cycle is an essential biogeochemical process within the sulphur-rich mangrove ecosystem. About twenty different types of bacterial genera were reported as SOBs and SRBs from the northern Kerala mangrove habitat. Temperature, redox potential, and sulphates were the significant factors influencing the microbial communities, in mangrove sediments (Li et al., 2021).

Sulphur-oxidizing bacteria (SOB) convert substances like hydrogen sulfide and elemental sulphur into sulphate, which can be used by plants. Oxidation of the sulphide by sulphur-oxidizing bacteria (includes photoautotrophs, chemolithotrophs and heterotrophs) shows major role in sulphur cycle (Li et al., 2021). The genus Thiomicrospira (27%) is reported as the most abundant SOB in the current study, followed by Sulfurimonas (18%). Thiomicrospira were found highest at Valapattanam (82%) in the current study. Sulfurimonas are chemolithoautotrophs, obtaining energy from oxidizing inorganic substances like sulphide, thiosulphate, and elemental sulphur, and use carbon dioxide as their carbon source and they have been reported from various mangrove habitats (Cui et al., 2025). Sulfurimonas were found highest at Kadalundi (90%) in the current study.

Sulphur-reducing bacteria (SRB) are a diverse group of microorganisms that thrive in anaerobic environments and use sulphate as a terminal electron acceptor, reducing it to hydrogen sulfide. They play a significant role in the sulphur cycle and can impact various industrial processes due to their ability to cause corrosion and fouling (Widdel and Hansen, 1992). In the current study, the genus Desulfatiglans (28%) was found to be the predominant SRB, followed by Desulfopila (23%), and both were found highest at Kadalundi (75%).

Desulfatiglans utilize various aromatic compounds like phenol and benzoate as electron donors in the process of sulphate reduction. They are commonly found in anaerobic environments such as river sediments and marine subsurface sediments and in mangrove habitats (Li et al., 2021). Desulfopila is a strictly anaerobic, mesophilic, sulphate-reducing bacterial strain, particularly in anaerobic environments like marine sediments, oil fields, wastewater treatment plants, and various mangrove habitats (Isaza et al., 2021).

Nitrogen-fixing bacteria, ammonifying bacteria, nitrifying bacteria, and denitrifying bacteria were identified in the current study. Ten different nitrogen-fixing bacterial genera, five different ammonifying bacterial genera, seven different nitrifying bacterial genera and six different denitrifying bacterial genera were identified. The nitrogen cycle is vital for all life on earth, playing a key role in ecosystem health and sustainability. It ensures a continuous supply of nitrogen, a crucial component of DNA, RNA, proteins, and chlorophyll, which are essential for plant growth, animal development, and overall ecosystem function (Bernhard, 2010). Nitrogen-fixing bacteria, also known as diazotrophs, play a crucial role in mangrove ecosystems by converting atmospheric nitrogen into usable forms for plant growth. These bacteria can be found in various locations within the mangrove, including the rhizosphere (soil around the roots), within the roots themselves, and even in the sediment. Their activity is vital for maintaining the health and productivity of these coastal environments and is regulated by the nif regulon, which is a set of seven operons (Usman and Wali, 2024). Abundance of genus Bradyrhizobium (32%) is reported as the highest in the present study, followed by Mesorhizobium (23%).

Bradyrhizobium were found highest at Kadalundi (29%), and Mesorhizobium at Pazhayangadi (66%). Bradyrhizobium are common soil-dwelling bacteria that can form symbiotic relationships with leguminous plant species, where they fix nitrogen. Studies have reported Bradyrhizobium as PGPR and can positively affect symbiotic nitrogen fixation by enhancing both root nodule number or mass, dry weight of nodules, yield components, grain yield, soil nutrient availability, and increasing the nitrogenase activity. It has been isolated from different mangrove habitats (Inoue et al., 2020).

Ammonifying bacteria are microorganisms that play a vital role in the nitrogen cycle. Ammonification is essential for recycling nitrogen in ecosystems with the help of genes encoding enzymes like proteases, hydratases, and nitrite reductases (NrfA). These bacteria break down organic matter, releasing ammonia, which can then be used by plants and other microorganisms (Zhao et al., 2015). Genus Pseudomonas (10%) was found in highest abundance among ammonifying bacteria in the current study. It was found highest at Kasaragod (40%). Previous studies have found that Pseudomonas sp. capable of dissimilatory nitrate reduction to ammonium (DNRA) under aerobic conditions (usually anaerobic) and have been reported as plant growth promoters with nitrogen-fixing and hydrocarbon degradation abilities from different mangrove habitats (Saha and Sen, 2024).

Nitrifying bacteria play a crucial role in the nitrogen cycle within mangrove habitats by facilitating the oxidation of ammonia to nitrite and then to nitrate. This process, known as nitrification, is vital for making nitrogen available for plant uptake (Fiencke et al., 2005). Nitrification in bacteria is a two-step process involving ammonia oxidation and nitrite oxidation. Genes responsible for these processes include amoA (or bamoA), nxrA, and nxrB. Ammonia-oxidizing bacteria (AOB) possess amoA for ammonia oxidation, while nitrite-oxidizing bacteria (NOB) contain nxrA and nxrB for nitrite oxidation (Medina et al., 2024). Nitrifying bacteria, together with denitrifying bacteria and anammox bacteria, play a crucial role in removing this excess nitrogen, reducing its potential negative impact on the ecosystem. This process is essential for providing mangrove plants with readily available nitrogen compounds for growth and for mitigating the effects of nitrogen pollution in these coastal environments.

Genus Nitrospira (55%) was found in highest abundance among nitrifying bacteria in the current study, followed by Idiomarina (22%) and Nitrosomonas (19%), and found highest at Kasaragod (40%). "Nitrospira" refers to a genus of bacteria that includes diverse species capable of nitrite oxidation and, in some cases, comammox (complete ammonia oxidation). These bacteria are found in various environments, including freshwater, soils and wastewater treatment plants, and they are characterized by their adaptation to oligotrophic conditions and isolation from various mangrove habitats (Mubaraq et al., 2024). Nitrifying Idiomarina and Nitrosomonas have been reported from mangrove habitats (Galisteo et al., 2024).

Denitrifying bacteria play a crucial role in mangrove ecosystems by removing excess nitrogen through a process called denitrification. This process converts nitrate and nitrite into nitrogen gas, which then returns to the atmosphere, preventing nutrient overload and promoting a balanced ecosystem. Genus Paracoccus (59%) was found in highest abundance among denitrifying bacteria in the current study, followed by Candidatus Solibacter (34%), and was found highest at Valapattanam (47%). Paracoccus is a facultative anaerobe with denitrification ability and has been isolated from different mangrove habitats (Yu et al., 2024). Denitrification in bacteria is a stepwise process where nitrate is converted to dinitrogen gas, with four key enzymes involved, each encoded by specific genes. These enzymes are nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos).

Methanogenesis, the microbial production of methane, is a significant process in mangrove habitats, playing a crucial role in the carbon cycle and influencing greenhouse gas emissions. While mangroves act as a sink for carbon dioxide, methanogenesis can offset some of these benefits by releasing methane, a potent greenhouse gas and organic matter decomposition, producing methane (Ulumuddin et al., 2023). In the current study eleven different bacterial genera were reported to have the ability for methanogenesis. Genus Methyloceanibacter (64%) was found in highest abundance among methanotrophs, followed by Methylophaga (23%) and the highest was found at Chettuva (33%). Methyloceanibacter is a moderately thermophilic, methanol-oxidizing bacterium isolated from different mangrove habitats (Yang et al., 2023). Methylophaga is a haloalkaliphilic restricted facultative methylotroph, isolated from different marine habitats, including mangroves (Das et al., 2024).

Eight different bacterial genera were found in the study reported to have the ability for iron reduction in the iron cycle. Iron-reducing bacteria play a crucial role in the mangrove ecosystem by facilitating nutrient cycling, particularly the transformation of iron and carbon. These bacteria help detoxify the environment by preventing the accumulation of toxic sulfide. In microbial iron cycling, genes responsible for iron reduction include those encoding c-type cytochromes, such as OmcS and OmcZ, and those involved in electron transport, like the MtrCAB operon (Cobacho et al., 2024).

Genus Desulfuromusa (23%) was found in highest abundance among iron-reducing bacteria, followed by Gallionella (20%), and the highest was found at Kadalundi (26%). Desulfuromusa can use iron (III) as a terminal electron acceptor in their energy metabolism, oxidizing organic compounds like acetate, lactate, or hydrogen, and is isolated from different marine habitats (Magnuson et al., 2023). Gallionella is a genus of iron-oxidizing bacteria that plays a significant role in iron redox cycling, particularly in freshwater and wetlands (Hribovsek et al., 2024).

The functional annotation studies indicated that the synthesis and degradation pathways of various significant compounds were frequently observed in Valapattanam, Kadalundi, and Kasaragod. This suggests that the habitat is abundant in metabolically active bacterial communities, which are also experiencing stress. The presence of bacteria involved in the nitrogen, iron, methanogenesis and sulphur cycles in mangrove ecosystems indicates a complex biogeochemical environment. These bacteria are crucial for nutrient cycling and ecosystem health. Their diversity highlights mangroves effectiveness in transforming essential nutrients, emphasizing their role in maintaining ecosystem stability.

Chapter 5

SCREENING FOR HYDROLYTIC ENZYME PRODUCTION

Chapter - 5

SCREENING FOR HYDROLYTIC ENZYME PRODUCTION

5.1 INTRODUCTION

The distinctive features of mangrove habitat stimulate microorganisms to develop distinctive adaptations in their ecology, morphology, biology and physiology. Bacteria in mangroves employ specialized metabolic pathways to synthesize vital macromolecules that enable their survival in this unique environment (Kachiprath et al., 2019).

Microorganisms play a vital role in regulating biogeochemical processes, enhancing production, and sustaining the equilibrium within the mangrove ecosystem (Alongi et al., 1993). The widespread presence of microorganisms illustrates their capacity to endure and flourish in various ecosystems. Their existence in extreme habitats that nearly reach the recognized thresholds of adaptable life underscores their significance in those ecosystems. Various microbial communities, often exhibiting significant extracellular enzymatic activities, inhabit microbial aggregates and are found throughout the environment. These remarkable abilities fuel our interest in comprehending the metabolites produced by microorganisms and the ways in which these substances engage with their surrounding materials (Bano et al., 2023).

Studies of these bacterial hydrolytic enzymes in mangrove ecosystems could lead to a better understanding of their ecological roles. Despite being the largest category of enzymes, hydrolytic enzymes are also the most commercially viable. Mangrove bacterial enzymes within this category have been the subject of extensive research (Gupta et al., 2002).

5.2 REVIEW OF LITERATURE

Studies about microbial enzymes are expanding rapidly, with investigations moving beyond typical terrestrial ecosystems to biodiverse environments that have minimal or no human presence. The microbes were found to produce exceptional

metabolites with heat tolerance, psychrotropic, halotolerance and resilience to environmental pressures that are typically intolerable for more common microorganisms (Seo, 2024). The cycling of organic matter was carried out through the production of extracellular enzymes by heterotrophic bacteria. Microbes primarily contribute to the industrial demand for various enzymes. Owing to their rapid growth rates, short life spans, and ease of genetic manipulation, microorganisms are favored in the industry for enzyme production (Rombouts and Pilnik, 1986).

Microbial enzymes serve as economical and sustainable catalysts that are essential to biochemical and metabolic processes. Enzymes derived from various microbial ecosystems exhibit a wide range of applications across multiple industries, such as agriculture, cosmetics, textiles and pharmaceuticals (Thulasisingh et al., 2024). The mangrove ecosystem is highly intricate in terms of its physicochemical and microbial characteristics, as the estuarine environment serves as a junction between marine waters and freshwater from rivers. Consequently, a wide array of physicochemical conditions and nutrient variations can be observed, influenced by seasonal changes and river water influx, where only a limited number of organisms can thrive, either as facultative or obligate forms. The distinctive ecosystem of mangroves, marked by extreme physicochemical conditions (Singh, 2020), encompasses high salinity, specific pH levels, elevated temperatures and low oxygen levels, which together foster a unique microbial community, including bacteria that produce various enzymes with potential applications in industry and medicine.

Bacterial enzymes are notable for their robustness, exhibiting thermostability, pH stability and high catalytic efficiency at diverse and often harsh conditions, which is highly advantageous for industrial applications. They also possess high substrate specificity (Nigam, 2013). Enzymes sourced from microorganisms are known to exhibit higher potency and durability compared to those obtained from plants and animals. Bacterial enzymes from mangrove habitats are known for their halotolerance and ability to degrade complex organic matter such as lignocellulose and often exhibit high activity and stability for various biotechnological applications in fields like detergents, textiles and waste treatment (Kharadi et al., 2019). These enzymes are diverse, including proteases, amylases, cellulases, lipases, and laccases, and their

functional capabilities are shaped by the unique, fluctuating, and nutrient-rich conditions of mangrove environments.

Mangrove ecosystems are enriched with a diverse range of complex carbon sources like starch (from leaves and plant detritus), proteins (from decaying organic matter), cellulose (from plant debris) and lipids (from plant tissue). This diverse range of complex organic compounds provides a rich food source for bacteria, stimulating their metabolism and promoting the production of various enzymes (Hu et al., 2024).

5.2.1 Protease

Protease plays a crucial role in the breakdown of organic nitrogen, and bacteria that produce it have been identified as the primary microflora responsible for regulating nitrogen levels in ecosystems. Microbial systems yield three types of proteases: acidic, neutral and alkaline (Vijayalakshmi et al., 2011). Among various proteases derived from different sources, microbial proteases are favoured for applications due to their rapid growth, effective production, extensive diversity, prolonged shelf life and the possibility of genetic manipulation of microorganisms, in contrast to plant or animal sources (Song et al., 2023). The protease enzyme, characterized by its proteolytic activity, has been identified in various bacterial isolates, including Bacilli, Pseudomonas and Clostridium.

Numerous studies from mangrove ecosystems have isolated protease-producing bacterial strains, including those by Kutty et al. (2020a) from Kadalundi, Kerala; Paul et al. (2020) from Northern Kerala; Varghese et al. (2020) from the Ayiramthengu mangrove ecosystem in Kerala; and Anand et al. (2025) from the coastal region of Kerala; Castro et al. (2014) from the Brazilian mangrove ecosystem; Ntabo et al. (2018) from Kenya; and Mamangkey et al. (2021) from a mangrove station in North Sumatra, Indonesia.

Previous studies have demonstrated that proteases are highly effective in deproteinization processes for the chitin derived from shrimp waste. The enzyme protease finds extensive applications in various industries, such as detergents, leather, textiles, food processing, pharmaceuticals, biotechnology, bioremediation, waste treatment sectors and medicine (Iber et al., 2022).

5.2.2 Lipase

Lipases are hydrolytic enzymes that break down lipids (fats) into fatty acids and glycerol and are also capable of synthesizing lipids in non-aqueous environments. They are ubiquitous across microorganisms, plants and animals and are characterized by a catalytic triad of serine, histidine and an acidic residue (aspartate/glutamate). Lipases exhibit interfacial activity. Bacterial lipases are characterized by their ability to hydrolyze and synthesize esters, their stability at extreme pH and temperature, their tolerance to organic solvents, and their selectivity for certain substrates and reactions. Bacterial lipases offer advantages over animal and plant sources due to their high yields, ease of genetic modification and consistent, rapid production (Ali et al., 2023).

Bacterial lipases in mangrove habitats are important because they are crucial for nutrient cycling through lipid breakdown and support the ecosystem's health by degrading detritus and maintaining the food web. Lipases are produced by diverse bacteria from mangrove habitats. *Bacillus* sp., *Staphylococcus* sp., and *Pseudomonas* sp. are the common lipase producers (Yao et al., 2021).

Bacterial lipases have diverse applications across industries, including food processing (flavor development, fat modification), detergents (stain removal), biodiesel production, pharmaceutical synthesis (creating specialty chemicals and resolving enantiomers), cosmetics and perfumery, the pulp and paper industry, agrochemicals, fine chemicals and bioremediation. Their ability to catalyze esterification and transesterification reactions makes them valuable, eco-friendly biocatalysts for modifying fats and oils in both aqueous and non-aqueous environments (Patel et al., 2019). Research in mangrove ecosystems is ongoing to discover novel lipases with unique and beneficial characteristics for various biotechnological uses.

5.2.3 Amylase

Amylases represent a category of hydrolytic enzymes that break down the glycosidic bonds in starch and similar polysaccharide compounds, resulting in the formation of simpler sugars like glucose, maltose and dextrin. These enzymes are crucial for their targeted application in the industrial process of starch conversion (My et al., 2022).

Numerous bacteria have been identified and recorded as amylase-producing organisms, including the genera Bacillus, Vibrio, Arthrobacter, Escherichia, Micrococcus, Proteus, Pseudomonas, Serratia and Streptomyces (Joshi et al., 2021). Klinfoong et al. (2022) isolated amylase-producing bacteria from mangrove soil in Thailand; Farshid and Faranak (2015) from mangrove forests in the south of Iran; Kutty et al. (2020a) from Kadalundi, Kerala; and Paul et al. (2020) from Northern Kerala. The amylases derived from *Bacillus paralicheniformis* have been demonstrated to be an exceptionally effective enzyme for digesting raw starch (Bozic et al. 2011). *Geobacillus* sp. isolated from the thermal waters of a geothermal spring showed the capability to produce highly thermostable alpha-amylase (Gurumurthy et al., 2012).

Microbial amylases are the most prevalent and preferred choice for industrial applications compared to other sources due to their benefits, including cost-effectiveness, high productivity, thermostability and ease of optimization (My et al. 2022). Bacterial amylases have diverse applications across various industries, including the production of high fructose corn syrup (HFCS) and other sweeteners, as a desizing agent in the textile industry to remove starch from fabrics, and in brewing and baking processes to aid in sugar fermentation and dough preparation. They are also used in the paper and detergent industries, for enzyme replacement therapy in patients with pancreatic insufficiency, and even in the treatment of bacterial infections by reducing biofilm formation (Saini et al., 2017).

5.2.4 Cellulase

Generally, cellulase consists of a mixture of three enzymes: exoglucanases, endoglucanases, and β-glucanases. Cellulolytic bacteria are capable of hydrolyzing cellulose into simple sugar molecules (Bamrungpanichtavorn et al., 2023).

Bacterial strains from genera such as Micrococcus, Bacillus, Pseudomonas, Xanthomonas, Vibrio and Brucella, which are found in mangrove ecosystems, possess the ability to hydrolyze cellulose (Behera et al., 2014). Previous studies have identified ninety-nine bacterial species from coastal mangrove sediment at Logending Beach, Kebumen, Indonesia, of which 88% exhibit cellulolytic activity (Pramono et al., 2021). In the mangrove ecosystem of Kuala Simbur village, three bacterial isolates exhibited the highest cellulolytic activity, which includes Bacillus, Cellulomonas and

Micrococcus (Batubara et al., 2022). From the mangrove soil located on the northern coast of Aceh Province, Indonesia, 22 out of 39 bacterial isolates displayed cellulase activity (Dewiyanti et al., 2023).

Cellulase enzymes rank as the third most significant enzymes for industrial applications. The thermophilic cellulase enzymes hydrolyze cellulose across different industries encompasses the generation of bioethanol and the creation of valuable organic compounds derived from renewable agricultural waste (Hardiman et al., 2010). Cellulases are enzymes that decompose processing waste from seaweeds and can function as biofertilizers. Cellulases from microbial origin are found to be superior to others due to their higher stability and performance, despite the substantial cost associated with enzyme production (Bamrungpanichtavorn et al., 2023).

5.2.5 Chitinase

Nature is abundant in chitin, a biopolymer that resembles cellulose. The ongoing shedding of marine zooplankton contributes to a considerable quantity of chitin in seawater. Chitinase enzyme hydrolyze chitin, which is a polymer of N-acetyl-D-glucosamine (Gonfa et al., 2023). Chitinases can be classified into two main types: endochitinases and exochitinases (Rishad et al., 2017).

Previous studies reported chitinolytic bacteria from mangrove habitats, including Rishad et al. (2017) from Valanthakad backwaters of Cochin, Kachiprath et al. (2019) from Malippuram, Ernakulam, Castro et al. (2014) from Brazilian mangrove forests and Li et al. (2020) from China. Chitinase producing bacteria were reported as *Vibrio* sp., *Bacillus* sp. and *Serratia* sp. (Gonfa et al., 2023).

Bacterial chitinases are valuable biocatalysts, used in diverse applications such as agriculture as a biopesticide, in waste management for breaking down organic waste and in the pharmaceutical industry for producing valuable chitin derivatives. Bacterial chitinases are often more stable and cost-effective compared to enzymes from plants or animals (Gonfa et al., 2023).

5.2.6 Laccase and Ligninase

Ligninase enzyme system includes three oxidative enzymes: lignin peroxidase, manganese peroxidase and laccase (Robinson and Nigam, 2008). Lignocellulose,

comprises cellulose, hemicellulose and lignin. It is also a major source of organic matter. These ligninolytic enzymes possess remarkable versatility and play a crucial role in breaking down various xenobiotic and recalcitrant polymers (Chowdhary et al., 2016).

One significant advantage of lignin-degrading enzymes from bacteria, compared to those from fungi, is that bacteria, especially *Escherichia coli*, are much easier to genetically engineer and modify (Chang et al., 2014). However, it is noteworthy that research on microbial ligninolytic enzymes has predominantly concentrated on fungal enzymes, with bacterial enzymes receiving comparatively little in-depth investigation.

Chantarasiri (2021) successfully isolated ligninolytic *Shewanella* sp. from mangrove wetland soils in Thailand, while Das et al. (2025) isolated ligninolytic *Stenotrophomonas maltophilia* from the Sundarban mangrove ecosystem in India. Studies have indicated that *Bacillus* sp., *Aeromonas* sp., *Serratia* sp., *Pseudomonas* sp., and *Enterobacter* sp. are capable of producing lignin-degrading enzymes (Bharagava et al., 2018; Falade et al., 2019). Das et al. (2025) successfully isolated laccase-producing bacteria from the mangrove habitat of Sundarban, West Bengal, India, and Basha et al. (2024) from Parangipettai in Tamil Nadu. Mangrove wetland soils are abundant in organic carbon and lignin, primarily due to the accumulation of plant leaf litter, decaying wood and the underground roots of vegetation. These soils provide an ideal environment for various ligninolytic bacteria, including *Lysinibacillus sphaericus*, *Mangrovibacterium lignilyticum*, *Streptomyces* sp. and several species of Bacillus (Kachiprath et al., 2019; Chantarasiri, 2021). Consequently, isolating novel and effective ligninolytic bacteria from mangrove wetland ecosystems presents a significant challenge.

Laccase and ligninase enzymes have proven uses in bioremediation and pollution management. Laccase and ligninase enzymes are utilized in various industries. The pulp and paper sector employs these enzymes for biobleaching and biopulping processes (Moldes and Vidal, 2012). These enzymes are essential in the textile industry for the degradation of azo dyes. Furthermore, the distillery sector uses them for bioremediation and the decolorization of effluent. In the petroleum industry,

laccase and ligninase enzymes contribute to the degradation of phenanthrene and pyrene. In agriculture, these enzymes assist in breaking down pesticides (Agrawal et al., 2018).

5.2.7 DNase

DNases facilitate the hydrolysis of deoxyribonucleic acid (DNA) by cleaving phosphodiester bonds. As a result, DNase is regarded as playing a crucial role in the utilization of DNA and the cycling of nutrients within the environment (Mulcahy et al., 2010). DNase, also known as deoxyribonuclease, is an enzyme that cleaves DNA in a non-specific manner, resulting in the release of di-, tri-, and oligonucleotide products with a 5'-phosphorylated end. The elimination of extracellular DNA plays a vital role in reducing the inflammatory response and maintaining homeostasis. Furthermore, DNases could be implicated in bacterial growth and biofilm maturation, in addition to enabling bacteria to evade the immune system (Berends et al., 2010).

A study by Al-Wahaibi et al. (2019) on marine bacteria from North Sea sediments exhibited extracellular DNase activity, with 28% identified as *Bacillus* spp. Paul et al. (2020) documented DNase-producing bacterial isolates from the mangrove habitat in Northern Kerala. The production of DNase has been documented in various bacterial species, such as *Serratia marcescens*, *Vibrio* sp., *Pseudomonas aeruginosa*, Myroides, Planococcus, Sporosarcina, Halomonas, as well as *Bacillus licheniformis* (Mulcahy et al., 2010).

DNase finds extensive applications in the field of biomedicine, particularly in the treatment of diseases such as cystic fibrosis and chronic obstructive pulmonary diseases. Certain bacteria generate DNases as a virulence factor, which assists in evading the immune system or facilitating biofilm maturation. Extracellular enzymes produced by bacteria are widespread in oceanic environments and are crucial for the cycling and fate of organic matter. Nevertheless, the understanding of DNase diversity among marine bacteria, particularly within marine aggregates, is still in its early stages (Haile and Ayele, 2022).

5.2.8 Pectinase

Pectinases catalyze the hydrolysis of pectin (polysaccharide present in plant cell walls) (Hassan et al., 2023). The primary components of heterogeneous pectic substances include pectins, protopectins and polygalacturonic acids. According to Ward et al. (1989), pectinase enzymes help plants make their cell walls bigger, soften tissues during development and storage and break down and recycle plant waste in an environmental friendly way.

Previous studies reported pectinase-producing microbes from mangrove sediment, which includes Maria et al. (2005) from the southwest coast of India, Arijit et al. (2013) from Valapattanam, Kerala and Ungcharoenwiwat and Sakayaroj (2022) from Thailand. Alqahtani et al. (2022) successfully isolated the pectinolytic *Bacillus subtilis* strain from a horticulture field in Bangalore, India.

The primary sources of pectinase are microorganisms, predominantly bacteria, fungi and yeast. The commercial utilization of pectinases was first noted in the 1930s. Pectinase enzymes are widely employed in the food industry, especially for the extraction and clarification of fruit juices, liquefaction, saccharification of plant biomass, paper manufacturing and the fermentation of coffee and tea. Various industries, including food processing, textiles and biofuel production, widely utilize pectinase enzymes due to their high effectiveness in breaking down pectin (Haile and Ayele, 2022). Bacterial isolates from mangrove sediments in Indonesia and Kenya produce these enzymes, which are essential for degrading pectin, a complex polysaccharide present in plant cell walls (Ntabo et al., 2018). Various methods can be implemented to enhance pectinase production; however, due to the instability of enzymes, the overall cost for widespread application tends to be elevated.

The microbial community in the mangrove forests of Northern Kerala is rich in diversity and remains largely unexplored. Within this community, there are bacteria that possess the remarkable ability to produce a wide range of extracellular hydrolytic enzymes. Studies of these bacterial hydrolytic enzymes in mangrove ecosystems could lead to better understanding of the ecological roles these enzymes play. The present study investigates the enzyme production capability of northern Kerala mangrove bacteria. The production of protease, amylase, lipase, cellulase, ligninase, DNase, laccase, chitinase and pectinase by the bacterial isolates were determined.

5.3 MATERIALS AND METHODS

The bacterial isolates were screened for their ability to produce the following enzymes

5.3.1 Protease, amylase, lipase and laccase

Nutrient agar medium supplemented with casein (2%), starch (1%), tributyrin (1%) and guaiacol (0.01%) was prepared for the detection of protease, amylase, lipase and laccase, respectively. Plates were spot inoculated and incubated at 28 ± 2°C for 24 hours. Presence of clearance zone was noted as positive, and the diameter of the zone was recorded. In the case of amylase, plates were flooded with Gram's iodine solution (Iodine-1 g and potassium iodide-2 g in 300 ml distilled water) and the presence of a clearance zone was noted. The formation of a brown colour around the colonies in the Guaiacol medium was considered positive for laccase enzyme activity (Aunstrup, 1979).

5.3.2 Cellulase

Carboxymethyl cellulose (CMC) media was used for testing cellulase production. The plates were spot inoculated and incubated at 28 ± 2ºC for 24 hours. After incubation, the plates were flooded with 1% congo red solution and kept for 15 minutes at room temperature, thereby adding 1M NaCl (58.44 g NaCl in 1 L distilled water). The zone of clearance around the colonies was noted as positive result (Udhardt et al., 2005).

Carboxymethyl cellulose (CMC) medium

Peptone	-	10 g
Carboxymethyl cellulose	-	10 g
KH_2PO_4	-	2 g
Agar	-	12 g
$MgSO_4.7H_2O$	-	0.3 g
$(NH_4)_2SO_4$	-	2.5 g
Gelatin	-	2 g

Congo red	-	1%
Distilled water	-	1000 ml
pH	-	6.8

5.3.3 DNase

The isolates were spot inoculated on DNase agar. After incubation at 28 ± 2ºC for 24 hours, the plates were flooded with 1N HCl. Clearance zone around the colonies was recorded as positive result (Sanchez-Porro et al., 2003).

DNase agar medium

Enzymatic digest of casein	-	15 g
Enzymatic digest of animal tissue	-	5 g
Sodium chloride	-	5 g
Deoxyribonucleic Acid	-	2 g
Agar	-	15 g
Distilled water	-	1000 ml
pH	-	7.3

5.3.4 Ligninase

Crawford's agar was used as the basal medium for testing lignin degradation. The plates were spot inoculated and incubated at 28 ± 2ºC for 24 hours. Formation of halo zone or brown colour around the colonies was considered as positive result (Kameshwar and Qin, 2017).

Crawford's agar medium

Glucose	-	10 g
Yeast extract	-	1.5 g
Na_2HPO_4	-	4.5 g
KH_2PO_4	-	1g
Tannic acid	-	5%

$MgSO_4$	-	0.02 g
$CaCl_2$	-	0.5 g
Agar	-	20 g
Distilled water	-	1000 ml
pH	-	7

5.3.5 Chitinase

Colloidal chitin agar was used for testing chitinase production. The plates were spot inoculated and incubated at 28 ± 2ºC for 24 hours. The zone of clearance around the colonies was noted as positive result (Kuddus and Ahmad, 2013).

Colloidal chitin agar medium

Na_2HPO_4	-	6 g
KH_2PO_4	-	3 g
NH_4Cl	-	1 g
NaCl	-	0.5 g
Yeast extract	-	0.05 g
Agar	-	15 g
Colloidal chitin	-	1%
Distilled water	-	1000 ml
pH	-	7

Preparation of colloidal chitin

About 40g of chitin powder was added to an Erlenmeyer flask. Slowly, 600 ml of concentrated HCl was added, and the mixture was shaken at 30°C for 60 minutes. Chitin was precipitated as a colloidal suspension when added to 2 litres of water at 4 to 10°C. The suspension was filtered through Whatman filter paper and washed with 5 L of distilled water, this process was repeated three times until the pH reached 3.5.

5.3.6 Pectinase

Pectin agar was used for testing the production of pectinase. The plates were spot inoculated and incubated at 28 ± 2ºC for 24 hours. After incubation the plates were flooded with 1% Cetyltrimethylammonium bromide (CTAB) and the zone of clearance was noted as positive result (Bibi et al., 2018).

Pectin agar medium

Pectin	-	5.0 g
$CaCl_2$	-	0.2g
NaCl	-	20 g
$FeCl_3$. $6H_2O$	-	0.01g
Yeast Extract	-	1.0 g
Agar	-	20.0 g
Distilled water	-	1000 ml
pH	-	7

5.4 RESULTS

5.4.1 Total hydrolytic enzyme production

The plate method was employed for qualitative screening the bacterial isolates for enzyme production. Qualitative screening was carried out for nine industrially important enzymes, such as protease, amylase, lipase, cellulase, laccase, ligninase, DNase, chitinase and pectinase (Appendix II).

A total of 708 bacterial isolates were screened for hydrolytic enzyme production. All the isolates showed enzyme activity for at least one of the tested enzymes except pectinase. The positive isolates showed a zone of clearance or brown colouration around the colonies on specific substrates (Fig 5.1). The highest percentage of bacterial isolates were found to be lipase producers (69%), followed by protease (55%), amylase (48%), cellulase (43%), DNase (27%), chitinase (3.5%), ligninase (3%) and laccase (1%) (Fig. 5.2).

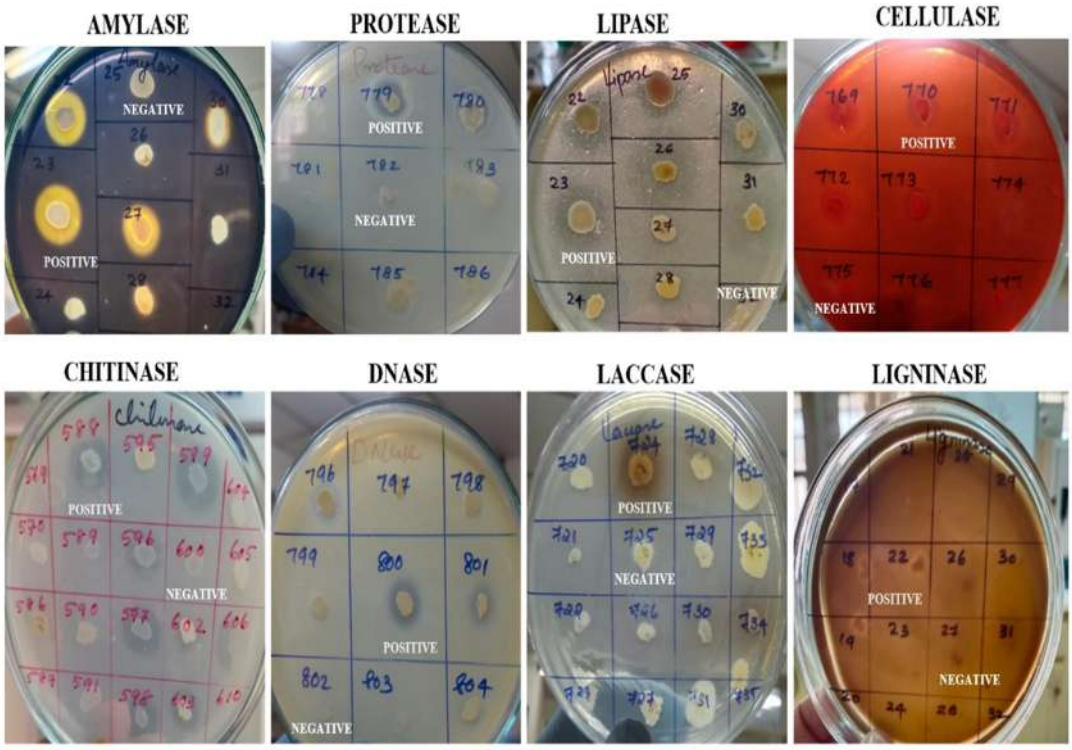


Fig. 5.1 Bacterial isolates showing hydrolytic enzyme activity

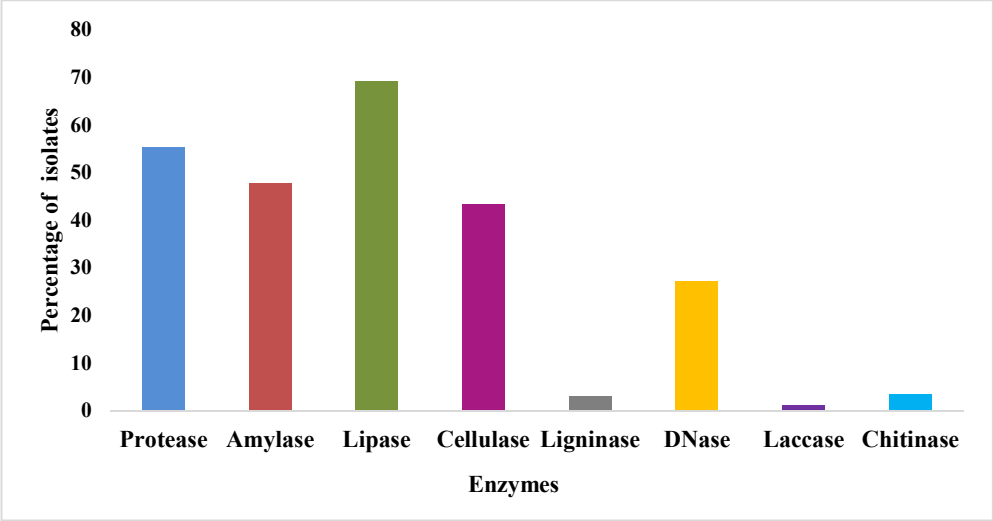


Fig. 5.2 Total percentage of bacterial isolates with hydrolytic enzyme production during the two-year sampling period

5.4.2 Year-wise hydrolytic enzyme production

During the first year of sampling, the majority of bacterial isolates produced lipase (52%), followed by protease (48%), amylase (47%), DNase (38%), cellulase (30%), chitinase (2%), ligninase (1%) and laccase (0.4%) (Fig. 5.3A). During the second year of sampling, 80% of isolates produced lipase, followed by protease (60%), cellulase (52%), amylase (48%), DNase (20%), chitinase (5%), ligninase (4%) and laccase (2%) (Fig. 5.3B).

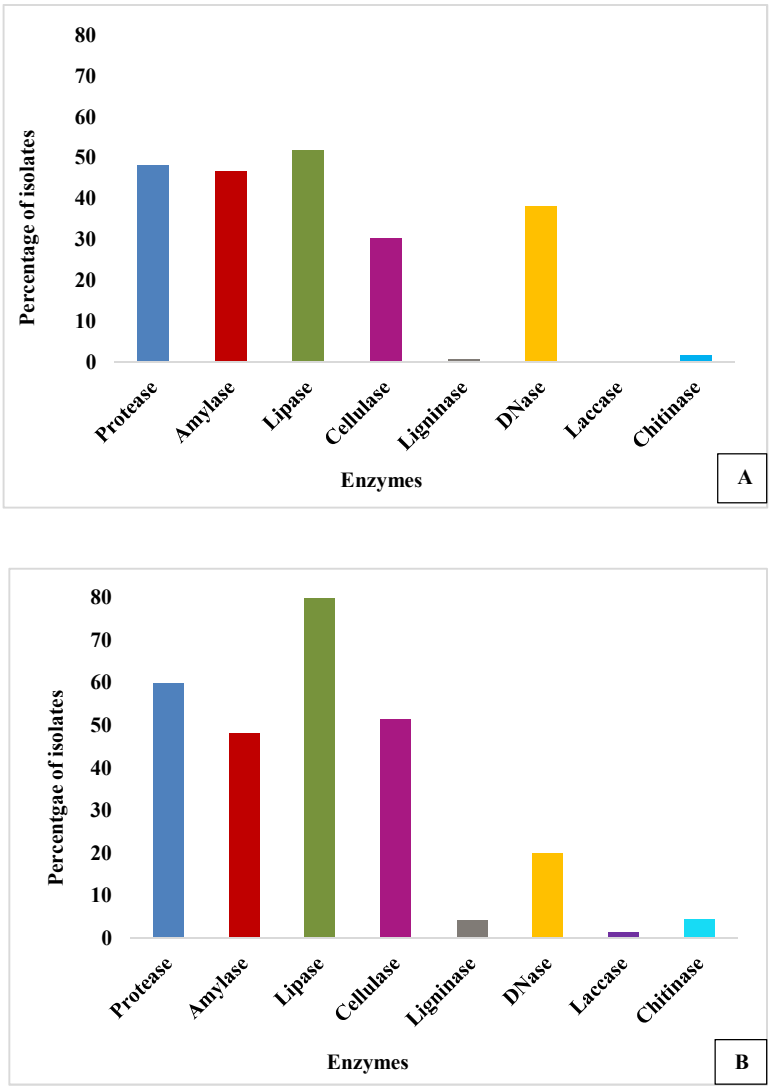


Fig. 5.3 Percentage of bacterial isolates with hydrolytic enzyme production: during the first year (A) and second year (B) of sampling

5.4.3 Season-wise hydrolytic enzyme production

In the first year, during the monsoon sampling, the order of percentage of positive isolates was amylase > protease, lipase > cellulase > DNase > ligninase > laccase. However, during the second year of the monsoon season, it was lipase > protease > amylase > cellulase > DNase > ligninase > laccase > chitinase.

Lipase > protease > cellulase > amylase > DNase was the order of the percentage positive isolates during post-monsoon sampling in the first year. In the second year, it was lipase > cellulase > protease > amylase > DNase > ligninase > laccase.

As for the pre-monsoon sampling, the enzyme activity order for the first-year isolates was amylase > lipase > DNase > protease > cellulase > chitinase, and for the second-year isolates, it was lipase > protease > cellulase > amylase > chitinase > DNase > ligninase.

During the first year monsoon sampling (Fig 5.4 A), the highest percentage of isolates produced amylase (62%), followed by protease (60%), lipase (60%), cellulase (42%), ligninase (3%) and laccase (2%) enzymes. The highest percentage of DNase (46%) and chitinase producers (4%) were obtained during the pre-monsoon season. During the first year of sampling, ligninase and laccase producing bacterial isolates were obtained only in the monsoon season and chitinase producers in the pre-monsoon season.

Majority of the lipase (87%), amylase (57%), ligninase (5.3%), and laccase (4%)-producing isolates were found during the monsoon season of the second year of sampling (Fig 5.4B). Maximum cellulase (63%), protease (62.5%) and DNase (34%) producers were obtained during the post-monsoon season. Chitinase producers (13%) were found to be maximum during the pre-monsoon season of second year sampling.

Bacterial isolates producing lipase, protease, amylase, cellulase and DNase enzymes were obtained during all the sampling seasons. Chitinase producing isolates were obtained only during the pre-monsoon season (5%) of the first year of sampling and the monsoon (1%) and pre-monsoon season (19%) of the second year of sampling. Ligninase producing bacteria were isolated during all seasons except the first-year pre-monsoon and post-monsoon seasons. Laccase producers were obtained only in the first-year monsoon (1%), second year monsoon (6%) and post-monsoon seasons (1%).

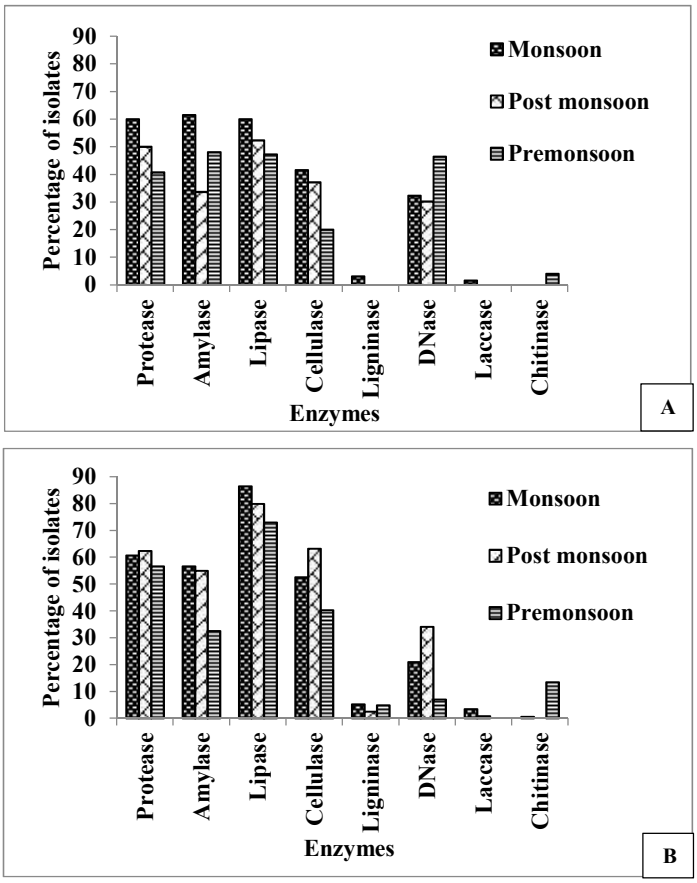


Fig. 5.4 Season-wise percentage of bacterial isolates showing hydrolytic Enzyme production during the first (A) and second (B) year of sampling

5.4.4 Station-wise hydrolytic enzyme production

The total hydrolytic enzyme production at each station was analyzed during the first and second years of sampling, as shown in figures 5.5 and 5.6, respectively. Protease, amylase, lipase and cellulase producers were isolated in all the seasons from Kasaragod station. Amylase, lipase and DNase producers were consistently isolated across all seasons from the Edat station. No chitinase producers were obtained from Edat. At the Valapattanam station, protease, amylase, lipase and DNase producers were consistently isolated throughout all seasons. Protease, amylase and lipase producers were consistently isolated throughout all seasons from the Pazhayangadi, Elathur and Ponnani stations. No ligninase producers were obtained from Ponnani station. Protease, amylase, lipase and cellulase producers were consistently isolated throughout all seasons from Chettuva station. Laccase producers were not obtained from Kasaragod, Pazhayangadi and Valapattanam stations.

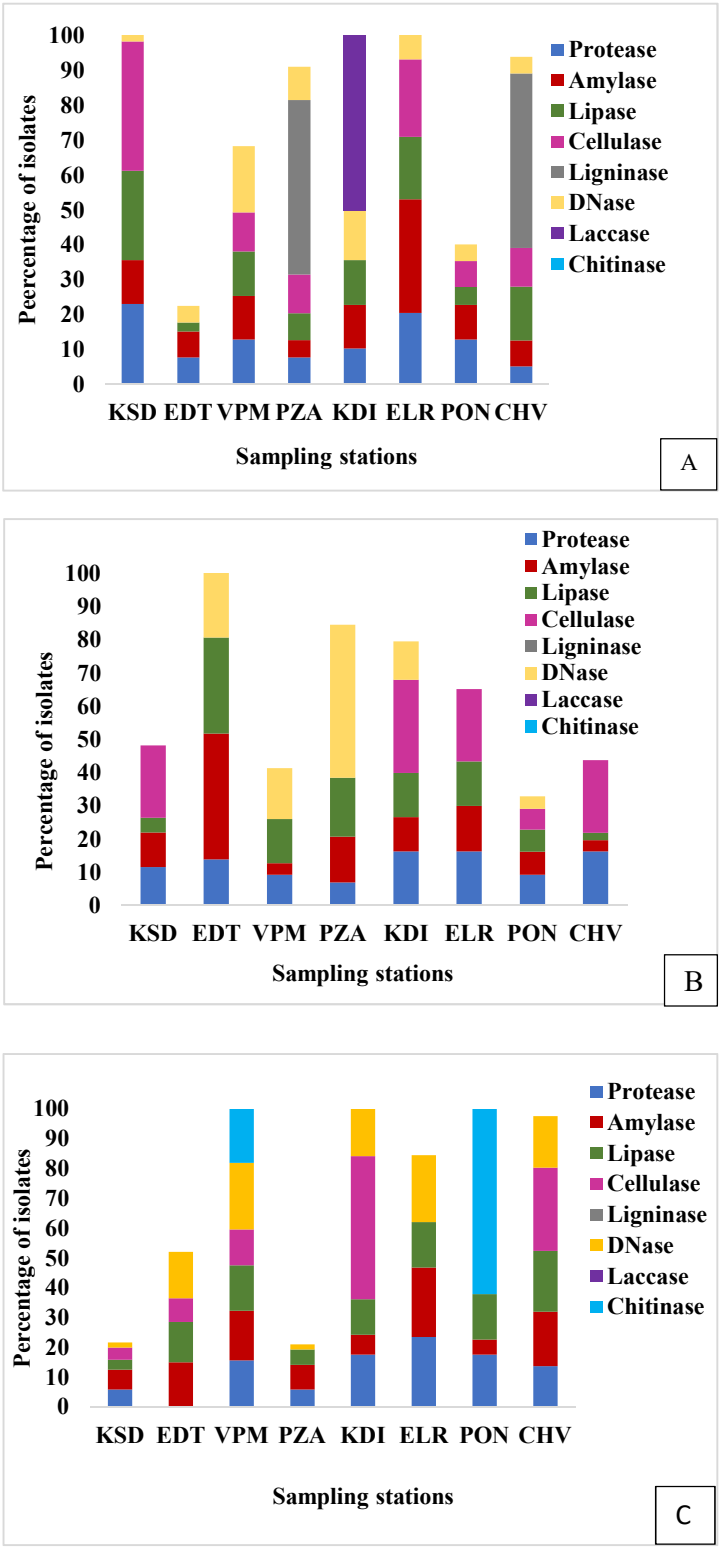


Fig. 5.5 **Sampling station-wise percentage of bacterial isolates showing hydrolytic enzyme production during the first year of sampling A) Monsoon B) Post-monsoon C) Pre-monsoon**

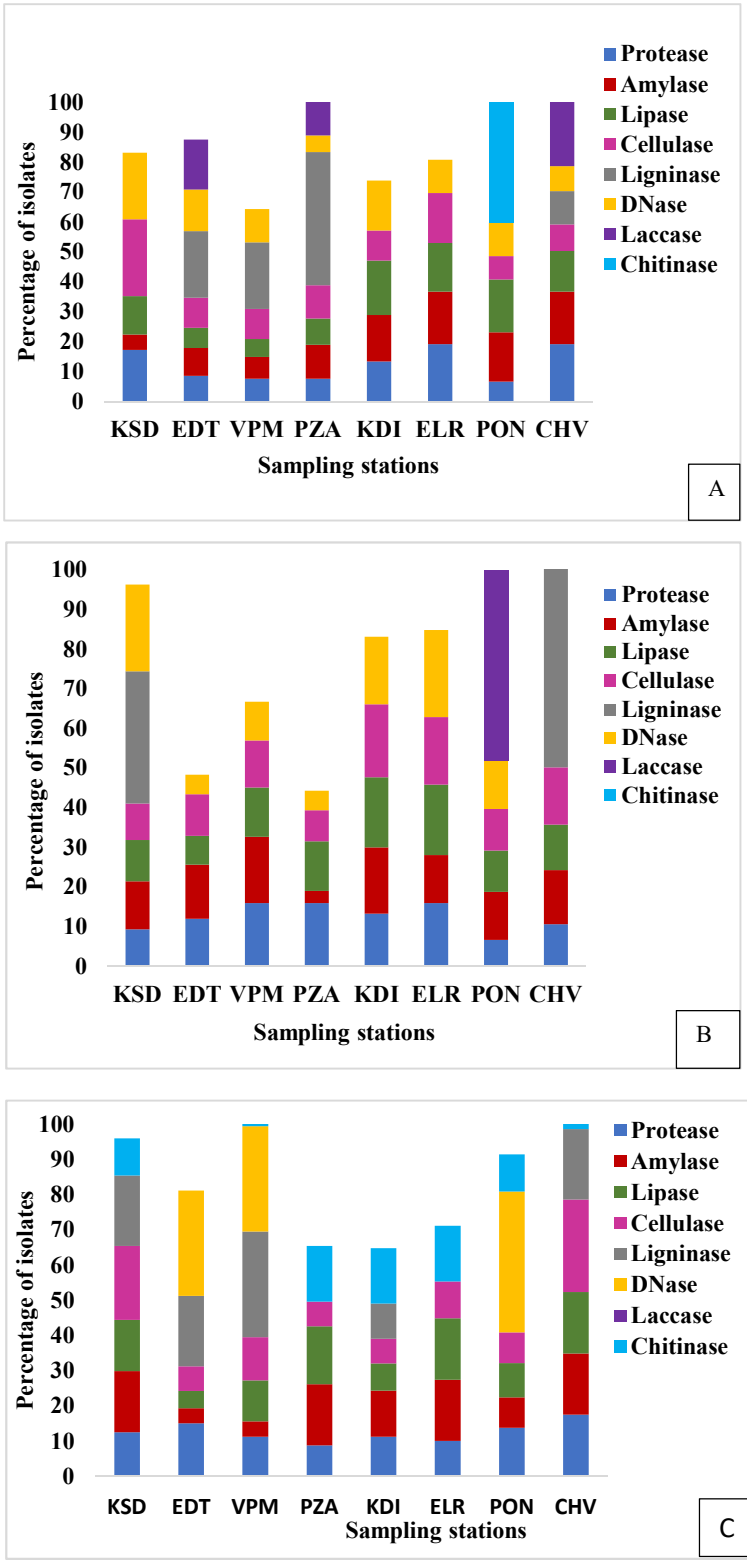


Fig. 5.6 **Sampling station-wise percentage of bacterial isolates showing hydrolytic enzyme production during the second year of sampling A) Monsoon B) Post-monsoon C) Pre-monsoon**

5.4.5 Genera-wise percentage of bacterial isolates showing enzyme production

Over a two-year sampling period, 17 genera were identified from eight different mangrove habitats using morpho-biochemical methods. The total enzyme production for each genus obtained during the entire sampling period is presented in Fig. 5.7. All the bacterial genera isolated in this study were capable of producing protease, amylase, lipase and cellulase enzymes. Chitinase enzyme was produced by all the isolates except Klebsiella, Citrobacter, Lactobacillus, Lysobacter, Proteus and Escherichia. Ligninase enzyme was produced only by Bacillus, Klebsiella, Pseudomonas, Xanthobacter, Alcaligenes and Acinetobacter. The DNase enzyme was produced by all genera except Aeromonas. The laccase enzyme was produced only by Bacillus, Pseudomonas, Enterococcus and Serratia.

Comparing the total enzyme production exhibited by bacterial isolates, the highest production was shown by Bacillus (45%), followed by Pseudomonas (16%), Xanthobacter (6%) and Vibrio (5%) and the least by Escherichia (0.5%). Bacillus exhibited the highest percentage of all enzyme production.

Protease (24%), amylase (23%), lipase (31%), chitinase (1%), cellulase (19%), ligninase (1%), DNase (13%), and laccase (0.4%) were all produced by most of the Bacillus isolates. Pseudomonas isolates were the second-most common. They showed protease (10%), amylase (7%), cellulase (6%), ligninase (0.7%), DNase (5%) and lipase (12%) enzyme production. Isolates belonging to the genus Citrobacter (0.1%) showed the least protease and amylase production. Isolates belonging to the genus Escherichia showed the least amylase (0.1%), DNase (0.1%) and lipase (0.3%) production. Isolates belonging to the genera Klebsiella, Citrobacter, Lactobacillus, Lysobacter, Proteus and Escherichia were not found to produce lipase enzyme. Isolates belonging to the genera Enterococcus, Enterobacter, Staphylococcus, Vibrio, Aeromonas, Citrobacter, Lactobacillus, Acinetobacter, Serratia, Lysobacter, Proteus and Escherichia did not show ligninase production. Aeromonas isolates were not found to produce the DNase enzyme.

Pseudomonas (0.7%) showed the second most laccase production. Isolates belonging to the genera Bacillus, Pseudomonas, Enterococci and Serratia were the only ones to produce the laccase enzyme. Bacterial isolates belonging to Bacillus and

Pseudomonas were able to produce the eight enzymes tested. The majority of the isolates belonging to Bacillus produced lipase enzyme (31%), followed by protease (24%), amylase (23%), cellulase (19%), DNase (13%), chitinase (1.3%), ligninase (1.1%) and laccase (0.4%). 12% of Pseudomonas isolates produced lipase enzyme, followed by protease (10%), amylase (7%), cellulase (6%), DNase (5%), ligninase (0.7%), laccase (0.3%) and chitinase (0.1%). The majority of the isolates belonging to Enterococcus produced the lipase enzyme (3%), followed by protease and cellulase (2%), DNase and amylase (1%), chitinase (0.3%) and laccase (0.1%). The majority of the isolates belonging to the genus Klebsiella produced cellulase (1%), and the least produced enzymes were amylase and ligninase (0.3%). 5% of the Xanthobacter isolates produced the lipase enzyme, and the least produced enzyme was chitinase (0.1%). The majority of the isolates belonging to the genus Alcaligenes produced lipase (2%), and the least produced enzymes were chitinase and ligninase (0.3%). The majority of the Enterobacter isolates produced lipase (2.7%), and the least produced enzyme was chitinase (0.1%). The majority of the isolates belonging to the genus Staphylococcus produced lipase (3.2%), and the least produced enzymes were chitinase and DNase (0.7%). Most of the Vibrio isolates showed protease production (3.7%), and the least produced enzyme was chitinase (0.1%). The majority of isolates belonging to the genus Aeromonas produced protease and amylase (0.7%), and the least produced chitinase (0.1%). Most of the Citrobacter isolates showed lipase production (0.6%), and the least produced enzymes were amylase and protease (0.1%). The majority of the isolates belonging to the genus Lactobacillus, showed cellulase enzyme production (1.4%), and the least produced enzyme was DNase (0.6%). Most of the isolates belonging to the genus Acinetobacter produced the protease enzyme (0.7%), and the least produced enzyme was chitinase (0.1%). 2.5% of Serratia isolates showed lipase production, and the least produced enzymes were chitinase and DNase (0.1%). Most of the Lysobacter isolates showed lipase production (1.6%), and the least produced enzyme was DNase (0.4%). The majority of the isolates belonging to Proteus produced protease, amylase and lipase (0.4%), and the least produced enzyme was cellulase (0.14%). Most of the isolates belonging to the genus Escherichia showed production of protease, cellulase and lipase (0.3%), and the least produced enzymes were amylase and DNase (0.1%).

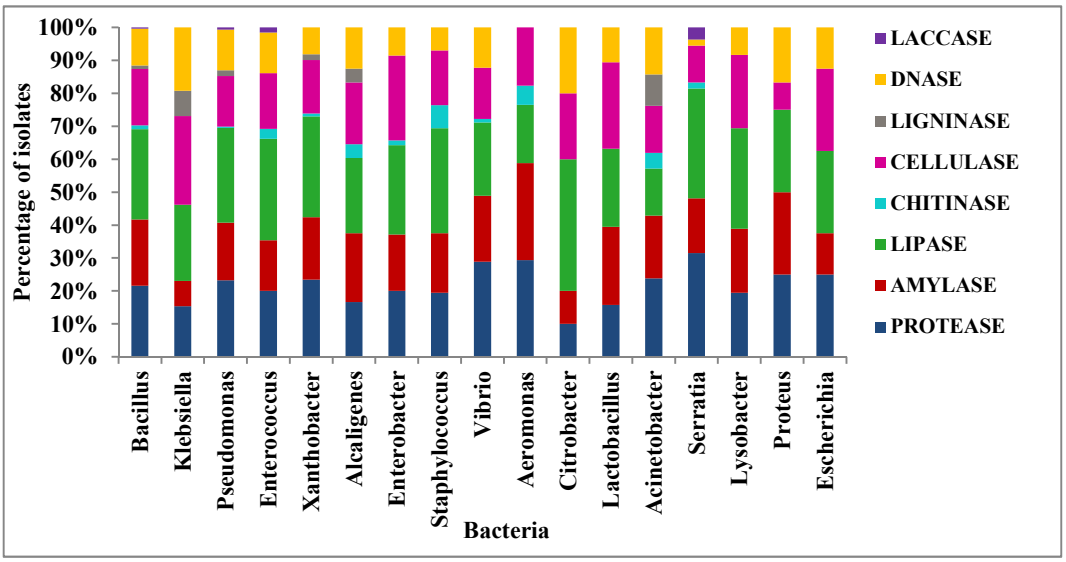


Fig. 5.7 The total percentage of isolates from different genera showing hydrolytic enzyme production during the study period

5.4.6 Year-wise bacterial genera showing enzyme production

During the first year of sampling, isolates belonging to Bacillus were found to produce all the enzymes except ligninase (Fig. 5.8). Pseudomonas produced all enzymes except ligninase and chitinase during the first year of sampling.

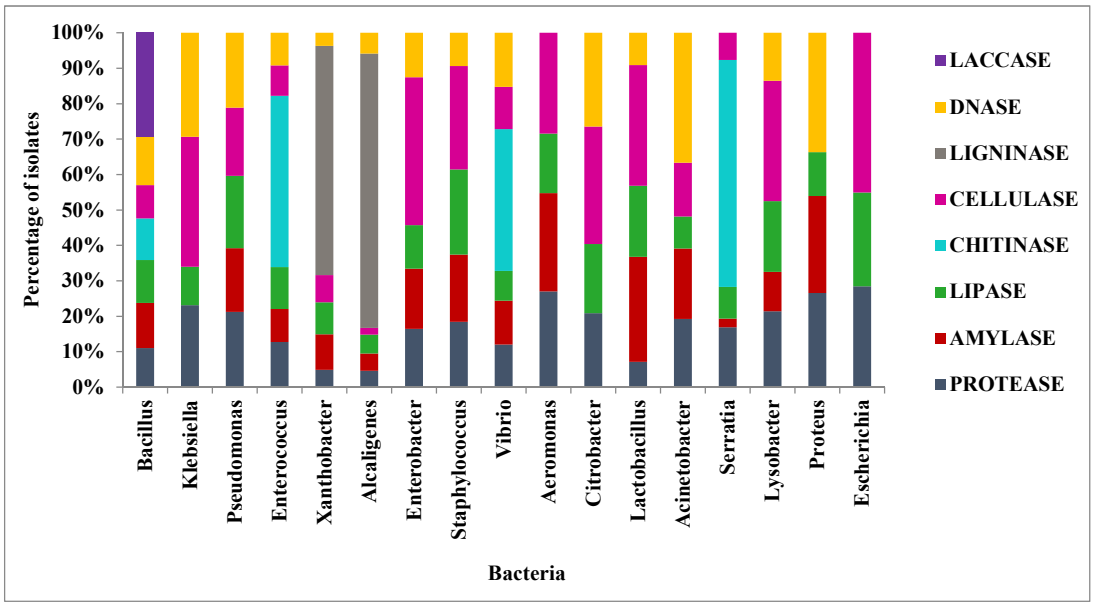


Fig. 5.8 The percentage of isolates from different genera showing hydrolytic enzyme production during the first year of sampling

During the second year of sampling (Fig. 5.9), the maximum production of protease, amylase, lipase, chitinase, cellulase, DNase and ligninase enzymes were shown by bacterial isolates belonging to the genus Bacillus. Bacillus, Pseudomonas, and Serratia isolates produced the laccase enzyme. All the isolates, except Citrobacter, Proteus, and Escherichia, produced protease and amylase enzymes. The percentage of isolates from different genera showing hydrolytic enzyme production during the two years and seasonally during the sampling period is detailed in appendix III and IV.

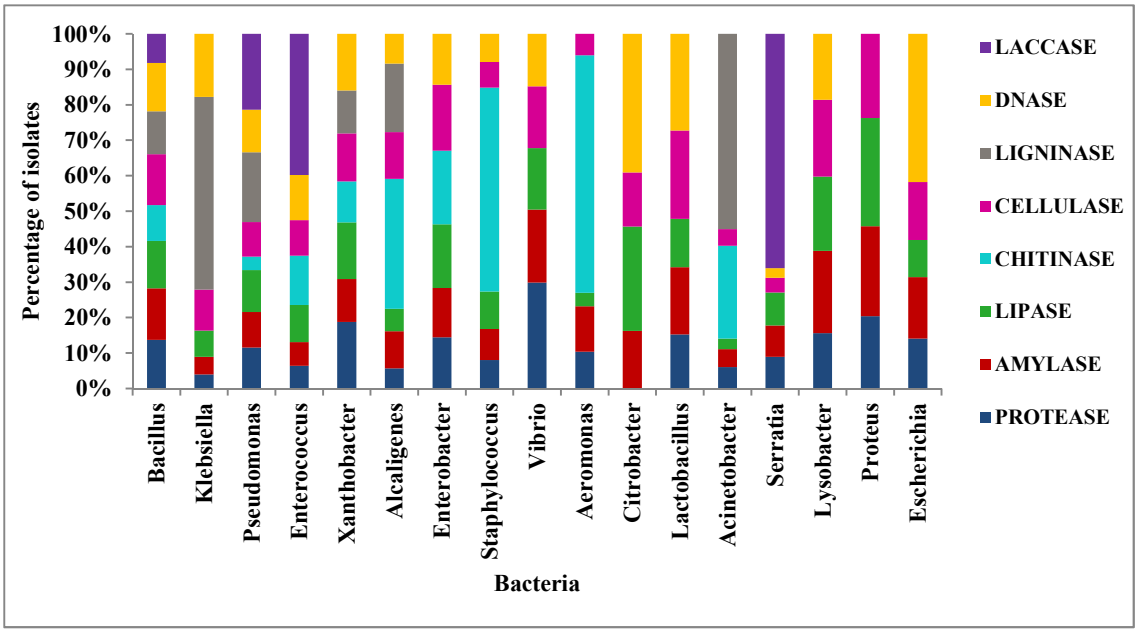


Fig. 5.9 The percentage of isolates from different genera showing hydrolytic enzyme production during the second year of sampling

5.5 DISCUSSION

Seventeen different bacterial genera were identified across eight mangrove stations from northern Kerala. These bacteria were found to produce eight important industrial enzymes, including protease, amylase, lipase, cellulase, ligninase, DNase, laccase and chitinase. All of the bacterial isolates obtained were capable of producing at least one of these enzymes. Previous studies found diverse bacterial communities in mangrove sediments with unique enzyme-producing capabilities. Mangrove bacteria possess traits that allow them to thrive in harsh environments, making them promising candidates for biotechnology (Alves et al., 2020). Variations in climate, organic matter, carbon sources, diversity, nutrient concentration, pH, temperature, depth,

anthropogenic influence, environmental stressors and the presence of pollutants can interfere with the enzyme activity of mangrove bacteria (Sinha and Parli, 2020).

Limited research has been conducted on enzyme production by mangrove bacteria in northern Kerala. In the current study, the highest percentage of bacterial isolates were found to be lipase producers (69%), followed by protease (55%), amylase (48%), cellulase (43%), DNase (27%), chitinase (3.5%), ligninase (3%) and laccase (1%). Kutty et al. (2020) studied the enzyme hydrolytic potential of bacterial isolates from the Kadalundi (Kerala) mangrove forest and found that 76% of isolates produced lipase, followed by protease (75%), amylase (72%), cellulase (62%) and ligninase (20%). However, Varghese et al. (2020) studied bacterial populations in decaying leaf litter of Ayiramthengu mangrove in Kerala. They found that about 31% of the isolates produced lipase, with cellulase (23%) and amylase (18%) being the next most common enzymes. Kachiprath et al. (2019) studied hydrolytic enzymes from microbes in mangroves of Malippuram, Ernakulam, Kerala. They found that over 90% of the isolates had both amylolytic and proteolytic activity. Castro et al. (2014) investigated the diversity and enzymatic analysis of endophytic bacteria in the Brazilian mangrove ecosystem. They found that 75% of the isolates had protease activity and 62% had endoglucanase activity. Feller and Lovelock (2017) found lipase, protease, amylase and cellulase enzymes producing bacteria from Carrie Bow Cay, Florida and reported their importance in nutrient cycling.

The unique characteristics of mangroves, such as high salinity, tidal variation and high biodiversity, make them promising agents for bioprospecting enzymes. The complexity of the ecosystem, combined with human impacts, may lead to a variety of extracellular enzyme production (Mohandas and Kathiresan, 2016). Organic matter from both terrestrial and aquatic sources, such as leaf litter and detritus, is essential for bacterial growth and enzyme production (Alongi, 2014). Lauber et al. (2009) and Donato et al. (2011) reported that the decomposition of complex organic compounds in mangrove sediments, like cellulose, lignin and chitin, requires a variety of enzymes for decomposition, and bacteria producing enzymes like cellulases, ligninases and chitinases aid in decomposition. Bacteria with the ability to produce enzymes like amylase, protease, cellulase and lipase have a competitive advantage in utilizing different carbon sources. Mangroves receive a continuous supply of nutrients,

including nitrogen and phosphorus, from tidal waters. This nutrient-rich environment promotes the growth of bacteria and their enzymatic functions. The bacterial enzymes play a crucial role in breaking down complex organic compounds like lipids, proteins, starch and cellulose found in the mangrove habitat into simpler forms that bacteria can utilize as a carbon and energy source, thereby aiding in biogeochemical cycling (Kachiprath et al., 2019).

The isolates belonging to Bacillus and Pseudomonas were found to produce all eight tested enzymes, such as protease, amylase, lipase, cellulase, chitinase, ligninase, laccase and DNase. These genera are known to thrive in diverse environments such as terrestrial, freshwater, soil and marine habitats (Hernandez-Gonzalez et al., 2018). The current study found that bacterial isolates from Klebsiella and Enterococcus produced seven enzymes, with the exception of chitinase and ligninase, respectively. Serratia isolates produced all enzymes except ligninase. Xanthobacter, Alcaligenes and Acinetobacter isolates produced all tested enzymes except laccase. Enterobacter, Staphylococcus, and Vibrio isolates produced six enzymes, except ligninase and laccase. Citrobacter, Proteus, Lactobacillus, Lysobacter and Escherichia isolates produced five enzymes, except chitinase, ligninase, and laccase. Previous studies have indicated that *Bacillus* sp., *Pseudomonas* sp., *Acinetobacter* sp., and *Vibrio* sp. are involved in carbohydrate breakdown in estuarine sediment and benthic biofilms (Imran and Ghadi, 2019). Dias et al. (2009) found that the orders Vibrionales and Bacillales were prevalent in Brazilian mangrove sediments, and these organisms were capable of producing various extracellular enzymes such as amylases, proteases, esterases and lipases. Castro et al. (2014) found that *Bacillus* sp. had high enzymatic indices for amylase and protease enzymes in the Brazilian mangrove ecosystem. Khianngam et al. (2013) screened endophytic bacteria from mangrove plants in Thailand and found that twenty isolates had activities related to proteases, lipases, amylases or cellulases. Additionally, Alves et al. (2020) reported the presence of xylanase and α-amylase enzymes in bacteria from Bertioga mangrove soil in Brazil. Mayanglambam et al. (2020) found that enzymes from mangrove endophytes have various activities like lipolytic, cellulolytic, proteolytic and amylolytic.

In the present study seasonal variations were observed in bacterial enzyme activity. The variations are frequently observed and are linked to factors such as

macroclimatic and microclimatic changes, alterations in nutrient availability, bacterial growth and fluctuations in leaf litter inputs (Morrissey et al., 2014). Microbial enzyme activity is prominent not just in the monsoon season but also in other seasons in the current study. In a study conducted by Luo and Gu (2014), seasonal fluctuations in extracellular enzymatic activity of coastal mangrove sediment at the Mai Po nature reserve in Hong Kong, China, was investigated. The results indicated a wide range of enzyme activities throughout the seasons, with a negative correlation between oxidative and hydrolytic enzymes. Additionally, factors such as pH, temperature, soluble phenolics, total organic carbon, phosphorus and nitrogen were found to significantly impact enzyme activities.

Bacteria possess the capability to adjust to seasonal changes in order to efficiently decompose organic matter, recycle nutrients and uphold the ecological equilibrium of mangrove ecosystems. During the monsoon, mangrove habitats often face heightened levels of precipitation and an influx of freshwater from rivers and estuaries. This rise in freshwater can lead to a decline in salinity levels in the mangrove sediments, as evidenced in a study by Santos et al. (2011) on bacterial diversity and the effects of oil contamination in untouched mangrove sediment in Brazil. The decrease in salinity during the monsoon season can influence the composition of the bacterial community and their microbial functions. Some bacteria may decrease their production of enzymes, especially those adapted to saltier conditions. Additionally, the increased availability of organic matter during the monsoon season may cause a change in the types of organic compounds accessible for bacterial enzymatic activity. In the pre-monsoon season, there is a clear change in temperatures and weather patterns, which leads to higher evaporation rates and increased salinity levels in mangrove sediments. This rise in salinity creates a favourable environment for salt-tolerant bacteria, allowing them to produce enzymes that are well-suited to these conditions. Furthermore, the presence of organic matter and detritus can affect the activity of bacterial enzymes, as demonstrated in a review study by Kristensen et al. (2008) on the dynamics of organic carbon in different mangrove ecosystems. Fernandes et al. (2012) investigated denitrification in tropical mangrove sediments in Goa, India. Their findings revealed that this region experiences reduced rainfall and the onset of a drier period during the post-monsoon season. Despite these changes, salinity levels tend to

remain stable during this season. This stable salinity creates a favourable environment for bacteria that produce enzymes. The presence of organic matter, nutrient dynamics and the composition of detritus can all impact the enzymatic activity of bacteria during the post-monsoon season. Seasonal weather patterns, such as monsoon, pre-monsoon and post-monsoon, can affect the physicochemical properties of mangrove sediments. This, in turn, has impacted the bacterial population and their enzyme production in the current study.

In the present study, variations were not observed between sampling stations because of factors like natural variability within the northern Kerala mangrove ecosystems and also the interconnectedness of environmental conditions. However, changes were observed in the distribution of enzyme-producing bacteria. Amylase and lipase-producing bacteria were isolated from all the sampling stations during all seasons, but protease-producing bacteria were isolated from all stations except from Edat during the first year of pre-monsoon sampling. Cellulase-producing bacteria were isolated during monsoon sampling from all stations except Edat and Kadalundi and chitinase-producing bacteria were isolated from Ponnani only during the monsoon season and during the pre-monsoon of the second year of sampling from all sampling stations except Edat. Differences in moisture, pH, plant biomass, temperature, salinity, nutrient availability and organic matter content between mangrove stations were identified as reasons for changes in hydrolytic potential of the bacterial isolates (Zhang et al., 2014). Wei et al. (2021) compared microbial community structure and function in sediment between natural regenerated and original mangrove forests in South China, finding higher protease and cellulase enzyme activities in the original forest. Wang et al. (2021) found that physicochemical factors, along with spatial variables, significantly influence the metabolic function of bacterial communities in mangrove sediments.

Lipase producers were predominant (69%) in the current study. The presence of lipase enzyme-producing bacteria in mangrove environments may be affected by lipid-rich organic material and ecological factors. Furthermore, in this study, the carbohydrate, protein, and lipid concentrations were observed to be between 0.01 and 0.25 mg/g, 0.1 and 9.9 mg/g, and 0.2 and 9.3 mg/g, respectively. The elevated lipid content in sediment may be associated with an increase in the activity of

microorganisms that produce lipase. Lipids act as a source of carbon and energy for bacterial communities. Lipid-rich organic matter is supplied to ecosystems from fallen leaves, woody debris and nearby vegetation detritus, serving as energy sources for bacteria (Vanwonterghem et al., 2016).

All the bacterial isolates under all genera were found to produce lipase enzyme, and maximum production was exhibited by Bacillus (31%), followed by Pseudomonas (12%). In a study by Kathiresan et al. (2011) on microbial enzyme activity in decomposing leaves of the Vellar estuary mangrove forest in India, lipase-producing bacteria such as *Bacillus* sp*., Azotobacter* sp., *Acinetobacter* sp. and *Pseudomonas* sp. were found. Mishra and Gupta (2014) also discovered various bacterial genera, including Bacillus, Enterobacter, Bertioga, Pantoea and Streptomyces, in the Bhitarkanika mangroves, which showed promising lipase enzyme activities. Mayanglambam et al. (2020) studied enzymes from mangrove endophytes and found that lipases have a hydrophobic binding pocket that aids in their interaction with lipid substrates, breaking them down into fatty acids and glycerol through hydrolysis. This process is essential for organic matter decomposition and nutrient cycling in mangrove ecosystems. Bacterial lipases in mangroves have a wide tolerance to temperature and pH, allowing them to thrive in diverse environmental conditions, and the abundance of lipase-producing bacteria in mangroves is influenced by various factors such as the presence of lipid-rich substrates, different aerobic and anaerobic conditions, nutrient cycling, microbial diversity, sediment characteristics and detritus input (Kristensen et al., 2008).

Lipase enzyme, has diverse applications in bioremediation, food industry, biotechnology, pharmaceuticals and more. Nyyssonen et al. (2010) investigated the degradation ability of mangrove bacteria, emphasizing the potential of mangrove bacterial lipases in bioremediation efforts by breaking down complex organic pollutants, including surfactants and polyethylene glycols. The application of lipases from mangrove bacteria extends to the food industry, contributing to the production of baked goods, dairy products and flavour enhancement, as noted by Nawani et al. (2002). Mangrove bacterial lipases are promising for a range of biotechnological uses, such as producing biofuels, biodiesel and bioplastics (Santos et al., 2017). Moreover,

lipases are essential in pharmaceutical applications, including the development of pharmaceutical intermediates and drug formulations (Fickers et al., 2009).

Protease enzyme production was observed in all genera in the current research and maximum production was shown by isolates belonging to Bacillus (24%), followed by Pseudomonas (10%). Protease-producing bacteria play a crucial role in the mangrove ecosystem by breaking down complex proteins in organic matter into simpler peptides and amino acids. This process provides carbon and nitrogen sources for other microorganisms, supporting nutrient cycling (Bhattacharyya et al., 2017). Protein degradation by bacteria can enhance nutrient availability for plants and other microorganisms in the ecosystem. Mangrove litter and other organic materials contribute to a significant proportion of proteins in the sediment, which offers a suitable environment for proteolytic bacteria to flourish (Sneha et al., 2021).

Nursyirwani et al. (2021) isolated proteolytic *Bacillus* sp. from sediments in the mangrove ecosystem at the Dumai marine station in Riau Province, Indonesia. Additionally, Gusman et al. (2022) isolated proteolytic *Vibrio* sp. from mangrove sediments of Probolinggo City, Indonesia. Parvathi et al. (2017) discovered that protease-producing bacteria found in the sediments of a tropical mangrove forest on the southwest coast of India can assist in breaking down toxic proteins or peptides, potentially reducing the impact of pollutants or toxic compounds in mangrove environments.

Hamza (2017) conducted a comprehensive review on the industrial applications of bacterial protease, emphasizing its uses in detergents, leather, food, pharmaceuticals, textiles and bioremediation. Microbe-derived protease is favoured over plant-derived protease due to its ability to be rapidly produced in large quantities, cost-effectiveness and sustainability. The proteases commonly utilized in various industries are derived from Bacillus species, including *B. subtilis, B. cereus, B. licheniformis, B. megaterium, B. halodurans and B. alkaliphilus* (Hanan, 2012).

In the current study, it was observed that isolates belonging to all genera produced the amylase enzyme, and the maximum was exhibited by Bacillus (23%) and Pseudomonas (7%) isolates. The production of this enzyme by mangrove bacteria plays a significant role in breaking down organic matter, particularly starch

mineralization. This enzymatic process is important for nutrient cycling in mangrove ecosystems, as it releases carbon and energy for other organisms (Kristensen et al., 2008). Amylolytic bacteria, which have the ability to decompose starch, are also prevalent in mangrove sediments due to the presence of carbohydrates in the sediment (litter resulting from the degradation of dead mangrove stems and leaves is rich in starch). These bacteria are essential for nutrient cycling and decomposition processes within the mangrove ecosystem (Silitonga et al., 2019).

Fasa et al. (2019) discovered 28 actinomycete isolates from mangrove litter in Pramuka Island, Indonesia, that exhibited amylase activity. Various bacterial genera, such as Bacillus, Enterobacter, Vibrio, Desulfurella, Peribacillus, Priestia and Pseudomonas, have been identified as potential sources for commercializing amylase enzymes. *Bacillus proteolyticus*, *Desulfurella* sp., *Pseudomonas entomophila* and *Pseudomonas putida* were reported as amylase-producing bacteria in a study conducted by Klinfoong et al. (2022) on mangrove soil in Thailand. Furthermore, a study on mangrove-associated bacteria demonstrated the potential role of various bacterial isolates, including *Vibrio alginolyticus*, in amylase production (Mamangkey et al., 2021). The study conducted by Kachiprath et al. (2019) in Ernakulam, which focused on bacteria from Malippuram mangroves, found that more than 90% of the isolates exhibited amylolytic activity. Notable species such as *Bacillus subtilis, Bacillus amyloliquefaciens* and *Bacillus megaterium* were identified. Castro et al. (2014) conducted a comprehensive study on the diversity and enzymatic analysis of endophytic bacteria from various mangrove tree branches, revealing that *Bacillus* sp. exhibited the highest activity for amylase and endoglucanase. Similarly, Putri et al. (2021) discovered that *Bacillus paramycoides* and *Enterobacter cloacae* were amylase-producing bacteria in their study at Dumai Marine Station Mangrove in Indonesia.

Furthermore, Mamangkey et al. (2021) emphasized in their research on mangrove plants in North Sumatra, Indonesia, that the ability of bacteria to produce amylase enzymes is significant for various industries, including agriculture, pulp and paper, medicine, baking, food processing, starchy waste utilization, and other biotechnological advancements.

In the present investigation, isolates belonging to all genera exhibited cellulolytic activity and maximum production was shown by Bacillus (19%) and Pseudomonas (6%) isolates. Cellulase enzymes play a crucial role in breaking down cellulose into simpler sugars, aiding in the conversion of cellulosic materials into usable products and contributing to nutrient cycling (Kachiprath et al., 2019). Nakagiri (1998) studied the factors influencing the distribution of halophytophthoras in subtropical mangroves and highlighted the enzymatic reactions that transform plant debris into a nutritious source for organisms at higher trophic levels. Behera et al. (2017) conducted a comprehensive review on cellulose-producing bacteria in mangrove ecosystems, emphasizing that the sediment in mangrove forests provides an ideal environment for cellulase production due to the continuous input of cellulosic carbon in the form of litter, which serves as a substrate for decomposition by fungi and bacteria. Cellulolytic bacteria, such as Bacillus, Klebsiella and Fictibacillus, have been discovered from various mangrove ecosystems. These include the mangrove habitat in Hanura village (Sumardi et al., 2021), the mangrove ecosystem in North Sumatra (Mamangkey et al., 2021), and the coastal mangrove sediments in Logending Beach, Kebumen, Indonesia (Pramono et al., 2021). Mangrove areas have also been found to harbour cellulolytic bacteria such as *Citrobacter freundii, Vibrio alginolyticus* and *Actinomyces bovis* (Saha, 2018). In Philippines, Tabao and Moasalud (2010) evaluated the bioprospecting potential of bacterial communities in mangroves and identified promising cellulase-producing Bacillus species, including *B. cereus, B. licheniformis* and *B. pumilus*.

Cellulases are widely used in various industries, including food, animal feed, fermentation, agriculture, pulp and paper, biofuel production and textiles (Mayanglambam et al., 2020). Exploring cellulolytic enzymes from mangrove bacteria has the potential to contribute to the development of more efficient and novel cellulases for industrial applications and renewable energy generation.

In the current research, all the isolates have the ability to produce the chitinase enzyme, except those belonging to Klebsiella, Citrobacter, Lactobacillus, Lysobacter and Escherichia. Chitinases are known for their ability to break down chitin into low-molecular-weight oligosaccharides, with various applications in industries such as agriculture, medicine, and more (Keyhani and Roseman, 1999). Rishad and Jisha

(2017) conducted a study where they isolated and characterized potent mangrove microflora, including *Bacillus subtilis, Bacillus amyloliquefaciens* and *Candida parapsilopsis,* from Valanthakad Mangroves, Kochi, Kerala, which displayed chitinase activity. Moderately halophilic bacteria, including *Bacillus aerophilus, Bacillus pumilus* and *Pseudomonas plecoglossicida*, were isolated from the Pichavaram mangrove ecosystem in Tamil Nadu by Paranetharan et al. (2018) and were found to produce chitinase enzymes. Chitinase enzymes have important applications in industries such as medicine, agriculture and environmental remediation (Rishad et al., 2017). Antimicrobial and biopesticide properties of bacterial chitinase enzymes make them useful for biocontrol in many fields (Mamangkey et al., 2021).

The present study found that bacterial isolates from the genera Bacillus, Klebsiella, Pseudomonas, Xanthobacter, Alcaligenes and Acinetobacter produced the ligninase enzyme. Ligninase enzymes play a crucial role in the degradation of lignin in plant material (Kumar and Chandra, 2020). Chandra et al. (2011) specifically isolated ligninolytic *Klebsiella* sp. while studying bacterial decolorization in paper pulp. Various researchers have conducted studies and found that bacterial strains such as *Bacillus* sp., *Pseudomonas* sp., *Citrobacter* sp., *Klebsiella pneumoniae* and *Serratia marcescens* produce extracellular peroxidases that aid in lignin degradation (Yadav et al., 2009). Additionally, Kachiprath et al. (2019) identified potent strains of bacteria, including *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus megaterium, Bacillus mojavensis, Streptomyces galbus* and *Streptomyces* sp., that produce ligninase enzymes in the mangrove ecosystem of Malippuram, Ernakulam, Kerala. Chantarasiri (2021) discovered a ligninolytic bacterium, *Lysinibacillus sphaericus,* in the Bueng Samnak Yai coastal wetland of Thailand. Meanwhile, Mamangkey et al. (2021) found a variety of bacteria with diverse enzyme properties, including ligninase, in the mangrove ecosystem in North Sumatra.

Ligninase enzymes have promising potential in bioremediation, breaking down lignin-based pollutants in the environment. Additionally, in the pulp and paper industry, ligninase enzymes can enhance the bleaching process by eliminating lignin from pulp fibers, resulting in brighter, more environmentally friendly paper products (Underkofler et al., 1958).

In the current study, bacterial isolates belonging to all genera, with the exception of Aeromonas, were found to produce DNase enzyme. DNase enzymes work on DNA to release nucleotides, which include nitrogen and phosphorus compounds. Bacteria and other microorganisms utilize these nucleotides making it easier for nutrients to move around in ecosystems (Giambastiani and Moreno, 2008). Bacteria can use DNase enzymes to outcompete other microorganisms by breaking down their DNA, aiding in resource acquisition and niche colonization. Additionally, some bacteria produce DNase enzymes as a defense mechanism against pathogens; these enzymes can degrade the genetic material of invading microorganisms, diminishing their virulence. Molecular biology widely uses DNase enzymes for tasks like DNA purification, RNA extraction and the removal of genomic DNA contamination in research and diagnostics. Abdessamad et al. (2023) discovered DNase-producing *Bacillus* sp. in the Khnifiss lagoon in Morocco, while Meena et al. (2023) isolated DNase-positive actinomycetes from the mangroves of the Andaman Islands, India.

Moreover, DNase enzymes have potential applications in agriculture for soil remediation, improving nutrient availability and enhancing plant growth. In wastewater treatment, DNase enzymes can assist in breaking down organic matter and improving nutrient removal efficiency. They also play a crucial role in genetic and genomic research, as well as in the development of biopharmaceuticals (Meena et al., 2023).

The laccase enzyme was produced only by isolates belonging to the genera Bacillus, Klebsiella, Pseudomonas, Enterococcus and Serratia. Laccase is an enzyme belonging to the group of blue oxidases, which can be found in plants, insects, fungi, and bacteria (Thurston, 1994). Previous studies have identified various bacteria from mangrove areas that exhibit laccase activity. *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus megaterium, Bacillus mojavensis* and *Streptomyces galbus* are some of the bacteria known to produce laccase (Kachiprath et al., 2019). Also, actinomycetes found in an Egyptian mangrove area have been shown to produce laccase (Atalla et al., 2013). Luo et al. (2017) investigated the diversity and abundance of bacterial laccase like genes in the sediments of the Mai Po Nature Reserve, a subtropical mangrove ecosystem in Hong Kong, China. The researchers found that Proteobacteria, Actinobacteria, Bacteroidetes and unknown species were the

predominant laccase-producing bacteria in this ecosystem. They observed significant variations in the diversity and abundance of these genes across different sediment types and sampling seasons. The study found that the main factors that caused the differences in bacterial laccase-like genes between ecosystems were the different amounts of carbon and nitrogen in the soils and sediments. The researchers also investigated the variety of laccase-producing bacteria in the rhizosphere of rice plants in paddy fields and obtained laccase producing Pseudomonas and Lysinibacillus (Niladevi and Prema, 2005).

Laccase enzymes have demonstrated potential applications in various industries, including paper and pulp, textiles, food, beer, wine, and juices. They have promising potential in bioremediation processes for the degradation of pesticides and herbicides, as well as the removal of toxic pollutants (Verma et al., 2020). Because these enzymes are very stable at high temperatures and don't react with organic compounds, they could be used to break down and remove pollutants from the environment (Zhang et al., 2019).

The current investigation revealed that mangrove bacteria have the ability to produce a wide range of extracellular enzymes that are well-suited for industrial purposes. The production of bacterial enzymes in the mangrove ecosystem is influenced by various biotic and abiotic factors.

Chapter 6

SELECTION, MOLECULAR IDENTIFICATION OF POTENTIAL BACTERIAL ISOLATES AND DEVELOPMENT OF BACTERIAL CONSORTIA

Chapter - 6

SELECTION, MOLECULAR IDENTIFICATION OF POTENTIAL BACTERIAL ISOLATES AND DEVELOPMENT OF BACTERIAL CONSORTIA

6.1 INTRODUCTION

Identifying bacteria in mangroves is crucial because these bacteria have a high potential for industrial applications (such as producing enzymes for detergents, food, and medicine), play essential roles in ecosystem functions like nutrient cycling and carbon sequestration, and offer unique opportunities to discover novel species and functionalities with high biotechnological potential. This identification provides a baseline for understanding their specific metabolic activities and contributions to the health of this vital ecosystem, as well as for conservation efforts (Thatoi et al., 2013).

Molecular identification and phylogenetic studies provide a detailed understanding of species identification, evolutionary relationships, genetic diversity and population structure by comparing genetic sequences. These techniques enable the accurate classification of organisms, the reconstruction of their evolutionary history through a phylogenetic tree, and the discovery of new species, making them valuable tools in fields like biodiversity research, medicine and conservation (Roch, 2006).

To select bacterial strains for a consortium, understanding the consortium's functional goals and then screening strains for desired traits, such as enzyme activity and metabolite production, are carried out. Key criteria include high performance in the target application, compatibility between strains to ensure synergistic function and prevent negative interactions, stability under environmental conditions, and genetic identity for reproducibility (Nunes et al., 2024).

Optimizing *in-vitro* bacterial growth using various parameters like temperature, pH, nutrient sources, agitation and incubation time results in the identification of ideal

conditions for maximum bacterial biomass, metabolic activity and desired product (e.g., secondary metabolites) yield. This process helps determine specific, optimized cultivation parameters to improve strain performance for research or industrial applications, leading to more reliable and efficient bacterial cultivation and production (Morabandza et al., 2021).

Bacterial consortia offer advantages like broader metabolic capabilities and enhanced stability over monocultures, enabling the degradation of diverse contaminants and complex compounds. Their applications include bioremediation of pollutants such as plastics and petroleum, agriculture for crop growth, disease suppression and soil health, and biotechnology for producing biofuels, biohydrogen and other valuable products from waste materials (Cao et al., 2022).

6.2 REVIEW OF LITERATURE

Mangrove ecosystems serve as vital habitats for numerous marine microorganisms, particularly bacteria, which are essential to the health and sustainability of these environments. Microbes not only contribute significantly to the formation and preservation of the mangrove biosphere but also represent a source of biotechnologically significant products (Thatoi et al., 2013). While extensive studies have been conducted on various aspects of mangroves, including biogeography, botany, zoology, ichthyology, environmental pollution, and their economic implications, there remains a gap in understanding the microbial activities within mangrove waters and sediments. Furthermore, the phylogenetic and functional characterization of microbes in these ecosystems has not received as much attention as in other environments.

According to Mignard and Flandrois (2006), 16S rDNA sequencing has emerged as the standard method for bacterial taxonomy and identification. This data encompasses morphological, behavioural, or molecular characteristics and the phylogenetic analysis aims to reconstruct the historical relationships among genes or species. Regardless of the specific application, all phylogenetic analysis necessitates a criterion for evaluating the fit of data to potential trees, a strategy for exploring possible solutions to identify the tree that best aligns with the data, and a method for gauging the reliability of the results. A widely utilized criterion is maximum

likelihood, where the optimal tree is the one that renders the observed data most probable under a specified evolutionary model (Woese, 2000).

Microbial degradation offers significant benefits for environmental restoration, particularly in addressing hazardous waste because of its economic viability, eco-friendliness and safety. Recently, the use of microbial consortia has become the preferred method among researchers for developing bioremediation agents. The process of bioremediation utilizing bacterial consortia involves microorganisms breaking down harmful substances into less toxic or non-toxic forms, proving to be more effective than relying on a single bacterial strain (Kaya et al., 2024).

Microbial consortium enhances the degradation efficiency of complex compounds by cross-feeding among bacteria and helps to reduce feedback inhibition (Bharti and Grimm, 2021). A consortium comprises multiple strains with unique physiological characteristics that provide a range of degradation capabilities. Typically, a bacterial consortium is formed by combining individual strains in equal proportion (Dai et al., 2020). Microbial consortia exhibit significant adaptability and stability in complex environments. When various cells with distinct functions are integrated, a dynamic equilibrium is achieved through intricate interactions among the cells, resulting in a system that is more resilient and stable in the face of environmental changes (McCarty and Ledesma-Amaro, 2019).

Numerous factors influence the efficiency and rate of biodegradation. It includes the environmental variables, attributes of microbes and the kind of pollutants, and optimization will provide a better result (Kebede et al., 2021). Biotic (competition, antagonism or predation) and abiotic factors (environmental variables) influence bioremediation (Abatenh et al., 2017). These factors impact microbial activities, the efficiency of degradative enzymes and overall degradation processes.

In the process of assembling microbial consortia for the degradation of complex compounds, selecting appropriate strains with effective catalytic capabilities is crucial. It is also essential to evaluate whether these strains can coexist harmoniously. The selection criteria for potent strains typically include non-antagonistic interactions, compatibility, low mutation rates, non-haemolytic behaviours, non-toxic byproducts and high tolerance (Jawed et al., 2019). The potential

virulence or pathogenicity of the consortium raises significant concerns during environmental release. It is essential to assess the pathogenicity of bacteria prior to their application in bioremediation to avert unforeseen health risks for both humans and the environment. Pathogenic bacteria have the potential to induce diseases, and their introduction into the environment, especially in areas where humans or other organisms may interact with the treated zone, can present a significant danger (Darmawati et al., 2021). Haemolytic activity is recognized as a major virulence marker, as the presence of haemolysin can lead to the lysis of the host's red blood cells, aiding in the pathogen's dissemination through the bloodstream (Dadheech et al., 2016). Blood agar plates are commonly utilized as enriched media to cultivate fastidious organisms and to distinguish bacteria based on their haemolytic properties. Haemolysin acts as a toxic enzyme that causes red blood cell lysis and enhances cell permeability, increasing vulnerability to infectious agents. The application of hydrolytic bacteria as agents for bioremediation is recognized; however, it is crucial to verify their compliance with safety standards. Numerous studies have utilized blood agar plates to assess bacterial pathogenicity (Darmawati et al., 2021; Purwaningrum et al., 2021).

The competitive behaviour of bacteria, where one bacterium suppresses the growth of another, plays a crucial role in selecting bacteria for bioremediation purposes. Gaining insight into this antagonistic relationship enables researchers to identify bacteria that effectively break down pollutants while simultaneously inhibiting the proliferation of competing microorganisms that could obstruct the remediation process (Nunes et al., 2024). In bacterial consortia, the antagonistic effects of certain bacteria may hinder the effectiveness of bacterial remediation. Thus, employing a consortium of potential microbes that possess a wide range of catabolic enzymes and genes may lead to a more rapid, efficient and complete biodegradation. Kebede et al. (2021) conducted a study on bioremediation, which indicated that identifying the antagonistic characteristics of bacteria is a significant factor in the formation of bacterial consortia. Microbial consortia typically exhibit both multifunctionality and resistance, as various species collaborate to optimize the utilization of all available substrates, thus enhancing bioremediation efficiency in comparison to individual microorganisms.

Microbial enzymes serve as essential metabolic catalysts, which has led to their widespread application across various industries. This study focuses on assessing several industrially significant enzymes, including protease, lipase, amylase, chitinase, ligninase and cellulase. Each bacterial species exhibits unique optimal conditions that enhance enzyme activity (Cao et al., 2022). To maximize enzyme production, it is crucial to optimize the growth conditions of bacteria by adjusting parameters such as temperature, pH, nutrient composition, agitation speed and incubation duration. Key factors to consider include the specific bacterial strain, the desired enzyme and the optimal growth conditions for that organism. Such an endeavour often requires a balance between vigorous bacterial growth and substantial enzyme secretion (Morabandza et al., 2021). The optimization process involve turbidity analysis, where the cloudiness of a bacterial culture is monitored over time to identify the best conditions for cell growth, which is directly linked to enzyme production. This goal is typically achieved by modifying factors such as temperature, pH, salinity, nutrient composition and incubation time, while turbidity is measured to evaluate cell density and pinpoint the peak phase of enzyme production (Pylak et al., 2021).

The present study focuses on hydrolytic enzyme characteristics of bacterial isolates from mangroves. The objectives include molecular identification and phylogenetic analysis of the selected isolates, compatibility assessments, haemolysis detection, optimization of *in-vitro* conditions and the development of consortia

6.3 MATERIALS AND METHODS

6.3.1 Primary screening for bacterial isolates

The potential isolates were selected by evaluating their ability to produce various enzymes and the extent of zone of clearance obtained during the qualitative enzyme analysis (Refer section 5.4.1).

6.3.2 Molecular characterization

6.3.2.1 Extraction of bacterial genomic DNA

Bacterial isolates were cultured in Nutrient broth (HI MEDIA) for 24 hrs at 28± 2°C. The genomic DNA was extracted using DNA extraction kit (Origin DNA Isolation Kit) according to manufacturer's instruction. The quality and amount of

extracted DNA was evaluated by Nano-Drop 2000c (Thermo Scientific, Massachusetts, USA). The extracted DNA samples were kept at −20°C for further analysis.

6.3.2.2 Amplification of 16S rRNA gene

A pair of universal primers for the 16S rRNA gene, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) (Weisburg et al., 1991) was used for the amplification of 16S rRNA gene (≈1500 bp). Polymerase chain reaction (PCR) was performed in 25 µl reaction volume, containing 0.25 µl Taq DNA polymerase (5 U per µl), 0.5 µl of each primer, 0.5 µl of dNTP (1.25 mM), 2.5 µl of 10X PCR buffer and $MgCl_2$ (50 mM), 0.5 µl (50–100 ng) of genomic DNA and 20.75 µl molecular grade distilled water. Thermal conditions for PCR were as follows: Initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 10 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a 3 min final extension at 72°C. PCR products were electrophoresed in 1% w/v agarose gel and visualized by Ultraviolet (UV) transilluminator (MEDOX, Chennai, India). All amplified PCR products were subjected to purification using PCR Purification kit (Origin, India) following the manufacturer's instruction.

6.3.2.3 DNA sequencing and phylogenetic analysis

Purified PCR products were sequenced by Sanger's dideoxy chain termination sequencing method (Sanger and Coulson, 1975) at AgriGenome Labs Pvt. Ltd., Kochi, India. Sequenced DNA data were compiled and analyzed. The sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) at NCBI (www.ncbi.nlm. nih.gov). The sequences were multiple aligned using the programme Clustal W and the phylogenetic tree was generated using the maximum likelihood method in MEGA software (version 11.0; Biodesign Institute, Tempe, USA) (Tamura et al., 2021).

6.3.3 Secondary screening for potential bacterial isolates

From the among the short-listed bacterial isolates, potential candidates for consortia development were ultimately selected based on their ability to produce larger zones of enzyme activity after 24 hours of incubation.

6.3.4 Determination of pathogenicity of the selected bacterial strains

Pathogenicity tests were conducted by examining the type of haemolysis on Blood agar media. Blood agar plate was prepared using sterile Nutrient agar medium that had been melted and cooled to between 45 and 50°C, to which sterile defibrinated blood, warmed to room temperature, was added at a concentration of 5% (v/v). The medium was dispensed into sterile plates carefully to prevent bubbles and froth. A loopful of a purified bacterial colony was inoculated onto the surface of a sheep blood agar plate (BAP), followed by incubation at 37°C for 18-24 hours (Buxton, 2005). Haemolysis characteristics were evaluated after 18-24 hours of incubation, based on the type of haemolysis zone around bacterial colonies. If there is the formation of a greenish colour around the colony it indicates α-haemolysis; clear zones indicate β-haemolysis and no lysis or colour change indicate gamma haemolysis. Haemolytic activity indicates pathogenicity.

6.3.5 Compatibility checking of the selected isolates *in-vitro*

The antagonistic activity was assessed to evaluate the compatibility between the selected bacterial strains using cross-streaking method before consortium preparation. In the middle of a Nutrient agar plate, a single bacterial strain was vertically streaked (1.5 cm wide streak diametrically across the plate) and the other strains were streaked perpendicular to the initial strain. The plate was incubated at 28±2°C for 24 hrs, and the plates were observed for any inhibition zones between the strains (Lertcanawanichakul and Sawangnop, 2008).

6.3.6 Growth optimization of the potential isolates

Preparation of inoculum:

Nutrient broth tubes were prepared and sterilized at 121.5°C for 15 minutes in an autoclave. The bacterial isolates were inoculated into Nutrient broth tubes. Incubation was done at room temperature (28± 2°C) for 24 hours. The cell density was calibrated using the 0.5 McFarland standard (0.05 ml of 1% $BaCl_2$ mixed with 9.95 ml of H_2SO_4), which contains approximately 1.5×10^8 cells that served as the inoculum (Roessler and Brewer, 1967). Optical density of the culture suspension

was taken at 600 nm in a UV-VIS spectrophotometer (Thermo Fisher Scientific, USA).

Preparation of medium:

Temperature:

Nutrient broth with 15 ppt salinity was used to determine the optimum growth temperature of the selected isolates. The culture tubes were incubated at different temperature (20, 30, 40, 50, 60°C).

Salinity:

Nutrient broth was prepared with different salinities (0, 5, 10, 15, 20, 25, 30, 35, 40 ppt) for testing the optimum growth of the selected isolates.

pH:

Nutrient broth with 15 ppt salinity at different at different pH (5, 6, 7, 8, 9 and 10) was prepared to determine the optimum growth pH of the selected isolates. The pH of the medium was adjusted using 1 N HCl and 1N NaOH.

Agitation:

The selected isolates were inoculated into nutrient broth tubes of 15 ppt salinity and incubated at different agitation speeds of 50, 100, 150 and 200 rpm.

Inoculation and incubation:

Nutrient broth tubes were prepared in triplicates and 10 µl of 0.1 OD cell suspension was inoculated. Incubation was done at room temperature (28 ± 2°C) for 24 hours in the case of determination of optimum growth at different pH and salinity.

Determination of growth:

Bacterial growth was estimated by measuring the optical density at 600 nm using UV-VIS spectrophotometer (Thermo Fisher Scientific, USA).

6.3.7 Development of bacterial consortia

The bacterial strains which showed the same growth parameters were selected for the preparation of consortia. The microbial consortia were prepared by permutation and combination by analyzing their concomitant enzyme production, compatibility and haemolytic activity (Masurkar and Pathade, 2023).

6.4 RESULTS

6.4.1 Selection of potential isolates

A total of 708 bacterial isolates were isolated from 8 mangrove stations along North Kerala during the two-year study period. Out of 708 total bacterial isolates, 35 were chosen as they exhibited the production of most of the enzymes tested and yielding maximum clearance zone in qualitative enzyme analysis.

6.4.2 Molecular identification of selected bacterial isolates

Molecular identification of the selected 35 potential bacterial isolates was carried out using 16S rRNA gene sequencing using universal bacterial primers (Fig. 6.1). The sequences obtained were deposited in the NCBI database. Table 6.1 provides details on the enzyme activity of the selected isolates, their molecular identification and Gen Bank accession numbers.

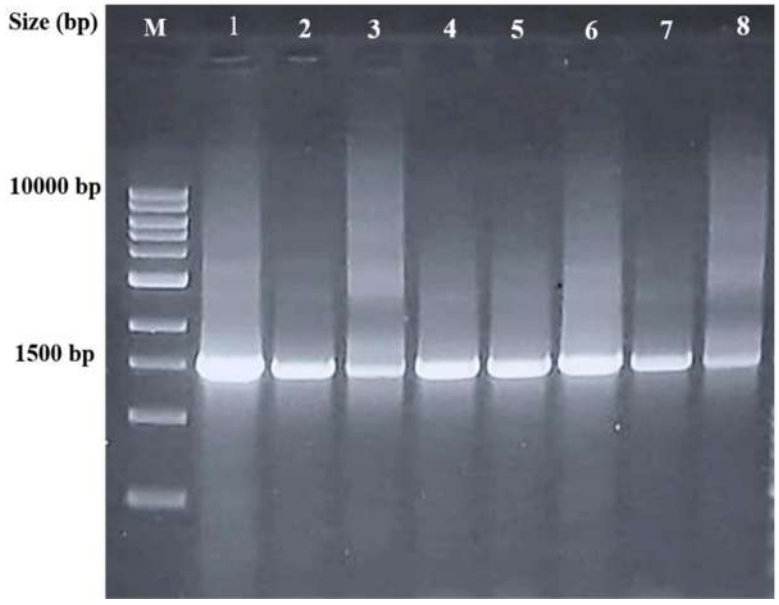


Fig. 6.1 Agarose gel electrophoresis of the PCR product. M represents molecular marker. 1-8 bacterial strains.

Table 6.1 Molecular identification of 35 selected bacterial isolates and their hydrolytic potential

Sl. No.	Bacterial strain	GenBank Accession No.	Collection site	Season of collection	Zone of diameter (mm)							
					Protease	Amylase	Lipase	Chitinase	Cellulase	Ligninase	Laccase	DNase
1	*Alcaligenes faecalis*	OK605873	Kadalundi	Monsoon	12	10	10	-	-	-	6	10
2	*Alcaligenes faecalis*	OK605752	Pazhayangadi	Monsoon	-	-	5	-	8	5	-	11
3	*Alcaligenes faecalis*	OK605555	Valapattanam	Pre-monsoon	10	8	-	5	-	-	8	10
4	*Alcaligenes faecalis*	OK605545	Pazhayangadi	Monsoon	11	10	6	-	8	6	-	-
5	*Pseudomonas aeruginosa*	OK605804	Pazhayangadi	Monsoon	11	11	7	-	6	-	6	-
6	*Pseudomonas fulva*	MT994551	Kadalundi	Pre-monsoon	10	-	10	-	10	-	-	10
7	*Stutzerimonas stutzeri*	MT994548	Kadalundi	Pre-monsoon	8	10	-	8	11	-	-	-
8	*Klebsiella pneumoniae*	OK605872	Elathur	Monsoon	12	11	8	-	-	-	-	12
9	*Klebsiella pneumoniae*	OK605556	Valapattanam	Monsoon	11	13	10	-	8	-	-	10
10	*Klebsiella pneumoniae*	OK605546	Kasaragod	Post-monsoon	10	12	11	-	6	5	-	-
11	*Klebsiella aerogenes*	OK605774	Ponnani	Pre-monsoon	-	11	-	8	-	-	8	12
12	*Klebsiella aerogenes*	OK605541	Chettuva	Monsoon	-	8	8	-	11	5	-	-
13	*Klebsiella aerogenes*	OK605544	Valapattanam	Monsoon	12	14	8	-	6	8	6	12
14	*Bacillus subtilis*	OK605298	Valapattanam	Pre-monsoon	10	11	5	-	6	-	-	-
15	*Bacillus subtilis*	MT994519	Kadalundi	Post-monsoon	-	12	6	-	8	-	-	10
16	*Bacillus albus*	MT994599	Kadalundi	Post-monsoon	12	8	10	6	9	-	-	-
17	*Bacillus cereus*	OK605552	Chettuva	Monsoon	10	10	9	-	-	-	6	-

18	*Bacillus cereus*	ON479459	Elathur	Monsoon	13	11	5	-	8	-	6	-
19	*Bacillus cereus*	MT994521	Pazhayangadi	Monsoon	10	12	7	-	10	-	-	14
20	*Bacillus cereus*	MT994518	Edat	Monsoon	8	14	11	-	6	-	-	11
21	*Bacillus aryabhattai*	MT994514	Edat	Monsoon	10	11	8	-	8	-	-	10
22	*Bacillus amyloliquefaciens*	MT994481	Pazhayangadi	Monsoon	12	10	5	-	10	-	-	-
23	*Serratia marcescens*	OK605549	Pazhayangadi	Monsoon	15	19	10	13	20	-	6	11
24	*Serratia marcescens*	OK605554	Edat	Monsoon	-	-	12	6	-	-	8	10
25	*Serratia marcescens*	OK605548	Chettuva	Post-monsoon	15	11	6	-	7	-	6	14
26	*Serratia marcescens*	OK605553	Chettuva	Post-monsoon	23	20	17	5	20	6	-	13
27	*Serratia marcescens*	OK605543	Edat	Monsoon	14	15	17	10	9	6	10	12
28	*Providencia vermicola*	OK605287	Pazhayangadi	Monsoon	13	11	5	7	8	-	-	-
29	*Providencia rettgeri*	OK605300	Pazhayangadi	Monsoon	-	-	8	-	-	5	-	10
30	*Enterobacter cloacae*	ON479456	Kadalundi	Pre-monsoon	17	15	12	5	13	7	6	15
31	*Enterobacter asburiae*	ON479460	Elathur	Monsoon	15	10	5	-	10	-	6	12
32	*Enterobacter asburiae*	ON479462	Kadalundi	Pre-monsoon	15	18	13	18	14	6	-	17
33	*Lysinibacillus xylanilyticus*	MT994589	Kadalundi	Pre-monsoon	-	11	7	5	11	5	-	-
34	*Lysinibacillus macroides*	MT994559	Kadalundi	Monsoon	12	10	6	-	8		-	-
35	*Aeromonas caviae*	MT994523	Pazhayangadi	Monsoon	10	12	10	-	10	-	6	-

The selected 35 isolates had higher hydrolytic potential, indicated by a greater diameter, and they produced a greater number of the enzymes tested than the other isolates.16S rRNA gene sequencing of bacterial isolates showed that they belonged to the genera Klebsiella, Bacillus, Lysinibacillus, Stutzerimonas, Priestia, Aeromonas, Serratia, Enterobacter, Providencia, Pseudomonas and Alcaligenes. It included five

different strains of *Serratia marcescens*, four different strains of *Alcaligenes faecalis* and *Bacillus cereus*, three different strains of *Klebsiella pneumoniae* and *Klebsiella aerogenes*, two different strains of *Bacillus subtilis* and *Enterobacter asburiae,* and one each of *Pseudomonas aeruginosa, Pseudomonas fulva, Bacillus albus, Stutzerimonas stutzeri, Priestia aryabhattai, Bacillus amyloliquefaciens, Providencia vermicola, Providencia rettgeri, Enterobacter cloacae, Lysinibacillus xylanilyticus, Lysinibacillus macroides* and *Aeromonas caviae*.

6.4.3 Phylogenetic analysis of selected bacterial strains

Using the 16S rRNA gene sequence of 35 isolates and one outgroup, a phylogenetic tree was constructed using MEGA 11 software. Phylogenetic tree models with the lowest BIC scores (Bayesian Information Criterion) were found. The phylogenetic tree was constructed using 36 bacterial strains, of which *Chloroflexus aurantiacus* (NR043411) from the NCBI database was taken as the outgroup (Fig. 6.2). In the current study, the selected isolates belonged to 11 species under the phylum Firmicutes and 24 under the phylum Proteobacteria.

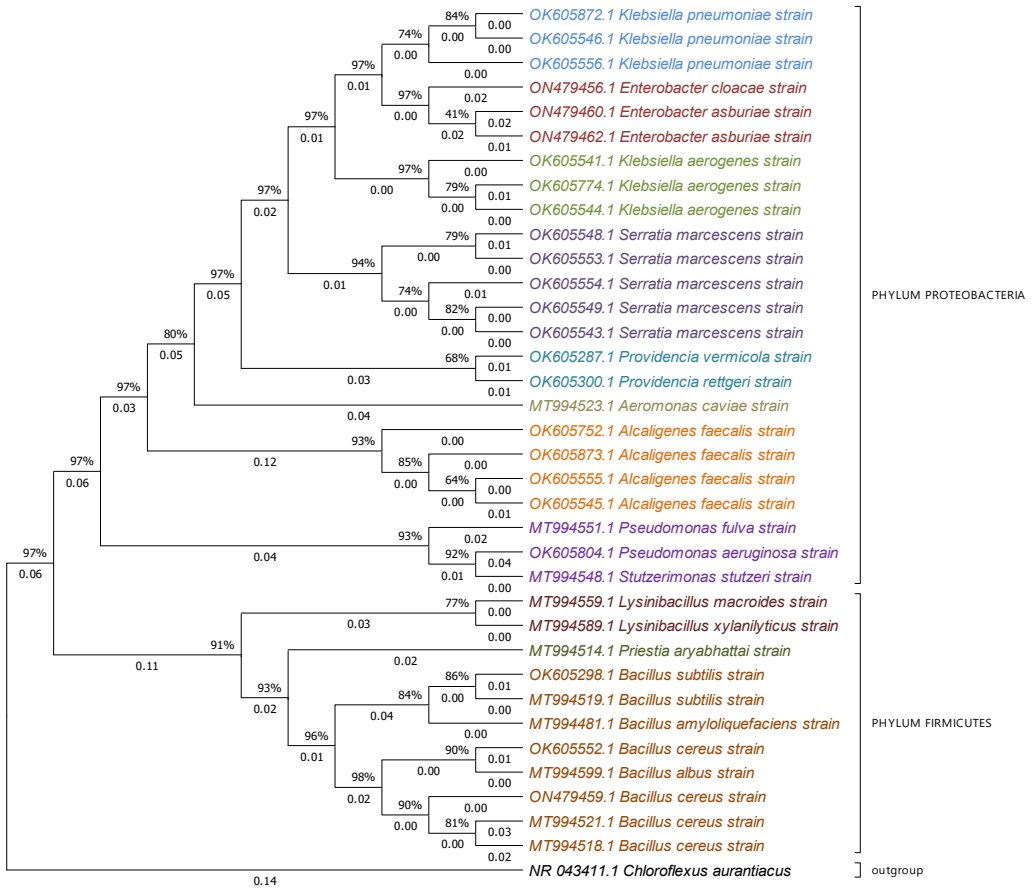


Fig. 6.2 Maximum likelihood tree showing the phylogenetic relationships among the selected bacterial isolates

The phylogenetic tree revealed three main clades, comprising two subclades and one major clade. The subclade belonged to the phylum Proteobacteria and the phylum Firmicutes. The selected bacterial isolates belonged to these subclades. In the subclade phylum Proteobacteria, gram-negative bacteria belonging to the genera Klebsiella, Enterobacter, Serratia, Providencia, Aeromonas, Alcaligenes, Pseudomonas and Stutzerimonas were present. In the subclade phylum Firmicutes, gram-positive bacteria belonged to the genera Lysinibacillus, Priestia, and Bacillus. The selected isolates, which belonged to phyla Proteobacteria and Firmicutes, were found to be arising from a single node and have a common ancestor. Klebsiella, Enterobacter, Serratia, Providencia, Aeromonas, Alcaligenes, Pseudomonas, Stutzerimonas, Lysinibacillus, Priestia and Bacillus have separate clades and internodes.

6.4.4 Final selection of potential bacterial isolates

For the development of consortia, potential bacterial strains with comparatively higher hydrolytic enzyme potential, compatible and non-haemolytic properties have to be selected. Out of 35 bacterial isolates, 8 potential candidates (ST 01-08) were finally chosen for consortia development because they exhibited a higher zone of enzyme activity after 24 hours of incubation (Fig. 6.3). The selected strains were ST 01, ST 02, ST 03, ST 04, ST 05, ST 06, ST 07 and ST 08 (Table 6.2).

ST 01 and ST 06 produced eight enzymes; ST 04, 05, 07, and 08 produced seven; and ST 02 and 03 produced six enzymes. The selected strains were identified as *Enterobacter cloacae* (ST 01), *Enterobacter asburiae* (ST 03 and ST 07), Serratia marcescens (ST 02, ST 04, ST 05, and AT 06), and *Klebsiella aerogenes* (ST 08). Strain-wise variations in the enzymatic potential were observed in the case of *Enterobacter asburiae* and *Serratia marcescens.* All the selected strains were found to be gram-negative.

Table 6.2 Details of the selected bacterial strains for consortia development

Strain No.		ST 01	ST 02	ST 03	ST 04	ST 05	ST 06	ST 07	ST 08
Bacteria		*Enterobacter cloacae*	*Serratia marcescens*	*Enterobacter asburiae*	*Serratia marcescens*	*Serratia marcescens*	*Serratia marcescens*	*Enterobacter asburiae*	*Klebsiella aerogenes*
Gram Property		Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Motility		Motile	Motile	Motile	Motile	Motile	Motile	Motile	Non- motile
Growth characteristics on Nutrient Agar plate		1-4 mm medium-sized, circular or irregular with entire margins, smooth, shiny and slightly raised greyish to white or pale yellow-coloured colonies.	1–5 mm diameter sized, circular or irregular-shaped, slightly yellowish-red, pigmented, smooth, shiny, and somewhat raised colonies.	1-3 mm diameter sized, circular or irregular-shaped, greyish-white or white creamy-coloured, and rough-textured colonies.	1–5 mmdiameter sized circular or irregular-shaped, slightly yellowish-red, pigmented, smooth, shiny, and somewhat raised colonies.	1–5 mm-diameter sized, circular or irregular-shaped, slightly yellowish-red, pigmented, smooth, shiny, and somewhat raised colonies.	1–5 mm-diameter sized, circular or irregular-shaped, slightly yellowish-red, pigmented, smooth, shiny, and somewhat raised colonies.	1-3 mm diameter sized, circular or irregular-shaped, greyish-white or white creamy-coloured, and rough-textured colonies.	1-4 mm diameter sized circular or irregular colonies, as well as smooth, shiny and entire margin colonies that were greyish white or pale yellow in colour.
GenBank Accession		ON479456	OK605548	ON479460	OK605549	OK605553	OK605543	ON479462	OK605544
Collection site		Kadalundi	Chettuva	Elathur	Pazhayangadi	Chettuva	Edat	Kadalundi	Valapattanam
Collection season		Pre-monsoon	Post- monsoon	Monsoon	Monsoon	Post- monsoon	Monsoon	Pre-monsoon	Monsoon
Zone of diameter of enzyme production(mm)	**Protease**	17	15	15	15	23	14	15	12
	Amylase	15	11	10	19	20	15	18	14
	Lipase	12	6	5	10	17	17	13	8
	Chitinase	5	-	-	13	5	10	18	-
	Cellulase	13	7	10	20	20	9	14	6
	Ligninase	7	-	-	-	6	6	6	8
	Laccase	6	6	6	6	-	10	-	6
	DNase	15	14	12	11	13	12	17	12

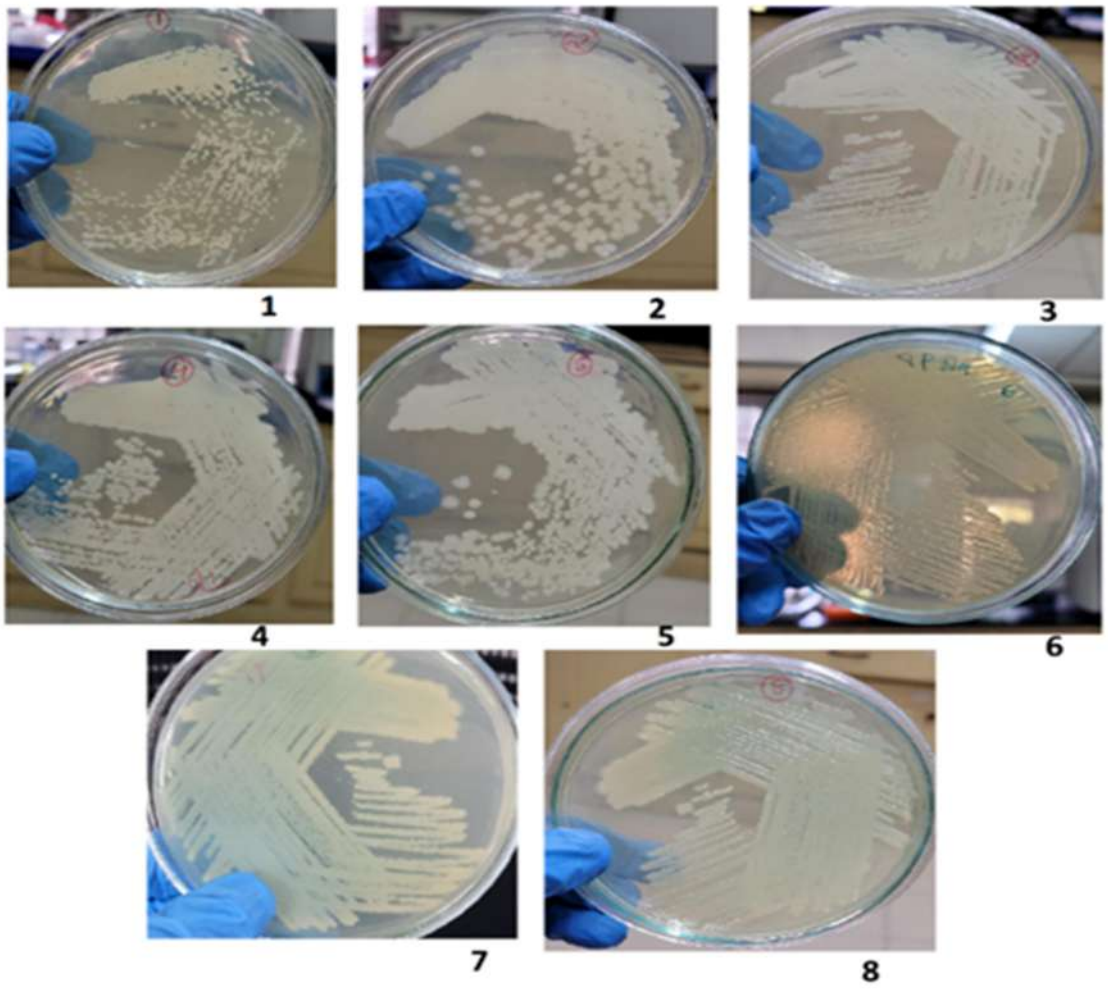


Fig. 6.3 The selected bacterial strains on Nutrient agar plates: 1- ST 01; 2-ST 02; 3- ST 03; 4- ST 04; 5- ST 05; 6- ST 06; 7- ST 07 and 8- ST 08.

6.4.5 Haemolytic activity of the selected strains

After 24 hours of incubation, all the selected bacterial strains were non-haemolytic (Fig. 6.4). The haemolytic activity is a characteristic of pathogenic strains. So, as none of the selected strains were found to be haemolytic, they were determined as good candidates for consortia development.

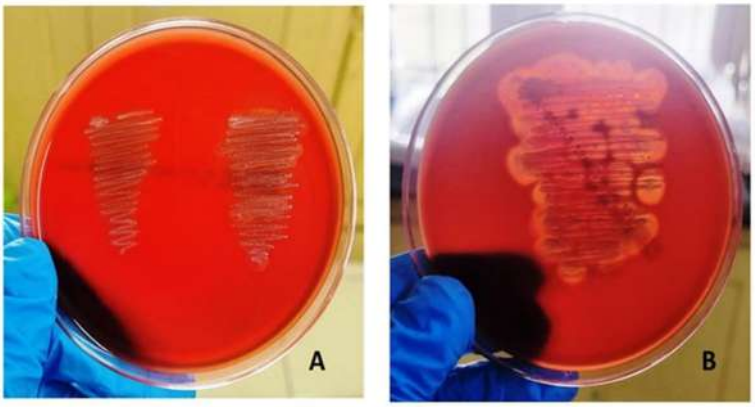


Fig. 6.4 Twenty-four hours incubated sheep blood agar plate: A) Non-hemolytic blood agar plate B) Positive control (Hemolytic plate)

6.4.6 Compatibility checking

Compatibility checking of the selected bacterial strains were determined using the cross-streaking method. After 24 hours of incubation, no growth inhibition or antagonistic activity was observed among the 8 selected bacterial strains, and they were found to be compatible with each other (Fig. 6.5).

Fig 6.5 Compatibility testing of the selected bacterial strains

6.4.7 Growth optimization of the selected bacterial isolates

Growth optimization of the selected bacterial isolates was done for physico-chemical parameters, which included temperature, pH, salinity and agitation. The optimum temperature, salinity, pH and agitation for maximum growth turbidity of each of the selected bacterial isolates are depicted in Table 6.3. Optimum growth temperature for the selected bacterial isolates ranged from 20 to 40ºC, salinity from 15 to 25 ppt, pH from 5 to 8 and agitation at 150 rpm.

Table 6.3 Optimum range of growth conditions for the selected bacterial strains

Bacterial strain		ST 01	ST 02	ST 03	ST 04	ST 05	ST 06	ST 07	ST 08
Temperature (°C)	20	+	+	-	+	++	+	-	+
	30	++	+	+	++	++	+++	++	+++
	40	++	-	+	++	+	+++	++	+++
	50	+	-	-	+	+	+	-	+
	60	-	-	-	+	-	+	-	-
Salinity (ppt)	10	-	+	+	-	-	-	-	++
	15	+	+	++	+	+	+	-	+
	20	+	+++	++	+	+++	+	++	++++
	25	-	+++	+	-	+++	-	++	++++
	30	-	+	+	-	+	-	+	+++
pH	5	++	-	+++	-	-	-	+	-
	6	++	+	+++	+	-	-	+	++
	7	++	+	+++	+	+	+	+	++
	8	+	-	+	-	+	+	-	-
	9	+	-	-	-	-	-	-	-
Agitation (rpm)	100	-	-	+	-	+	+	-	-
	150	++	+	+++	+	++	+++	+	+
	200	-	-	+	-	+	++	-	-

6.4.8 Development of bacterial consortia

The compatible strains showing almost similar growth requirements were selected for consortia development, five different combinations of consortia were developed (CS 01-05) (Table 6.4).

Table 6.4 Developed bacterial consortia

Sl. No.	Consortia	Bacterial strains in each consortia
1	CS 01	ST 02, ST 04, ST 07, ST 08
2	CS 02	ST 01, ST 03, ST 07
3	CS 03	ST 04, ST 05, ST 06
4	CS 04	ST 02, ST 06
5	CS 05	ST 01, ST 03, ST 07, ST 08

CS 01 contained 4 different strains of bacteria showing similar growth range which includes 2 strains of Serratia, one Enterobacter and one Klebsiella; CS 02 with 3 different strains of Enterobacter; CS 03 with 3 different strains of Serratia; and CS 05 with 4 different strains of which 3 are Enterobacter and one Klebsiella.

6.5 DISCUSSION

Thirty-five potential bacterial strains were selected by analysing the hydrolytic enzyme production of the bacterial isolates in the current study, and molecular identification was done. 16S rRNA sequencing is a culture-free, cost-effective method for identifying bacterial species by analysing their unique genetic signatures within the highly conserved 16S ribosomal RNA gene (Mignard and Flandrois, 2006).

The selected isolates included five strains of *Serratia marcescens*, four strains each of *Alcaligenes faecalis* and *Bacillus cereus*, three strains each of *Klebsiella pneumoniae* and *Klebsiella aerogenes*, two strains each of *Bacillus subtilis* and *Enterobacter asburiae* and one strain each of *Pseudomonas aeruginosa, Pseudomonas fulva, Bacillus albus, Stutzerimonas stutzeri (*formerly known as *Pseudomonas stutzeri), Priestia aryabhattai (*formerly known as *Bacillus aryabhattai), Bacillus amyloliquefaciens, Providencia vermicola, Providencia rettgeri, Enterobacter cloacae, Lysinibacillus xylanilyticus, Lysinibacillus macroides* and *Aeromonas caviae*.

There are reports of these bacteria isolated from different mangrove ecosystems globally. *Serratia marcescens* strain was isolated by Patel et al. (2016) from the coastal area of South Gujarat, Pereira et al. (2023) from urban mangroves in Raposa, Brazil, and Neto et al. (2020) from mangrove sediments of the Anil River, Sao Luis, Brazil. The *Alcaligenes faecalis* strain was isolated by Behera et al. (2017) from mangroves of

the Mahanadi delta, Odisha, and by Quach et al. (2023) from mangrove sediment of Vietnam. *Bacillus amyloliquefaciens* strain was isolated by Kurniawan et al. (2018) from mangrove sediments in Bangka Island; Feng et al. (2009) from the leaf of *Rhizophora stylosa*; Hastuti et al. (2015) from decayed mangrove stems in the Waai seashore of Ambon Island, Indonesia; and Tabao and Moasalud (2010) from mangroves in the Philippines. Feng et al. (2009) isolated *Bacillus sp.* from the leaf of Kandal *Rhizophora stylosa*. *Bacillus aryabhattai* was isolated by Kashif et al. (2023) from marine mangroves; Liang et al. (2022) from mangrove soil of Techeng Island in Zhanjiang, China; Foophow and Tangjitjaroenkun (2014) from mangrove forests in Chanthaburi province, Thailand; and Lu et al. (2023) from the National Shankou natural reserve of mangroves in Beihai, Guangxi, China. *Enterobacter cloacae* were isolated by Tam et al. (2018) from mangrove ecosystems in Vietnam. A *Pseudomonas aeruginosa* strain was isolated by Kurniawan et al. (2018) from mangrove sediments in Bangka Island and by Anna and Parthasarathi (2014) from mangrove ecosystems in Pichavaram, Tamil Nadu, India. Bhadania et al. (2015) isolated *Providencia* sp. from Kutch, India. Pringgenies and Setyati (2023) isolated *Alcaligenes faecalis* and *Bacillus cereus* strains from mangrove sediments in Semarang and Karimunjawa waters, Indonesia. Effendi et al. (2020) isolated *Bacillus amyloliquefaciens*, *Bacillus cereus,* and *Klebsiella pneumoniae* from Riau University campus in Indonesia. Kutty et al. (2020) isolated *Bacillus amyloliquefaciens, Bacillus subtilis, Enterobacter* sp.*, Klebsiella aerogenes*, *Pseudomonas stutzeri, Lysinibacillus macrolides and Lysinibacillus xylanilyticus* from mangrove sediments at Kadalundi, Kerala. Chantarasiri (2015) isolated *Bacillus amyloliquefaciens*, *Enterobacter* sp., *Klebsiella pneumoniae,* and *Lysinibacillus sphaericus* strains from mangrove swamps in Rayong River, Thailand. Prihanto et al. (2020) isolated *Lysobacillus* sp. and *Enterobacter* sp. from the Indonesian mangrove ecosystem. Ningsih et al. (2014) isolated *Bacillus* sp. and *Pseudomonas* sp. from the mangrove area of Peniti, West Kalimantan, Indonesia. Bamrungpanichtavorn et al. (2023) isolated *Aeromonas* sp., *Pseudomonas* sp., and *Bacillus* sp. from mangrove forests in Eastern Thailand. Castro et al. (2014) isolated *Bacillus* sp., *Enterobacter* sp., and *Xanthomonas* sp. from the Brazilian mangrove ecosystem. Rudiansyah et al. (2017) isolated *Aeromonas* sp. and *Pseudomonas* sp. from the mangrove area of Peniti, West Kalimantan, Indonesia. Behera et al. (2014)

isolated *Xanthomonas* sp., *Pseudomonas* sp. and *Bacillus* sp. from mangrove soil in the Mahanadi River Delta in Odisha, India. Geraldi et al. (2022) isolated *Bacillus subtilis and Bacillus aryabhattai* from the coastal sand dunes of Parangkusumo, Indonesia.

Phylogenetic analysis determined the evolutionary relationship between the potential isolates. In the current study, 24 bacterial strains belonged to phylum Proteobacteria, and the remaining 11 belonged to phylum Firmicutes. According to Gupta (2018), Proteobacteria is diverse and among one of the largest groups within prokaryotes. Woese and coworkers first defined the group Proteobacteria based on 16S rRNA analyses (Woese, 1987). In the current study, the genera Klebsiella, Enterobacter, Serratia, Providencia, Aeromonas, Alcaligenes and Pseudomonas or Stutzerimonas belong to the phylum Proteobacteria. The genera Lysinibacillus, Priestia, and Bacillus belong to the phylum Firmicutes and the class Bacilli (Graumann, 2007).

The study by Janssen (2006) found that Proteobacteria is the most prevalent phylum, which is consistent with the common findings in soil libraries. This phylum showcases an impressive variety of morphological, physiological and metabolic characteristics, significantly contributing to the global cycles of carbon, nitrogen and sulphur. Gammaproteobacteria represents the largest and most varied group within the Proteobacteria phylum, encompassing approximately 50% of all identified species. This class comprises at least 180 genera and 750 species, organised into 15 distinct orders (NCBI Taxonomy database, 2024). Firmicutes are an important marine phylum of gram-positive bacteria. The broad ecological spectrum and the unique physiological, biochemical, and molecular traits of Firmicutes have attracted significant interest from researchers (Younas, 2024). Phylum Firmicutes is widespread in nature. The group includes numerous spore-forming gram-positive bacteria that are vital to the microbial ecosystem responsible for breaking down lignocellulosic biomass and carbohydrate polymers. In the present study, four different species were identified in the genus Bacillus: *B. cereus*, *B. subtilis*, *B. amyloliquefaciens* and *B. albus.* All of these strains diverged from the same clade.

Providencia species are reported to be ubiquitous in the environment, commonly found in water, soil, and animal reservoirs (Yuan et al., 2020). *Aeromonas*

caviae strains were included in the Aeromonadaceae family. *Alcaligenes faecalis* showed the highest genetic divergence (0.12) from the clade in phylum Proteobacteria because it is the only bacteria that comes under class Betaproteobacteria, family Alcaligenaceae, among the selected strains.

The genus Pseudomonas showed 0.04 genetic divergence from the entire phylum Proteobacteria and is placed in a separate clade. With 313 species, this genus showcases considerable metabolic diversity, allowing for the colonization of numerous ecological niches (Madigan and Martinko, 2005). As one of the most widely distributed groups of gram-negative bacteria, Pseudomonas can be found in virtually every natural environment, such as soil, water and air, where they have established strong associations with various plants and animals (Andreani and Fasolato, 2017). *P. aeruginosa* has been studied for use in bioremediation and in processing polyethylene in municipal solid waste (Hu et al., 2024). In the present study, the genera Pseudomonas and Stutzerimonas share a recent common ancestor and diverged from the same clade. Stutzerimonas is a recently proposed genus within the family Pseudomonadaceae. This genus includes certain species previously classified under the genus Pseudomonas. Specifically, *Stutzerimonas stutzeri* corresponds to the former *Pseudomonas stutzeri* group. *S. stutzeri* exhibits a wide range of distribution in the environment and plays significant ecological roles, such as nitrogen fixation and the degradation of pollutants (Ge et al., 2023).

The current study mainly focused on the implication of bacterial enzymes for bioremediation purposes using developed consortia. When creating microbial consortia for the degradation of complex compounds, it is essential to choose chassis strains that demonstrate adequate catalytic capabilities. Furthermore, the compatibility of these strains with others in the consortium is a significant consideration. The selection criteria for potent strains typically include non-antagonistic interactions, compatibility, low mutation rates, non-haemolytic behaviours, non-toxic byproducts and high tolerance (Darmawati et al., 2021). The final selected strains in the current study showed higher hydrolytic enzyme potential, compatibility, non-antagonistic and non-pathogenic properties and the selected isolates were found to be gram-negative. Although both gram-positive and gram-negative bacteria exhibit distinct characteristics, gram-negative bacteria may present specific benefits in bioremediation

due to their unique cell wall composition and metabolic functions. The presence of an outer membrane in gram-negative bacteria can confer greater resistance to biocides and certain antibiotics, which may render them more effective for bioremediation in polluted environments. Furthermore, they have a broader array of catabolic genes and demonstrate greater adaptability in cohabiting with other organisms, which could enhance the process of biodegradation (Maher and Hassan, 2023).

Several studies have proven that the haemolytic behaviour of bacteria is one of the preliminary ways to determine pathogenicity (Ethica et al., 2018; Sumardi et al., 2021). Pathogenic bacteria are not safe to use as bioremediation agents, considering public safety. It is therefore important to set simple yet effective and affordable pathogenicity tests so that they could be used as bioremediation agents. According to Jawetz et al. (1982), α-haemolysin can lyse rabbit erythrocytes and inflict damage on platelets, which may subsequently harm the circulatory system and various tissues, such as muscle and renal cortex. Strains within a species can differ in the diseases they cause, their ecological environments and numerous other attributes, including genetic factors and plasmids.

Many reports about bacterial consortia have shown that mixing non-antagonistic agents improves biodegradation. However, there are also findings that suggest certain combinations of bioagents do not improve activity compared to their individual counterparts. Incompatibility among co-inoculants can occur, as these bioagents may inhibit each other and other microorganisms. Consequently, the compatibility of the microorganisms being co-inoculated is a vital requirement for the effective development of microbial consortia (James and Mathew, 2017). The present study employed the cross-streaking method to ascertain the compatibility of the strains. None of the strains were found to be antagonistic. The selection of an optimal bioinoculant must consider the adaptability of bacterial strains to stressful conditions and their interactions with one another. Such consideration is important because a single strain may not operate effectively as a bioremediator when competing against a diverse group of antagonistic strains. Cross-streaking methods were previously employed to assess antagonistic activity (Ali et al., 2023). In the present study, all combinations showed compatibility with each other. The main factor that bacteria need to be able to work well together is synergism (Sarkar and Chourasia, 2017).

Compatibility and synergism are crucial, as the presence of antagonistic relationships among microorganisms can destabilise the consortium, preventing it from achieving its desired functions.

Selection of bacteria for bioremediation is the crucial step in the current study. The selected strains include *Enterobacter cloacae, Enterobacter asburiae, Serratia marcescens* and *Klebsiella aerogenes.* The presence of all the strains was already reported from various environmental sources.

Enterobacter sp. are facultative anaerobic gram-negative bacilli belonging to the family of Enterobacteriaceae. *Enterobacter cloacae* and *Enterobacter asburiae* were included in *the Enterobacter cloacae* complex (Paauw et al., 2008). *Enterobacter cloacae* were reported from the Pichavaram mangrove forest in India (Rameshkumar et al., 2010); wetland soil from Thailand (Chantarasiri, 2020); Matang mangrove estuaries (Ghaderpour et al., 2014); and mangrove sediments in the east coast peninsula, Malaysia (Jalal et al., 2010). *E. cloacae* occur as a commensal in water, sewage, soil, meat, hospital environments the skin and in the intestinal tracts of humans and animals (Grimont and Grimont, 2006). The *Enterobacter cloacae* complex is an opportunistic pathogen, but strain variations were present and are also part of the commensal enteric flora of the human gastrointestinal tract (Chantarasiri, 2020). Several Enterobacter species could be isolated from the rhizosphere of black mangroves (*Avicennia germinans* L.) and the branches of red mangroves (*Rhizophora mangle*) (Castro et al., 2014). They were considered as phosphate-solubilizing bacteria and mangrove-associated endophytic bacteria with high levels of nitrogen-fixing capacity from the rhizosphere. *E. cloacae* caused an increase in photosynthetic pigments, enhanced nutrient absorption, promoted vegetative plant growth, facilitated the degradation of benzene, supported the biodegradation of explosives and contributed to the biological control of plant diseases (Chandarana and Amaresan, 2023).

Enterobacter asburiae is a gram-negative, facultatively anaerobic, oxidase-negative, non-motile and non-pigmented rod-shaped bacterium of the Enterobacteriaceae family that has been isolated from soil, water and food products (Lau et al., 2013). It is also known as the epiphytic bacterium and also reported as a

quorum sensing (QS) bacterium. The process of successful colonization is characterized by the activation of several key activities, such as the secretion of exoenzymes, establishment of symbiotic relationships, formation of biofilms, sporulation, expression of virulence, production of antibiotics, bioluminescence and conjugation. Some studies showed that *E. asburiae* can degrade polyethylene plastics, freeing up phosphorus and other minerals for plants, and act as probiotics for aquatic animals and in biohydrogen production. Enterobacter species have been reported with the ability to produce amylase, protease, lipase and ligninase (Chantarasiri, 2020).

Genus Serratia is capable of thriving in diverse environments, including water, soil and the digestive tracts of various animals. *Serratia marcescens* are gram-negative bacteria in the family Enterobacteriaceae. Serratia are widespread in the environment but are not a common component of the human faecal flora (Gillespie and Hawkey, 2006). Some variants of *S. marcescens* can synthesize a pigment referred to as prodigiosin, which can appear in shades from dark red to pale pink, depending on the colony's age. This pigment has been recognized for its potential anticancer effects. *Serratia marcescens* reported as producing lipase, cellulase and ligninase, can be used for in situ and ex situ bioremediation of hexavalent chromium (Jasu et al., 2023; Kumar et al., 2023).

Enterobacter aerogenes was recently renamed as *Klebsiella aerogenes*. Genetic characteristics of the species led to its reclassification, revealing its closest relationship to *Klebsiella pneumoniae* (Alvarez-Marin et al., 2021). It is widely distributed in the environment and is found in the human gastrointestinal tract; also, certain strains can act as opportunistic pathogens in hospitals. The *Klebsiella aerogenes* strain was found to have the potential to remove cadmium ions, improve the effective phosphorus and potassium contents in soil and degrade xenobiotic compounds and reported as producing ligninase, hydrogen, protease, and cellulase (Tu et al., 2024).

Key physicochemical parameters such as temperature, pH, salinity and agitation were optimized in the current study. These factors were optimized to reach maximum growth, which highlighted the optimal combinations for subsequent analysis. Previous studies have indicated that optimising production conditions is crucial for achieving the highest possible yield and productivity while reducing

production costs (Abdel-Fattah et al., 2005). Each organism or strain has its own special conditions for maximum enzyme production. As for the extracellular enzymes, *in-vitro* conditions strongly affected many enzymatic processes and the transportation of various components across the cell membrane; and growth turbidity and metabolic activity vary with the source of isolation, type of strain, genetic makeup of strain and cultivation condition.

In the current study bacterial growth turbidity is analysed to determine metabolic activity. Previous studies reported that growth turbidity indicates enzyme activity and metabolic action because an increase in cell mass, which causes turbidity, requires a high level of metabolic energy and the synthesis of enzymes to support cell division and multiplication. When bacteria actively grow, their metabolic machinery works overtime to produce new cells, enzymes and cellular components, which results in a denser and cloudier culture. But, decreased metabolic activity or cell death leads to a decline in turbidity. It was found that the conditions, such as pH, temperature, salinity, agitation speed, inoculum volume and nutrient source, influence enzyme production greatly from one bacterial strain to another, as reported by Gaur et al. (2018).

Isolates ST 01, 03, 04, 06, 07 and 08 showed optimum growth at 30 and 40°C; ST 02 and 05 at 20 and 30°C. Temperature is a vital environmental factor that controls the growth and production of metabolites by microorganisms; its level usually varies among different organisms and influences extracellular enzyme secretion, possibly by altering the physical properties of the cell membrane (Zhou et al., 2018). It is believed that the stabilization of the metabolic network during enzyme production at different temperatures occurs through the folding of its proteins, most likely aided by the presence of chaperones.

The optimum minimum pH range was 5-7 for ST 01, 03, and 07; 6-7 for ST 02, 04, and 08; and 7-8 for ST 05 and 06. Most aquatic bacteria thrive at a pH level between 7 and 8.5, as noted by Padan et al. (2005). Changes in the pH of the medium can impact the ionization of nutrients, thereby limiting their accessibility to microorganisms and resulting in a decline in their metabolic functions. The initial pH of a culture medium is important as it promotes and regulates the synthesis of the enzyme by microorganisms (Rafique et al., 2022).

The optimum salinity was 15 and 20 ppt for ST 01, 03, 04, and 06 and 20 and 25 ppt for ST 02, 05, 07, and 08. Salinity critically affects bacterial growth and metabolic action, with optimal growth occurring within a specific salt range, while excessive or insufficient levels inhibit cellular functions by causing osmotic stress and ion toxicity (Zahra et al., 2023). The selected isolates showed the highest bacterial growth at 150 rpm. Previous reports suggested that agitation improves dissolved oxygen levels and nutrient distribution, which are essential for aerobic bacteria. It provides uniform mixing and heat/mass transfer, ensuring consistent conditions and an efficient supply of resources. However, excessive agitation can damage bacterial cells and inhibit metabolic activity, so an optimal agitation rate must be determined for each specific strain and product (Wriahusna et al., 2020).

According to Khusro et al. (2017), the marked decrease in enzyme activity that followed was primarily due to the ongoing depletion of essential nutrients in the culture medium, resulting in an environment that was not conducive to bacterial growth. Additionally, as the inoculum size increased, enzyme production further declined because of nutrient scarcity, which in turn reduced the metabolic activities of the microorganisms (Patel et al*.,* 2009). Hence, for economical enzyme production, it is of immense importance that conditions should be optimized.

The isolated strains exhibiting the same range of growth requirements were selected for further study, and five different combinational consortia were developed, suggesting that they can grow efficiently together without one species dominating the other or producing toxins, leading to a stable consortium with synergistic interactions and ultimately higher, more consistent enzyme production. This ensures that each member of the community contributes effectively, preventing the disruption of the microbial community ratio and optimizing yields (Nunes et al., 2024).

Consortium CS 01 contained strains of *Serratia marcescens, Enterobacter asburiae,* and *Klebsiella aerogenes*. CS 02 contained strains of *Enterobacter cloacae* and *Enterobacter asburiae*. CS 03 contained only three strains of *Serratia marcescens*. CS 04 contained only two strains of *Serratia marcescens*. CS-05 contained strains of *Enterobacter cloacae*, *Enterobacter asburiae* and *Klebsiella aerogenes*. The co-cultivation as consortium is more effective than utilizing single bacterial strains,

leading to quicker pollutant degradation and a significant improvement in the biodegradation of soil contaminants due to their better adaptability and tolerance to variable and complicated environments (Varjani et al., 2021). In the current study, a top-down approach was the principle used for designing artificial consortiums, according to Lawson et al. (2019). This strategy utilizes specifically selected environmental parameters that drive an existing microbiome, either native or inoculated, through ecological selection to carry out the desired biological activities. The effective use of microbial metabolites is poised to significantly benefit humanity.

Previous studies used different combinations of consortia and reported more efficient enzyme activity and degradation than individual ones, which include hydrocarbons, paroxetine, bezafibrate, e-waste, roxarsone, endosulfan, chrysene, crude oil and plastic (Perdigao et al., 2020; Qi et al., 2022)

In In the present study, the enzymatic potential of mangrove isolates was evaluated with the aim of bioremediation. Masurkar and Pathade (2023) developed microbial consortia aimed at producing amylase, protease, gelatinase and lipase from isolates derived from organic kitchen waste, resulting in rapid waste degradation within a 15-day period, thereby showcasing significant potential for compost formation when utilized in plant growth. According to the microbial degradation study conducted by Mori et al. (2023), assessing the enzyme activity of bacteria is essential for comprehending their degradation abilities, as enzymes serve as the primary mechanisms through which bacteria decompose complex molecules. The pollutant substrate can interact with various microbial enzymes under optimal conditions (temperature/pH/contact time/concentration) to effectively convert them into less harmful products. The key enzymes involved in this process include cytochrome P450s, dehydrogenases, ligninase, laccases, proteases, hydrolases, lipases, amylase, cellulase, chitinase, peroxidases and dehalogenases. These enzymes facilitate a range of reactions to decompose pollutants, including agrochemicals, aromatic hydrocarbons, dyes, polymers, and halogenated compounds (Nour et al., 2024). Microbial enzymes perform various functions, such as oxidation, reduction, transformation, degradation and mineralization, to reduce or eliminate environmental pollution. The synchronised operation of multiple enzymes enables these consortia to efficiently break down a broad spectrum of pollutants, rendering them a significant approach for environmental

cleanup, exhibiting greater metabolic resilience and demonstrating superior performance in field applications (Artanti et al., 2024).

In the present study the advantages of amylase, lipase, protease, cellulase, chitinase, and ligninase enzymes were utilized aiming to develop a high-performing consortium for effective degradation. The protease enzymes are utilized in the bioremediation of marine crustacean waste (Jellouli et al., 2011). Laccase and ligninases are effective in removing and detoxifying textile dyes and phenolic compounds generated by the textile industry (Sondhi et al., 2015). Lipase has been employed to mitigate oil spills, including n-alkanes, aromatic hydrocarbons and PAHs (Deng et al., 2023). α-amylase is employed in the bioremediation of n-alkanes (Pinto et al., 2020). Cellulose-degrading bacteria were utilized for the degradation of sanitary napkins (Panicker et al., 2024). Chitinase, an enzyme responsible for breaking down chitin, contributes to bioremediation by degrading chitin-based pollutants and materials, thereby enhancing nutrient cycling, improving soil health, facilitating wastewater treatment, emulsifying hydrocarbons and bioremediation and potentially aiding in oil spill cleanup (Nour et al., 2024).

The molecular identification, phylogenetic analysis and optimization of *in-vitro* growth conditions will enhance our understanding of mangrove bacterial enzyme activity and can be utilized as consortia for bioremediation.

Chapter 7

APPLICATION OF FREE AND IMMOBILIZED BACTERIAL CONSORTIA FOR BIOREMEDIATION

Chapter - 7

APPLICATION OF FREE AND IMMOBILIZED BACTERIAL CONSORTIA FOR BIOREMEDIATION

7.1 INTRODUCTION

Microbial enzyme studies help researchers understand their function by quantifying enzyme production, characterizing enzymes, assessing bacterial viability and metabolism, and evaluating the effect of these enzymes on industrial processes and ecological systems. These studies provide crucial data for optimizing industrial processes, understanding microbial communities and developing new biotechnological applications (Lu et al., 2024).

Bacterial consortia enhance enzyme activity through synergistic interactions, where different species work together, producing a wider range of enzymes or providing necessary conditions for other enzymes to function more efficiently. This leads to improved enzyme production or activity in processes such as bioremediation, biocatalysis and maintaining soil health (Cao et al., 2022). Immobilized bacterial cells help enzyme production by making cells more stable and resistant to environmental stress, allowing their reusability, which reduces costs and simplifies separation from products (Mehrotra et al., 2021). This makes consortia a powerful, cost-effective, and sustainable tool for treating industrial wastewater, polluted soil, and contaminated sediment (Massot et al., 2022).

7.2 REVIEW OF LITERATURE

The widespread presence of microorganisms, particularly bacteria, illustrates their capacity to endure and flourish in various ecological environments. Their existence in mangrove ecosystems highlights their significant role within these habitats (Verma and Shah, 2022).

Microbial enzymes are increasingly recognized for their extensive applications in both industry and medicine, primarily due to their stability, catalytic efficiency, simpler and cost-effective production, optimization compared to plant and animal enzymes, potential for genetic enhancement, non-toxic and environmental friendly nature. Microbial enzymes are linked to the biodegradation of intricate natural toxic substances. These compounds serve as excellent agents for bioremediation, making this approach a viable and environmental friendly biotechnology. Recently, advancements have been made in this domain, and the expansion of research areas is essential at this time (Dave and Das, 2021).

Microorganisms are recognized for their essential contribution to technology, particularly in the industrial-scale production of both intracellular and extracellular enzymes and involved in biocatalysis (Gupta et al., 2002). Microbial enzymes provide benefits compared to traditional chemical catalysts, because of higher catalytic activity and substrate specificity (Baweja et al., 2016). The exploration of microbial diversity, alongside contemporary molecular methodologies like metagenomics and genomics, is facilitating the identification of novel microbial enzymes.

Enzyme assays typically assess either the depletion of substrates or the generation of products over time (Schnell et al., 2006). The predominant technique employed is spectrophotometry, which utilizes light absorption to quantify the concentration of substrates or products (Urvoy et al., 2020). Multiple factors influence the results of the assay, and several parameters must be monitored to ensure its proper functioning. These include composition of media, salt concentration, suppressors, promoters, temperature effects, pH levels, enzyme saturation and the degree of macromolecular crowding (Daniel et al., 2010). The ideal conditions for enzyme activity typically differ across species and even among various strains, requiring targeted research to enhance enzyme production and effectiveness (Patel et al., 2005). Enzymes exhibit variations in characteristics, including substrate specificity, active site configuration, catalytic mechanisms, as well as optimal pH and temperature ranges (Sumantha et al., 2005).

Proteases, referred to as peptidases or proteinases, are enzymes that facilitate the hydrolysis of peptide bonds in proteins, which are essential for various

physiological and industrial applications (Gupta et al., 2002). The assay for sigma non-specific protease activity serves as a standardized method for assessing protease activity (Cupp-Enyard, 2008). During the protease assay, casein (substrate) releases tyrosine and other peptides. The interaction of Folin's reagent with free tyrosine gives a blue-coloured compound, and the substance can be quantified spectrophotometrically.

Amylases are enzymes that hydrolyze starch by breaking down the internal alpha 1–4 glycosidic bonds in polysaccharides (Amin et al., 2021). The quantitative assessment of amylase activity typically involves measuring the rate at which starch is hydrolyzed into simpler sugars, often employing a colorimetric technique. The method entails utilizing a substrate such as starch along with an indicator like iodine or assessing the generation of reducing sugars through a reagent such as DNS (3,5-dinitrosalicylic acid). The variation in colour or the quantity of reducing sugars generated is subsequently linked to the activity of amylase, with the reaction products being identified based on their absorbance at a designated wavelength, such as 540 nm (Mulimani and Lalitha, 1996).

In order to evaluate the activity of bacterial cellulases, researchers generally employ a mix of qualitative and quantitative techniques, such as Congo red staining, DNS assay, Nelson and Somogyi copper methods, and occasionally fluorometric approaches. These techniques measure the enzyme's capacity to decompose cellulose, a complex carbohydrate, into simpler sugars (Dashtban et al., 2010).

The chitinase enzyme can hydrolyse chitin into its simple sugar molecules. A chitinase assay generally entails quantifying the reducing sugars liberated from colloidal chitin by the chitinase enzyme. This process is frequently conducted through a colorimetric approach, such as the dinitrosalicylic acid (DNS) method, wherein the reducing sugars interact with DNS, and the resultant colour is assessed at a designated wavelength (Bailey, 1988). The alkaline 3,5-dinitrosalicylic acid (DNS) method, along with the Nelson and Somogyi copper methods, is among the most recognized techniques, noted for its speed and simplicity. Although the copper method developed by Nelson and Somogyi is less user-friendly compared to the DNS technique, it offers greater reliability and sensitivity (Al Talebi et al., 2022).

For lignin degradation, enzymes such as laccase, lignin peroxidase and manganese peroxidase are involved (Pollegioni et al., 2015). Commonly employed methods for the quantitative assessment of lignin peroxidase include the azure B assay, the veratryl alcohol assay and the phenol red assay (Kameshwar and Qin, 2017). Veratryl alcohol assay and Azure B assay are used for ligninase activity analysis (Archibald et al., 1992). This assay quantifies lignin peroxidase (LiP) activity by assessing the oxidation of Azure B dye, which results in a reduction of absorbance at 651 nm. The underlying principle of the assay is that LiP can oxidize various substrates, including Azure B, in the presence of hydrogen peroxide. This oxidation alters the dye's optical characteristics, enabling the quantification of the enzyme's activity.

Lipases, classified as triacylglycerol hydrolases, represent a significant category of enzymes with substantial biotechnological importance, finding extensive applications across food, dairy, detergent, and pharmaceutical sectors (Gupta et al., 2004). The distinctive lipase enzymes, known as triacylglycerol acyl hydrolases, facilitate hydrolysis, esterification, and alcoholysis reactions (Chandra et al., 2020). Given the broad substrate specificity of lipases, various assay protocols are utilized for lipase assessment, which includes titration, colorimetry, turbidity analysis, etc. (Thomson et al., 1999). Olive oil or tributyrin is commonly used as a substrate for lipase assay because they are simple, accurate and reproducible (Stoytcheva et al., 2012).

Environmental pollution represents one of the most critical challenges faced by the world today, primarily caused by the unregulated release of untreated waste and contaminants into the ecosystem. Although various contemporary remediation technologies, including physical, chemical and biological methods, are currently employed, they remain inadequate for effectively restoring the environment. Enzymatic bioremediation is an efficient, straightforward, eco-friendly and socially endorsed method for addressing environmental contamination. Microbial enzymes facilitate various processes such as oxidation, reduction, transformation, degradation and mineralization to reduce or eliminate pollutants. Nevertheless, the limited production of these enzymes pose a challenge to their broader application. The utilization of bacterial consortia, genetic engineering, immobilization techniques,

nanoenzymes, biosensors, bioleaching and various other methods can enhance the effectiveness, activity, stability, substrate selectivity and longevity of enzymes, thereby facilitating the removal of environmental contaminants (Yagnik et al., 2023). The significant heterogeneity within naturally occurring microbial communities, along with their synergistic and antagonistic interactions, interdependent growth patterns, and complex metabolic behaviors, presents considerable challenges in achieving targeted remediation objectives (Leenen et al., 1996).

The bioremediation process relies mainly on these microorganisms, which enzymatically degrade multiple pollutants and convert them into less or non-toxic substances or metabolites that could serve as beneficial products (Syed et al., 2023). Bioremediation refers to the process by which microorganisms convert or degrade pollutants into less harmful or non-toxic substances (Sharma et al., 2018). This process can be quite time-intensive. Certain bacteria and fungi have been shown to degrade multiple pollutants effectively (Zhang et al., 2019). Therefore, the efficiency and stability of this process are influenced by various factors, including pH levels, temperature, oxygen availability, the properties of the medium (e.g., water or soil), humidity and sufficient nutrient content. Recent developments in enzymology offer promising methods to improve biodegradation processes.

Utilizing microbial consortia for the degradation of complex compounds offers greater benefits than employing isolated bacteria, as the former demonstrates enhanced adaptability and stability within their growth environment, thereby creating an optimal catalytic setting for each enzyme necessary in the biodegradation process. Advances in synthetic biology and gene-editing technologies enable the design of artificial microbial consortia systems that are more efficient, stable and resilient, allowing for the production of high-value-added products due to their robust degradation capabilities (Cao et al., 2022). Artificial microbial consortia are developed by distributing catalytic enzyme expression pathways among different strains which are co-cultivated for optimal results. These consortia can be engineered to effectively degrade various complex compounds, allowing for the modular assembly and improvement of metabolic pathways through structural modifications and overcomes feedback inhibition (Patowary et al., 2016). The subsequent consideration involves the categorization of degradation pathways. Two frequently employed artificial design

strategies to ensure complex stability are cross-feeding and quorum sensing (QS). In microbial consortia with a limited number of strains, symbiotic interactions predominantly rely on the cross-feeding of single metabolites, such as amino acids (Harcombe et al., 2018). The fundamental molecular mechanism governing synergistic metabolism within microbial consortia remains undisclosed. While the process of intercellular communication in extensive microbial consortia is not yet fully understood and the regulatory approaches are still lacking, it is anticipated that as research progresses, the enhanced metabolic capabilities and resilience of engineered microbial consortia will facilitate their application in the degradation of complex compounds. Recent studies have indicated that various microorganisms, including bacteria, fungi and algae, are capable of effectively remediating multiple pollutants (AbuQamar et al., 2025). A bacterial consortium can effectively bioremediate contaminated sites, such as those with petroleum hydrocarbons (Hu et al., 2020) and sewage (Wu et al., 2020).

Immobilized enzymes are becoming recognized as potent agents for both biodegradation and biosynthesis. Over the course of a few decades, this progress garnered interest, leading various research teams to modify the technology by immobilizing bacteria for the remediation of organic pollutants (Ramakrishna and Prakasham, 1999). Utilizing free cells for remediation presents certain drawbacks, such as a low concentration of biomass at specific locations and times, the potential for microbial washout during application and vulnerability to external stressors. The advantages of using immobilized cells over free forms include efficient biomass segregation, low contamination risk, reusability and resilience (Mehrotra et al., 2021).

Choosing an appropriate supporting matrix or carrier for cell immobilization is a critical factor (Mehrotra et al., 2019). The materials utilized for the immobilization of different cell types are categorized into inorganic substances and organic polymers (natural and synthetic) (Cassidy et al., 1996). Natural organic carriers are readily accessible, cost-effective, biodegradable and non-toxic, typically exhibiting greater absorptive properties than their inorganic counterparts. The diverse functional groups present on the surfaces of organic matrices further enhance their absorption capacity, and agar, chitosan, gelatin, collagen and alginate are regarded as natural organic polymers (Mehrotra et al., 2021). Bacteria can effectively maintain the immobilization

process and thrive within matrices created from natural polymers such as carrageenan and alginate (Lozinskiy et al., 2001). Cells immobilized in alginate experience minimal physicochemical alterations and produce porous and clear gel, making it a widely utilized material (de-Bashan and Bashan, 2010).

The management of waste has emerged as a vital concern for both environmental sustainability and public health. A significant focus is the environmental effects of using disposable sanitary napkins. Consortia composed of various microbial species typically demonstrate enhanced metabolic functions relative to individual strains, facilitating the degradation of resistant plastics and other waste materials. The use of poly-coated paper bags poses environmental issues mainly because the plastic coating is non-biodegradable, complicating recycling efforts and adding to plastic waste. Although the paper is biodegradable, the plastic coating obstructs this process and may release microplastics into the environment. Recently, paper bags have gained popularity as an alternative to plastic bags. Nevertheless, paper bags also present their own environmental issues (Wang and Li, 2021). Although paper products are biodegradable and recyclable, certain paper bags are treated with plastic to enhance their durability and water resistance. This treatment complicates the recycling process and raises concerns about plastic contamination (Gaylarde et al., 2021). If these types of paper bags enter the environment, the associated plastics may contribute to plastic pollution, potentially breaking down into microplastics (less than 5 mm) and even nanoplastics (less than 1000 nm). Regrettably, there is a scarcity of reports in this domain regarding the tracking of plastic sources in paper bags. Used sanitary napkins present considerable environmental challenges due to their non-biodegradable properties and the chemicals they contain, which contribute to prolonged pollution and potential health hazards. These products may require hundreds of years to decompose, leading to their accumulation in landfills and the release of toxic substances such as dioxins and furans into the soil and water. Approximately 200 tonnes of sanitary napkin waste are generated daily, with studies indicating that around 12.3 billion sanitary napkins are currently present in landfills across India. A significant concern regarding disposable menstrual napkins is the raw materials utilized, particularly polyethylene. Polyethylene is a plastic material frequently found in disposable sanitary napkins and tampons (Peberdy et al., 2019).

The present study examined the enzymatic activity of both free and immobilized bacterial consortia, focusing on their application in bioremediation, which includes the degradation of sanitary napkins and poly-coated paper bags.

7.3 MATERIALS AND METHODS

7.3.1 Preparation of bacterial inoculum

Nutrient broth tubes were prepared and sterilized at 121°C for 15 minutes in an autoclave. A loopful of purified bacterial isolates were inoculated on to nutrient broth tubes. Incubation was done at room temperature (28± 2°C) for 24 hours. The cells were harvested at the mid-logarithmic phase. The cell density was calibrated using the 0.5 McFarland standard (0.05 ml of 1% $BaCl_2$ mixed with 9.95 ml of H_2SO_4), which contains approximately 1.5×10^8 cells that served as the inoculum (Roessler and Brewer, 1967). Optical density of the culture suspension was taken at 600 nm in a UV-VIS spectrophotometer (Thermo Fisher Scientific, USA).

7.3.2 Preparation of immobilized bacteria

Bacterial cells were entrapped in alginate according to Johnsen and Flink (1986). Sodium alginate was dissolved in boiling water (1.5%, w/v) and autoclaved at 121°C for 15 min. For immobilized bead preparation, 1 ml each of 0.1 OD adjusted bacterial culture were mixed together and poured into sodium alginate solution (sodium alginate- 1.5%, w/v and autoclaved at 121°C for 15 min). The resulting suspension was extruded drop by drop through a hypodermic syringe into a gently stirred cold sterile $CaCl_2$ (7%, w/v) solution to obtain gel beads of approximately 2 mm in diameter. The gel beads of calcium alginate with entrapped bacterial cells were cured and hardened in the same solution for 30-60 min, assuring the completion of the gelling process. The beads were then thoroughly rinsed with sterile physiological saline (0.9% NaCl solution) to remove excess calcium ions and cells that are not trapped, which are then stored at 4°C in fresh sterile saline until used.

7.3.3 Experimental set up for analysing enzyme activity

The enzyme activity of bacterial consortia was compared with that of single strains. Both free state and immobilized state bacterial enzyme activity were measured. Four distinct types of experimental setups were used for this purpose (Table 7.1).

Table 7.1 Experimental set up

Sl. No	Bacterial type	Description
1	FS 01	Free state ST 01 Bacterial strain
2	FS 02	Free state ST 02 Bacterial strain
3	FS 03	Free state ST 03 Bacterial strain
4	FS 04	Free state ST 04 Bacterial strain
5	FS 05	Free state ST 05 Bacterial strain
6	FS 06	Free state ST 06 Bacterial strain
7	FS 07	Free state ST 07 Bacterial strain
8	FS 08	Free state ST 08 Bacterial strain
9	IS 01	Immobilized state ST 01 Bacterial strain
10	IS 02	Immobilized state ST 02 Bacterial strain
11	IS 03	Immobilized state ST 03 Bacterial strain
12	IS 04	Immobilized state ST 04 Bacterial strain
13	IS 05	Immobilized state ST 05 Bacterial strain
14	IS 06	Immobilized state ST 06 Bacterial strain
15	IS 07	Immobilized state ST 07 Bacterial strain
16	IS 08	Immobilized state ST 08 Bacterial strain
17	FCS 01	Free state CS 01 Bacterial Consortium
18	FCS 02	Free state CS 02 Bacterial Consortium
19	FCS 03	Free state CS 03 Bacterial Consortium
20	FCS 04	Free state CS 04 Bacterial Consortium
21	FCS 05	Free state CS 05 Bacterial Consortium
22	ICS 01	Immobilized state CS 01 Bacterial Consortium
23	ICS 02	Immobilized state CS 02 Bacterial Consortium
24	ICS 03	Immobilized state CS 03 Bacterial Consortium
25	ICS 04	Immobilized state CS 04 Bacterial Consortium
26	ICS 05	Immobilized state CS 05 Bacterial Consortium

7.3.4 Physico-chemical parameters for enzyme activity analysis

Optimal growth conditions were provided to analyse the enzyme activity of free and immobilized state bacterial cells of single strain and consortia (Table 7.2 and 7.3).

Table 7.2 ***Invitro*** **conditions for enzyme activity analysis of selected bacterial isolates**

Bacterial strain	Experiment No.	Temperature (ºC)	Salinity (ppt)	pH	Agitation Speed (rpm)	State of Bacterial strain
ST 01	1	30	15	5	150	FS 01
						IS 01
	2	30	15	6	150	FS 01
						IS 01
	3	30	15	7	150	FS 01
						IS 01
	4	30	20	5	150	FS 01
						IS 01
	5	30	20	6	150	FS 01
						IS 01
	6	30	20	7	150	FS 01
						IS 01
	7	40	15	5	150	FS 01
						IS 01
	8	40	15	6	150	FS 01
						IS 01
	9	40	15	7	150	FS 01
						IS 01
	10	40	20	5	150	FS 01
						IS 01
	11	40	20	6	150	FS 01
						IS 01
	12	40	20	7	150	FS 01
						IS 01
ST 02	1	20	20	6	150	FS 02
						IS 02
	2	20	20	7	150	FS 02
						IS 02
	3	20	25	6	150	FS 02
						IS 02
	4	20	25	7	150	FS 02
						IS 02
	5	30	20	6	150	FS 02
						IS 02

	6	30	20	7	150	FS 02
						IS 02
	7	30	25	6	150	FS 02
						IS 02
	8	30	25	7	150	FS 02
						IS 02
ST 03	1	30	15	5	150	FS 03
						IS 03
	2	30	15	6	150	FS 03
						IS 03
	3	30	15	7	150	FS 03
						IS 03
	4	30	20	5	150	FS 03
						IS 03
	5	30	20	6	150	FS 03
						IS 03
	6	30	20	7	150	FS 03
						IS 03
	7	40	15	5	150	FS 03
						IS 03
	8	40	15	6	150	FS 03
						IS 03
	9	40	15	7	150	FS 03
						IS 03
	10	40	20	5	150	FS 03
						IS 03
	11	40	20	6	150	FS 03
						IS 03
	12	40	20	7	150	FS 03
						IS 03
ST 04	1	30	15	6	150	FS 04
						IS 04
	2	30	15	7	150	FS 04
						IS 04
	3	30	20	6	150	FS 04
						IS 04
	4	30	20	7	150	FS 04
						IS 04
	5	40	15	6	150	FS 04
						IS 04
	6	40	15	7	150	FS 04
						IS 04
	7	40	20	6	150	FS 04
						IS 04

	8	40	20	7	150	FS 04
						IS 04
ST 05	1	20	20	7	150	FS 05
						IS 05
	2	20	20	8	150	FS 05
						IS 05
	3	20	25	7	150	FS 05
						IS 05
	4	20	25	8	150	FS 05
						IS 05
	5	30	20	7	150	FS 05
						IS 05
	6	30	20	8	150	FS 05
						IS 05
	7	30	25	7	150	FS 05
						IS 05
	8	30	25	8	150	FS 05
						IS 05
ST 06	1	30	15	7	150	FS 06
						IS 06
	2	30	15	8	150	FS 06
						IS 06
	3	30	20	7	150	FS 06
						IS 06
	4	30	20	8	150	FS 06
						IS 06
	5	40	15	7	150	FS 06
						IS 06
	6	40	15	8	150	FS 06
						IS 06
	7	40	20	7	150	FS 06
						IS 06
	8	40	20	8	150	FS 06
						IS 06
ST 07	1	30	20	5	150	FS 07
						IS 07
	2	30	20	6	150	FS 07
						IS 07
	3	30	20	7	150	FS 07
						IS 07
	4	30	25	5	150	FS 07
						IS 07
	5	30	25	6	150	FS 07
						IS 07

	6	30	25	7	150	FS 07
						IS 07
	7	40	20	5	150	FS 07
						IS 07
	8	40	20	6	150	FS 07
						IS 07
	9	40	20	7	150	FS 07
						IS 07
	10	40	25	5	150	FS 07
						IS 07
	11	40	25	6	150	FS 07
						IS 07
	12	40	25	7	150	FS 07
						IS 07
ST 08	1	30	20	6	150	FS 08
						IS 08
	2	30	20	7	150	FS 08
						IS 08
	3	30	25	6	150	FS 08
						IS 08
	4	30	25	7	150	FS 08
						IS 08
	5	40	20	6	150	FS 08
						IS 08
	6	40	20	7	150	FS 08
						IS 08
	7	40	25	6	150	FS 08
						IS 08
	8	40	25	7	150	FS 08
						IS 08

Table 7.3 ***Invitro*** **conditions for enzyme activity analysis of developed bacterial consortia**

Bacterial consortia	Experiment No.	Temperature (ºC)	Salinity (ppt)	pH	Agitation Speed (rpm)	State of Bacterial strain
CS 01	1	20	15	5	150	FCS 01
						ICS 01
	2	20	15	6	150	FCS 01
						ICS 01
	3	20	15	7	150	FCS 01
						ICS 01
	4	20	20	5	150	FCS 01
						ICS 01
	5	20	20	6	150	FCS 01
						ICS 01
	6	20	20	7	150	FCS 01
						ICS 01
	7	20	25	5	150	FCS 01
						ICS 01
	8	20	25	6	150	FCS 01
						ICS 01
	9	20	25	7	150	FCS 01
						ICS 01
	10	30	15	5	150	FCS 01
						ICS 01
	11	30	15	6	150	FCS 01
						ICS 01
	12	30	15	7	150	FCS 01
						ICS 01
	13	30	20	5	150	FCS 01
						ICS 01
	14	30	20	6	150	FCS 01
						ICS 01
	15	30	20	7	150	FCS 01
						ICS 01
	16	30	25	5	150	FCS 01
						ICS 01
	17	30	25	6	150	FCS 01
						ICS 01

	18	30	25	7	150	FCS 01
						ICS 01
	19	40	15	5	150	FCS 01
						ICS 01
	20	40	15	6	150	FCS 01
						ICS 01
	21	40	15	7	150	FCS 01
						ICS 01
	22	40	20	5	150	FCS 01
						ICS 01
	23	40	20	6	150	FCS 01
						ICS 01
	24	40	20	7	150	FCS 01
						ICS 01
	25	40	25	5	150	FCS 01
						ICS 01
	26	40	25	6	150	FCS 01
						ICS 01
	27	40	25	7	150	FCS 02
						ICS 02
CS 02	1	30	15	5	150	FCS 02
						ICS 02
	2	30	15	6	150	FCS 02
						ICS 02
	3	30	15	7	150	FCS 02
						ICS 02
	4	30	20	5	150	FCS 02
						ICS 02
	5	30	20	6	150	FCS 02
						ICS 02
	6	30	20	7	150	FCS 02
						ICS 02
	7	30	25	5	150	FCS 02
						ICS 02
	8	30	25	6	150	FCS 02
						ICS 02
	9	30	25	7	150	FCS 02
						ICS 02
	10	40	15	5	150	FCS 02
						ICS 02
	11	40	15	6	150	FCS 02
						ICS 02
	12	40	15	7	150	FCS 02
						ICS 02

	13	40	20	5	150	FCS 02
						ICS 02
	14	40	20	6	150	FCS 02
						ICS 02
	15	40	20	7	150	FCS 02
						ICS 02
	16	40	25	5	150	FCS 02
						ICS 02
	17	40	25	6	150	FCS 02
						ICS 02
	18	40	25	7	150	FCS 02
						ICS 02
CS 03	1	20	15	6	150	FCS 03
						ICS 03
	2	20	15	7	150	FCS 03
						ICS 03
	3	20	15	8	150	FCS 03
						ICS 03
	4	20	20	6	150	FCS 03
						ICS 03
	5	20	20	7	150	FCS 03
						ICS 03
	6	20	20	8	150	FCS 03
						ICS 03
	7	20	25	6	150	FCS 03
						ICS 03
	8	20	25	7	150	FCS 03
						ICS 03
	9	20	25	8	150	FCS 03
						ICS 03
	10	30	15	6	150	FCS 03
						ICS 03
	11	30	15	7	150	FCS 03
						ICS 03
	12	30	15	8	150	FCS 03
						ICS 03
	13	30	20	6	150	FCS 03
						ICS 03
	14	30	20	7	150	FCS 03
						ICS 03
	15	30	20	8	150	FCS 03
						ICS 03
	16	30	25	6	150	FCS 03
						ICS 03

	17	30	25	7	150	FCS 03
						ICS 03
	18	30	25	8	150	FCS 03
						ICS 03
	19	40	15	6	150	FCS 03
						ICS 03
	20	40	15	7	150	FCS 03
						ICS 03
	21	40	15	8	150	FCS 03
						ICS 03
	22	40	20	6	150	FCS 03
						ICS 03
	23	40	20	7	150	FCS 03
						ICS 03
	24	40	20	8	150	FCS 03
						ICS 03
	25	40	25	6	150	FCS 03
						ICS 03
	26	40	25	7	150	FCS 03
						ICS 03
	27	40	25	8	150	FCS 03
						ICS 03
CS 04	1	20	15	6	150	FCS 04
						ICS 04
	2	20	15	7	150	FCS 04
						ICS 04
	3	20	15	8	150	FCS 04
						ICS 04
	4	20	20	6	150	FCS 04
						ICS 04
	5	20	20	7	150	FCS 04
						ICS 04
	6	20	20	8	150	FCS 04
						ICS 04
	7	20	25	6	150	FCS 04
						ICS 04
	8	20	25	7	150	FCS 04
						ICS 04
	9	20	25	8	150	FCS 04
						ICS 04
	10	30	15	6	150	FCS 04
						ICS 04
	11	30	15	7	150	FCS 04
						ICS 04

	12	30	15	8	150	FCS 04
						ICS 04
	13	30	20	6	150	FCS 04
						ICS 04
	14	30	20	7	150	FCS 04
						ICS 04
	15	30	20	8	150	FCS 04
						ICS 04
	16	30	25	6	150	FCS 04
						ICS 04
	17	30	25	7	150	FCS 04
						ICS 04
	18	30	25	8	150	FCS 04
						ICS 04
	19	40	15	6	150	FCS 04
						ICS 04
	20	40	15	7	150	FCS 04
						ICS 04
	21	40	15	8	150	FCS 04
						ICS 04
	22	40	20	6	150	FCS 04
						ICS 04
	23	40	20	7	150	FCS 04
						ICS 04
	24	40	20	8	150	FCS 04
						ICS 04
	25	40	25	6	150	FCS 04
						ICS 04
	26	40	25	7	150	FCS 04
						ICS 04
	27	40	25	8	150	FCS 04
						ICS 04
CS 05	1	30	15	5	150	FCS 05
						ICS 05
	2	30	15	6	150	FCS 05
						ICS 05
	3	30	15	7	150	FCS 05
						ICS 05
	4	30	20	5	150	FCS 05
						ICS 05
	5	30	20	6	150	FCS 05
						ICS 05
	6	30	20	7	150	FCS 05
						ICS 05

	7	30	25	5	150	FCS 05
						ICS 05
	8	30	25	6	150	FCS 05
						ICS 05
	9	30	25	7	150	FCS 05
						ICS 05
	10	40	15	5	150	FCS 05
						ICS 05
	11	40	15	6	150	FCS 05
						ICS 05
	12	40	15	7	150	FCS 05
						ICS 05
	13	40	20	5	150	FCS 05
						ICS 05
	14	40	20	6	150	FCS 05
						ICS 05
	15	40	20	7	150	FCS 05
						ICS 05
	16	40	25	5	150	FCS 05
						ICS 05
	17	40	25	6	150	FCS 05
						ICS 05
	18	40	25	7	150	FCS 05
						ICS 05

7.3.5 Preparation of medium with substrate

Mineral salt medium was prepared and supplemented with 0.5% of specific substrates (Carboxymethylcellulose, casein, starch, tributyrin, colloidal chitin and lignin) in separate flasks.

Mineral salt medium

Sodium nitrate	-	2 g
Potassium chloride	-	0.5 g
Magnesium sulphate	-	0.5 g
Di-potassium hydrogen phosphate	-	1 g
Ferrous sulphate	-	0.01 g

Distilled water	-	1000 ml
pH	-	7.3

7.3.6 Inoculation of free and immobilized bacterial consortia to the medium

The bacterial strains used in the development of 5 different consortia are depicted in Table 6.4. Free state CS 01 was prepared by mixing 0.1 OD adjusted 1 ml each of ST 02, 04,07 and 08 bacterial inoculum into 100 ml modified Czapek mineral salt broth. The medium was incubated by providing optimum conditions (Table 7.3) for 72 hours. Samples were collected every 24 hours and quantitative assays were performed by withdrawing the medium aseptically (Nannipieri and Ruggiero, 2002). All steps were carried out under sterile conditions. Six separate flasks were kept for 6 different enzyme substrates. The procedure was followed as such for the free state consortia CS 02, 03, 04 and 05.

For the immobilized bacterial consortium, immobilized state CS 01 was prepared by mixing 0.1 OD adjusted 1 ml each ST 02, 04, 07 and 08 bacterial isolates into sodium alginate solution (sodium alginate- 1.5%, w/v and autoclaved at 121°C for 15 min) and followed the procedure mentioned in 7.3.2. Approximately 5 g of calcium alginate beads was added to a flask containing 100 ml of the modified Czapek mineral salt broth medium with 0.5% substrate mixed aseptically and incubated by providing optimum conditions (Table 7.3) for 72 hours. Samples were collected every 24 hours and quantitative assay was performed by withdrawing the medium aseptically. All the steps were carried out under sterile conditions. Six separate flasks were maintained for the analysis of 6 different enzyme assays. The procedure was followed same for immobilized state consortia CS 02, 03, 04 and 05.

7.3.7 Quantitative enzyme assay

Quantitative enzyme assay was performed at 24, 48 and 72 hours. For enzymatic assay, 5 ml of sample was withdrawn aseptically and centrifuged at 10,000 rpm. The supernatant was subjected to protease, amylase, cellulase, chitinase, ligninase and lipase enzyme assays. Standards were used and experiments were carried out in triplicate, and standard deviation was calculated. The enzyme activity was calculated using the formula (Bisswanger, 2014).

$$\text{Enzyme activity (U/ml)} = \frac{\text{Consumed substrate} \times \text{Total reaction volume}}{\text{Reaction time (minutes)} \times \text{Enzyme volume (ml)}}$$

7.3.7.1 Protease assay

Protease activity was measured by Sigma's universal protease activity assay using casein as substrate (Cupp-Enyard, 2008). 500 µl of supernatant of culture filtrate was added to 500 µl of 1% casein (SRL) prepared in 50 mM potassium phosphate buffer (pH 7.5) and incubated at 37ºC for 10 minutes. The reaction was stopped by the addition of 500 µl of 20% w/v tricholoroacetic acid (TCA). The mixture was incubated at 37°C for 30 minutes and then centrifuged at 10000 x g for 10 minutes. 1 ml of the supernatant was taken in a fresh test tube to which 5000 µl of 0.5 M sodium carbonate was added, followed by the quick addition of 500 µl of Folin- Ciocalteu reagent. The tubes were kept in dark for 20 minutes. The absorbance of the supernatant was measured at 660 nm spectrophotometrically UV-VIS spectrophotometer (Thermo Fisher Scientific, USA). Control consisted of reaction mixture without supernatant (enzyme). A standard curve was generated using tyrosine as standard (0.2 mg/ml). One unit (U) of protease activity is defined as the amount of enzyme required to liberate 1µg tyrosine per millilitre per minute under the standard assay conditions.

7.3.7.2 Amylase assay

Amylase activity was measured by DNSA (3,5-dinitrosalicylic acid) method using starch as substrate (Jain et al., 2020). 500 µl of supernatant of culture filtrate was added to 500 µl of 1% starch (SRL) prepared in 50 mM phosphate buffer (pH 7.0) and incubated at 37ºC for 10 minutes. After incubation, 1000 µl of DNS reagent was added and incubated at 100°C for 10- 15 minutes. The test tube containing the mixture was cooled to stop the reaction by rinsing it with running water and then ice water so that the test tube does not break. Absorbance of the supernatant was measured at 540 nm spectrophotometrically UV-VIS spectrophotometer (Thermo Fisher Scientific, USA). Control consisted of reaction mixture without supernatant (enzyme). A standard curve was generated using glucose as standard (1 mg/ml). One unit of enzyme activity (U) is defined as the amount of enzyme that releases 1 µmol of reducing sugar (glucose) per minute under assay conditions.

7.3.7.3 Cellulase assay

Cellulase activity was measured by DNSA (3,5-dinitrosalicylic acid) method using CMC (Carboxymethyl cellulose) as substrate (Jain et al., 2020). 500 µl of supernatant of culture filtrate was added to 500 µl of 1% CMC prepared in 50 mM phosphate buffer (pH 7.0) and incubated at 37ºC for 10 minutes. After incubation, 1000 µl of DNS reagent was added and incubated at 100°C for 10- 15 minutes. The test tube containing the mixture was cooled to stop the reaction by rinsing it with running water and then ice water so that the test tube does not break. Absorbance of the supernatant was measured at 540 nm spectrophotometrically UV-VIS spectrophotometer (Thermo Fisher Scientific, USA). Control consisted of reaction mixture without supernatant (enzyme). A standard curve was generated using glucose as standard (1 mg/ml). One unit of enzyme activity (U) is defined as the amount of enzyme that releases 1 µmol of reducing sugar (glucose) per minute under assay conditions.

7.3.7.4 Chitinase assay

Chitinase activity was measured by DNSA (3,5-dinitrosalicylic acid) method using colloidal chitin as substrate (Jain et al., 2020). 500 µl of supernatant of culture filtrate was added to 500 µl of 1% colloidal chitin prepared in 50 mM phosphate buffer (pH 7.0) and incubated at 45°C for 1 hour. After incubation, 3000 µl of DNSA reagent was added and incubated at 100°C for 5 minutes. The test tube containing the mixture was cooled to stop the reaction by rinsing it with running water and then ice water so that the test tube does not break. Absorbance of the supernatant was measured at 530 nm spectrophotometrically UV-VIS spectrophotometer (Thermo Fisher Scientific, USA). Control consisted of reaction mixture without supernatant (enzyme). A standard curve was generated using N-acetylglucosamine as standard (1 mg/ml). One unit of enzyme activity (U) is defined as the amount of enzyme that releases 1 µmol of N-acetyl glucosamine from chitin per minute under assay conditions.

7.3.7.5 Ligninase assay

Ligninase activity was measured by determining the production of lignin peroxidase enzyme using Azure B as substrate (Archibald, 1992). Lignin peroxidase

(LiP) enzyme was measured based on the oxidation of dye Azure B. 500 µl of the supernatant of culture filtrate, 1000 µl of 125 millimolar sodium tartrate buffer (pH 3), and 500 µl of 0.16 millimolar azure B were taken, and then 500 µl of 2 millimolar hydrogen peroxide was added to initiate the reaction. Absorbance was measured at 651 nm spectrophotometrically UV-VIS spectrophotometer (Thermo Fisher Scientific, USA). Control consisted of reaction mixture without supernatant (enzyme). A standard curve was generated using Veratraldehyde solutions standard (1 mg/ml). One unit of enzyme activity was expressed as an OD decrease at 651 nm of 0.1 units per minute per litre of the culture filtrate.

7.3.7.6 Lipase assay

Lipase activity was measured by titrimetric method using tributyrin oil as substrates (Naher, 1974). The reaction mixture was prepared by 1000 µl tributyrin oil emulsified in 3000 µl of 50mM Tris- HCl buffer (pH 8.0). 1000 µl of each supernatant of culture filtrate was added to the reaction mixture and incubated at 37°C for 30 min. Control consisted of reaction mixture without supernatant (enzyme). In the test and control 1-2 drops of 1% phenolphthalein indicator were added. The contents of each reaction mixture were titrated against 50 mM NaOH solution to an end point of pink colour at pH 10.0. Lipase activity was calculated as micromoles of free fatty acids formed from tributyrin oil per ml of crude lipase enzyme as per the equation. One unit (U) of lipase enzyme is defined as the amount of enzyme required to liberate 1µmol of fatty acids from triglycerides.

$$\text{Enzyme activity (U/ml)} = \frac{(Vs\text{-}V_B) \times N \times 1000}{S}$$

Where,

Vs: Volume of 50 mM NaOH solution consumed by the enzyme-substrate (ml)

V_B: Volume of 50 mM NaOH solution in the titration by the substrate (control) (ml)

N: Molar strength of the NaOH solution used for titration (50 mM)

S: Volume of reaction mixture

7.3.8 *In vitro* degradation of sanitary pads and polycoated carry bags using developed bacterial consortia

7.3.8.1 Samples used for the study

1. Regular synthetic sanitary napkins were obtained from nearby store (ultra-XL one piece consists 284 mm long sized, 23.5 x 9.2 x 13 cm dimension, ≈ 6 g weight). Synthetic sanitary napkins composed of a non-woven top sheet (polypropylene/polyethylene) for skin contact, an absorbent core with superabsorbent polymers (SAPs) and wood pulp, a waterproof polyethylene back sheet to prevent leaks, and synthetic adhesives for securing the layers. Other synthetic components include fragrances, and chemicals like BPA and BPS (Woeller and Hochwalt, 2015). One pad was cut into pieces and were added in one sterile glass jar for the study.

2. Poly coated paper carry bag was obtained from nearby store, with dimensions 25 x 35 x 13 cm; ≈ 45 g weight. Polycoated paper carry bag is composed of two main layers: a high-quality paper base, and a thin layer of polyethylene (poly) coating applied to one or both sides (Accinelli et al., 2012). 5 g each piece of polycoated paper bag was used in single batch of study.

7.3.8.2 Pre-treatment of the samples

The fresh samples were cut into small pieces (≈ 4 × 8 cm sized pieces). The pieces were weighed (= initial) and UV sterilized. All subsequent steps were carried out under sterile conditions. The dry, sterile sample pieces were transferred into sterile glass jar and 15 ml of modified Czapek mineral salt broth medium without substrate was added (Ellammal et al., 2022). The experimental set up is shown in Fig. 7.1.

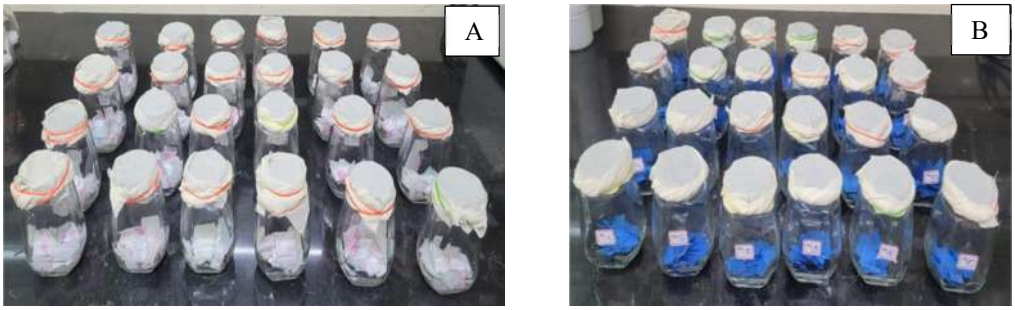


Fig. 7.1 Experimental set up for degradation of A) Sanitary napkin and B) Polycoated paper carry bag

7.3.8.3 Inoculation of bacterial consortia and *invitro* degradation of samples

Degradation efficiency of five bacterial consortia was analysed in the study, employing free state and immobilized state bacterial consortia. Free state CS 01 was prepared by mixing 0.1 OD adjusted 1 ml each ST 02, 04,07, and 08 bacterial inoculum into sterile glass jar with sample and 15 ml of modified Czapek mineral salt broth medium. The flasks were sealed with multiple layered muslin cloth and mixed well. The procedure was followed for each free state consortia CS 02-05.

For the immobilized bacterial consortium, immobilized state CS 01 was prepared by mixing 0.1 OD adjusted 1 ml each ST 02, 04, 07, and 08 bacterial inoculum into sodium alginate solution (sodium alginate- 1.5%, w/v and autoclaved at 121°C for 15 min) and followed the procedure mentioned in 7.3.2. Approximately 5 g of calcium alginate beads was added to a sterile glass jar with sample and 15 ml of modified Czapek mineral salt broth medium. The flasks were sealed with multiple layered muslin cloth and mixed well. The procedure was followed for each immobilized state consortia CS 02-05.

After mixing, the experiment jars were incubated at room temperature for one month (week 1- 4 analysis). At regular intervals (after each 7 days), samples were taken aseptically, UV sterilized, washed and dried (hot air oven at 50ºC). After complete drying, the samples were weighed again (=final) to determine the decrease in the weight. Weight loss was calculated using the following formula (Ellammal et al., 2022).

Weight loss (%) = (Initial weight- Final weight / Initial weight) ×100

7.4 RESULTS

The enzyme assay of consortia and single strains was carried out. The free and immobilized consortial activity was determined along with free and immobilized single strains, and the results were noted. The photograph of immobilized beads is presented in Fig. 7.2.

Fig. 7.2 Immobilized bacterial cells

7.4.1 Enzyme assay of individual bacterial strains

The eight strains selected were allowed to grow under conditions showing maximum growth obtained by optimization. The enzyme production was observed for 24-72 hours, and results were noted Appendix V and VI). The maximum enzyme production obtained in free and immobilized single strains is presented in table 7.4.

Table 7.4 The enzyme activity demonstrated by individual bacterial strains at the given physico-chemical conditions

SL. NO	ENZYME	TYPE OF BACTERIA	BACTERIAL STRAIN NAME	TEMPERATURE (°C)	SALINITY (ppt)	pH	ESTIMATED ENZYME CONCENTRATION (U/ML)	THE TIME AT WHICH HIGHEST ENZYME CONCENTRATION ESTIMATED (HOURS)
1	Protease	Free	ST 01	40	15	5	16 ± 0.15	24
			ST 02	30	20	7	29 ± 0.25	24
			ST 03	40	20	6	23 ± 0.19	24
			ST 04	40	15	6	8 ± 0.21	24
			ST 05	20	20	8	12 ± 0.11	24
			ST 06	40	15	7	20 ± 0.09	24
			ST 07	40	20	6	12 ± 0.24	24
			ST 08	30	20	6	20 ± 0.19	24
		Immobilized	ST 01	30	15	6	17 ± 0.31	72
			ST 02	30	20	6	30 ± 0.25	24
			ST 03	40	20	7	26 ± 0.12	24
			ST 04	40	20	6	21 ± 0.16	72
			ST 05	20	25	8	14 ± 0.21	24

			ST 06	40	20	8	21 ± 0.29	24
			ST 07	40	20	7	22 ± 0.17	72
			ST 08	30	20	7	27 ± 0.15	72
2	Amylase	Free	ST 01	30	20	6	142 ± 0.28	72
			ST 02	20	25	7	158 ± 0.31	48
			ST 03	40	20	6	151 ± 0.25	48
			ST 04	40	20	6	156 ± 0.22	48
			ST 05	30	25	8	108 ± 0.21	48
			ST 06	40	20	8	75 ± 0.17	72
			ST 07	30	20	6	20 ± 0.18	72
			ST 08	40	20	7	85 ± 0.11	48
		Immobilized	ST 01	40	20	5	167 ± 0.23	72
			ST 02	30	25	6	185 ± 0.16	72
			ST 03	40	20	6	115 ± 0.17	72
			ST 04	40	20	6	175 ± 0.16	72
			ST 05	30	25	8	146 ± 0.25	72
			ST 06	40	20	8	135 ± 0.15	72
			ST 07	40	20	7	34 ± 0.13	72
			ST 08	30	20	6	180 ± 0.14	72
3	Lipase	Free	ST 01	40	20	6	1 ± 0.16	24
			ST 02	20	20	6	2 ± 0.19	24
			ST 03	40	15	7	20 ± 0.23	24
			ST 04	30	20	6	2 ± 0.24	24
			ST 05	30	20	8	25 ± 0.21	24
			ST 06	40	15	7	64 ± 0.18	24
			ST 07	30	20	7	3 ± 0.14	24
			ST 08	40	20	6	1 ± 0.15	24
		Immobilized	ST 01	40	20	6	3 ± 0.13	24
			ST 02	30	25	7	4 ± 0.11	24
			ST 03	40	20	6	23 ± 0.18	24
			ST 04	40	15	6	5 ± 0.3	24
			ST 05	20	20	8	31 ± 0.2	48
			ST 06	30	20	7	56 ± 0.12	48
			ST 07	40	20	6	5 ± 0.16	24
			ST 08	40	20	6	5 ± 0.23	24
4	Chitinase	Free	ST 01	40	20	6	3 ± 0.21	24
			ST 02	20	20	6	0.12 ± 0.01	24
			ST 03	40	15	7	0.11 ± 0.03	24
			ST 04	40	15	6	1.3 ± 0.01	24
			ST 05	20	25	8	3 ± 0.05	24
			ST 06	40	20	7	2.4 ± 0.03	24
			ST 07	40	20	7	1.7 ± 0.04	24
			ST 08	40	20	6	0.5 ± 0.12	24
		Immobilized	ST 01	40	15	6	3.2 ± 0.19	24
			ST 02	20	25	7	0.23 ± 0.02	24

			ST 03	40	20	7	0.17 ± 0.13	24
			ST 04	40	15	6	2.2 ± 0.17	24
			ST 05	20	25	8	2.9 ± 0.15	24
			ST 06	30	20	8	3.3 ± 0.12	24
			ST 07	40	20	7	6.2 ± 0.19	24
			ST 08	40	20	6	0.24 ± 0.02	24
5	Cellulase	Free	ST 01	40	20	5	47 ± 0.17	48
			ST 02	30	25	6	63 ± 0.15	24
			ST 03	40	15	6	74 ± 0.12	24
			ST 04	40	20	6	38 ± 0.18	24
			ST 05	30	25	8	29 ± 0.23	48
			ST 06	40	20	7	39 ± 0.22	24
			ST 07	40	25	7	39 ± 0.17	24
			ST 08	40	25	6	27 ± 0.16	24
		Immobilized	ST 01	40	20	6	77 ± 0.15	48
			ST 02	20	25	6	97 ± 0.12	24
			ST 03	30	20	6	71 ± 0.17	24
			ST 04	40	20	7	61 ± 0.19	24
			ST 05	30	25	8	64 ± 0.21	24
			ST 06	40	20	7	70 ± 0.13	24
			ST 07	30	25	7	61 ± 0.11	24
			ST 08	40	20	7	57 ± 0.1	24
6	Ligninase	Free	ST 01	40	20	6	21 ± 0.09	72
			ST 02	20	25	6	3 ± 0.2	24
			ST 03	40	20	7	7 ± 0.18	24
			ST 04	30	15	6	12 ± 0.21	24
			ST 05	30	20	8	62 ± 0.15	48
			ST 06	40	20	7	66 ± 0.14	48
			ST 07	40	25	7	27 ± 0.3	48
			ST 08	40	20	6	12 ± 0.2	48
		Immobilized	ST 01	40	15	6	11 ± 0.31	24
			ST 02	20	25	7	15 ± 0.15	24
			ST 03	40	15	7	9 ± 0.12	48
			ST 04	40	15	6	11 ± 0.19	24
			ST 05	30	25	8	74 ± 0.09	72
			ST 06	30	20	7	75 ± 0.26	48
			ST 07	40	25	7	25 ± 0.23	48
			ST 08	40	20	6	31 ± 0.16	48

The maximum enzyme activity using individual strains was obtained for amylase (185 ± 0.16 U/ml), followed by cellulase (97 ± 0.12 U/ml), ligninase (75 ± 0.26 U/ml), lipase (64 ± 0.18 U/ml), protease (30 ± 0.25 U/ml), and chitinase (6.2 ± 0.19 U/ml). For amylase activity, all the strains except IS 03 showed maximum activity. The maximum amylase activity was exhibited by IS 02 (185 ± 0.16 U/ml) and the lowest by FS 07 (20 ± 0.18 U/ml). All immobilized strains, with the exception of the IS 03 strain, demonstrated increased activity for cellulase. IS 02 exhibited the highest cellulase activity (97 ± 0.12 U/ml), FS 08 displayed the lowest activity (27 ± 0.16 U/ml). For ligninase enzyme activity, FS01, FS04 and FS07 strains showed maximum enzyme production. Comparing the free and immobilized states, maximum ligninase production was obtained by IS 06 (75 ± 0.26 U/ml) and the least by FS 02 (3 ± 0.2 U/ml). The lipase production was found to be highest in FS 06 (64 ± 0.18 U/ml) and lowest in FS 01 and FS 08 (1 ± 0.16 U/ml). 56 ± 0.12 U/ml is the maximum amount of lipase enzyme that was produced by the IS 06 strain. Maximum protease production was shown by immobilized strains, where the highest enzyme activity was obtained by IS 02 (30 ± 0.25 U/ml) and the lowest by FS 04 (8 ± 0.21 U/ml). FS 05 and FS 08 produced maximum chitinase others in the immobilized state, but IS 07 showed maximum chitinase production (6.2 ± 0.19 U/ml) and the least by FS 03 (0.11± 0.03 U/ml).

7.4.2 Enzyme assay of free and immobilized state bacterial consortia

In consortia, member strains were allowed to grow in synthetic media by providing optimum conditions. The free and immobilized consortia enzyme activity in the given conditions was estimated (Table 7.5).

Table 7.5 The enzyme activity demonstrated by bacterial consortia at the given physico-chemical conditions

SL. NO	ENZYME	TYPE OF CONSORTIA USED	NAME OF CONSORTIA	TEMPERATURE (°C)	SALINITY (ppt)	pH	ESTIMATED ENZYME CONCENTRATION (U/ML)	THE TIME AT WHICH HIGHEST ENZYME CONCENTRATION ESTIMATED (HOURS)
1	Protease	Free	CS 01	40	25	5	27 ± 0.25	48
			CS 02	40	25	6	15 ± 0.13	24
			CS 03	40	15	8	16 ± 0.19	24
			CS 04	40	25	6	20 ± 0.21	24
			CS 05	40	25	5	22 ± 0.20	24
		Immobilized	CS 01	40	15	5	29 ± 0.23	48
			CS 02	40	20	6	14 ± 0.19	24
			CS 03	20	15	7	18 ± 0.17	24
			CS 04	20	15	6	14 ± 0.21	24
			CS 05	40	25	5	32 ± 0.19	24
2	Amylase	Free	CS 01	20	15	7	318 ± 0.28	24
			CS 02	40	20	6	286 ± 0.25	24
			CS 03	40	15	8	274 ± 0.19	72
			CS 04	40	15	6	230 ± 0.17	24
			CS 05	40	20	6	207 ± 0.18	24
		Immobilized	CS 01	40	25	6	355 ± 0.26	24
			CS 02	30	20	6	373 ± 0.34	24
			CS 03	20	25	8	241 ± 0.32	24
			CS 04	40	25	8	241 ± 0.29	24
			CS 05	40	20	5	239 ± 0.18	24
3	Lipase	Free	CS 01	40	20	5	39 ± 0.09	24
			CS 02	40	15	7	21 ± 0.17	48
			CS 03	40	20	8	34 ± 0.19	72
			CS 04	40	15	8	36 ± 0.23	24
			CS 05	30	15	6	39 ± 0.22	48
		Immobilized	CS 01	40	25	6	46 ± 0.21	48
			CS 02	40	20	6	34 ± 0.19	24
			CS 03	40	20	8	22 ± 0.15	72
			CS 04	40	20	6	42 ± 0.28	24
			CS 05	40	15	6	63 ± 0.23	48
4	Chitinase	Free	CS 01	40	20	6	11 ± 0.17	72
			CS 02	40	25	6	1 ± 0.14	24
			CS 03	40	20	8	3 ± 0.21	48

			CS 04	40	25	6	1 ± 0.15	24
			CS 05	40	20	6	16 ± 0.17	72
		Immobilized	CS 01	40	15	7	18 ± 0.19	72
			CS 02	40	20	6	24 ± 0.25	72
			CS 03	40	15	8	7 ± 0.22	24
			CS 04	40	15	7	7 ± 0.16	48
			CS 05	40	15	6	16 ± 0.23	72
5	Cellulase	Free	CS 01	40	20	6	176 ± 0.25	72
			CS 02	40	20	5	69 ± 0.11	24
			CS 03	20	25	6	116 ± 0.19	24
			CS 04	40	25	6	84 ± 0.14	72
			CS 05	40	25	6	56 ± 0.17	24
		Immobilized	CS 01	40	25	5	178 ± 0.18	72
			CS 02	30	15	6	75 ± 0.23	72
			CS 03	40	20	8	151 ± 0.24	72
			CS 04	40	15	6	84 ± 0.20	24
			CS 05	40	20	6	73 ± 0.19	72
6	Ligninase	Free	CS 01	40	25	5	227 ± 0.22	24
			CS 02	40	25	5	179 ± 0.19	72
			CS 03	40	25	7	134 ± 0.16	48
			CS 04	40	15	6	101 ± 0.20	24
			CS 05	40	20	7	120 ± 0.21	48
		Immobilized	CS 01	40	20	5	270 ± 0.22	24
			CS 02	30	15	6	198 ± 0.32	72
			CS 03	20	15	8	210 ± 0.28	48
			CS 04	40	25	8	127 ± 0.15	48
			CS 05	40	25	6	185 ± 0.31	48

It was found that immobilized consortia produced higher concentrations of each of the enzymes than free consortia. The maximum amount of enzyme produced was amylase (373 ± 0.34 U/ml), followed by ligninase (270 ± 0.22 U/ml), cellulase (178 ± 0.18 U/ml), lipase (63 ± 0.23 U/ml), protease (32 ± 0.19 U/ml), and chitinase (24 ± 0.25 U/ml). For the amylase enzyme, all consortia except ICS 03 exhibited the maximum activity in the immobilized condition. ICS 02 exhibited the highest amylase activity (373 ± 0.34 U/ml), while FCS 05 exhibited the lowest activity (207 ± 0.18 U/ml). In the case of the ligninase enzyme, the immobilized consortia exhibited superior output, with the greatest yield recorded at 270 ± 0.22 U/ml for ICS 01 and the lowest at 101± 0.20 U/ml for FCS 04. Cellulase activity was higher in the immobilized

condition, with significant activity shown by ICS 01. The maximum enzyme activity was recorded in ICS 01 (178 ± 0.18 U/ml), whereas the minimum was noted in FCS 05 (56 ± 0.17 U/ml). For protease, FCS 02 and FCS 04 yielded maximum activity while the other strains showed maximum activity in the immobilized state. Protease activity was highest in ICS 05 (32 ± 0.19 U/ml) and lowest in ICS 02 and ICS 04 (14 ± 0.21 U/ml). Chitinase activity was significantly higher in the immobilized condition, with maximum enzyme activity by ICS 02 (24 ± 0.25 U/ml), whereas the minimum was noted in FCS 02 and FCS 04 (1 ± 0.15 U/ml). Lipase activity was found to be higher in the immobilized condition, with maximum enzyme activity by ICS 05 (63 ± 0.23 U/ml), whereas the minimum was observed in FCS 02 (21 ± 0.17 U/ml).

7.4.3 Application using developed bacterial consortia

For analyzing the bioremediation capacity of developed consortia, weight reduction was determined by *invitro* degradation of commercially available sanitary napkins and polycoated carry bags.

7.4.3.1 Sanitary napkin degradation

Fig. 7.3 A depicts degradation result of sanitary napkin after week 4. Fig. 7.4 illustrates the observed sanitary napkin degradation results using free and immobilized-state consortia for a period of 4 weeks.

Fig. 7.3 Degradation image of A) Sanitary napkin and B) Polycoated paper carry bag after week 4.

It was observed that free-state consortia were more effective at degrading sanitary napkins than immobilized consortia. FCS 01 had the highest degradation rate (29%), followed by FCS 02 (27%), and ICS 01 achieved a 21% reduction. FCS 01 and FCS 02 consortia exhibited about 20% and 25% degradation during the first week, but immobilized consortia attained only 0% and 1%, respectively. Significant variations were observed within the bacterial consortia in both the free state (p= 0.001) and immobilized state (p= 0.001).

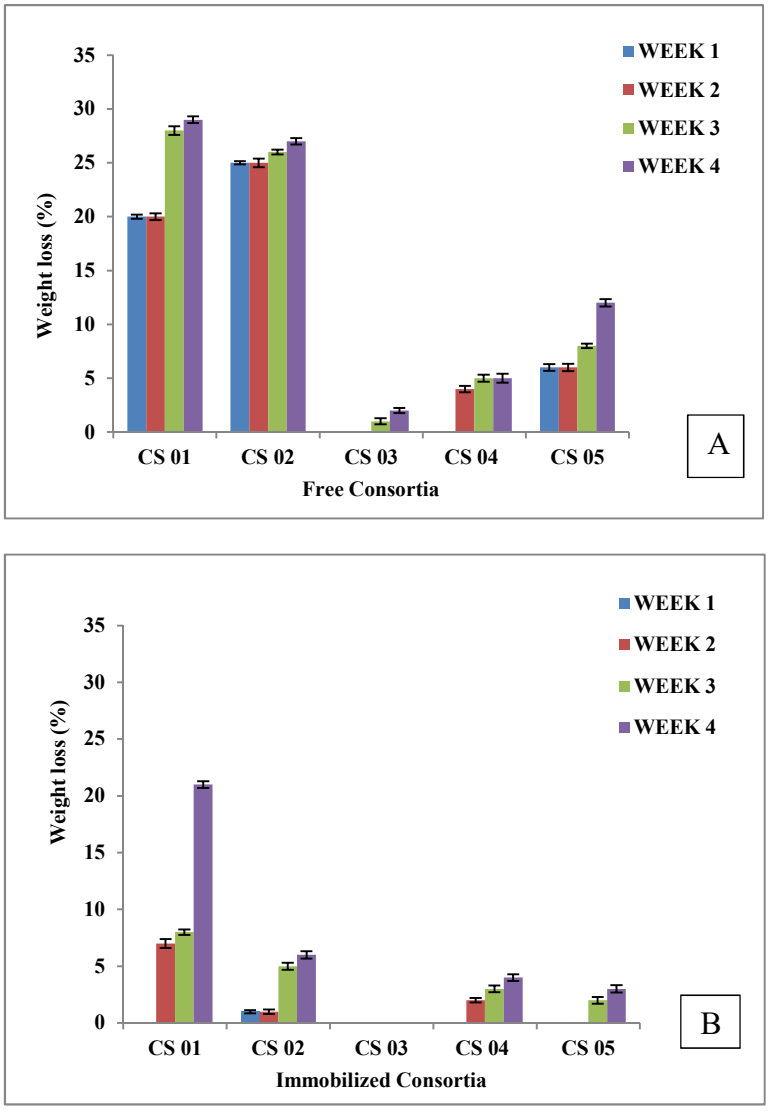


Fig. 7.4 **Sanitary napkin degradation using free state consortia (A) and immobilized state consortia (B)**

7.4.3.2 Polycoated carry bag degradation

Fig. 7.3 B depicts degradation result of polycoated paper carry bag after week 4. The observed results of poly-coated carry bag degradation using free-state and immobilized-state consortia for a period of 4 weeks are depicted in Fig. 7.5.

Maximum weight loss was exhibited by ICS 01 (8%) during the fourth week, followed ICS 02 and ICS 04 and FCS 03 and FCS 05 (5%). FCS 01, FCS 02 and FCS 04, along with ICS 03 and ICS 05, exhibited a weight loss of 3% during the fourth week. Significant variations were observed within the bacterial consortia in both the free state (p= 0.001) and immobilized state (p= 0.001).

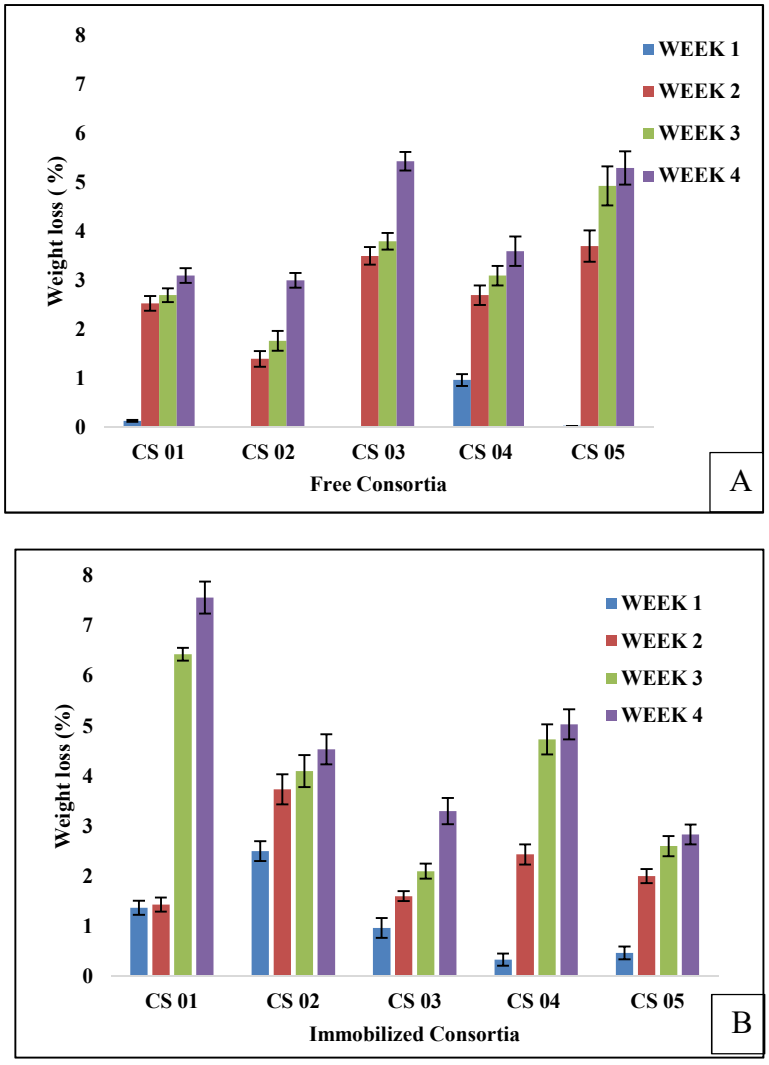


Fig. 7.5 Poly coated paper bag degradation using free state consortia (A) and immobilized state consortia (B)

7.5 DISCUSSION

This study was aimed at estimating the activity of six industrially important enzymes, such as protease, amylase, lipase, chitinase, cellulase and ligninase, from bacterial strains isolated from the Northern Kerala mangrove forest using free and immobilized consortia. The potential strains used included *Enterobacter cloacae*, *Enterobacter asburiae*, *Serratia marcescens,* and *Klebsiella aerogenes*. Previous studies have shown the hydrolytic enzyme potential of these selected strains (Indriati and Megahati, 2021; Osho et al., 2022; Asitok et al., 2023).

Masurkar and Pathade (2023) conducted a study on microbial consortia for amylase, protease and lipase production from isolates obtained from organic kitchen waste. The enzyme activity was higher for consortia than for single bacterial strains, which is consistent with the present study. The highest enzyme activity found in the current study by consortia was 373 ± 0.34 U/ml, 32 ± 0.19 U/ml, and 63 ± 0.23 U/ml for amylase, protease and lipase enzymes, respectively. But in Masurkar and Pathade's (2023) study, they obtained only a maximum activity of 18 U/ml, 22 U/ml and 18 U/ml, respectively, for the above-mentioned enzymes.

In a study by Msarah et al. (2020), *Bacillus licheniformis* HULUB1 and *Bacillus subtilis* SUNGB2 isolates showed the highest α-amylase activity of 18.15 U/ml and 22.14 U/ml, respectively. In the current study maximum amylase activity was exhibited by consortium ICS 02 (373 U/ml) at 24 hours of incubation. The amylase activity obtained in the present study exceeds the activity obtained by bacterial strains isolated from sugar factory waste at 48 hours of incubation (Sanjaya et al., 2024). Previous studies have proven that the consortia developed in the current study can produce a considerable amount of amylase enzyme within 24 hours (Pranay et al., 2019).

In the current study, the highest protease activity was exhibited by IS02 (30 ± 0.25 U/ml) and ICS 05 (32 ± 0.19 U/ml) at 24 hours of incubation in mineral salt broth. Masi et al. (2021) isolated alkaline protease-producing bacteria from leather industry effluent, which showed the highest protease activity of 19 U/ml. Maximum lipase activity was exhibited by FCS 05 (63 ± 0.23 U/ml) and FS 06 (64 ± 0.18 U/ml) in the present study. Earlier research has provided evidence that aligns with the

findings of the current study, as indicated by the works of Maciel (2013) and Ferreira et al. (2023). The heightened activity in the free state arises from factors like steric hindrance, reduced substrate accessibility, enzyme conformation changes during immobilization, diffusion limitations, enzyme conformational shifts, interactions with the immobilization support and microbial interactions (Mokhtar et al., 2020). Although immobilization provides advantages such as improved stability and reusability, it may also restrict substrate access to the enzyme's active site or modify its ideal conformation, particularly in intricate consortia.

In the present study, ICS 02 showed the highest chitinase activity (24 ± 0.25 U/mL) at 72 hours of incubation in mineral salt broth. Numerous studies have been conducted in bacteria showing chitinase activity, and it was found that the increase in incubation period yields more chitinase enzyme (Gonfa et al., 2023). Maximum cellulase activity was shown by ICS 01 (178 ± 0.18 U/ml) at 72 hours of incubation in mineral salt broth. A strain of *B. velezensis* M2 was isolated from pig manure, demonstrating cellulase activity of 61.50 U/mL under optimal conditions (Li et al., 2020). The cellulase activity of bacterial consortia developed from cellulolytic bacterial strains from landfill leachate collected from the Pulau Burung landfill site of Penang, Malaysia, obtained a maximum cellulase activity of 0.90 U/ml (Chukwuma et al., 2025). According to Long et al. (2024), the highest cellulase activity exhibited by consortia of Bacillus strains was found to be 156.63 U/ml. A strain of *Bacillus subtilis* K1, capable of producing cellulase, was isolated from crop straw, exhibiting a cellulase activity of 24.69 U/mL (He et al., 2023). The isolates obtained in the present study showed more cellulase activity than the previous studies conducted by various researchers. Maximum ligninase activity was exhibited by ICS 01 (270 ± 0.22 U/ml) at 24 hours of incubation in mineral salt broth. The results of the current study are proficient and surpass the results obtained in previous studies by Verma and Madamwar (2003), Peter et al. (2014) and Rajan et al. (2022).

The enzyme activity was higher with immobilized consortia than with free consortia, except for the lipase enzyme, in the present study. Rahman et al. (2006) reported that the immobilized cells are efficient, recoverable and reusable, thus lowering the cost of remediation. Furthermore, Wang et al. (2019) analyzed the remediation potential of an immobilized bacterial consortium with biochar as a carrier

in pyrene-Cr (VI) co-contaminated soil. The results showed that bacterial consortium entrapped in alginate beads and bacterial consortium adsorbed on biochar and sequential entrapment in alginate beads resulted in more efficient Cr (VI) removal compared with bacterial consortium adsorption onto biochar and free consortia.

Biodegradation efficiency of the consortium of free cells versus immobilized microbial cells showed the best oil biodegradation result for free cells with 73.3% and 69.4% for immobilized cells in a study conducted by Ferreira et al. (2023). The lower consortial enzyme activity may be because of metabolic hindrance. Diaz et al. (2002) showed that immobilized hydrocarbonoclastic bacteria can survive better and have 4-7 times better crude oil degradation efficiency than the free cell form in a high-saline environment. Furthermore, immobilized cells are recoverable and reusable, thus lowering the cost of remediation (Rahman et al., 2006). Bioremediation of crude oil has always been a popular research area, and several bacterial strains have been proved to be potential oil-degrading strains (Chen et al., 2017). Wang et al. (2007) worked on a microbial consortium for effectively degrading phenanthrene, and the results showed that the microbial consortium has a promising application in bioremediation of oil-contaminated environments and could be potentially used in microbial enhanced oil recovery (MEOR).

Amylase production was found to be the highest in the present study using bacterial consortia, followed by ligninase, cellulase, lipase, protease and chitinase. The selected enzymes in the current study have a variety of industrial applications. Microbial proteases have wide-ranging applications in several fields, including baking, brewing, detergents, leather making, pharmaceuticals, meat tenderizing, cosmetics, medical diagnosis, feed industries, hydrolytic applications to prepare active peptides and environmental protection applications, such as waste treatment and reuse (Song et al., 2023). With the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries (Rana et al., 2013). The lipase enzyme has been explored for industrial use, with promising results in oil displacement tests and detergent applications (Chandra et al., 2020). Bacterial chitinase enzymes hold significant industrial applications such as biocontrol of phytopathogenic fungi and harmful insects

in agriculture, biorefinery and biomedical research for eco-friendly chitin waste management (Siriporn et al., 2023). Bacterial chitinases are cost-effective and sustainable sources of proteins that can be utilized on an industrial scale (Annika et al., 2023). Cellulase enzyme has a crucial role in industries like biofuel production, paper and pulp manufacturing, textile processing, agriculture, and food processing (Bhardwaj et al., 2021). Bacterial cellulases are known for their efficient catalytic action, stability, and genetic versatility, making them advantageous for industrial use. These enzymes play a significant role in degrading cellulose-rich waste feedstock for bioconversion processes, aiding in the production of biofuels and other valuable compounds. Additionally, they are utilized in fruit and vegetable processing, food tenderization, juice clarification, and enhancing aroma and taste in food items. Bacterial ligninases offer environmentally friendly solutions for lignin removal from lignocellulosic biomass, aiding in bioethanol production (Kamimura et al., 2019). They can be utilized in processes such as pulp delignification, wastewater treatment, biofuel production, dye removal and biopulping. Additionally, these enzymes have applications in bioremediation of textile and pulp industry wastewater, aromatic compound degradation, and even in the production of bioplastics.

In the present study the bacterial hydrolytic potential was aimed to apply for bioremediation purposes. Bioremediation is usually carried out by the microbial consortium rather than by individual species in the natural environment, and different strains or species play different functional roles (Zhang et al., 2021). In the current study 5 different bacterial consortia were developed. The consortia were found to produce the maximum amount of protease, amylase, cellulase, chitinase and ligninase enzymes when compared to single strains. The present study found that the consortia of microorganisms performed better than single strains. Numerous studies indicate that achieving full degradation of substances using a single strain is challenging. In contrast, co-cultivating a microbial consortium proves to be more effective than relying on individual bacteria, as it accelerates the degradation of pollutants and can greatly improve the biodegradation process in soil (Zhang and Zhang, 2021). In a microbial consortium, interactions occur not only among microbial cells of the same species, typically facilitated by quorum sensing (QS), but also between different species. These interspecies interactions can include mutualism and competition for

resources within the same ecological niche. These mutual effects based on metabolites will affect metabolisms and the yield of the target product in the process (Jiang et al., 2017). According to Zhang and Zhang (2022), the microbial consortium achieves these activities by enhancing synergistic degradation, reducing the accumulation of intermediate products, generating crude enzymes and self-regulating the degradation process. Due to the synergistic interaction of different microorganisms in the population, bacterial consortia are more effective for environmental bioremediation due to their better adaptability and tolerance to variable and complicated environments (Wang et al., 2021).

Bacterial consortia were implemented in bioremediation or degradation of several compounds. Studies have proved the effective degradation of kitchen wastes using microbial consortia without any foul smell (Sarkar et al., 2011; Masurkar and Pathade, 2023). Masurkar and Pathade (2023) developed a microbial consortium that can effectively and rapidly bring about the degradation of kitchen wastes that can be used in agricultural soils, and this consortium showed rapid degradation of waste as compared to others in a 15-day duration. Chen et al. (2017) conducted a study on oil degradation using consortia, where five strains of bacteria isolated from the Zhejiang coast in China were used. The mixed flora of the five strains performed well, degrading 75.1% crude oil (1%, w/v) in 7 days. Ramsay et al. (2000) demonstrated a great capacity for polyaromatic hydrocarbon (PAH) degradation in vitro using a consortium.

The degradation efficiency of a poly-coated carry bag and sanitary napkin was analyzed *invitro*, and it was found that the developed bacterial consortia can degrade both. The degradation ability was higher for sanitary napkins than polycoated carry bags in a four-week period, and variations were observed in degradation using free-state and immobilized consortia. Free-state consortia showed maximum weight loss in degradation of sanitary napkins (29%), and immobilized consortia in polycoated carry bags (8%) in a period of 4 weeks. The observed variation in performance is probably attributed to the unimpeded mobility of bacteria and enzymes in their free state, which facilitates degradation. The rate of degradation fluctuates over the weeks. Furthermore, the stages of growth and characteristics of the sample can significantly affect the degradation process. During the stationary phase, the utilization of these components

diminishes, leading to a decrease in degradation (Meletiadis et al., 2001). Byproducts also cause metabolic obstruction, which affects the degradation process. Both samples, such as the carry bag and sanitary napkin, pose a significant environmental health risk. Jones et al. (2017) discovered that samples containing polyethylene (PE) and polypropylene (PP) served as carbon sources that facilitated microbial growth due to their saprotrophic nature. Panicker et al. (2024) explored the bioremediation of sanitary napkins through the use of cellulase-degrading bacteria. The study found that a bacterial consortium decreased 0.1 grams of sanitary napkin material to 0.03 grams in two days. Ellammal et al. (2022) found that Bacillus and Pseudomonas effectively degrade sanitary napkins, with Bacillus causing a 30–35% weight loss and Pseudomonas achieving a 45% reduction. However, understanding of the biodegradation processes for disposable hygiene products remains limited. Additionally, information on the composition of these products is scarce, and the labeling lacks clarity regarding product design. A study by Dinu et al. (2022) revealed that soil microbes had minimal influence on the absorption area of sanitary napkins, with noticeable effects limited to the surface of the top sheet after 40 days. Pawar et al. (2024) reported that Whisper sanitary napkins exhibited no signs of degradation after a period of 6–8 months, retaining their original weight. The components, such as propellant ethyl alcohol and triclosan, were estimated by the Ministry of Drinking Water and Sanitation, Government of India (2024), and require approximately 500 to 600 years for complete decomposition. The soil microbial community appears to have a minimal impact on the biodegradation process, with slight variations depending on the specific brand. While there are numerous biodegradable sanitary napkins on the market today, a considerable number of consumers still opt for non-biodegradable alternatives because of their comfort, durability and leak-proof features. This preference contributes to environmental issues. Consequently, the results of this study could provide substantial advantages to society.

The low-density plastic present in the polycoated carry bag is also difficult to degrade. The management and recycling of plastic waste have emerged as a significant global concern, impacting all living organisms across various ecosystems. The microorganisms exhibited a slow growth rate during the initial weeks of the current study, despite the ample availability of nutrients. This suggests that the organisms had not yet fully adapted to the carbon source provided, particularly plastics. Microorganisms degrade plastic at different rates due to the varying effectiveness of

their enzymes (Shah et al., 2008). The evidence suggests that biodegradation is affected by polymer properties, the organisms involved, and any pretreatment methods used. Variations in the capacity of different microorganisms to break down plastics may be because they are from the distinct environments in which they are sourced. The primary processes involved in the microbial degradation of polyethylene (PE) plastics include the oxidation of the polyethylene surface and the creation of carbonyl groups, both of which lead to the material's deterioration and fragmentation (Ali et al., 2022). Abrusci et al. (2011) employed a consortium of Bacillus strains and showed 7–10% mineralization in polyethylene films over 90 days. Usha et al. (2011) used *Pseudomonas* sp. from garbage soil and obtained 37% weight loss in PE bags during 6 months of incubation. Yang et al. (2014) used microbial consortia containing *Enterobacter* sp. and *Bacillus* sp. and obtained 6% degradation in low-density polyethylene (LDPE) films in 60 days.

The current study achieved an 8% weight loss within 4 weeks, which appears beneficial for addressing the plastic degradation concern. Numerous studies indicate that utilizing a combination of different microbial strains, rather than relying on individual microbes, can enhance plastic degradation. This approach takes into account the synergistic interactions between microorganisms and their associated enzymes, leading to improved efficiency in the degradation process (Mukherjee et al., 2016). One possible reason for this performance is that different enzymes and metabolic pathways from various strains within the microbial consortium collaborate and boost each other's efficiency in the microbial degradation of plastics.

The bacterial consortia developed in this study show promising bioremediation applications. Microbial consortia exhibit remarkable adaptability and stability in intricate environments. When diverse cells with distinct functions merge, they establish a dynamic equilibrium through intricate interactions, enhancing the overall system's resilience and stability in response to environmental changes. A group of microbes can achieve significantly more than an individual strain, especially when it comes to breaking down substances and producing byproducts.

Chapter - 8

SUMMARY AND CONCLUSION

Chapter - 8

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8.1 SUMMARY

Various environmental factors drive the abundance and diversity of bacteria in mangrove ecosystems, which make up the majority of the microbial biomass. They are crucial for various ecological functions, including nutrient cycling, decomposition and carbon dynamics, bioremediation and plant growth promotion. Despite their importance, these bacterial communities are still considered underexplored, particularly regarding their overall functional and taxonomic diversity, which are essential for mangrove health, productivity, and their role in combating climate change. Investigating the diversity of bacteria in mangroves aids in understanding the nutrient cycling, which is crucial for the health of these ecosystems. It offers ideas for developing biotechnological applications in agriculture, medicine and industry, and contributes to effective conservation efforts by identifying unique microbial communities and their functions, which ultimately helps protect these vital ecosystems.

The present study was focused on the impact of sediment characteristics on the occurrence of mangrove bacteria, the diversity of mangrove bacteria in the sediments of Northern Kerala, their enzyme properties, the development of consortia, immobilization and the application of the potential bacteria in bioremediation. Sediment samples were collected from 8 sampling stations, namely, Kasaragod, Edat, Valapattanam, Pazhayangadi, Kadalundi, Elathur, Ponnani, and Chettuva. Physico-chemical characteristics of the mangrove sediment samples were determined. The enumeration of the culturable bacterial population was done through plate count. The isolates were identified up to the generic level based on morphological, physiological and biochemical characteristics. Metagenomics and functional annotation studies were also performed. The hydrolytic enzyme potential of the isolates was studied, which includes amylase, cellulase, DNase, chitinase, laccase, ligninase, protease, lipase, and pectinase. Potential bacterial isolates were selected for consortia development based on hydrolytic enzyme potential

compatibility and haemolytic property. Growth of the selected isolates was examined at various pH, temperature, salinity and agitation to determine their optimal growth range. Developed bacterial consortia were subjected to estimation of enzyme activity, which includes amylase, cellulase, DNase, chitinase, ligninase, protease and lipase. The activity of enzymes was also examined in relation to the application of immobilization technology. Based on the enzyme activity observed, the isolates were screened and subjected to a biodegradation of sanitary napkins and polycoated paper carry bags.

The salient findings of the study are as follows:

- Sediment physicochemical parameters of 8 different mangrove stations along northern Kerala mangrove habitat were analyzed in the current study.
- Temperature of the sediment ranged from 21- 32ºC and pH from 6- 7.
- Salinity ranged from 3 to 38 ppt. Highest salinity was reported from Ponnani during post-monsoon and lowest from Valapattanam during monsoon.
- The sediment organic matter ranged from 0.2 ± 0.03 to 7 ± 0.04%. Highest organic matter was found at Ponnani during monsoon and lowest at Chettuva during pre- monsoon season.
- The carbohydrate content ranged from 0.01 ± 0.002 to 0.25 ± 0.02 mg/g. Highest value was obtained from Kadalundi during pre-monsoon and lowest from Ponnani during pre-monsoon season.
- The protein content ranged from 0.1 ± 0.02 to 9.9 ± 0.04 mg/g. Highest value was obtained from Kadalundi during pre-monsoon and lowest from Elathur during monsoon season.
- The lipid content ranged from 0.2 ± 0.01 to 9.3 ± 0.02 mg/g. Highest and lowest values were obtained from Kasaragod during monsoon and pre-monsoon, respectively.
- The sand content varied from 46 to 97%. Highest sand content was observed at Edat during monsoon and lowest at Pazhayangadi during monsoon season.

- The silt content varied from 0.3 to 55%. Highest silt content was observed at Valapattanam during monsoon and lowest at Pazhayangadi during pre-monsoon.
- The clay content varied from 0.3 – 35%. Highest and lowest clay content was observed at Ponnani during pre-monsoon and post-monsoon, respectively.
- In the first year of sampling, sediment texture was consistently sandy throughout all seasons. In the second year, it remained predominantly sandy during the pre-monsoon and monsoon periods, but changed to silty sand in the post-monsoon season.
- The bacterial population ranged from 6 ± 0.03 to 8 ± 0.04 $\log_{10}$ CFU/10g. Highest population was obtained from Valapattanam during monsoon and lowest from Ponnani during pre-monsoon season.
- The impact of environmental factors, like temperature, pH, salinity, organic matter, carbohydrates, proteins, lipids and particle size (sand, silt and clay), on bacterial population was analyzed and the results showed year-wise, seasonal and sampling station-wise variation ($p<0.05$) without exhibiting any trend. The findings indicated that these parameters are interconnected and have a combined effect. The particle size, organic matter, lipid and protein content were closely associated to the bacterial population.
- A total of 708 bacterial isolates were analyzed, leading to the identification of 17 bacterial genera through morpho-biochemical methods. The bacterial isolates belonged to the genera Bacillus, Klebsiella, Pseudomonas, Enterococcus, Xanthobacter, Alcaligenes, Enterobacter, Staphylococcus, Vibrio, Aeromonas, Citrobacter, Lactobacillus, Acinetobacter, Serratia, Lysobacter, Proteus and Escherichia.
- The highest bacterial diversity was observed during post-monsoon season during both years of sampling (16 genera), followed by the monsoon and pre-monsoon seasons. Bacillus was the predominant genera obtained in both sampling years and all seasons.

- Metagenomic analysis of the post monsoon sediment samples from the 8 stations revealed a total of 45 phyla, 121 classes, 308 orders, 507 families, 1036 genera and 1834 bacterial species.
- The predominant phylum obtained was Proteobacteria (57%), class was Gammaproteobacteria (40.4%), order was Oceanospirillales (11.2%), family was Shewanellaceae (5.48%), genus was Shewanella (5.47%), and species were *Alcanivorax* sp. *(3%)* and *Shewanella colwelliana* (2.8%).
- Functional annotation study showed the presence of genes associated with the metabolism of biomolecules as predominant (68%), followed by other metabolic functions (20%), environmental functions (11%), and stress response (1%), indicating a versatile and biochemically active microbiome.
- Genetic pathways in bacteria that are responsible for the synthesis and breakdown of crucial compounds have been uncovered, including the production of antibiotics, the degradation of various recalcitrant and xenobiotic substances, and the breakdown of hydrocarbons. Bacteria that play a major role in the sulphur cycle, nitrogen cycle, iron cycle and methanogenesis have been identified.
- Twenty different types of bacterial genera were identified as sulphate oxidizing bacteria (SOB) under nine orders and twenty different types of bacterial genera as sulphate reducing bacteria (SRB) under twelve orders. Genus Thiomicrospira (27%) was the most abundant SOB and genus Desulfatiglans (28%) were the most abundant SRB, identified.
- Metagenomic analysis from mangrove sediment samples revealed the presence of several bacterial genera involved in nitrogen fixation, ammonification, nitrification, and denitrification processes of the nitrogen cycle.
- About ten different bacterial genera under six orders were identified to have the ability of nitrogen fixation and the most abundant nitrogen fixing bacteria identified, belonged to Genus Mesorhizobium (32%).

- Five different bacterial genera under four orders were found which have the ability of ammonification. The most abundant ammonifying bacteria identified belonged to Genus Pseudomonas (40%).
- Seven different bacterial genera under five orders were identified in the sampling stations with the ability of nitrification. The most abundant nitrifying bacteria was identified under Genus Nitrospira (55%).
- Six different bacterial genera belonging to six orders were identified with the ability of denitrification. Most abundant denitrifying bacteria belonged to under Genus Paracoccus (59%).
- Eleven different bacterial genera under six orders were identified from the sampling stations which have the ability of methanogenesis. Genus Methyloceanibacter (64%) was the most abundant methanotroph.
- Eight different bacterial genera under seven orders were identified as iron redox cycling bacteria. Genus Desulfuromusa (23%) was the most abundant iron-reducing bacteria.
- The bacterial isolates were subjected to qualitative analysis for their extracellular hydrolytic enzyme potential, the highest percentage of bacterial isolates were found to be lipase producers (69%), followed by protease (55%), amylase (48%), cellulase (43%), DNase (27%), chitinase (3.5%), ligninase (3%) and laccase (1%) producers.
- Based on the extent of clearance zone by hydrolytic enzyme production, 35 bacterial isolates were selected and identified by molecular techniques. The selected isolates belonged to genera Klebsiella, Bacillus, Lysinibacillus, Stutzerimonas, Priestia, Aeromonas, Serratia, Enterobacter, Providencia, Pseudomonas and Alcaligenes. The molecular sequences were deposited in the NCBI database and they were phylogenetically analyzed, where 11 species belonged to phylum Firmicutes, and 24 belonged to phylum Proteobacteria.
- Eight bacterial strains (ST 01-08) were selected for consortia development based on their extracellular enzyme production. These strains were found to be compatible and exhibited non-haemolytic characteristics.

- The selected bacterial candidates were strains of *Enterobacter cloacae*, *Enterobacter asburiae*, *Serratia marcescens* and *Klebsiella aerogenes.*
- ST 01 was *Enterobacter cloacae* strain; ST 02, 04, 05, and 06 were *Serratia marcescens* strains; ST 03 and 07 were *Enterobacter asburiae* strains; and ST 08 was *Klebsiella aerogenes* strain. The strains within *Enterobacter asburiae* and *Serratia marcescens* exhibited variations due to their distinct enzymatic and biochemical properties, and all the selected strains were found to be gram-negative.
- ST 01 and ST 06 demonstrated the production of eight types of hydrolytic enzymes, while ST 04, 05, 07 and 08 produced seven types of enzymes and ST 02 and 03 produced six different enzymes.
- The growth of each selected bacterial isolates was optimized for various factors including temperature, salinity, pH and agitation.
- In temperature optimization, ST 01, 03, 04, 06, 07, and 08 demonstrated maximum growth turbidity at 30ºC and 40ºC, whereas ST 02 and 05 exhibited peak turbidity at 20ºC and 30ºC.
- In salinity optimization, ST 01, 03, 04, and 06 demonstrated maximum growth turbidity at 15 and 20 ppt, whereas ST 02, 05, 07 and 08 exhibited peak turbidity at 20 and 25 ppt.
- In pH optimization, ST 01, 03 and 07 demonstrated maximum growth turbidity at pH 5,6 and 7; whereas ST 02, 04, and 08 exhibited peak turbidity at pH 6 and 7; and ST 05 and 06 showed maximum turbidity at pH 7 and 8.
- In optimization of agitation speed, all the 8 strains demonstrated maximum growth turbidity at 150 rpm.
- The compatible strains that exhibited similar growth requirements were chosen for the development of consortia, resulting in the formation of five distinct consortia (CS 01, CS 02, CS 03, CS 04 and CS 05).

- Consortium CS 01 contained ST 02, 04, 07, and 08; CS 02 contained ST 01, 03 and 07; CS 03 contained ST 04, 05 and 06; CS 04 contained ST 02 and 06; and CS 05 contained ST 01, 03, 07 and 08.

- Single strains selected and the consortia developed, were immobilized in calcium alginate beads to check the effectiveness of immobilization technology in bioremediation.

- The enzymes that aid in bioremediation, including amylase, protease, cellulase, chitinase, lipase and ligninase, were subsequently analyzed quantitatively through specific standard enzyme assays. The free and immobilized consortial activity was compared with free and immobilized single strains, and the results were noted.

- The maximum enzyme activity using individual strains were as follows- amylase (185 U/ml), followed by cellulase (95 U/ml), ligninase (75 U/ml), lipase (64 U/ml), protease (30 U/ml), and chitinase (6.2 U/ml). It was found that immobilized consortia produced higher concentrations of each of the enzymes than free consortia except for lipase. The maximum amount of enzyme produced by immobilized consortia were amylase (373 U/ml), followed by ligninase (270 U/ml), cellulase (178 U/ml), lipase (63 U/ml), protease (32 U/ml) and chitinase (24 U/ml). Maximum lipase activity was exhibited by FST 06 (64 U/ml).

- Biodegradation of commercially available sanitary napkins and polycoated carry bags was done to analyze the bioremediation capacity of developed consortia. Weight reduction was determined by *in vitro* degradation of the samples for a period of 4 weeks.

- It was observed that free-state consortia were more effective at degrading sanitary napkins than immobilized consortia. FCS 01 showed the highest degradation rate (29%), followed by FCS 02 (27%), and ICS 01 achieved a 21% reduction after 4 weeks.

- In polycoated carry bags, the maximum weight loss was exhibited by ICS 01 (8%) after four weeks, followed by ICS 02, ICS 04, FCS 03 and FCS 05 (5%).

8.2 CONCLUSION

The study on bacteria from northern Kerala's mangrove sediments provided insights into the rich bacterial diversity and functional potential within these ecosystems, highlighting their crucial roles in nutrient cycling, organic matter degradation and potential applications in biotechnology and bioremediation. It reveals how environmental factors influence the distribution and composition of bacterial communities, leading to microbial populations adapted to the mangrove's unique conditions.

Developing bacterial consortia based on the analysis of enzyme potential offers significant benefits, such as enhanced and complete degradation of complex substrates like waste and pollutants. By selecting individual strains with distinct, complementary enzymatic activities, consortia can achieve synergistic effects, leading to more efficient, stable and robust degradation and bio-production compared to single cultures. This approach leverages the division of labour, allowing for the generation of diverse enzymes and overcoming limitations faced by single bacterial strains. Immobilizing bacterial enzymes significantly enhances their stability, activity and reusability by anchoring them to a support material, making them resistant to harsh conditions like temperature, pH and solvents. This approach simplifies enzyme recovery and allows for easier separation from reaction mixtures, leading to more cost-effective and sustainable industrial processes.

Studying the microbial populations and functions within mangrove ecosystems is crucial for effectively protecting them, as it allows for the development of targeted restoration measures based on a deep understanding of their current health, leading to a more informed and targeted approach to conservation. The unique adaptations of mangrove microbes to extreme conditions make them a significant source of biotechnological resources, offering solutions for environmental challenges and improving human well-being.

Chapter - 9

RECOMMENDATIONS

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RECOMMENDATIONS

FUTURE ASPECTS/RECOMMENDATIONS

- Functional metagenomics

  Functional metagenomics helps identify novel genes, enzymes and bioactive compounds from entire microbial communities, leading to discoveries in medicine and biotechnology.

- Purification and characterization of the enzymes obtained

  Purification and characterization of bacterial enzymes help understand their fundamental properties, such as stability, optimal operating conditions (pH and temperature) and molecular weight, which are crucial for their practical applications in industries like medicine, food and biotechnology.

- Transcriptomics

  Transcriptomics studies provide crucial insights into cellular function by revealing patterns of gene expression. It reveals which genes are active, how gene activity is regulated, and how gene expression changes in response to various conditions.

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APPENDIX

APPENDIX I

Principal component analysis of environmental factors: eigenvector values

Vector name	Variable	PC1	PC2	PC3	PC4	PC5
TEM	Temperature (°C)	**0.253**	**-0.387**	0.117	-0.028	0.219
PH	pH	**-0.331**	**-0.42**	0.122	0.228	-0.084
SLY	Salinity (PPT)	-0.208	0.11	0.184	**0.674**	**-0.419**
CHO	Carbohydrate (mg/g)	0.008	**-0.301**	**-0.559**	**-0.241**	**-0.411**
PTN	Protein (mg/g)	0.034	**0.395**	-0.001	**-0.372**	**-0.607**
LPD	Lipid (mg/g)	**0.246**	**0.413**	-0.212	0.057	**0.381**
OM	Organic matter (%)	**0.297**	**0.256**	**-0.361**	**0.472**	-0.028
Sand	Sand (%)	**-0.506**	0.184	**-0.246**	0.032	0.192
Silt	Silt (%)	**0.451**	**-0.344**	-0.093	0.138	-0.135
Clay	Clay (%)	**0.236**	0.166	**0.615**	-0.117	-0.112
CFU	Bacterial population (log_{10} CFU/10g)	**0.342**	0.008	-0.045	0.191	-0.138

APPENDIX II

Details of biochemical tests and hydrolytic potential of the bacterial Isolates

Isolate No	HYDROLYTIC ENZYME POTENTIAL								BIOCHEMICAL TEST									MORPHOLOGY		Genera Identified
	PROTEASE	AMYLASE	LIPASE	CHITINASE	CELLULASE	LIGNINASE	DNASE	LACCASE	Indole	MR	VP	Citrate	Chitinase	Urease	Catalase	Oxidase	TSI	Gram staining	Motility	
1	+	+	+				+		-	-	-	-	+	-	+	+	K/A	-	+	ALCALIGENES FAECALIS
2		+					+		+	+	+	-	-	+	+	-	K/A	+	+	BACILLUS
3			+					+	+	+	+	-	-	-	+	-	A/A	+	+	BACILLUS
4	+	+	+				+		+	-	-	+	-	-	+	-	A/A	-	+	ALCALIGENES FAECALIS
5	+	+	+						+	+	+	-	+	+	+	-	A/K	-	+	ALCALIGENES FAECALIS
6	+	+	+						+	-	+	-	-	+	+	-	A/A	-	+	PSEUDOMONAS
7	+	+					+		+	+	+	+	-	-	+	+	A/A	+	+	BACILLUS
8		+					+		+	+	+	+	+	+	+	-	A/A*	+	+	BACILLUS
9	+	+	+						+	-	+	+	-	+	+	+	K/A	-	+	VIBRIO
10	+	+							-	-	+	+	-	-	+	-	A/A	-	+	PSEUDOMONAS
11	+	+	+		+				-	-	+	-	+	+	+	+	A/A	-	+	VIBRIO
12	+	+	+		+		+		-	-	+	-	-	-	+	-	A/A	-	+	PSEUDOMONAS AERUGINOSA
13			+		+	+	+		-	-	+	-	-	-	+	-	K/A	-	+	ALCALIGENES FAECALIS
14	+								-	-	+	-	-	-	+	-	A/A	+	+	BACILLUS
15		+							-	-	+	-	+	+	+	-	A/A	-	+	PSEUDOMONAS
16	+	+	+		++		+		-	-	-	-	-	-	+	+	K/A	-	+	PSEUDOMOANS
17	+		+				+		-	-	+	-	-	-	+	-	A/A	-	+	PSEUDOMONAS
18			+				+		-	-	-	-	-	-	+	-	A/K	-	-	XANTHOBACTER
19	+	+			+		+		-	-	-	-	-	-	-	+	A/A	+	+	BACILLUS
20	+	+	+		+				-	-	+	-	+	-	-	+	A/K	+	+	ENTEROCOCCUS
21	+	+							-	+	-	-	-	-	-	-	A/A	-	+	ENTEROBACTER
22			+						-	-	-	-	+	+	-	-	A/K	-	+	XANTHOBACTER
23	+	+	+		+				-	+	+	-	-	+	-	+	K/A	+	-	STAPHYLOCOCCUS
24	+	+	+		+		+		-	+	+	-	-	-	+	-	A/K	+	+	BACILLUS
25	+		+		+		+		-	-	+	-	-	-	-	-	A/A	-	+	CITROBACTER
26	+	+	+		+		+		-	-	+	-	-	-	-	-	K/K	+	-	STAPHYLOCOCCUS
27	+		+		+		+		-	-	+	-	-	+	-	-	NC/NC	-	-	ENTEROBACTER
28	+	+	+		+		+		-	-	-	-	-	-	-	+	A/A	+	+	BACILLUS
29	+		+		+		+		-	-	-	-	-	-	+	-	K/K	-	-	KLEBSIELLA
30	+		+		+		+		-	-	-	-	-	-	+	-	K/A	+	+	BACILLUS
31	+	+	+		+				-	+	-	-	-	-	-	-	A/K	-	+	ENTEROBACTER
32			+		+				-	-	-	-	+	-	-	-	A/A*	+	-	STAPHYLOCOCCUS
33			+		+				-	-	+	-	+	+	-	-	K/A	+	+	BACILLUS
34			+			+	+		-		+	-	-	+	-	-	A/A	-	-	XANTHOBACTER
35	+	+	+		+				-	-	-	-	-	-	-	-	A/A	+	-	LACTOBACILLUS
36			+		+				+	-	-	+	-	-	+	+	A/A*	-	+	PSEUDOMONAS
37			+						-	+	-	-	-	+	-	-	A/K	+	+	BACILLUS
38		+	+						+	-	-	+	-	-	+	+	A/A*	+	+	BACILLUS
39	+	+							-	-	-	-	-	-	-	+	K/A	+	+	BACILLUS
40					+				-	-	-	-	-	+	+	-	K/K	-	+	VIBRIO
41	+		+						-	-	-	-	-	-	+	-	K/K	-	+	PSEUDOMONAS
42	+	+							-	+	-	-	+	-	+	-	A/A*	+	-	ENTEROCOCCUS

43			+					-	-	-	-	-	-	-	K/K	+	+	BACILLUS
44	+	+						+	-	-	-	+	+	A/A*	+	+	BACILLUS
45	+	+						-	-	-	-	-	+	K/A	-	-	ACINETOBACTER
46						+		-	-	-	+	+	-	K/K	-	+	VIBRIO
47	+	+	+					-	-	+	-	-	-	K/K	-	-	XANTHOBACTER
48	+		+					-	-	-	-	-	+	K/K	-	+	PSEUDOMONAS
49			+	+				-	-	+	-	-	+	K/K	+	-	ENTEROCOCCUS
50	+		+					-	-	+	+	+	+	K/K	-	-	AEROMONAS
51			+	+				-	-	+	-	+	+	K/K	-	+	PSEUDOMONAS
52		+	+					-	-	+	-	+	+	K/A	-	+	XANTHOBACTER
53		+	+	+				-	-	-	-	+	+	K/A	+	+	BACILLUS
54		+						-	-	+	-	+	+	K/A	-	+	PSEUDOMONAS
55	+	+		+				-	+	+	-	-	+	A/A	+	+	BACILLUS
56	+	+	+	+				-	-	+	-	+	+	A/A	-	+	PSEUDOMONAS
57	+	+	+			+		-	-	-	-	+	+	K/A	-	+	PSEUDOMONAS
58	+	+						-	+	+	-	-	-	A/A	-	+	PSEUDOMONAS
59	+	+						-	-	-	-	+	+	K/A*	-	-	XANTHOBACTER
60	+	+	+					-	-	+	-	+	+	K/A	+	-	STAPHYLOCOCCUS
61		+	+			+		-	-	-	+	+	+	K/K	+	+	BACILLUS
62	+	+					+	-	-	-	-	+	+	K/K	-	+	PSEUDOMONAS
63		+						-	-	-	+	-	-	K/K	-	-	AEROMONAS
64	+	+	+	+				-	-	-	-	+	+	K/A	+	-	STAPHYLOCOCCUS
65			+					-	-	-	-	+	+	K/A	+	-	ENTEROCOCCUS
66			+					-	-	-	+	+	+	K/A	+	+	BACILLUS
67		+	+	+			+	-	+	-	+	-	-	NC/NC	+	+	BACILLUS
68	+		+	+				-	-	+	-	-	-	K/A	-	-	PSEUDOMONAS
69		+		+				-	-	+	-	-	-	K/A*	-	-	XANTHOBACTER
70	+		+	+				-	+	+	-	-	+	K/A	-	+	ENTEROBACTER
71	+		+	+			+	-	-	+	-	-	-	K/A*	-	+	LYSOBACTER
72	+		+	+				-	-	+	-	+	-	K/A	-	+	ESCHERICHIA
73	+		+	+				-	+	-	-	-	-	K/A	+	+	BACILLUS
74							+	-	-	+	-	-	+	K/A	-	+	VIBRIO
75	+	+						-	-	-	-	+	+	K/A	-	+	VIBRIO
76			+	+				-	+	+	-	-	+	K/NC	-	+	ENTEROBACTER
77	+		+	+				+	-	+	-	+	+	K/A	-	+	PSEUDOMONAS
78	+		+					-	-	+	+	-	+	K/A*	-	+	PSEUDOMONAS
79		+		+				-	-	+	-	+	+	NC/NC	-	+	LACTOBACILLUS
80	+			+				-	-	+	+	-	+	K/A*	-	-	KLEBSIELLA
81	+			+				-	-	-	-	-	-	K/A	-	-	ACINETOBACTER
82			+	+				+	-	-	-	-	+	K/A	-	+	XANTHOBACTER
83	+		+	+				-	-	-	+	+	+	K/A	+	+	BACILLUS
84	+	+		+				-	-	+	+	-	+	K/K	+	+	BACILLUS
85	+	+		+				-	+	+	-	-	+	K/A	+	+	BACILLUS
86	+		+	+				-	-	+	-	+	+	K/A	-	+	PSEUDOMONAS
87	+			+				-	-	+	-	-	+	K/A	-	+	SERRATIA MARCESCENS
88	+		+	+				-	-	+	+	-	+	K/A*	-	+	VIBRIO

89	+	+		+			+	-	-	-	-	+	+	A/A*	-	+	ENTEROBACTER
90			+	+			-	-	+	-	-	+	+	K/NC	-	+	VIBRIO
91	+	+					-	-	+	-	-	+	+	NC/NC	-	-	ENTEROBACTER
92	+		+	+			-	-	+	-	-	+	+	K/A	-	+	SERRATIA MARCESCENS
93	+		+				-	+	+	-	-	+	+	A/A	+	+	BACILLUS
94	+		+	+			-	+	-	-	-	-	+	A/A	+	+	BACILLUS
95	+	+	+				-	-	-	-	-	+	+	A/A	+	+	BACILLUS
96	+			+			-	-	-	-	-	-	+	A/A	+	+	BACILLUS
97	+		+	+			-	-	+	-	-	-	-	NC/A	+	+	BACILLUS
98					+		-	-	+	+	-	-	+	K/A*	+	+	BACILLUS
99	+	+	+				-	+	+	+	-	-	+	NC/NC	-	+	SERRATIA MARCESCENS
100	+		+				-	-	-	-	-	-	+	K/NC	-	+	PSEUDOMONAS
101	+	+	+	+			-	-	-	-	-	+	-	K/A	-	+	PSEUDOMONAS
102	+	+	+	+			-	+	+	-	+	-	+	NC/NC	+	+	BACILLUS
103		+		+			-	-	-	-	-	+	+	K/NC	-	+	AEROMONAS
104	+			+			-	+	+	-	-	-	+	K/NC	+	+	BACILLUS
105	+		+	+			-	-	-	-	-	-	-	NC/NC	+	-	STAPHYLOCOCCUS
106	+		+	+			-	+	+	-	-	+	-	K/NC	-	+	PSEUDOMONAS
107				+			-	-	+	-	-	+	+	K/NC	+	+	BACILLUS
108	+	+	+				-	-	+	-	-	-	+	NC/NC	-	-	AEROMONAS
109	+		+				-	-	+	-	-	+	+	K/A	-	+	PSEUDOMONAS
110	+	+	+		+		-	+	+	-	-	+	+	A/A	-	+	PROTEUS
111	+	+			+		-	+	-	-	-	+	+	A/A	+	+	BACILLUS
112	+	+	+				-	-	-	-	+	+	-	A/A*	-	-	XANTHOBACTER
113		+	+		+		-	-	+	-	-	+	+	K/NC	-	+	PESUDOMONAS
114		+	+		+		-	-	+	-	-	+	+	K/A	-	-	ACINETOBACTER
115			+				-	+	-	+	-	+	+	K/NC	-	+	SERRATIA MARCESCENS
116	+	+	+		+		-	-	-	+	+	-	-	A/A*	-	+	VIBRIO
117			+				-	-	-	+	+	-	-	A/A*	-	+	PSEUDOMONAS
118	+	+			+		-	-	+	-	-	-	+	K/A	-	+	PROTEUS
119	+	+	+				-	-	+	-	+	+	+	K/A	-	-	XANTHOBACTER
120		+					-	-	+	-	+	-	-	K/A	-	-	XANTHOBACTER
121		+	+				-	-	+	-	+	-	-	K/A	-	+	PSEUDOMONAS
122			+				-	-	+	-	-	-	+	K/A	+	+	BACILLUS
123	+	+	+				+	-	+	+	+	+	+	A/A	+	+	BACILLUS
124			+				-	-	+	+	-	+	+	K/A	-	+	VIBRIO
125			+				-	-	-	+	-	+	+	K/A	-	+	PSEUDOMONAS
126	+		+				-	-	-	+	-	+	-	A/A	+	+	BACILLUS
127			+				-	-	-	+	-	+	+	K/NC	-	+	VIBRIO
128			+		+		-	-	-	+	-	-	-	A/A	-	+	PSEUDOMONAS
129					+		-	-	-	+	-	-	-	A/A	+	-	ENTEROCOCCUS
130	+	+			+		-	-	-	+	+	-	+	K/NC	+	+	BACILLUS
131					+		+	-	+	-	-	+	+	K/A	+	-	ENTEROCOCCUS
132	+		+		+		-	-	-	-	+	+	-	A/A*	+	-	ENTEROCOCCUS
133					+		-	-	-	-	+	+	-	A/A*	+	+	BACILLUS
134		+			+		+	-	-	-	-	+	+	K/NC	-	+	VIBRIO

135		+	+				-	+	+	-	-	-	+	+	A/NC	-	+	PSEUDOMONAS
136						+	-	-	+	+	-	-	-	+	A/A	-	+	PSEUDOMONAS
137						+	-	-	-	-	-	-	-	+	NC/NC	-	-	KLEBSIELLA
138	+	+	+			+	-	-	+	-	-	-	+	-	K/A	-	+	ALCALIGENES
139			+			+	-	-	+	+	-	-	+	+	NC/NC	-	+	PSEUDOMONAS
140		+	+			+	-	-	-	-	-	-	+	+	K/A	+	-	STAPHYLOCOCCUS
141							-	-	+	+	-	-	+	+	NC/NC	-	+	PSEUDOMONAS AERUGINOSA
142	+						-	+	-	+	-	-	-	+	NC/NC	-	+	VIBRIO
143						+	-	-	+	+	-	-	+	+	NC/NC	+	+	BACILLUS
144	+		+				-	+	+	+	-	-	+	+	NC/NC	+	+	BACILLUS
145			+			+	-	-	+	-	-	-	-	-	NC/NC	-	+	PSEUDOMONAS
146	+					+	-	+	-	-	-	-	-	-	K/A	+	+	BACILLUS
147		+				+	-	-	+	-	-	-	+	-	K/A	+	+	BACILLUS
148			+				-	+	-	-	+	-	+	-	K/A	-	+	PSEUDOMONAS
149			+				-	-	+	-	-	+	+	-	K/A	+	+	BACILLUS
150			+				-	+	-	-	-	-	+	-	K/A	+	+	BACILLUS
151			+				-	+	+	-	-	-	-	-	A/A	+	-	ENTEROCOCCUS
152	+		+				-	+	+	-	-	-	+	-	NC/NC	-	+	AEROMONAS
153	+	+					-	+	+	-	-	+	-	+	A/A	+	+	BACILLUS
154	+	+	+			+	-	-	+	-	-	-	+	+	K/NC	+	+	BACILLUS
155	+	+				+	-	-	-	-	-	-	+	-	NC/NC	-	+	PSEUDOMONAS
156		+					-	+	-	-	-	+	+	-	K/NC	+	+	ENTEROCOCCUS
157	+		+			+	-	+	+	-	-	-	+	-	NC/NC	+	+	BACILLUS
158		+	+				-	-	-	-	+	+	-	-	A/A*	+	+	BACILLUS
159						+	-	+	+	-	-	-	-	-	A/A	+	+	BACILLUS
160	+					+	-	+	+	-	-	-	+	+	NC/NC	+	+	BACILLUS
161			+			+	-	+	+	-	-	-	+	+	NC/NC	+	+	LACTOBACILLUS
162		+	+				-	+	+	-	-	-	+	-	NC/NC	-	+	PSEUDOMONAS
163	+	+				+	-	-	+	-	-	-	+	+	NC/NC	-	+	PSEUDOMONAS
164						+	-	+	-	-	-	+	+	-	K/NC	+	+	BACILLUS
165		+					-	+	-	-	-	-	-	-	NC/NC	+	+	LACTOBACILLUS
166			+			+	-	+	+	-	-	-	+	+	NC/NC	+	+	BACILLUS
167	+	+	+				-	+	-	-	-	-	+	+	A/A	+	+	BACILLUS MEGATERIUM
168	+	+	+				-	-	+	-	-	-	+	+	NC/NC	-	+	PSEUDOMONAS
169						+	-	+	-	-	-	-	+	-	K/NC	+	+	BACILLUS
170		+					-	-	+	+	-	-	-	+	NC/NC	+	+	BACILLUS
171		+					-	+	-	-	-	-	-	+	K/NC	+	+	BACILLUS
172			+			+	-	-	+	-	-	-	+	+	K/NC	+	+	BACILLUS
173	+					+	-	-	+	-	-	-	+	-	K/NC	-	+	PSEUDOMONAS
174			+				-	-	+	-	-	-	+	+	NC/NC	+	-	LACTOBACILLUS
175	+		+				-	+	+	-	-	-	+	+	K/NC	-	+	VIBRIO
176	+		+				-	+	+	-	-	-	+	+	K/NC	+	+	BACILLUS
177	+	+	+				-	+	+	-	-	-	-	-	NC/NC	+	+	BACILLUS
178	+	+	+				-	-	-	-	-	-	+	+	K/A	+	+	BACILLUS
179		+					-	-	+	+	-	-	+	+	K/NC	+	+	BACILLUS
180	+		+				-	-	+	+	-	+	+	-	K/NC	+	+	BACILUS

181	+	+	+					-	+	+	-	-	-	-	+	K/NC	+	+	BACILLUS
182	+	+	+			+		+	+	-	-	-	+	+	-	A/A	+	+	BACILLUS MEGATERIUM
183	+		+			+		-	-	+	-	-	+	-	+	A/A*	+	+	BACILLUS
184	+	+	+			+		-	-	+	-	-	-	+	-	A/A	+	+	BACILLUS
185		+	+			+		-	+	+	-	-	-	+	+	A/A*	+	+	BACILLUS
186		+				+		-	-	+	-	-	-	+	-	A/A	+	+	BACILLUS
187		+			+			-	-	-	-	-	+	+	+	NC/NC	-	-	ENTEROBACTER
188					+			-	-	+	-	-	+	+	+	K/A*	+	+	BACILLUS
189		+			+			-	-	+	-	+	-	+	-	A/A	-	-	XANTHOBACTER
190	+	+	+					-	+	-	-	-	+	-	-	A/A	+	+	BACILLUS
191	+		+				+	-	-	+	-	-	-	-	-	A/A	-	+	PSEUDOMONAS
192		+	+		+			-	-	+	-	-	-	+	+	A/A	+	+	LACTOBACILLUS
193			+		+			-	-	+	-	-	+	+	+	K/A	+	+	BACILLUS
194		+	+		+			-	-	-	-	-	-	+	+	K/A	-	+	PSEUDOMONAS
195			+		+			-	-	-	+	+	-	-	+	A/A*	+	+	BACILLUS
196	+		+					-	-	+	-	+	-	-	-	K/A	-	+	SERRATIA
197	+							-	-	-	-	-	-	+	+	K/A	-	+	SERRATIA MARCESCENS
198	+							-	-	+	-	-	-	+	+	A/A	+	+	BACILLUS
199		+						-	-	-	-	+	+	-	-	A/A*	+	+	BACILLUS
200	+		+					-	-	+	+	-	+	+	+	-	-	+	SERRATIA MARCESCENS
201	+		+					-	+	-	-	-	-	+	+	K/A	+	+	BACILLUS
202	+		+					-	-	+	-	-	+	+	-	A/A	+	-	ENTEROCOCCUS
203				+				-	+	+	+	-	-	+	-	A/A	-	+	SERRATIA
204				+				-	+	-	+	-	+	-	-	A/A	+	+	BACILLUS
205			+					-	-	+	-	-	+	+	+	A/A B	+	+	BACILLUS
206				+				-	-	-	+	+	-	-	-	A/A*	+	-	ENTEROCOCCUS
207	+		+					-	+	+	-	-	-	+	+	K/B	+	-	ENTEROCOCCUS
208	+		+					-	+	+	-	-	-	+	+	K/B	+	+	BACILLUS
209	+		+					-	-	-	+	-	+	+	+	K/B	+	+	BACILLUS
210		+	+					-	+	+	-	-	+	+	+	A/A	+	+	BACILLUS
211			+				+	-	+	-	+	-	+	+	+	K/B	+	+	BACILLUS
212			+				+	-	+	+	+	-	+	+	-	K/AB	+	+	BACILLUS
213	+	+	+					+	+	-	+	-	+	+	-	A/B	+	+	BACILLUS
214	+	+			+			+	+	+	+	-	-	-	-	K/AB	+	+	BACILLUS
215	+				+			-	-	+	-	-	-	+	-	K/B	+	+	BACILLUS
216			+		+			-	+	+	+	-	-	+	+	K/B	+	+	BACILLUS
217			+		+			-	+	-	+	-	-	+	+	K/B	-	+	PSEUDOMONAS
218			+		+			-	-	-	-	+	-	-	-	A/A*	-	+	ENTEROBACTER
219		+						-	+	+	-	-	+	+	-	K/B	-	+	VIBRIO
220								+	+	-	+	-	-	+	+	K/B	-	+	VIBRIO
221	+							-	+	-	-	-	-	+	-	K/B	+	+	BACILLUS
222	+		+		+			-	-	+	+	-	-	+	-	K/AB	-	+	PSEUDOMONAS
223					+			-	-	+	-	-	-	+	-	NC/NC	-	+	PSEUDOMONAS
224					+			-	-	-	-	+	+	-	-	A/A*	-	+	LYSOBACTER
225					+		+	-	-	-	+	-	+	+	+	K/AB	+	+	BACILLUS
226		+	+		+			-	-	-	-	-	-	-	-	K/AB	-	-	XANTHOBACTER

227	+		+			+		-	+	+	-	-	+	-	-	K/AB	-	-	XANTHOBACTER
228	+		+					-	+	-	-	-	-	+	+	A/A	-	+	PSEUDOMONAS
229	+				+			-	-	+	+	-	+	-	-	K/AB	-	+	PSEUDOMONAS
230	+				+			-	-	+	+	-	+	+	+	K/AB	-	+	PSEUDOMONAS
231		+				+		-	-	+	+	-	-	+	+	K/AB	+	+	BACILLUS
232			+		+			-	-	+	+	-	-	-	-	K/AB	+	+	BACILLUS
233		+				+		-	-	-	+	-	-	+	+	K/AB	-	-	ACINETOBACTER
234		+	+		+			-	-	-	-	-	-	+	+	K/AB	-	+	PSEUDOMONAS
235						+		-	-	-	-	-	+	-	-	K/AB	-	-	ACINETOBACTER
236			+			+		-	-	+	-	-	+	+	+	K/AB	-	+	PSEUDOMONAS
237		+	+			+		-	+	-	-	-	-	+	+	K/AB	+	+	BACILLUS
238		+				+		-	-	-	-	-	-	+	-	K/K	+	+	BACILLUS
239		+						-	-	+	-	-	+	-	-	K/AB	+	+	BACILLUS
240						+		-	+	-	-	-	+	-	-	K/AB	-	+	PSEUDOMONAS
241		+				+		-	-	+	-	-	+	+	+	K/AB	+	+	BACILLUS
242		+	+					-	-	+	-	-	-	+	+	A/A*	+	+	BACILLUS MEGATERIUM
243		+	+					-	-	+	-	-	+	-	-	K/AB	+	+	BACILLUS
244		+	+		+			-	-	+	-	-	-	-	-	K/AB*	+	+	BACILLUS
245						+		-	-	+	-	-	-	-	-	A/A	-	+	PSEUDOMONAS
246			+					-	-	-	-	-	-	+	-	K/AB	-	+	XANTHOBACTER
247							+	-	-	+	-	-	-	+	-	K/AB	-	+	PSEUDOMONAS
248	+		+		+			-	-	-	-	-	+	+	+	K/AB	+	+	BACILLUS SUBTILIS
249		+	+			+		-	-	-	-	+	-	-	-	A/A*	+	+	BACILLUS
250	+		+			+		-	+	+	-	-	+	+	+	A/A	+	+	BACILLUS
251						+		-	-	-	-	-	+	-	-	K/AB	-	+	VIBRIO
252	+	+	+					-	-	-	-	-	+	-	-	NC/NC	+	+	ENTEROCOCCUS
253	+	+					+	-	+	-	-	-	-	+	+	K/NC	-	+	VIBRIO
254		+	+					-	-	+	-	-	+	+	+	NC/NC	-	+	PSEUDOMONAS
255					+		+	-	-	+	-	-	+	+	+	A/A	+	+	ENTEROCOCCUS
256	+	+	+					-	-	+	-	+	-	-	-	A/A	-	+	LYSOBACTER
257	+	+		+			+	-	-	+	-	-	+	-	-	A/A	-	+	VIBRIO
258							+	-	-	+	-	+	-	+	+	K/AB*	-	+	PSEUDOMONAS
259			+				+	-	-	+	-	-	+	+	+	K/NC	+	+	BACILLUS
260		+	+					-	-	+	-	-	+	+	+	NC/NC*	-	+	ENTEROBACTER
261	+	+			+			-	-	+	-	-	+	+	+	NC/NC*	+	+	BACILLUS
262	+	+			+			-	-	+	-	-	+	-	-	NC/NC*	-	+	PSEUDOMONAS
263			+		+			-	+	+	-	-	+	+	+	A/A*	+	+	BACILLUS
264		+	+					-	-	+	+	-	+	+	+	K/NC	-	+	PSEUDOMONAS
265		+						-	+	-	-	-	-	+	+	A/NC	+	+	BACILLUS
266		+	+					+	-	-	+	-	+	+	+	A/NC	+	+	BACILLUS
267	+						+	-	-	-	+	-	+	+	-	K/NC	+	+	BACILLUS
268	+	+						-	-	+	+	-	-	+	+	A/A*	+	-	ENTEROCOCCUS
269	+	+	+					-	-	+	-	-	+	+	+	K/NC	-	+	XANTHOBACTER
270					+			-	+	-	-	-	-	+	+	NC/NC	-	+	XANTHOBACTER
271		+						-	-	-	-	-	-	+	+	NC/NC	+	+	BACILLUS
272	+		+					-	-	-	-	-	+	-	-	K/K*	-	+	SERRATIA

273	+	+					-	+	-	+	+	+	-	NC/NC	+	+	BACILLUS
274		+	+				-	-	-	+	-	+	K/NC	+	+	BACILLUS
275		+			+		-	-	-	-	-	-	K/NC	+	+	BACILLUS
276			+				-	-	-	-	+	+	A/NC	+	+	BACILLUS
277	+						-	-	-	-	+	+	NC/NC	+	+	BACILLUS
278			+				+	-	+	-	-	+	K/A	+	+	BACILLUS
279			+				-	+	-	-	-	+	A/AB*	-	+	LYSOBACTER
280	+		+		+		-	-	+	-	-	-	A/A*	-	+	LYSOBACTER
281	+	+	+				-	-	-	-	+	-	K/NC	+	+	BACILLUS
282			+				-	-	+	-	+	-	K/A	-	+	SERRATIA MARCESCENS
283	+		+		+		-	-	-	+	+	-	A/A*	+	+	BACILLUS
284	+		+		+		-	-	-	-	-	-	A/A*	-	-	XANTHOBACTER
285		+	+		+		-	-	+	-	+	+	K/A*	-	+	LYSOBACTER
286	+		+		+		-	-	-	+	+	-	A/A*	+	+	BACILLUS
287			+		+		-	+	+	-	-	+	K/A*	+	+	BACILLUS MEGATERIUM
288			+		+		-	-	+	-	+	+	K/K	-	+	LYSOBACTER
289			+				-	+	+	-	-	-	K/A*	-	-	XANTHOBACTER
290		+	+				-	+	+	-	-	+	K/A*	+	+	BACILLUS
291	+		+				-	-	+	-	-	+	K/A	-	+	SERRATIA MARCESCENS
292			+				-	-	+	-	-	+	K/A*	+	+	BACILLUS
293	+	+	+				-	-	+	-	+	+	A/NC	+	+	BACILLUS
294		+	+			+	-	-	-	-	-	-	K/K	-	+	LYSOBACTER
295	+	+	+				-	-	+	-	-	-	K/NC	-	+	LYSOBACTER
296	+					+	-	-	+	-	+	-	A/A*	+	+	BACILLUS
297	+		+				-	-	-	+	-	+	A/AB*	-	+	SERRATIA MARCESCENS
298		+					-	-	-	-	+	+	K/NC	-	+	PSEUDOMONAS
299		+	+				-	-	-	-	+	-	K/NC	+	+	BACILLUS
300	+		+		+		-	+	+	-	+	+	K/A	+	+	BACILLUS
301		+					-	+	+	-	+	+	A/A*	-	+	SERRATIA MARCESCENS
302	+	+				+	-	+	+	-	+	+	A/A*	+	+	BACILLUS
303		+	+			+	-	-	+	-	+	-	A/A*	+	+	BACILLUS
304		+	+	+		+	-	-	+	-	-	-	NC/NC	+	+	BACILLUS
305			+				-	-	+	+	+	-	A/A*	-	-	XANTHOBACTER
306		+	+				+	-	+	+	-	+	A/A*	+	+	BACILLUS
307			+				-	-	+	+	-	-	A/A*	-	+	PSEUDOMONAS
308		+	+		+		-	-	+	-	-	+	A/A*	+	+	BACILLUS
309	+	+	+				-	-	+	-	-	+	A/A*	-	+	LYSOBACTER
310	+					+	-	-	-	+	-	-	A/A	+	+	BACILLUS
311	+		+		+		-	-	+	-	-	+	K/AB*	+	+	BACILLUS
312	+		+		+		-	+	+	+	-	+	A/AB*	+	-	ENTEROCOCCUS
313	+	+	+				-	-	-	-	+	-	A/A*	+	+	BACILLUS
314	+	+	+		+		-	-	+	-	+	-	A/NC	+	+	BACILLUS
315					+		-	-	-	+	+	-	A/A*	+	+	ENTEROCOCCUS
316	+	+	+		+		-	+	+	-	-	+	K/NC	+	+	BACILLUS
317	+						-	-	-	-	-	+	A/A	-	+	VIBRIO
318	+	+	+		+		-	-	+	-	-	+	A/A*	+	+	BACILLUS

319		+	+			+		-	-	+	-	+	+	K/A*	+	-	STAPHYLOCOCCUS
320	+	+	+					-	+	+	-	+	+	A/A	+	+	BACILLUS
321	+	+	+					-	-	+	-	-	-	K/A*	+	+	BACILLUS
322	+		+					-	-	-	-	+	+	K/A	+	+	BACILLUS
323	+	+	+			+		-	-	-	+	-	+	NC/NC	+	+	BACILLUS
324	+	+	+					-	-	-	-	-	-	A/A*	+	+	BACILLUS
325	+		+					-	-	-	+	-	-	A/A*	+	+	BACILLUS
326	+	+	+					-	+	-	+	-	+	A/NC	-	+	VIBRIO
327	+	+	+					-	-	+	-	+	-	A/A*	-	+	PSEUDOMONAS
328			+		++			-	+	-	-	+	+	A/A*	+	-	BACILLUS
329	+	+	+			+		-	-	+	-	+	+	NC/NC	-	+	XANTHOBACTER
330		+	+					-	-	-	-	+	+	K/A*	-	-	XANTHOBACTER
331	+	+	+		+			-	-	+	+	+	+	K/K	-	+	PSEUDOMONAS
332	+	+	+		+			-	-	-	-	+	-	A/A*	+	+	BACILLUS
333	+	+	+		+		+	-	-	+	-	+	+	NC/NC	+	+	BACILLUS
334			+					-	-	+	-	+	+	K/A*	-	+	SERRATIA MARCESCENS
335	+		+					-	-	+	-	+	+	K/K	+	+	BACILLUS
336			+					-	+	-	-	-	-	NC/NC	-	+	PSEUDOMONAS
337	+	+	+					-	+	-	+	+	+	K/A*	-	+	SERRATIA MARCESCENS
338	+	+	+					-	+	-	-	+	-	K/K	+	+	BACILLUS
339	+	+	+				+	-	-	-	-	+	-	A/A*	+	+	BACILLUS CEREUS
340	+	+	+					-	-	+	-	+	-	NC/NC	+	+	BACILLUS
341			+					-	-	-	+	+	+	K/A*	+	+	BACILLUS
342	+		+			+		-	+	-	+	+	+	K/K*	+	+	BACILLUS
343	+	+				+		-	+	+	+	-	+	K/K*	+	+	BACILLUS
344	+		+					-	-	-	+	+	-	K/K*	+	+	BACILLUS
345	+	+	+					-	+	-	-	+	-	A/A*	+	+	BACILLUS
346		+	+					-	+	+	+	+	+	K/K*	+	+	BACILLUS
347	+	+	+			+								A/A*	+	+	BACILLUS
348	+		+				+	-	-	+	-	+	+	A/A*	-	+	VIBRIO
349	+	+	+					-	-	+	-	+	+	K/K	-	+	SERRATIA MARCESCENS
350	+							-	+	+	-	+	-	A/A	-	+	VIBRIO
351			+					-	+	-	-	+	-	K/A	-	+	PSEUDOMONAS
352	+	+				+		-	-	+	-	-	+	A/A*	+	+	BACILLUS
353	+	+	+			+		-	-	-	+	+	-	K/A*	-	-	XANTHOBACTER
354	+	+	+					-	+	-	-	+	+	K/K*	-	+	PSEUDOMONAS
355			+				+	-	-	+	-	+	-	K/K*	-	+	PSEUDOMONAS
356		+	+			+		-	+	-	+	+	-	A/K*	-	+	PSEUDOMONAS
357	+	+	+			+		-	+	-	-	+	-	A/K	+	-	LACTOBACILLUS
358	+		+					-	+	+	-	+	-	K/K	-	+	PSEUDOMONAS
359	+	+	+					-	-	-	+	+	-	K/A*	+	+	BACILLUS
360	+	+	+			+		-	-	-	+	+	+	K/K	-	+	PSEUDOMONAS
361	+	+						-	-	+	-	+	+	K/K	+	+	BACILLUS
362		+	+					-	-	+	-	+	-	K/A*	+	+	BACILLUS
363	+	+	+					-	+	+	-	+	-	K/K	+	+	BACILLUS
364							+	-	+	-	-	+	+	K/K*	+	+	BACILLUS

365			+				-	+	-	-	+	-	K/A*	+	+	BACILLUS
366	+	+					-	-	+	-	+	+	K/K	+	+	BACILLUS
367	+		+			+	-	-	-	-	+	+	K/A*	-	+	VIBRIO
368		+	+				-	+	-	-	+	+	K/A*	+	+	BACILLUS
369		+	+				-	-	+	-	+	-	K/K	+	+	BACILLUS
370			+				-	-	-	-	+	-	K/A*	+	+	BACILLUS
371		+	+			+	-	-	-	+	-	-	K/A*	+	-	ENTEROCOCCUS
372	+		+	+	+		-	-	-	+	-	-	K/A*	+	+	BACILLUS
373		+	+				-	-	-	-	+	-	A/A*	-	+	CITROBACTER
374	+	+	+				-	-	-	-	+	-	K/A	-	+	PSEUDOMONAS
375			+				-	-	-	-	+	-	K/A*	-	+	XANTHOBACTER
376			+				-	-	+	-	-	-	K/A*	+	+	BACILLUS
377			+		+		-	+	-	-	+	-	K/A*	+	+	BACILLUS
378			+				-	-	+	-	+	-	A/A*	-	+	VIBRIO
379			+				-	-	-	+	+	+	K/A	-	+	CITROBACTER
380		+	+				-	-	+	-	+	+	K/A*	+	+	BACILLUS
381	+	+	++				-	-	+	-	+	+	A/A*	+	+	BACILLUS
382		+	+				-	-	-	-	+	-	A/A*	-	+	PSEUDOMONAS
383			+				-	-	-	-	+	-	A/A*	-	+	SERRATIA MARCESCENS
384	+	+	+		+		-	-	-	+	+	-	A/A*	+	+	BACILLUS
385			+				-	-	-	-	+	-	A/A*	-	+	ENTEROBACTER
386		+	+		+		-	-	+	-	+	+	A/A*	+	+	BACILLUS
387		+	+		+		-	-	-	+	-	+	A/A	+	+	BACILLUS
388			+				-	-	+	-	-	-	A/A*	+	+	BACILLUS
389		+	+		+		-	+	-	-	-	-	A/A*	+	+	BACILLUS
390					+		-	+	-	+	+	+	A/A*	-	-	KLEBSIELLA
391							-	+	-	-	-	+	A/A*	+	-	LACTOBACILLUS
392	+	+	+		+		-	-	-	-	+	-	A/A*	-	-	XANTHOBACTER
393	+		+		+		-	-	+	-	+	-	A/A*	-	-	XANTHOBACTER
394		+					-	-	-	+	+	-	A/A	-	+	XANTHOBACTER
395	+				+		-	-	-	-	-	-	A/A	-	-	XANTHOBACTER
396	+	+	+		+		-	+	-	+	+	-	A/A	+	+	BACILLUS
397	+		+		+		-	-	-	+	-	+	K/A	+	+	BACILLUS
398	+		+		+		-	-	+	-	-	+	K/A*	+	+	BACILLUS
399	+		+		+		-	-	-	+	-	+	A/A	+	+	BACILLUS
400	+		+		+		-	-	+	+	-	-	A/A*	+	+	BACILLUS
401	++		+		+		-	-	+	-	-	+	K/A*	+	+	BACILLUS
402	+				+		-	-	-	-	-	+	A/A*	-	-	XANTHOBACTER
403	+		+		+	+	-	-	+	-	+	+	A/A	+	+	BACILLUS
404	+		+		+		-	-	-	-	-	+	A/A	-	-	XANTHOBACTER
405	+		+		+	+	-	-	+	-	+	+	A/A	+	+	BACILLUS
406			+		+		-	+	-	-	+	-	A/A	-	-	KLEBSIELLA
407			+		+		-	+	-	-	+	-	A/A*	-	+	PSEUDOMONAS
408	+		+		+	+	-	-	-	-	-	+	K/A	+	+	BACILLUS
409			+		+		-	-	+	-	+	+	A/A	+	+	LACTOBACILLUS
410	+	+	+		+		-	-	+	-	-	+	A/A	+	+	BACILLUS

411	+	+	+					-	-	-	-	-	+	+	A/A	+	+	BACILLUS
412	+		+					-	-	-	-	+	+	-	A/A	+	+	BACILLUS
413	+		+			+		-	-	+	+	-	+	+	A/A*	+	+	BACILLUS
414			+					-	-	-	-	+	+	-	A/A	+	+	BACILLUS
415			+					-	-	-	-	+	-	+	A/A	+	-	ENTEROCOCCUS
416			+					-	+	-	-	+	-	+	A/A	-	+	XANTHOBACTER
417			+			+		-	-	-	-	+	-	+	A/A	-	+	ALCALIGENES FAECALIS
418	+	+	+		+		+	-	-	+	-	+	+	-	A/A*	-	+	SERRATIA MARCESCENS
419	+	+	+		+			-	+	+	-	+	-	-	K/A	-	+	AEROMONAS
420			+			+		-	-	-	-	+	+	+	A/A	-	+	ALCALIGENES
421	+	+	+		+			-	+	-	-	+	-	+	A/A	-	+	PSEUDOMONAS
422	+		+			+		-	+	-	-	+	-	-	A/A	+	+	BACILLUS
423	+	+	+		+			-	-	-	-	-	-	-	K/A*	-	+	XANTHOBACTER
424			+				+	-	-	+	-	+	+	+	A/A	-	+	PSEUDOMONAS
425	+	+	+		+		+	-	+	+	-	-	-	+	K/A	+	+	BACILLUS CEREUS
426	+	+	+		+	+		-	-	-	-	+	-	+	A/K	-	+	PSEUDOMONAS
427		+	+		+			-	+	-	-	-	+	-	A/A*	+	+	BACILLUS
428			+			+		-	+	+	-	-	+	+	K/A	-	+	PSEUDOMONAS
429	+	+	+		+	+		-	+	+	-	-	+	-	K/A	+	+	BACILLUS
430	+	+	+		+	+		+	+	-	+	-	-	-	K/K	-	+	PSEUDOMONAS
431	+	+	+		+			+	+	-	-	-	+	+	K/K	-	+	LYSOBACTER
432	+	+	+		+		+	+	+	-	+	-	+	+	A/K	+	+	BACILLUS
433	+	+	+		+			+	+	+	-	-	+	+	A/K	-	+	PSEUDOMONAS
434	+	+	+		+	+		+	+	-	+	-	+	-	A/K	+	+	BACILLUS
435					+			+	+	-	+	-	-	-	A/K	+	+	BACILLUS
436	+	+	+		+		+	+	+	-	-	-	+	+	A/K	+	+	BACILLUS
437	+		+		+			-	-	-	-	-	+	+	A/K	+	+	BACILLUS
438	+	+	+		+		+	-	-	-	-	+	+	+	NC/NC	-	+	XANTHOBACTER
439	+	+	+		+		+	-	+	-	+	+	+	-	A/K	-	+	PSEUDOMONAS
440	+	+	+		+			-	+	-	+	-	+	-	A/K	+	+	BACILLUS CEREUS
441	+	+	+		+		+	-	-	-	-	-	+	+	A/NC	+	+	BACILLUS
442	+	+	+		+		+	-	-	-	-	+	+	+	A/K	+	+	BACILLUS
443	+	+	+		+	+		-	+	-	+	+	+	-	A/K	+	+	BACILLUS
444	+	+	+		+	+		-	+	-	-	-	+	+	A/K	+	+	BACILLUS ARYABHATTAI
445			+				+	-	-	-	-	-	+	-	A/K	-	+	SERRATIA MARCESCENS
446	+	+	+		+		+	-	-	-	-	+	+	-	A/NC	+	+	BACILLUS
447	+	+	+		+			-	-	-	-	-	+	+	A/K	+	+	BACILLUS
448	+	+	+		+			-	-	-	-	-	+	-	A/NC	+	+	BACILLUS
449	+	+	+		+			-	-	-	-	-	+	-	A/NC	+	+	ENTEROBACTER
450		+	+		+		+	-	-	-	-	-	-	-	A/NC	-	+	PSEUDOMONAS
451		+	+		+			-	-	-	-	-	-	-	A/NC	-	+	ALCALIGENES
452	+	+	+		+			-	+	-	-	-	-	+	A/A	-	+	ALCALIGENES
453			+		+		+	-	+	-	-	-	+	-	A/A*	-	+	ALCALIGENES
454	+	+	+		+		+	-	+	-	-	+	+	-	A/A*	-	+	ENTEROBACTER
455	+	+	+		+		+	-	-	-	-	-	-	-	K/A*	-	+	ESCHERICHIA
456	+	+	+		+			-	-	-	-	-	-	+	A/A	+	+	BACILLUS

457	+	+	+	+				-	+	+	-	+	-	-	K/K	+	+	BACILLUS
458		+						-	-	-	-	-	+	NC/NC	+	+	BACILLUS
459	+		+	+		+		-	-	-	-	-	+	A/NC	-	+	PSEUDOMONAS
460	+	+	+	+		+		-	+	-	-	+	+	NC/NC	+	+	BACILLUS
461			+					-	-	-	-	+	+	A/A	+	+	BACILLUS
462	+		+	+		+		-	-	+	-	+	+	A/A	-	+	LYSOBACTER
463			+					-	-	-	-	+	+	A/NC	-	-	ENTEROBACTER
464		+	+	+				-	-	-	-	-	+	A/NC	+	+	BACILLUS MEGATERIUM
465	+	+	+	+				-	+	-	-	-	-	A/A	+	+	BACILLUS
466			+					-	+	-	-	-	+	A/A	-	-	XANTHOBACTER
467								-	-	-	-	+	+	A/A	-	+	VIBRIO
468		+				+		-	+	-	-	-	+	A/A	-	+	ENTEROBACTER
469		+				+		-	+	-	-	-	-	A/NC	-	+	XANTHOBACTER
470		+	+			+		-	+	-	-	-	+	A/A	+	-	STAPHYLOCOCCUS
471	+		+	+		+		-	-	-	-	+	+	A/A	-	+	PSEUDOMONAS
472			+					-	+	-	-	+	-	A/NC	+	+	BACILLUS
473	+		+					-	-	-	-	-	+	A/NC	+	+	BACILLUS
474	+							-	-	-	-	-	+	A/NC	-	+	VIBRIO
475	+	+				+		-	+	-	-	+	-	A/NC	-	-	XANTHOBACTER
476	+	+				+		-	-	-	-	-	+	A/NC	-	+	VIBRIO
477		+						-	-	-	-	+	+	A/A	+	-	STAPHYLOCOCCUS
478		+	+	+				-	+	+	-	-	+	K/A	+	+	BACILLUS
479		+	+				+	-	-	-	-	+	+	A/NC	+	+	BACILLUS
480	+	+	+	+	+		+	-	-	-	-	-	+	A/A	-	-	KLEBSIELLA PNEUMONIAE
481			+					-	-	-	-	-	-	K/A*	+	-	STAPHYLOCOCCUS
482			+					-	-	-	-	-	-	A/A*	-	+	SERRATIA
483	+		+	+		+		-	-	-	-	-	-	A/A*	-	+	ALCALIGENES
484			+					-	+	-	-	-	-	NC/NC	-	+	PSEUDOMONAS
485	+	+	+	+		+		-	-	-	-	-	+	A/NC	+	+	BACILLUS
486		+	+	+				-	-	-	-	-	-	K/A*	+	+	BACILLUS
487	+	+	+	+				-	-	-	-	-	-	K/A*	+	-	STAPHYLOCOCCUS
488	+	+		+		+		-	+	-	-	-	+	NC/NC	-	+	VIBRIO
489								-	-	-	-	-	+	A/NC	+	+	BACILLUS
490	+		+	+		+		-	-	-	-	-	+	A/A	+	+	BACILLUS
491	+		+	+		+		-	-	-	-	+	+	A/A	+	+	BACILLUS
492			+	+		+		-	-	-	-	-	+	A/NC	-	+	CITROBACTER
493	+	+	+	+				-	-	-	-	-	+	A/A	+	+	BACILLUS
494	+		+	+				-	-	+	-	-	+	A/A*	+	+	BACILLUS
495	+	+	+	+				-	+	-	-	-	+	K/NC	+	+	LACTOBACILLUS
496	+		+			+		-	+	-	-	-	+	K/NC	+	-	STAPHYLOCOCCUS
497	+	+	+	+				-	-	-	+	-	+	K/A	+	+	LACTOBACILLUS
498			+	+				-	+	+	-	+	-	K/A	+	+	BACILLUS MEGATERIUM
499			+					-	+	-	-	+	+	K/A	-	+	VIBRIO
500	+	+	+					-	+	+	-	+	-	K/NC	+	-	STAPHYLOCOCCUS
501	+		+	+		+		-	+	+	-	+	+	K/NC	+	+	BACILLUS
502			+					-	+	-	-	+	+	K/A	+	-	STAPHYLOCOCCUS

503		+	+				-	-	+	-	-	-	+	K/K	-	+	ENTEROBACTER
504		+	+			+		-	-	+	-	-	+	K/A	+	+	BACILLUS
505			+					-	+	-	-	-	+	K/NC	+	+	BACILLUS
506	+	+	+					-	+	+	-	+	+	K/A*	-	+	PSEUDOMONAS
507	+	+	+					-	+	+	-	-	+	K/A	-	+	SERRATIA MARCESCENS
508			+					-	+	+	-	-	+	K/K*	-	+	ENTEROBACTER
509	+		+					-	+	+	-	-	+	K/K	+	+	BACILLUS
510	+	+	+			+		-	-	+	-	+	+	K/K*	-	+	SERRATIA MARCESCENS
511	+	+	+		+			-	+	-	-	-	+	K/K*	-	+	ACINETOBACTER
512	+	+			+			-	-	+	-	-	+	K/K	-	+	ACINETOBACTER
513								-	-	-	-	-	+	K/K	+	+	BACILLUS
514	+		+					-	+	+	-	+	+	K/A	+	+	BACILLUS
515		+				+		-	+	+	+	+	+	K/A	+	+	BACILLUS
516	+	+	+					-	+	-	-	+	+	K/A	-	+	ENTEROBACTER
517			+			+		-	+	-	-	-	+	K/A*	+	-	ENTEROCOCCUS
518		+	+				+	+	+	+	-	-	+	A/A	+	-	ENTEROCOCCUS
519			+					+	+	+	+	-	+	K/A*	+	+	BACILLUS MEGATERIUM
520	+		+			+		+	+	+	-	-	+	K/A*	-	+	PSEUDOMONAS
521		+	+					+	-	+	+	-	+	K/A*	++	-	ENTEROCOCCUS
522	+	+	+			+		+	-	+	+	-	+	A/A*	-	+	PSEUDOMONAS
523		+	+			+		+	+	+	+	-	+	K/A*	+	+	BACILLUS
524								+	+	+	+	-	+	K/A*	+	-	ENTEROCOCCUS
525			+				+	+	-	+	-	-	+	K/A*	+	-	ENTEROCOCCUS
526	+	+	+					+	-	+	-	-	+	K/A*	-	+	VIBRIO
527	+	+				+		+	+	+	-	-	+	K/A	+	+	BACILLUS
528		+	+					+	+	+	-	-	+	K/A*	+	+	BACILLUS MEGATERIUM
529	+	+				+		+	+	+	-	-	+	K/A*	+	+	BACILLUS
530	+	+	+					-	-	-	-	+	+	K/A	+	+	BACILLUS
531		+						-	-	-	-	+	+	K/A	+	+	BACILLUS
532	+	+	+					-	-	-	+	-	+	A/A	-	+	SERRATIA
533	+							+	+	+	-	-	+	K/A*	+	+	BACILLUS
534	+	+	+			+		+	+	+	+	-	+	K/A*	+	+	BACILLUS
535	+	+	+			+		+	+	+	-	-	+	K/A*	-	+	PROTEUS
536		+	+			+		+	+	+	-	-	+	A/A	+	+	LACTOBACILLUS
537	+							-	+	+	-	-	+	A/A	+	+	BACILLUS
538	+	+	+			+		-	-	+	-	-	+	K/K	+	+	LACTOBACILLUS
539								-	-	+	-	-	+	A/A*	+	+	BACILLUS
540	+	+						+	-	+	+	-	+	K/A	+	+	BACILLUS
541			+					+	-	+	+	-	+	A/A	-	+	PROTEUS
542	+	+	+			+			-	-	-	+	+	K/A	+	+	BACILLUS MEGATERIUM
543	+		+					-	-	+	+	-	+	K/K	+	+	BACILLUS
544	+		+					-	-	+	+	-	+	A/A*	-	+	VIBRIO
545	+	+	+					-	-	-	-	+	+	K/A	+	+	BACILLUS
546	+		+					-	-	+	+	-	+	A/A	-	+	PSEUDOMONAS
547	+		+					-	-	+	+	-	+	K/A	-	+	VIBRIO
548	+		+			+		-	-	+	+	-	+	K/NC	+	+	BACILLUS MEGATERIUM

549	+		+					-	-	+	+	-	+	-	K/K	-	+	PSEUDOMONAS
550	+		+					-	-	-	-	+	-	+	K/A	+	-	ENTEROCOCCUS
551	+		+					-	-	+	-	+	+	+	A/A	-	-	ENTEROBACTER
552	+		+					-	-	+	-	+	+	+	K/A*	+	+	BACILLUS
553	+		+		+			-	-	+	-	+	+	+	K/A	+	+	BACILLUS
554	+					+		-	-	+	-	+	-	-	A/A	-	-	KLEBSIELLA
555	+	+	+		+			-	-	-	-	+	-	-	A/A	-	+	PSEUDOMONAS
556	+	+	+		+		+	-	-	-	-	+	-	-	A/A	+	+	BACILLUS
557	+	+	+		+		+	-	-	+	-	+	+	-	A/A	+	+	BACILLUS
558	+	+	+		+			-	-	+	-	+	-	-	A/A	-	+	ENTEROBACTER
559	+	+	+		+			-	-	+	-	+	-	-	A/A*	-	+	VIBRIO
560	+	+	+		+			-	-	+	-	+	-	-	A/A*	+	+	BACILLUS
561	+	+	+		+		+	-	-	+	-	+	-	-	A/A*	-	+	PSEUDOMONAS
562	+		+		+			-	-	-	-	+	-	-	K/A	-	+	VIBRIO
563	+							-	-	-	-	+	-	-	K/A	-	+	VIBRIO
564	+	+	+					-	-	+	-	+	-	-	K/A	-	+	SERRATIA
565	+	+	+					-	-	+	-	+	-	-	K/A	+	+	BACILLUS
566		+	+				+	-	-	+	-	+	-	-	K/A	-	+	ENTEROBACTER
567	+	+	+		+			-	-	+	-	+	-	-	K/A	-	+	PSEUDOMONAS
568	+	+	+		+			-	-	+	-	+	-	-	K/A*	+	+	BACILLUS
569			+					-	-	+	-	+	-	-	K/NC	+	+	BACILLUS
570		+	+	+				-	-	+	-	+	-	-	NC/NC	-	+	ALCALIGENES FAECALIS
571	+	+		+				-	-	+	-	+	-	-	NC/NC	-	-	AEROMONAS
572	+	+			+			-	-	-	+	+	+	-	K/A	-	+	VIBRIO
573	+							-	-	-	-	+	-	-	A/A	-	+	VIBRIO
574		+	+					-	-	-	-	+	-	-	A/A*	+	+	BACILLUS
575			+					-	-	-	-	+	-	-	A/A*	-	+	VIBRIO
576	+		+			+		-	-	-	-	+	-	-	A/A*	-	+	VIBRIO
577	+	+						-	-	-	-	+	-	-	A/A*	-	-	XANTHOBACTER
578		+	+					-	-	-	-	+	-	-	A/A*	-	-	XANTHOBACTER
579	+			+				-	-	-	-	+	-	-	A/NC	-	-	XANTHOBACTER
580	+							-	-	-	-	+	-	-	A/NC	-	+	VIBRIO
581	+		+		+			-	-	-	-	+	-	-	A/NC	-	+	VIBRIO
582	+	+		+	+	+		-	-	-	-	+	-	-	A/B	-	+	ALCALIGENES FAECALIS
583			+					-	-	-	-	+	-	-	A/A*	-	+	PSEUDOMONAS AERUGINOSA
584			+					-	-	-	-	+	-	-	K/K	-	+	XANTHOBACTER
585			+					-	-	-	-	+	-	-	K/K	+	-	ENTEROCOCCUS
586			+					-	-	-	-	+	-	-	K/K*	+	-	ENTEROCOCCUS
587			+					-	-	-	-	+	-	-	K/A	-	+	PSEUDOMONAS
588	+	+	+		+			-	-	-	-	+	-	-	K/K	-	+	PSEUDOMONAS
589	+		+		+			-	-	-	-	+	-	-	K/A	+	-	ENTEROCOCCUS
590		+	+					-	-	-	-	+	-	-	K/A	+	-	ENTEROCOCCUS
591			+					-	-	-	-	+	-	-	K/A	-	-	XANTHOBACTER
592			+		+			-	-	-	-	+	-	-	K/A*	-	+	PSEUDOMONAS
593		+	+					-	-	-	-	+	-	-	K/A*	+	+	BACILLUS
594	+	+	+					-	-	-	-	+	-	-	K/NC	+	+	BACILLUS

595		+	+					-	-	-	-	+	-	NC/NC	-	+	PSEUDOMONAS
596	+	+	+					-	-	-	-	+	-	NC/NC	+	-	ENTEROCOCCUS
597			+					-	-	-	-	+	-	K/A*	+	-	ENTEROCOCCUS
598	+		+	+				-	-	-	-	+	+	K/A	+	-	STAPHYLOCOCCUS
599	+	+	+	+	+			-	-	+	-	+	-	A/A	+	+	BACILLUS
600		+	+	+	+			-	-	+	-	+	-	K/A	+	+	BACILLUS
601	+		+		+			-	-	+	-	+	-	NC/NC	-	+	SERRATIA
602	+		+					-	-	-	-	+	+	K/A	+	+	BACILLUS
603			+					-	-	-	-	+	+	K/A	+	+	BACILLUS
604			+					-	-	-	-	+	+	K/A	+	+	BACILLUS
605		+	+					-	-	-	-	+	+	K/A	-	-	KLEBSIELLA
606	+		+		+			-	-	-	-	+	+	K/A	-	-	ENTEROBACTER
607	+		+	+	+			-	-	-	-	+	+	K/A	-	-	ACINETOBACTER
608	+	+	+		+			-	-	-	-	+	+	K/A	+	+	BACILLUS
609	+	+	+		+			-	-	-	-	+	+	K/A	+	+	BACILLUS
610	+	+	+		+			-	-	-	-	+	+	K/A	+	+	BACILLUS
611	+		+		+			-	-	-	-	+	+	K/A*	-	-	ENTEROBACTER
612		+	+		+	+		-	-	-	-	+	+	K/K	+	+	BACILLUS
613			+		+	+		-	-	-	-	+	+	K/K	-	-	KLEBSIELLA
614		+	+					-	-	-	-	+	+	K/K	+	-	BACILLUS
615			+		+			-	-	-	-	+	+	K/K	-	-	KLEBSIELLA
616			+					-	-	-	-	+	+	K/NC	+	+	BACILLUS
617		+	+		+			-	-	-	-	+	+	K/NC	-	+	ENTEROBACTER
618	+	+						-	-	-	-	+	+	K/NC	+	+	BACILLUS
619	+		+		+			-	-	-	-	+	+	A/A	-	+	ALCALIGENES FAECALIS
620	+	+						-	-	-	-	+	+	A/A	-	-	XANTHOBACTER
621	+		+	+	+			-	-	-	-	+	+	A/A	-	-	XANTHOBACTER
622	+		+		+			-	-	-	-	+	+	A/A	-	+	PSEUDOMONAS
623	+		+		+			-	-	-	-	+	+	A/A	-	+	XANTHOBACTER
624	+	+	+	+				-	-	-	-	+	+	A/A	+	+	BACILLUS
625	+		+		+			-	-	-	-	+	+	A/B	-	+	PSEUDOMONAS
626	+		+		+			-	-	-	-	+	+	A/B	-	-	XANTHOBACTER
627			+					-	-	-	-	+	+	A/NC	-	+	PSEUDOMONAS
628			+					-	-	-	-	+	+	A/NC	+	+	BACILLUS
629		+	+	+				-	-	-	-	+	+	A/NC	+	+	BACILLUS
630			+					-	-	-	-	+	+	A/NC	-	+	XANTHOBACTER
631	+		+					-	-	-	-	+	+	A/NC	-	+	PSEUDOMONAS
632								-	-	-	-	+	+	A/NC	-	-	XANTHOBACTER
633	+		+				+	-	-	-	-	+	+	A/NC	-	+	PSEUDOMONAS
634	+		+		+		+	-	-	-	-	+	+	A/NC	+	+	BACILLUS
635	+			+	+			-	-	-	-	+	+	A/NC	+	+	BACILLUS
636					+		+	-	-	-	-	+	+	A/NC	+	+	BACILLUS
637	+							-	-	-	-	+	+	NC/NC	-	-	XANTHOBACTER
638	+		+					-	-	-	-	+	+	NC/NC	-	+	XANTHOBACTER
639	+		+				+	-	-	-	-	+	+	NC/NC	-	-	XANTHOBACTER
640	+							-	-	-	+	+	+	K/A	-	+	PSEUDOMONAS

641	+						-	-	-	-	-	-	+	A/A	+	+	BACILLUS
642							-	-	-	-	-	-	+	A/A	+	+	BACILLUS
643			+				-	-	-	-	-	-	+	A/A	+	+	BACILLUS
644	+		+		+		-	-	-	-	-	-	+	A/A	+	+	BACILLUS
645	+		+		+		-	-	-	-	-	-	+	A/A	-	+	PSEUDOMONAS
646	+		+		+		-	-	-	-	-	-	+	A/A	-	+	PSEUDOMONAS
647	+						-	-	-	-	-	-	+	A/A	+	+	BACILLUS
648	+					+	-	-	-	-	-	-	+	A/A	-	+	PSEUDOMONAS
649			+				-	-	-	-	-	-	+	A/NC	-	+	PSEUDOMONAS
650					+		-	-	-	-	-	-	+	K/K	-	-	XANTHOBACTER
651	+						-	-	-	-	-	-	+	K/A	+	+	BACILLUS
652			+				-	-	-	-	-	-	+	K/A	-	+	XANTHOBACTER
653		+					-	-	-	-	-	-	+	K/A	+	+	BACILLUS
654	+					+	-	-	-	-	-	-	+	K/K	-	-	XANTHOBACTER
655		+	+			+	-	-	-	-	-	-	+	K/K	-	+	PSEUDOMONAS
656		+	+				-	-	-	-	-	-	+	K/NC	+	-	STAPHYLOCOCCUS
657			+				-	-	-	-	-	-	+	NC/NC	+	-	STAPHYLOCOCCUS
658			+		+		-	-	-	-	-	-	+	NC/NC	+	-	STAPHYLOCOCCUS
659		+	+				-	+	-	-	-	-	-	K/NC	-	+	PSEUDOMONAS
660	+	+	+				-	+	-	-	-	-	-	NC/NC	-	+	PSEUDOMONAS
661		+	+				-	+	-	-	-	-	-	NC/NC	-	+	PSEUDOMONAS
662	+	+	+				+	-	+	-	-	-	+	K/A*	+	+	BACILLUS
663			+				+	-	+	+	-	-	-	K/K	+	+	BACILLUS
664	+		+	+			+	-	+	+	-	-	+	K/K	+	-	STAPHYLOCOCCUS
665	+	+	+	+	+		+	-	-	+	-	+	+	K/K	+	-	STAPHYLOCOCCUS
666		+	+	+	+		+	-	+	-	-	-	+	K/K	+	-	STAPHYLOCOCCUS
667	+		+		+		+	-	-	-	-	-	+	K/NC	+	-	STAPHYLOCOCCUS
668	+						+	-	-	-	-	-	+	K/NC	+	+	BACILLUS
669			+				+	-	+	+	-	-	+	K/NC	+	+	BACILLUS
670			+				+	-	+	+	+	-	+	A/A	+	+	BACILLUS
671		+	+		+		-	-	+	-	-	+	+	A/A	-	+	PSEUDOMONAS
672	+		+		+									A/A	-	+	PSEUDOMONAS
673	+		+	+	+									A/A	+	-	STAPHYLOCOCCUS
674	+	+	+		+		+	+	-	-	-	-	-	A/A	+	+	BACILLUS
675	+	+	+		+		+	+	+	+	-	-	-	A/A	+	+	BACILLUS
676	+	+	+		+		-	+	+	+	+	-	+	A/K	+	+	BACILLUS
677	+		+		+		+	+	+	+	+	-	+	A/K	+	+	BACILLUS
678		+	+		+	+	+	+	+	+	-	-	+	A/NC	+	+	BACILLUS
679			+		+		+	+	-	+	+	-	+	A/NC	+	+	BACILLUS
680			+		+		+	+	+	+	+	-	+	A/NC	+	+	BACILLUS
681			+				-	+	+	+	+	-	+	A/NC	-	+	PSEUDOMONAS
682		+	+		+		+	+	-	+	-	-	-	A/NC	+	+	BACILLUS
683	+	+					+	+	-	+	-	-	+	A/NC	-	+	PSEUDOMONAS
684	+		+		+		-	+	+	+	-	-	-	A/NC	+	+	BACILLUS
685	+	+					+	+	+	+	-	-	-	K/K	+	-	STAPHYLOCOCCUS
686	+		+	+			+	+	+	+	-	-	+	K/A	-	+	PSEUDOMONAS

687	+		+		+	+			+						K/A	+	+	BACILLUS
688	+		+		+				+	-	-	-	+	+	K/A	-	+	PSEUDOMONAS
689	+	+	+	+					+	-	-	-	+	+	K/K	-	-	ENTEROBACTER
690	+		+		+				+	-	-	-	+	+	K/K	-	8	PSEUDOMONAS
691	+		+		+				-	+	+	+	-	+	K/NC	-	-	ENTEROBACTER
692			+						-	+	+	+	-	+	NC/NC	-	-	ENTEROBACTER
693			+						-	+	+	+	-	+	NC/NC	+	+	BACILLUS
694		+	+	+					-	+	+	+	-	+	K/NC	+	+	BACILLUS
695			+						-	-	+	+	-	+	NC/NC	+	-	ENTEROCOCCUS
696	+		+						-	-	+	+	-	+	NC/NC	+	-	ENTEROCOCCUS
697									-	-	-	-	-	+	K/A"	+	-	ENTEROCOCCUS
698	+		+				+		-	-	-	-	-	+	K/K	-	+	PSEUDOMONAS
699	+		+		+		+		-	-	-	-	-	+	K/K	-	+	PSEUDOMONAS
700	+			+	+				-	-	-	-	-	+	K/K	+	-	ENTEROCOCCUS
701			+		+		+		-	-	-	-	-	+	K/K	+	-	ENTEROCOCCUS
702	+								-	-	-	-	-	+	K/K	-	+	PSEUDOMONAS
703	+		+						-	-	-	-	-	+	K/NC	+	+	BACILLUS
704	+		+				+		-	-	-	-	-	+	NC/NC	+	+	BACILLUS
705	+		+						-	-	-	-	-	+	NC/NC	-	+	PSEUDOMONAS
706		+			+				-	+	-	-	-	-	K/K	-	+	PSEUDOMONAS
707	+	+					+		+	-	+	-	+	+	K/NC	+	-	LACTOBACILLUS
708	+		+		+				+	-	+	-	+	+	NC/NC	+	+	BACILLUS

***A-Acidic , K-Alkaline ,O-Gas, NC- No change**

APPENDIX III

A. Genera wise percentage of bacterial isolates showing hydrolytic enzyme production during the first year of sampling

Genera name	PROTEASE	AMYLASE	LIPASE	CHITINASE	CELLULASE	LIGNINASE	DNase	LACCASE
Bacillus	38	43	41	40	32	0	47	100
Klebsiella	2	0	1	0	2	0	2	0
Pseudomonas	21	18	20	0	19	0	21	0
Enterococcus	5	4	5	20	4	0	4	0
Xanthobacter	4	8	7	0	6	50	3	0
Alcaligenes	3	3	3	0	1	50	4	0
Enterobacter	4	4	3	0	10	0	3	0
Staphylococcus	4	4	5	0	6	0	2	0
Vibrio	6	6	4	20	6	0	8	0
Aeromonas	2	2	1	0	2	0	0	0
Citrobacter	1	0	1	0	1	0	1	0
Lactobacillus	1	3	2	0	4	0	1	0
Acinetobacter	2	2	1	0	1	0	3	0
Serratia	5	1	3	20	2	0	0	0
Lysobacter	2	1	1	0	2	0	1	0
Proteus	2	2	1	0	0	0	2	0
Escherichia	1	0	1	0	1	0	0	0

B. Genera wise percentage of bacterial isolates showing hydrolytic enzyme production during the second year of sampling

Genera name	PROTEASE	AMYLASE	LIPASE	CHITINASE	CELLULASE	LIGNINASE	DNase	LACCASE
Bacillus	47	51	46	35	50	42	47	29
Klebsiella	1	1	1	0	2	11	3	0
Pseudomonas	15	13	16	5	13	26	16	29
Enterococcus	2	2	4	5	4	0	5	14
Xanthobacter	8	5	7	5	6	5	7	0
Alcaligenes	2	3	2	10	4	5	2	0
Enterobacter	3	3	4	5	4	0	3	0
Staphylococcus	3	4	5	25	3	0	3	0
Vibrio	7	5	4	0	4	0	3	0
Aeromonas	1	1	0	5	0	0	0	0
Citrobacter	0	0	1	0	0	0	1	0
Lactobacillus	2	2	2	0	3	0	3	0
Acinetobacter	1	1	1	5	1	11	0	0
Serratia	4	4	4	0	2	0	1	29
Lysobacter	2	3	3	0	3	0	2	0
Proteus	0	0	1	0	0	0	0	0
Escherichia	0	0	0	0	0	0	1	0

APPENDIX IV

A. Genera wise percentage of bacterial isolates showing hydrolytic enzyme production during the monsoon season of the first year of sampling

MONSOON I	ENZYMES							
GENERA	Protease	Amylase	Lipase	Chitinase	Cellulase	Ligninase	DNase	Laccase
Bacillus	21	28	26	0	30	0	29	100
Klebsiella	3	0	3	0	4	0	5	0
Pseudomonas	28	25	21	0	15	0	24	0
Enterococcus	5	5	5	0	7	0	0	0
Xanthobacter	3	8	8	0	0	50	10	0
Alcaligenes	8	8	10	0	4	50	14	0
Enterobacter	8	5	5	0	7	0	5	0
Staphylococcus	10	10	13	0	15	0	5	0
Vibrio	5	5	5	0	7	0	5	0
Aeromonas	3	3	0	0	4	0	0	0
Citrobacter	3	0	3	0	4	0	5	0
Lactobacillus	3	3	3	0	4	0	0	0
Acinetobacter	3	3	0	0	0	0	0	0
Serratia	0	0	0	0	0	0	0	0
Lysobacter	0	0	0	0	0	0	0	0
Proteus	0	0	0	0	0	0	0	0
Escherichia	0	0	0	0	0	0	0	0

B. Genera wise percentage of bacterial isolates showing hydrolytic enzyme production during the post - monsoon season of the first year of sampling

POST MONSOON I	ENZYMES							
GENERA	Protease	Amylase	Lipase	Chitinase	Cellulase	Ligninase	DNase	Laccase
Bacillus	35	31	27	0	34	0	31	0
Klebsiella	2	0	0	0	3	0	4	0
Pseudomonas	16	10	29	0	16	0	19	0
Enterococcus	2	0	4	0	0	0	12	0
Xanthobacter	5	14	7	0	6	0	0	0
Alcaligenes	2	3	2	0	0	0	4	0
Enterobacter	5	3	2	0	9	0	0	0
Staphylococcus	2	3	4	0	3	0	4	0
Vibrio	7	10	7	0	6	0	12	0
Aeromonas	5	7	4	0	3	0	0	0
Citrobacter	0	0	0	0	0	0	0	0
Lactobacillus	0	3	0	0	3	0	0	0
Acinetobacter	2	3	2	0	3	0	4	0
Serratia	7	3	4	0	6	0	0	0
Lysobacter	2	0	2	0	3	0	4	0
Proteus	5	7	2	0	0	0	8	0
Escherichia	2	0	2	0	3	0	0	0

C. Genera wise percentage of bacterial isolates showing hydrolytic enzyme production during the pre - monsoon season of the first year of sampling

PRE MONSOON I	ENZYMES							
GENERA	**Protease**	**Amylase**	**Lipase**	**Chitinase**	**Cellulase**	**Ligninase**	**DNase**	**Laccase**
Bacillus	53	60	63	40	32	0	60	0
Klebsiella	0	0	0	0	0	0	0	0
Pseudomonas	20	17	14	0	28	0	21	0
Enterococcus	8	5	5	20	4	0	2	0
Xanthobacter	4	5	7	0	12	0	2	0
Alcaligenes	0	0	0	0	0	0	0	0
Enterobacter	0	3	2	0	12	0	3	0
Staphylococcus	0	0	0	0	0	0	0	0
Vibrio	6	5	2	20	4	0	7	0
Aeromonas	0	0	0	0	0	0	0	0
Citrobacter	0	0	0	0	0	0	0	0
Lactobacillus	0	3	3	0	4	0	2	0
Acinetobacter	0	0	0	0	0	0	3	0
Serratia	8	0	3	20	0	0	0	0
Lysobacter	2	2	2	0	4	0	0	0
Proteus	0	0	0	0	0	0	0	0
Escherichia	0	0	0	0	0	0	0	0

D. Genera wise percentage of bacterial isolates showing hydrolytic enzyme production during the monsoon season of the second year of sampling

MONSOON II	ENZYMES							
GENERA	**Protease**	**Amylase**	**Lipase**	**Chitinase**	**Cellulase**	**Ligninase**	**DNase**	**Laccase**
Bacillus	63	61	57	**100**	61	56	69	33
Klebsiella	0	0	1	0	2	0	3	0
Pseudomonas	11	13	13	0	10	44	6	33
Enterococcus	1	2	1	0	2	0	3	0
Xanthobacter	10	7	9	0	9	0	8	0
Alcaligenes	0	2	0	0	2	0	0	0
Enterobacter	0	0	1	0	0	0	0	0
Staphylococcus	0	1	1	0	1	0	3	0
Vibrio	5	1	3	0	1	0	3	0
Aeromonas	1	1	1	0	1	0	0	0
Citrobacter	0	1	1	0	0	0	0	0
Lactobacillus	1	1	1	0	3	0	3	0
Acinetobacter	0	0	0	0	0	0	0	0
Serratia	5	4	5	0	1	0	0	33
Lysobacter	4	5	5	0	6	0	3	0
Proteus	0	0	0	0	0	0	0	0
Escherichia	0	0	0	0	0	0	0	0

E. Genera wise percentage of bacterial isolates showing hydrolytic enzyme production during the post - monsoon season of the second year of sampling

POST MONSOON II	ENZYMES							
GENERA	**Protease**	**Amylase**	**Lipase**	**Chitinase**	**Cellulase**	**Ligninase**	**DNAse**	**Laccase**
Bacillus	41	41	40	0	46	0	34	0
Klebsiella	3	2	1	0	1	33	5	0
Pseudomonas	13	9	13	0	12	0	17	0
Enterococcus	1	2	5	0	4	0	5	**100**
Xanthobacter	1	2	1	0	1	0	5	0
Alcaligenes	3	3	4	0	5	0	5	0
Enterobacter	7	8	8	0	8	0	7	0
Staphylococcus	4	5	6	0	1	0	5	0
Vibrio	11	12	6	0	7	0	5	0
Aeromonas	0	0	0	0	0	0	0	0
Citrobacter	0	0	1	0	1	0	2	0
Lactobacillus	4	5	4	0	5	0	2	0
Acinetobacter	3	3	1	0	1	67	0	0
Serratia	5	6	5	0	3	0	2	0
Lysobacter	1	2	1	0	1	0	2	0
Proteus	1	2	2	0	1	0	0	0
Escherichia	1	2	1	0	1	0	2	0

F. Genera wise percentage of bacterial isolates showing hydrolytic enzyme production during the pre - monsoon season of the second year of sampling

PRE MONSOON II	ENZYMES							
GENERA	**Protease**	**Amylase**	**Lipase**	**Chitinase**	**Cellulase**	**Ligninase**	**DNAse**	**Laccase**
Bacillus	33	43	37	32	37	43	20	0
Klebsiella	0	2	3	0	4	14	0	0
Pseudomonas	24	20	23	5	19	14	50	0
Enterococcus	5	4	6	5	5	0	10	0
Xanthobacter	13	7	9	5	7	14	10	0
Alcaligenes	3	4	2	11	4	14	0	0
Enterobacter	5	4	6	5	7	0	0	0
Staphylococcus	8	9	9	26	9	0	0	0
Vibrio	6	2	4	0	5	0	0	0
Aeromonas	1	2	0	5	0	0	0	0
Citrobacter	0	0	0	0	0	0	0	0
Lactobacillus	1	2	0	0	0	0	10	0
Acinetobacter	1	0	1	5	2	0	0	0
Serratia	1	0	1	0	2	0	0	0
Lysobacter	0	0	0	0	0	0	0	0
Proteus	0	0	0	0	0	0	0	0
Escherichia	0	0	0	0	0	0	0	0

APPENDIX V

Enzyme activity of selected bacterial isolates at different physicochemical conditions

ST 01

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	30	15	5	150	F	ND	5 ± 0.3	8 ± 0.1
							I	06 ± 0.1	8 ± 0.2	14 ± 0.6
		2	30	15	6	150	F	09 ± 0.6	11 ± 0.02	14 ± 0.6
							I	ND	ND	17 ± 0.31
		3	30	15	7	150	F	ND	5 ± 0.05	9 ± 0.8
							I	ND	5 ± 0.6	14 ± 0.6
		4	30	20	5	150	F	ND	9 ± 0.05	11 ± 0.1
							I	02 ± 0.1	14 ± 0.2	23 ± 0.2
		5	30	20	6	150	F	ND	ND	5 ± 0.6
							I	ND	2 ± 0.05	7 ± 0.3
		6	30	20	7	150	F	04 ± 0.3	4 ± 0.3	8 ± 0.5
							I	11 ± 0.5	14 ± 0.4	15 ± 0.3
		7	40	15	5	150	F	16 ± 0.15	14 ± 0.3	12 ± 0.5
							I	11 ± 0.5	9 ± 0.5	4 ± 0.07
		8	40	15	6	150	F	10 ± 0.7	5 ± 0.6	ND
							I	ND	05 ± 0.3	6 ± 0.02
		9	40	15	7	150	F	06 ± 0.2	08 ± 0.3	10 ± 0.4
							I	08 ± 0.4	04 ± 0.4	12 ± 0.3
		10	40	20	5	150	F	09 ± 0.3	11 ± 0.3	9 ± 0.5
							I	11 ± 0.5	12 ± 0.5	14 ± 0.4
		11	40	20	6	150	F	ND	ND	6 ± 0.1
							I	12 ± 0.1	14 ± 0.2	16 ± 0.3
		12	40	20	7	150	F	03 ± 0.3	05 ± 0.04	8 ± 0.08
							I	ND	ND	4 ± 0.07

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	30	15	5	150	F	42 ± 0.2	58 ± 0.2	76 ± 0.3
							I	52 ± 0.1	53 ± 0.3	110 ± 0.7
		2	30	15	6	150	F	39 ± 0.6	49 ± 0.4	85 ± 0.7
							I	ND	52 ± 0.5	75 ± 0.6
		3	30	15	7	150	F	ND	ND	12 ± 0.3
							I	14 ± 0.7	48 ± 0.8	18 ± 0.2
		4	30	20	5	150	F	18 ± 0.6	53 ± 0.6	82 ± 0.3
							I	ND	95 ± 0.1	110 ± 0.3
		5	30	20	6	150	F	52 ± 0.2	79 ± 0.3	142 ± 0.28
							I	ND	52 ± 0.07	123 ± 0.6
		6	30	20	7	150	F	25 ± 0.8	56 ± 0.3	102 ± 0.3
							I	23 ± 0.5	75 ± 0.8	114 ± 0.06
		7	40	15	5	150	F	14 ± 0.5	12 ± 0.3	10 ± 0.3
							I	29 ± 0.5	49 ± 0.3	89 ± 0.5
		8	40	15	6	150	F	11 ± 0.6	19 ± 0.7	74 ± 0.9
							I	59 ± 0.3	62 ± 0.4	112 ± 0.8
		9	40	15	7	150	F	31 ± 0.1	17 ± 0.2	15 ± 0.2
							I	75 ± 0.4	19 ± 0.5	24 ± 0.1
		10	40	20	5	150	F	42 ± 0.4	98 ± 0.3	134 ± 0.5
							I	42 ± 0.1	79 ± 0.6	167 ± 0.23
		11	40	20	6	150	F	14 ± 0.4	20 ± 0.2	45 ± 0.6
							I	53 ± 0.2	75 ± 0.2	124 ± 0.1
		12	40	20	7	150	F	19 ± 0.3	17 ± 0.4	25 ± 0.05
							I	18 ± 0.6	19 ± 0.8	19 ± 0.07

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	30	15	5	150	F	0.2 ± 0.01	0.6 ± 0.05	1 ± 0.3
							I	ND	ND	0.3 ± 0.01
		2	30	15	6	150	F	ND	ND	ND
							I	ND	ND	ND
		3	30	15	7	150	F	ND	ND	ND
							I	ND	0.3 ± 0.02	0.6 ± 0.01
		4	30	20	5	150	F	ND	ND	ND
							I	ND	ND	ND
		5	30	20	6	150	F	ND	ND	0.5 ± 0.03
							I	ND	ND	ND
		6	30	20	7	150	F	ND	ND	ND
							I	0.2 ± 0.05	1 ± 0.04	1 ± 0.04
		7	40	15	5	150	F	ND	ND	ND
							I	ND	ND	ND
		8	40	15	6	150	F	ND	0.2 ± 0.02	ND
							I	ND	ND	ND
		9	40	15	7	150	F	ND	ND	ND
							I	ND	ND	ND
		10	40	20	5	150	F	ND	ND	ND
							I	ND	ND	0.8 ± 0.07
		11	40	20	6	150	F	1 ± 0.16	1 ± 0.08	0.9 ± 0.04
							I	3 ± 0.13	2 ± 0.03	2.5 ± 0.2
		12	40	20	7	150	F	1 ± 0.1	1 ± 0.03	1.6 ± 0.4
							I	ND	ND	ND

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	30	15	5	150	F	ND	0	1.2 ± 0.03
							I	ND	0.9 ± 0.02	1.5 ± 0.1
		2	30	15	6	150	F	ND	ND	0.4 ± 0.05
							I	1.2 ± 0.05	1.5 ± 0.06	2 ± 0.05
		3	30	15	7	150	F	ND	ND	ND
							I	ND	ND	0.7 ± 0.06
		4	30	20	5	150	F	2 ± 0.6	2.1 ± 0.07	1.8 ± 0.05
							I	2.3 ± 0.1	2.5 ± 0.2	2.9 ± 0.01
		5	30	20	6	150	F	ND	ND	ND
							I	ND	ND	0.7 ± 0.05
		6	30	20	7	150	F	ND	1.1 ± 0.4	1.5 ± 0.3
							I	ND	ND	1.4 ± 0.04
		7	40	15	5	150	F	ND	ND	ND
							I	ND	ND	1.5 ± 0.4
		8	40	15	6	150	F	1.9 ± 0.6	2 ± 0.07	2.3 ± 0.6
							I	3.2 ± 0.19	2.9 ± 0.4	2.5 ± 0.3
		9	40	15	7	150	F	2 ± 0.2	2.1 ± 0.3	1.9 ± 0.2
							I	1 ± 0.03	1 ± 0.04	1.4 ± 0.3
		10	40	20	5	150	F	ND	1.4 ± 0.04	ND
							I	1.1 ± 0.05	1.2 ± 0.6	1.6 ± 0.4
		11	40	20	6	150	F	3 ± 0.21	2.7 ± 0.5	1.9 ± 0.4
							I	2.3 ± 0.1	2.4 ± 0.02	2.9 ± 0.1
		12	40	20	7	150	F	1 ± 0.03	0.9 ± 0.04	1.2 ± 0.3
							I	1.7 ± 0.7	1.6 ± 0.8	1.9 ± 0.06

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	30	15	5	150	F	19 ± 0.6	12 ± 0.7	11 ± 0.5
							I	12 ± 0.7	34 ± 0.8	42 ± 0.06
		2	30	15	6	150	F	14 ± 0.3	16 ± 0.4	27 ± 0.3
							I	16 ± 0.3	32 ± 0.4	37 ± 0.3
		3	30	15	7	150	F	17 ± 0.6	19 ± 0.7	21 ± 0.5
							I	ND	24 ± 0.2	29 ± 0.1
		4	30	20	5	150	F	19 ± 0.7	21 ± 0.8	26 ± 0.6
							I	21 ± 0.2	32 ± 0.3	39 ± 0.2
		5	30	20	6	150	F	14 ± 0.5	16 ± 0.6	19 ± 0.5
							I	16 ± 0.4	32 ± 0.5	34 ± 0.3
		6	30	20	7	150	F	25 ± 0.4	35 ± 0.5	39 ± 0.3
							I	32 ± 0.6	34 ± 0.7	37 ± 0.5
		7	40	15	5	150	F	14 ± 0.7	16 ± 0.8	32 ± 0.6
							I	19 ± 0.2	29 ± 0.3	41 ± 0.2
		8	40	15	6	150	F	25 ± 0.7	26 ± 0.8	32 ± 0.7
							I	31 ± 0.2	41 ± 0.3	45 ± 0.2
		9	40	15	7	150	F	27 ± 0.1	32 ± 0.2	29 ± 0.1
							I	42 ± 0.7	39 ± 0.8	41 ± 0.7
		10	40	20	5	150	F	32 ± 0.5	47 ± 0.17	46 ± 0.5
							I	35 ± 0.7	41 ± 0.8	45 ± 0.6
		11	40	20	6	150	F	16 ± 0.3	32 ± 0.4	42 ± 0.3
							I	25 ± 0.6	48 ± 0.15	47 ± 0.5
		12	40	20	7	150	F	14 ± 0.6	21 ± 0.7	24 ± 0.5
							I	19 ± 0.7	32 ± 0.8	36 ± 0.6

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	30	15	5	150	F	ND	ND	4 ± 0.03
							I	ND	2 ± 0.06	6 ± 0.04
		2	30	15	6	150	F	ND	ND	ND
							I	2 ± 0.07	5 ± 0.8	10 ± 0.6
		3	30	15	7	150	F	7 ± 0.6	7 ± 0.07	6 ± 0.05
							I	9 ± 0.7	8 ± 0.08	6 ± 0.06
		4	30	20	5	150	F	8 ± 0.6	7 ± 0.07	4 ± 0.06
							I	2 ± 0.5	5 ± 0.6	8 ± 0.04
		5	30	20	6	150	F	1 ± 0.02	ND	4 ± 0.02
							I	9 ± 0.4	9 ± 0.05	12 ± 0.04
		6	30	20	7	150	F	8 ± 0.6	10 ± 0.7	19 ± 0.6
							I	7 ± 0.7	8 ± 0.8	10 ± 0.6
		7	40	15	5	150	F	ND	ND	4 ± 0.03
							I	6 ± 0.5	4 ± 0.6	2 ± 0.04
		8	40	15	6	150	F	7 ± 0.6	8 ± 0.7	12 ± 0.5
							I	11 ± 0.31	10 ± 0.2	9 ± 0.1
		9	40	15	7	150	F	3 ± 0.6	14 ± 0.7	16 ± 0.5
							I	5 ± 0.3	6 ± 0.4	9 ± 0.2
		10	40	20	5	150	F	ND	ND	9 ± 0.6
							I	6 ± 0.3	6 ± 0.4	7 ± 0.3
		11	40	20	6	150	F	7 ± 0.8	12 ± 0.9	21 ± 0.09
							I	4 ± 0.06	9 ± 0.7	10 ± 0.6
		12	40	20	7	150	F	ND	8 ± 0.5	15 ± 0.4
							I	1 ± 0.05	9 ± 0.6	7 ± 0.4

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

ST 02

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	20	20	6	150	F	ND	9 ± 0.04	12 ± 0.3
							I	ND	7 ± 0.3	11 ± 0.1
		2	20	20	7	150	F	7 ± 0.07	8 ± 0.6	5 ± 0.6
							I	10 ± 0.6	12 ± 0.6	8 ± 0.6
		3	20	25	6	150	F	9 ± 0.6	4 ± 0.06	6 ± 0.5
							I	11 ± 0.8	12 ± 0.7	11 ± 0.8
		4	20	25	7	150	F	ND	11 ± 0.6	8 ± 0.5
							I	ND	ND	11 ± 0.1
		5	30	20	6	150	F	15 ± 0.3	16 ± 0.4	14 ± 0.2
							I	30 ± 0.25	19 ± 0.6	21 ± 0.6
		6	30	20	7	150	F	29 ± 0.25	23 ± 0.4	26 ± 0.03
							I	19 ± 0.5	15 ± 0.6	16 ± 0.5
		7	30	25	6	150	F	14 ± 0.3	16 ± 0.4	18 ± 0.3
							I	16 ± 0.6	13 ± 0.4	16 ± 0.05
		8	30	25	7	150	F	5 ± 0.6	8 ± 0.7	10 ± 0.7
							I	2 ± 0.04	1 ± 0.04	9 ± 0.3

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	20	20	6	150	F	25 ± 0.3	35 ± 0.4	63 ± 0.2
							I	53 ± 0.4	26 ± 0.5	24 ± 0.4
		2	20	20	7	150	F	52 ± 0.4	69 ± 0.4	71 ± 0.03
							I	75 ± 0.6	81 ± 0.2	53 ± 0.5
		3	20	25	6	150	F	53 ± 0.6	69 ± 0.5	52 ± 0.4
							I	85 ± 0.2	86 ± 0.03	85 ± 0.1
		4	20	25	7	150	F	85 ± 0.4	158 ± 0.31	120 ± 0.3
							I	112 ± 0.8	142 ± 0.8	150 ± 0.8
		5	30	20	6	150	F	83 ± 0.7	81 ± 0.3	75 ± 0.7
							I	75 ± 0.8	64 ± 0.7	76 ± 0.8
		6	30	20	7	150	F	72 ± 0.4	131 ± 0.4	89 ± 0.3
							I	100 ± 0.4	126 ± 0.5	143 ± 0.3
		7	30	25	6	150	F	82 ± 0.7	91 ± 0.6	75 ± 0.6
							I	92 ± 0.2	162 ± 0.3	185 ± 0.16
		8	30	25	7	150	F	25 ± 0.8	68 ± 0.7	98 ± 0.04
							I	37 ± 0.3	96 ± 0.4	45 ± 0.2

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	20	20	6	150	F	2 ± 0.19	ND	1 ± 0.01
							I	ND	ND	ND
		2	20	20	7	150	F	ND	ND	ND
							I	ND	ND	ND
		3	20	25	6	150	F	ND	1 ± 0.07	ND
							I	1 ± 0.03	ND	ND
		4	20	25	7	150	F	ND	ND	ND
							I	ND	ND	ND
		5	30	20	6	150	F	1 ± 0.02	ND	ND
							I	ND	ND	ND
		6	30	20	7	150	F	ND	ND	ND
							I	ND	ND	ND
		7	30	25	6	150	F	ND	ND	ND
							I	2 ± 0.07	ND	ND
		8	30	25	7	150	F	1 ± 0.07	ND	1 ± 0.05
							I	4 ± 0.11	1 ± 0.02	ND

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	20	20	6	150	F	0.12 ± 0.01	ND	ND
							I	ND	ND	ND
		2	20	20	7	150	F	ND	ND	ND
							I	ND	ND	ND
		3	20	25	6	150	F	ND	ND	ND
							I	ND	0.1 ± 0.01	0.1 ± 0.01
		4	20	25	7	150	F	0.1 ± 0.2	ND	ND
							I	0.23 ± 0.02	0.19 ± 0.01	0.17 ± 0.02
		5	30	20	6	150	F	ND	ND	ND
							I	ND	ND	ND
		6	30	20	7	150	F	ND	ND	ND
							I	ND	ND	ND
		7	30	25	6	150	F	0.1 ± 0.02	ND	0.1 ± 0.03
							I	ND	ND	ND
		8	30	25	7	150	F	ND	ND	ND
							I	ND	ND	ND

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	20	20	6	150	F	25 ± 0.7	16 ± 0.6	8 ± 0.6
							I	32 ± 0.4	52 ± 0.3	69 ± 0.3
		2	20	20	7	150	F	12 ± 0.07	26 ± 0.7	59 ± 0.7
							I	52 ± 0.04	61 ± 0.5	84 ± 0.4
		3	20	25	6	150	F	19 ± 0.9	36 ± 0.8	51 ± 0.4
							I	97 ± 0.12	89 ± 0.9	91 ± 0.7
		4	20	25	7	150	F	36 ± 0.5	39 ± 0.5	57 ± 0.5
							I	46 ± 0.6	51 ± 0.6	72 ± 0.4
		5	30	20	6	150	F	52 ± 0.5	58 ± 0.2	46 ± 0.4
							I	61 ± 0.2	56 ± 0.3	52 ± 0.1
		6	30	20	7	150	F	29 ± 0.3	52 ± 0.1	34 ± 0.6
							I	53 ± 0.7	25 ± 0.6	14 ± 0.6
		7	30	25	6	150	F	63 ± 0.15	35 ± 0.4	14 ± 0.5
							I	59 ± 0.9	89 ± 0.8	91 ± 0.9
		8	30	25	7	150	F	52 ± 0.3	56 ± 0.4	49 ± 0.2
							I	54 ± 0.5	69 ± 0.1	41 ± 0.4

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	20	20	6	150	F	1 ± 0.03	2 ± 0.04	1 ± 0.02
							I	2 ± 0.05	ND	6 ± 0.04
		2	20	20	7	150	F	ND	ND	ND
							I	ND	ND	5 ± 0.3
		3	20	25	6	150	F	3 ± 0.2	2 ± 0.4	2 ± 0.03
							I	5 ± 0.02	6 ± 0.5	13 ± 0.5
		4	20	25	7	150	F	2 ± 0.02	2 ± 0.03	ND
							I	15 ± 0.15	13 ± 0.4	14 ± 0.3
		5	30	20	6	150	F	ND	2 ± 0.06	2 ± 0.05
							I	ND	5 ± 0.5	13 ± 0.5
		6	30	20	7	150	F	ND	ND	ND
							I	ND	ND	9 ± 0.4
		7	30	25	6	150	F	1 ± 0.09	1 ± 0.08	2 ± 0.8
							I	7 ± 0.7	9 ± 0.06	11 ± 0.7
		8	30	25	7	150	F	1 ± 0.03	ND	2 ± 0.03
							I	ND	1 ± 0.08	10 ± 0.4

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

ST 03

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	30	15	5	150	F	12 ± 0.5	15 ± 0.7	20 ± 0.6
							I	16 ± 0.1	21 ± 0.3	18 ± 0.3
		2	30	15	6	150	F	11 ± 0.12	9 ± 1.2	17 ± 1.3
							I	16 ± 0.9	19 ± 1.1	21 ± 1.2
		3	30	15	7	150	F	9 ± 0.8	15 ± 1.0	21 ± 1.1
							I	12 ± 1.3	16 ± 1.5	19 ± 1.7
		4	30	20	5	150	F	6 ± 0.01	9 ± 0.3	18 ± 1.4
							I	9 ± 0.1	11 ± 0.3	8 ± 0.2
		5	30	20	6	150	F	ND	14 ± 0.5	21 ± 0.5
							I	ND	18 ± 1.3	22 ± 1.4
		6	30	20	7	150	F	12 ± 0.5	8 ± 0.07	10 ± 0.7
							I	7 ± 0.7	9 ± 0.9	19 ± 1.0
		7	40	15	5	150	F	5 ± 0.4	4 ± 0.6	18 ± 0.6
							I	4 ± 0.9	6 ± 1.1	9 ± 1.1
		8	40	15	6	150	F	13 ± 1.2	21 ± 1.4	18 ± 1.5
							I	14 ± 0.5	16 ± 0.7	21 ± 0.6
		9	40	15	7	150	F	2 ± 0.03	5 ± 0.05	9 ± 0.4
							I	9 ± 0.06	12 ± 0.8	19 ± 0.7
		10	40	20	5	150	F	11 ± 0.5	14 ± 0.7	19 ± 0.6
							I	14 ± 0.9	16 ± 1.1	24 ± 1.1
		11	40	20	6	150	F	23 ± 0.19	18 ± 0.9	21 ± 0.9
							I	19 ± 0.2	24 ± 0.4	17 ± 0.3
		12	40	20	7	150	F	14 ± 0.5	16 ± 0.7	20 ± 0.7
							I	26 ± 0.12	21 ± 1.6	19 ± 1.7

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	30	15	5	150	F	18 ± 1.1	32 ± 1.3	75 ± 1.4
							I	7 ± 1.3	56 ± 1.5	63 ± 1.6
		2	30	15	6	150	F	23 ± 0.5	34 ± 0.7	29 ± 0.7
							I	19 ± 0.5	25 ± 0.7	39 ± 0.7
		3	30	15	7	150	F	26 ± 1.1	42 ± 1.3	86 ± 1.4
							I	46 ± 0.2	86 ± 0.4	75 ± 0.3
		4	30	20	5	150	F	42 ± 1.3	43 ± 1.5	79 ± 1.7
							I	53 ± 0.3	26 ± 0.5	85 ± 0.4
		5	30	20	6	150	F	29 ± 1.0	75 ± 1.2	93 ± 1.3
							I	33 ± 0.7	79 ± 0.9	116 ± 0.9
		6	30	20	7	150	F	13 ± 0.6	75 ± 0.8	92 ± 0.9
							I	29 ± 1.0	76 ± 1.2	98 ± 1.3
		7	40	15	5	150	F	34 ± 1.3	75 ± 1.5	91 ± 1.7
							I	47 ± 0.3	96 ± 0.5	126 ± 0.5
		8	40	15	6	150	F	51 ± 1.4	68 ± 1.6	48 ± 1.8
							I	63 ± 0.3	75 ± 0.5	99 ± 0.4
		9	40	15	7	150	F	82 ± 0.1	89 ± 0.3	74 ± 0.2
							I	32 ± 1.4	69 ± 1.6	85 ± 1.8
		10	40	20	5	150	F	49 ± 1.0	56 ± 1.2	71 ± 1.3
							I	96 ± 1.3	110 ± 1.5	98 ± 1.7
		11	40	20	6	150	F	75 ± 0.5	151 ± 0.25	125 ± 0.6
							I	45 ± 1.1	79 ± 1.3	115 ± 0.17
		12	40	20	7	150	F	56 ± 1.1	62 ± 1.3	48 ± 1.4
							I	29 ± 1.3	31 ± 1.5	42 ± 1.7

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	30	15	5	150	F	11 ± 0.5	12 ± 0.7	10 ± 0.7
							I	ND	ND	5 ± 1.2
		2	30	15	6	150	F	ND	4 ± 0.04	3 ± 0.03
							I	ND	ND	8 ± 0.6
		3	30	15	7	150	F	13 ± 1.1	14 ± 1.3	18 ± 1.4
							I	ND	18 ± 1.6	21 ± 1.7
		4	30	20	5	150	F	ND	14 ± 1.4	12 ± 1.5
							I	ND	ND	8 ± 0.2
		5	30	20	6	150	F	ND	ND	6 ± 0.4
							I	ND	12 ± 1.0	4 ± 1.0
		6	30	20	7	150	F	16 ± 1.2	19 ± 1.4	17 ± 1.5
							I	19 ± 1.3	21 ± 1.5	18 ± 1.7
		7	40	15	5	150	F	7 ± 0.6	11 ± 0.8	14 ± 0.8
							I	ND	15 ± 1.1	22 ± 1.2
		8	40	15	6	150	F	16 ± 1.1	18 ± 1.3	14 ± 1.4
							I	ND	12 ± 0.3	9 ± 0.3
		9	40	15	7	150	F	20 ±0.23	19 ± 1.2	15 ± 1.3
							I	12 ± 0.4	10 ± 0.6	9 ± 0.6
		10	40	20	5	150	F	ND	2 ± 0.04	9 ± 1.5
							I	19 ± 0.6	20 ± 0.8	22 ± 0.8
		11	40	20	6	150	F	16 ± 1.5	18 ± 1.7	15 ± 1.9
							I	23 ± 0.18	19 ± 1.4	21 ± 1.5
		12	40	20	7	150	F	12 ± 0.8	14 ± 1.0	19 ± 1.0
							I	9 ± 0.8	8 ± 1.0	15 ± 1.1

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	30	15	5	150	F	0.09 ± 0.01	0.05 ± 0.01	0.08 ± 0.01
							I	ND	ND	0.07 ± 0.02
		2	30	15	6	150	F	0.07 ± 0.01	ND	0.05 ± 0.01
							I	ND	0.02 ± 0.001	0.1 ±0.014
		3	30	15	7	150	F	ND	0.08 ± 0.01	0.05 ± 0.015
							I	ND	0.03 ± 0.001	0.06 ± 0.018
		4	30	20	5	150	F	0.06 ± 0.02	ND	0.09 ± 0.015
							I	0.05 ± 0.02	0.04 ± 0.018	ND
		5	30	20	6	150	F	ND	ND	0.06 ± 0.001
							I	ND	0.06 ± 0.01	0.12 ± 0.019
		6	30	20	7	150	F	ND	0.1 ± 0.02	0.08 ± 0.013
							I	ND	0.05 ± 0.01	0.09 ± 0.02
		7	40	15	5	150	F	ND	0.09 ± 0.02	0.02 ± 0.01
							I	ND	ND	0.05 ± 0.012
		8	40	15	6	150	F	0.09 ± 0.001	0.06 ± 0.013	0.03 ± 0.001
							I	0.12 ± 0.04	0.13 ± 0.06	0.16 ± 0.016
		9	40	15	7	150	F	0.11 ± 0.03	0.09 ± 0.01	0.07 ± 0.01
							I	0.13 ± 0.01	0.16 ± 0.02	0.14 ± 0.02
		10	40	20	5	150	F	ND	0.02 ± 0.01	0.06 ± 0.01
							I	ND	0.09 ± 0.02	0.02 ± 0.012
		11	40	20	6	150	F	0.05 ± 0.14	0.03 ± 0.001	0.05 ± 0.012
							I	ND	0.02 ± 0.001	0.09 ± 0.01
		12	40	20	7	150	F	ND	0.1 ± 0.01	0.02 ±0.001
							I	0.17 ± 0.013	0.09 ± 0.02	0.15 ± 0.02

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	30	15	5	150	F	15 ± 1.4	21 ± 1.6	36 ± 1.8
							I	16 ± 0.6	26 ± 0.8	59 ± 0.9
		2	30	15	6	150	F	18 ± 0.9	56 ± 1.1	62 ± 1.1
							I	14 ± 0.6	16 ± 0.8	25 ± 0.8
		3	30	15	7	150	F	7 ± 0.05	16 ± 0.7	54 ± 0.7
							I	6 ± 0.07	45 ± 0.9	36 ± 0.9
		4	30	20	5	150	F	8 ± 0.02	12 ± 1.2	19 ± 1.3
							I	12 ± 0.7	62 ± 0.9	45 ± 0.9
		5	30	20	6	150	F	11 ± 0.8	19 ± 1.0	25 ± 1.0
							I	71 ± 0.17	56 ± 0.3	63 ± 0.2
		6	30	20	7	150	F	26 ± 0.8	32 ± 1.0	54 ± 1.1
							I	62 ± 0.9	65 ± 1.1	69 ± 1.2
		7	40	15	5	150	F	19 ± 0.6	19 ± 0.8	32 ± 0.8
							I	51 ± 1.1	62 ± 1.3	53 ± 1.4
		8	40	15	6	150	F	74 ± 0.12	71 ± 0.5	64 ± 0.5
							I	42 ± 0.6	44 ± 0.8	49 ± 0.8
		9	40	15	7	150	F	39 ± 1.1	25 ± 1.3	35 ± 1.5
							I	41 ± 0.6	56 ± 0.8	69 ± 0.8
		10	40	20	5	150	F	38 ± 1.2	42 ± 1.4	58 ± 1.5
							I	21 ± 0.8	32 ± 1.0	48 ± 1.0
		11	40	20	6	150	F	17 ± 0.5	21 ± 0.7	33 ± 0.6
							I	6 ± 0.4	10 ± 0.6	29 ± 0.5
		12	40	20	7	150	F	18 ± 0.5	31 ± 0.7	58 ± 0.7
							I	20 ± 0.3	46 ± 1.2	32 ± 1.3

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	30	15	5	150	F	1 ± 0.02	0	5 ± 0.08
							I	3 ± 0.05	6 ± 0.7	5 ± 0.07
		2	30	15	6	150	F	6 ± 0.02	2 ± 0.4	4 ± 0.03
							I	ND	5 ± 0.2	7 ± 0.3
		3	30	15	7	150	F	6 ± 0.1	4 ± 0.3	6 ± 0.04
							I	ND	ND	3 ± 0.8
		4	30	20	5	150	F	6 ± 0.1	5 ± 0.3	3 ± 0.5
							I	2 ± 0.02	8 ± 0.7	7 ± 0.9
		5	30	20	6	150	F	ND	ND	5 ± 0.6
							I	ND	ND	4 ± 0.8
		6	30	20	7	150	F	1 ± 0.01	5 ± 0.3	2 ± 0.04
							I	5 ± 0.07	4 ± 0.9	8 ± 0.9
		7	40	15	5	150	F	3 ± 0.05	6 ± 0.7	5 ± 0.6
							I	ND	8 ± 0.4	7 ± 0.3
		8	40	15	6	150	F	ND	2 ± 0.3	4 ± 1.4
							I	7 ± 0.06	5 ± 0.8	7 ± 0.8
		9	40	15	7	150	F	6 ± 0.02	5 ± 0.4	2 ± 0.04
							I	2 ± 0.02	9 ± 0.12	7 ± 0.4
		10	40	20	5	150	F	4 ± 0.07	6 ± 0.9	4 ± 0.9
							I	ND	ND	8 ± 1.0
		11	40	20	6	150	F	ND	2 ± 0.6	4 ± 0.5
							I	2 ± 0.02	6 ± 0.09	8 ± 0.9
		12	40	20	7	150	F	7 ± 0.18	5 ± 0.5	6 ± 0.7
							I	4 ± 0.05	5 ± 0.06	8 ± 0.6

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

ST 04

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	30	15	6	150	F	2 ± 0.4	6 ± 0.6	7 ± 0.3
							I	6 ± 0.1	5 ± 0.2	14 ± 0.1
		2	30	15	7	150	F	6 ± 0.9	3 ± 0.2	6 ± 0.07
							I	ND	12 ± 1.1	20 ± 0.6
		3	30	20	6	150	F	7 ± 0.7	5 ± 0.03	2 ± 0.6
							I	10 ± 1.2	18 ± 1.5	19 ± 0.09
		4	30	20	7	150	F	ND	5 ± 0.3	7 ± 0.07
							I	ND	12 ± 0.2	18 ± 0.1
		5	40	15	6	150	F	8 ± 0.21	7 ± 0.4	5 ± 0.02
							I	12 ± 0.9	11 ± 1.2	16 ± 0.07
		6	40	15	7	150	F	ND	6 ± 0.06	7 ± 0.4
							I	ND	12 ± 0.9	15 ± 0.5
		7	40	20	6	150	F	5 ± 0.3	6 ± 0.5	7 ± 0.3
							I	9 ± 0.7	12 ± 0.32	21 ± 0.16
		8	40	20	7	150	F	7 ± 1.0	5 ± 0.3	4 ± 0.08
							I	14 ± 0.4	12 ± 0.6	19 ± 0.3

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	30	15	6	150	F	75 ± 0.2	91 ± 0.4	110 ± 0.2
							I	69 ± 0.5	78 ± 0.7	96 ± 0.4
		2	30	15	7	150	F	82 ± 0.4	86 ± 0.6	120 ± 0.3
							I	96 ± 0.07	110 ± 1.0	89 ± 0.6
		3	30	20	6	150	F	19 ± 0.6	68 ± 0.8	135 ± 0.5
							I	56 ± 0.2	89 ± 0.3	135 ± 0.1
		4	30	20	7	150	F	26 ± 0.5	52 ± 0.7	41 ± 0.4
							I	35 ± 1.2	54 ± 1.6	92 ± 0.9
		5	40	15	6	150	F	37 ± 1.0	62 ± 1.3	96 ± 0.8
							I	45 ± 1.1	68 ± 1.5	120 ± 0.9
		6	40	15	7	150	F	49 ± 0.4	78 ± 0.6	134 ± 0.3
							I	51 ± 0.5	96 ± 0.7	152 ± 0.4
		7	40	20	6	150	F	75 ± 0.3	156 ± 0.22	148 ± 0.7
							I	95 ± 0.2	124 ± 0.3	175 ± 0.16
		8	40	20	7	150	F	15 ± 1.1	52 ± 1.5	79 ± 0.9
							I	36 ± 0.3	62 ± 0.4	85 ± 0.2

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	30	15	6	150	F	1 ± 0.02	0	1 ± 0.07
							I	1 ± 0.06	1 ± 0.2	ND
		2	30	15	7	150	F	ND	ND	1 ± 0.04
							I	1 ± 0.09	1 ± 0.4	ND
		3	30	20	6	150	F	2 ± 0.24	1 ± 0.3	ND
							I	2 ± 0.3	ND	1 ± 0.03
		4	30	20	7	150	F	1 ± 0.2	1 ± 0.3	ND
							I	2 ± 0.3	3 ± 0.4	4 ± 0.2
		5	40	15	6	150	F	ND	4 ± 0.2	3 ± 0.1
							I	5 ± 0.3	4 ± 0.6	4 ± 0.9
		6	40	15	7	150	F	ND	ND	1 ± 0.07
							I	ND	1 ± 0.05	3 ± 0.09
		7	40	20	6	150	F	1 ± 0.4	ND	1 ± 0.03
							I	3 ± 1.0	2 ± 1.3	4 ± 0.08
		8	40	20	7	150	F	ND	1 ±0.13	1 ± 0.08
							I	1 ± 0.3	ND	4 ± 0.09

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	30	15	6	150	F	0.9 ± 0.5	0.2 ± 0.012	0.5 ± 0.04
							I	1.1 ± 0.8	0.9 ± 0.05	1.2 ± 0.06
		2	30	15	7	150	F	0.8 ± 0.2	0.9 ± 0.03	1.2 ± 0.1
							I	1.2 ± 1.1	1.9 ± 0.15	2.1 ± 0.09
		3	30	20	6	150	F	0.5 ± 0.01	0.8 ± 0.13	1.1 ± 0.08
							I	0.9 ± 0.02	1 ± 0.16	2.1 ± 0.9
		4	30	20	7	150	F	0.3 ± 0.01	0.2 ±0.14	0.9 ± 0.08
							I	1.1 ± 0.08	0.9 ± 0.10	2 ± 0.06
		5	40	15	6	150	F	1.3 ± 0.01	0.9 ± 0.03	1.2 ± 0.2
							I	2.2 ± 0.17	2 ± 0.09	1.9 ± 0.05
		6	40	15	7	150	F	0.2 ± 0.05	0.19 ± 0.02	0.17 ± 0.001
							I	0.4 ± 0.012	0.3 ± 0.03	1.8 ± 0.09
		7	40	20	6	150	F	0.3 ± 0.06	0.2 ± 0.04	1.2 ± 0.04
							I	ND	1.2 ±0.1	0.8 ± 0.06
		8	40	20	7	150	F	1.2 ± 0.3	1.1 ± 0.2	0.9 ± 0.07
							I	0.3 ± 0.1	1.2 ± 0.3	1.1 ± 0.1

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	30	15	6	150	F	28 ± 0.9	32 ± 1.2	25 ± 0.7
							I	31 ± 0.4	42 ± 0.5	58 ± 0.3
		2	30	15	7	150	F	26 ± 1.0	30 ± 1.3	34 ± 0.8
							I	25 ± 0.5	26 ± 0.7	44 ± 0.4
		3	30	20	6	150	F	12 ± 0.3	25 ± 1.7	30 ± 1.0
							I	36 ± 0.4	42 ± 1.3	51 ± 0.8
		4	30	20	7	150	F	14 ± 0.7	16 ± 0.9	29 ± 0.5
							I	26 ± 0.7	16 ± 1.0	34 ± 0.6
		5	40	15	6	150	F	22 ± 0.5	34 ± 0.8	28 ± 0.4
							I	27 ± 0.2	34 ± 0.3	18 ± 0.1
		6	40	15	7	150	F	32 ± 0.8	27 ± 1.1	14 ± 0.6
							I	28 ± 0.9	32 ± 1.2	13 ± 0.7
		7	40	20	6	150	F	38 ± 0.18	28 ± 1.4	32 ± 0.8
							I	27 ± 1.2	31 ± 1.6	45 ± 1.0
		8	40	20	7	150	F	26 ± 0.3	25 ± 0.5	32 ± 0.3
							I	61 ± 0.19	58 ± 0.8	41 ± 0.4

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	30	15	6	150	F	12 ± 0.21	11 ± 0.4	8 ± 0.02
							I	9 ± 0.06	8 ± 0.8	10 ± 0.05
		2	30	15	7	150	F	8 ± 0.1	9 ± 0.3	11 ± 0.01
							I	10 ± 0.07	9 ± 0.9	7 ± 0.05
		3	30	20	6	150	F	2 ± 0.03	1 ± 0.05	6 ± 0.03
							I	10 ± 0.6	9 ± 0.9	7 ± 0.05
		4	30	20	7	150	F	9 ± 0.1	10 ± 0.2	8 ± 0.1
							I	6 ± 0.4	5 ± 0.06	9 ± 0.3
		5	40	15	6	150	F	4 ± 0.7	2 ± 0.02	8 ± 0.6
							I	11 ± 0.19	10 ± 0.9	6 ± 0.5
		6	40	15	7	150	F	3 ± 0.3	2 ± 1.7	9 ± 1.0
							I	2 ± 0.6	7 ± 0.8	5 ± 0.05
		7	40	20	6	150	F	9 ± 1.2	7 ± 1.6	6 ± 0.09
							I	7 ± 0.04	5 ± 1.3	10 ± 0.08
		8	40	20	7	150	F	5 ± 0.03	4 ± 0.5	8 ± 0.3
							I	4 ± 0.06	3 ± 0.8	7 ± 0.05

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

ST 05

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	20	20	7	150	F	9 ± 0.05	10 ± 0.4	13 ± 0.4
							I	11 ± 0.1	13 ± 0.2	9 ± 0.1
		2	20	20	8	150	F	12 ± 0.11	10 ± 0.8	8 ± 0.09
							I	10 ± 1.0	11 ± 0.7	9 ± 0.09
		3	20	25	7	150	F	8 ± 0.9	10 ± 0.7	11 ± 0.8
							I	7 ± 1.4	12 ± 1.0	13 ± 1.3
		4	20	25	8	150	F	4 ± 0.1	6 ± 0.8	9 ± 1.0
							I	14 ± 0.21	12 ± 0.2	7 ± 0.1
		5	30	20	7	150	F	10 ± 0.3	11 ± 0.3	6 ± 0.03
							I	6 ± 1.1	8 ± 0.8	4 ± 0.02
		6	30	20	8	150	F	2 ± 0.05	9 ± 0.5	11 ± 0.5
							I	8 ± 0.8	10 ± 0.6	13 ± 0.7
		7	30	25	7	150	F	11 ± 0.4	9 ± 0.4	11 ± 0.4
							I	8 ± 0.9	9 ± 0.7	12 ± 0.8
		8	30	25	8	150	F	9 ± 1.2	8 ± 0.9	9 ± 0.1
							I	13 ± 0.5	12 ± 0.4	8 ± 0.4

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	20	20	7	150	F	25 ± 0.3	65 ± 0.3	94 ± 0.3
							I	56 ± 0.6	71 ± 0.5	101 ± 0.5
		2	20	20	8	150	F	18 ± 0.5	59 ± 0.4	73 ± 0.4
							I	36 ± 0.9	85 ± 0.7	125 ± 0.8
		3	20	25	7	150	F	72 ± 0.7	85 ± 0.6	46 ± 0.7
							I	81 ± 0.2	98 ± 0.2	102 ± 0.2
		4	20	25	8	150	F	16 ± 0.6	32 ± 0.5	49 ± 0.5
							I	39 ± 1.5	49 ± 1.0	78 ± 1.3
		5	30	20	7	150	F	51 ± 1.2	62 ± 0.9	81 ± 1.1
							I	62 ± 1.4	89 ± 1.0	132 ± 1.2
		6	30	20	8	150	F	48 ± 0.5	70 ± 0.4	98 ± 0.5
							I	96 ± 0.6	102 ± 0.5	139 ± 0.5
		7	30	25	7	150	F	57 ± 1.1	65 ± 0.8	89 ± 1.0
							I	52 ± 0.2	79 ± 0.2	109 ± 0.2
		8	30	25	8	150	F	49 ± 1.4	108 ± 0.21	98 ± 1.2
							I	98 ± 0.3	97 ± 0.3	146 ± 0.25

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	20	20	7	150	F	12 ± 1.0	18 ± 0.8	20 ± 0.9
							I	18 ± 0.7	16 ± 0.6	17 ± 0.6
		2	20	20	8	150	F	21 ± 0.7	23 ± 0.5	19 ± 0.6
							I	27 ± 1.1	31 ± 0.2	25 ± 0.9
		3	20	25	7	150	F	19 ± 1.4	16 ± 1.0	20 ± 1.3
							I	14 ± 0.4	16 ± 0.4	19 ± 0.3
		4	20	25	8	150	F	19 ± 1.5	21 ± 1.0	23 ± 1.3
							I	21 ± 0.3	28 ± 0.3	30 ± 0.3
		5	30	20	7	150	F	17 ± 0.1	19 ± 0.2	22 ± 0.1
							I	25 ± 1.5	28 ± 1.0	30 ± 1.3
		6	30	20	8	150	F	25 ± 0.21	22 ± 0.8	18 ± 0.9
							I	28 ± 1.4	30 ± 1.0	27 ± 1.2
		7	30	25	7	150	F	9 ± 0.05	12 ± 0.4	19 ± 0.4
							I	12 ± 1.2	20 ± 0.9	14 ± 1.1
		8	30	25	8	150	F	11 ± 1.2	15 ± 0.9	21 ± 1.1
							I	17 ± 1.4	24 ± 1.0	28 ± 1.3

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	20	20	7	150	F	ND	2 ± 0.5	1.2 ± 0.2
							I	1.2 ± 0.09	1 ± 0.02	0.9 ± 0.08
		2	20	20	8	150	F	ND	1.7 ± 0.2	2.3 ± 0.2
							I	2.5 ± 0.14	2.1 ± 0.2	2.4 ± 0.2
		3	20	25	7	150	F	1 ± 0.12	0.5 ± 0.3	0.9 ± 0.1
							I	1.9 ±0.15	1.5 ± 0.4	2 ± 0.3
		4	20	25	8	150	F	3 ± 0.01	1.9 ± 0.3	2.1 ± 0.1
							I	2.9 ± 0.15	2.5 ± 0.7	2 ± 0.3
		5	30	20	7	150	F	2.4 ± 0.2	2.1 ± 0.3	1.8 ± 0.2
							I	1.6 ± 0.08	1.4 ± 0.6	2 ± 0.1
		6	30	20	8	150	F	ND	2 ± 0.02	1.8 ± 0.3
							I	2 ± 0.14	1.8 ±0.3	2.1 ±0.2
		7	30	25	7	150	F	1.7 ± 0.07	2 ± 0.05	2.3 ± 0.4
							I	ND	0.9 ± 0.01	1.4 ± 0.09
		8	30	25	8	150	F	2.4 ± 0.12	1.9 ± 0.08	2.2 ± 0.02
							I	2 ± 0.1	1.9 ± 0.02	1.7 ± 0.1

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	20	20	7	150	F	12 ± 1.1	14 ± 0.8	18 ± 0.9
							I	25 ± 0.4	32 ± 0.4	49 ± 0.4
		2	20	20	8	150	F	17 ± 1.2	19 ± 0.9	27 ± 1.1
							I	39 ± 0.6	51 ± 0.5	61 ± 0.6
		3	20	25	7	150	F	11 ± 1.6	14 ± 1.1	21 ± 1.4
							I	15 ± 1.2	29 ± 0.9	52 ± 1.1
		4	20	25	8	150	F	27 ± 0.8	19 ± 0.6	16 ± 0.7
							I	14 ± 0.9	26 ± 0.7	32 ± 0.8
		5	30	20	7	150	F	13 ± 0.7	19 ± 0.5	22 ± 0.6
							I	49 ± 0.2	51 ± 0.2	62 ± 0.2
		6	30	20	8	150	F	15 ± 1.0	17 ± 0.7	14 ± 0.8
							I	7 ± 1.1	15 ± 0.8	37 ± 0.2
		7	30	25	7	150	F	6 ± 1.3	19 ± 0.9	26 ± 1.1
							I	12 ± 1.5	45 ± 1.1	32 ± 1.3
		8	30	25	8	150	F	9 ± 0.04	29 ± 0.23	31 ± 0.3
							I	64 ± 0.21	34 ± 0.5	49 ± 0.6

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	20	20	7	150	F	12 ± 0.3	16 ± 0.3	21 ± 0.2
							I	24 ± 0.7	35 ± 0.6	51 ± 0.6
		2	20	20	8	150	F	36 ± 0.2	25 ± 0.2	18 ± 0.1
							I	48 ± 0.8	62 ± 0.6	58 ± 0.7
		3	20	25	7	150	F	52 ± 0.4	58 ± 0.4	46 ± 0.4
							I	19 ± 0.8	24 ± 0.6	38 ± 0.7
		4	20	25	8	150	F	51 ± 0.1	49 ± 0.2	21 ± 0.1
							I	13 ± 0.5	29 ± 0.4	49 ± 0.4
		5	30	20	7	150	F	48 ± 0.9	60 ± 0.7	57 ± 0.8
							I	41 ± 0.8	58 ± 0.6	71 ± 0.7
		6	30	20	8	150	F	17 ± 1.6	62 ± 0.15	47 ± 1.4
							I	29 ± 0.7	35 ± 0.6	29 ± 0.6
		7	30	25	7	150	F	33 ± 1.5	38 ± 1.0	41 ± 1.3
							I	15 ± 1.2	36 ± 0.9	37 ± 1.1
		8	30	25	8	150	F	19 ± 0.4	29 ± 0.4	45 ± 0.4
							I	31 ± 0.7	52 ± 0.6	74 ± 0.1

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

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Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	30	15	7	150	F	6 ± 0.05	12 ± 0.05	14 ± 0.4
							I	12 ± 0.02	16 ± 0.2	8 ± 0.1
		2	30	15	8	150	F	18 ± 1.2	9 ± 0.08	10 ± 0.9
							I	16 ± 1.1	15 ± 0.8	14 ± 0.9
		3	30	20	7	150	F	9 ± 0.2	11 ± 0.7	15 ± 0.8
							I	11 ± 1.6	14 ± 1.1	16 ± 1.3
		4	30	20	8	150	F	5 ± 1.3	8 ± 0.9	19 ± 1.0
							I	14 ± 0.1	12 ± 0.2	20 ± 0.1
		5	40	15	7	150	F	20 ± 0.09	18 ± 0.4	13 ± 0.3
							I	15 ± 1.3	18 ± 0.9	17 ± 1.0
		6	40	15	8	150	F	16 ± 0.6	17 ± 0.5	19 ± 0.5
							I	18 ± 0.9	20 ± 0.7	22 ± 0.7
		7	40	20	7	150	F	11 ± 0.5	18 ± 0.4	16 ± 0.4
							I	9 ± 1.0	11 ± 0.7	14 ± 0.8
		8	40	20	8	150	F	16 ± 1.4	11 ± 1.0	18 ± 1.1
							I	24 ± 0.29	21 ± 0.5	19 ± 0.4

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	30	15	7	150	F	12 ± 0.3	46 ± 0.3	65 ± 0.3
							I	24 ± 0.6	32 ± 0.5	41 ± 0.5
		2	30	15	8	150	F	35 ± 0.5	62 ± 0.5	73 ± 0.4
							I	42 ± 1.0	65 ± 0.7	98 ± 0.8
		3	30	20	7	150	F	17 ± 0.8	52 ± 0.6	67 ± 0.7
							I	11 ± 0.2	54 ± 0.2	48 ± 0.2
		4	30	20	8	150	F	16 ± 0.6	39 ± 0.5	51 ± 0.5
							I	26 ± 1.6	59 ± 1.1	37 ± 1.3
		5	40	15	7	150	F	37 ± 1.3	64 ± 0.9	58 ± 1.1
							I	39 ± 1.5	72 ± 1.1	105 ± 1.2
		6	40	15	8	150	F	25 ± 0.6	57 ± 0.5	62 ± 0.5
							I	26 ± 0.6	36 ± 0.5	74 ± 0.5
		7	40	20	7	150	F	35 ± 1.3	48 ± 0.9	59 ± 0.5
							I	38 ± 0.2	85 ± 0.3	120 ± 0.2
		8	40	20	8	150	F	26 ± 1.6	51 ± 1.1	75 ± 0.17
							I	41 ± 0.3	64 ± 0.3	135 ± 0.15

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	30	15	7	150	F	23 ± 1.2	32 ± 0.8	56 ± 0.9
							I	32 ± 0.8	41 ± 0.6	29 ± 0.6
		2	30	15	8	150	F	19 ± 0.8	26 ± 0.6	37 ± 0.6
							I	24 ± 1.2	32 ± 0.8	29 ± 0.9
		3	30	20	7	150	F	18 ± 1.6	25 ± 1.1	35 ± 1.3
							I	36 ± 0.4	56 ± 0.12	52 ± 0.3
		4	30	20	8	150	F	41 ± 1.7	51 ± 1.1	57 ± 1.3
							I	28 ± 0.3	32 ± 0.3	41 ± 0.3
		5	40	15	7	150	F	64 ± 0.18	52 ± 0.2	54 ± 0.1
							I	42 ± 1.7	47 ± 1.1	52 ± 1.3
		6	40	15	8	150	F	32 ± 1.2	45 ± 0.8	60 ± 0.9
							I	47 ± 1.6	49 ± 1.1	52 ± 1.2
		7	40	20	7	150	F	49 ± 0.5	51 ± 0.5	47 ± 0.4
							I	29 ± 1.4	32 ± 0.9	41 ± 1.1
		8	40	20	8	150	F	27 ± 1.3	36 ± 0.9	48 ± 1.1
							I	35 ± 1.6	47 ± 1.1	29 ± 1.3

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	30	15	7	150	F	1.8 ± 0.06	0.9 ± 0.05	2 ± 0.5
							I	2 ± 0.1	2.1 ± 0.8	3 ± 0.08
		2	30	15	8	150	F	0.9 ± 0.02	1 ± 0.02	1.2 ± 0.2
							I	0.7 ± 0.05	2.9 ± 0.3	1.7 ± 0.2
		3	30	20	7	150	F	1 ± 0.13	0.9 ± 0.02	2.1 ± 0.1
							I	ND	2.8 ± 0.1	1.8 ± 0.3
		4	30	20	8	150	F	ND	2.1 ± 0.04	1.7 ± 0.3
							I	3.3 ± 0.12	2.7 ± 0.08	0.9 ± 0.08
		5	40	15	7	150	F	1.5 ± 0.3	1 ± 0.03	2 ± 0.02
							I	ND	2.1 ± 0.07	2.7 ± 0.07
		6	40	15	8	150	F	ND	1.9 ± 0.12	2 ± 0.02
							I	ND	ND	2.7 ± 0.1
		7	40	20	7	150	F	2.4 ± 0.03	1.9 ± 0.06	2 ± 0.06
							I	1.4 ± 0.2	0.9 ± 0.08	1.5 ± 0.02
		8	40	20	8	150	F	2 ± 0.03	2.5 ± 0.9	1.9 ± 0.3
							I	1.6 ± 0.2	2.1 ± 0.2	2.4 ± 0.1

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	30	15	7	150	F	32 ± 1.2	28 ± 0.9	19 ± 0.9
							I	14 ± 0.5	25 ± 0.4	52 ± 0.4
		2	30	15	8	150	F	16 ± 1.4	21 ± 1.0	35 ± 1.1
							I	21 ± 0.7	35 ± 0.5	47 ± 0.6
		3	30	20	7	150	F	29 ± 1.8	35 ± 1.2	29 ± 1.4
							I	31 ± 1.4	42 ± 1.0	62 ± 1.1
		4	30	20	8	150	F	27 ± 0.9	21 ± 0.7	32 ± 0.7
							I	29 ± 1.0	31 ± 0.7	44 ± 0.8
		5	40	15	7	150	F	36 ± 0.7	29 ± 0.6	42 ± 0.6
							I	51 ± 0.2	62 ± 0.3	68 ± 0.2
		6	40	15	8	150	F	28 ± 1.1	32 ± 0.8	37 ± 0.8
							I	49 ± 1.3	33 ± 0.9	28 ± 1.0
		7	40	20	7	150	F	39 ± 0.22	25 ± 1.0	14 ± 1.1
							I	70 ± 0.13	52 ± 1.2	54 ± 1.3
		8	40	20	8	150	F	27 ± 0.4	30 ± 0.4	35 ± 0.3
							I	56 ± 0.7	46 ± 0.6	51 ± 0.6

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	30	15	7	150	F	25 ± 0.3	36 ± 0.3	56 ± 0.2
							I	14 ± 0.8	35 ± 0.6	44 ± 0.6
		2	30	15	8	150	F	26 ± 0.2	38 ± 0.2	52 ± 0.1
							I	38 ± 0.9	54 ± 0.7	47 ± 0.7
		3	30	20	7	150	F	42 ± 0.4	52 ± 0.4	62 ± 0.4
							I	29 ± 0.9	75 ± 0.14	59 ± 0.7
		4	30	20	8	150	F	14 ± 0.1	29 ± 0.2	38 ± 0.1
							I	35 ± 0.5	42 ± 0.4	57 ± 0.4
		5	40	15	7	150	F	42 ± 1.0	52 ± 0.7	61 ± 0.8
							I	54 ± 0.9	48 ± 0.7	36 ± 0.7
		6	40	15	8	150	F	17 ± 1.8	25 ± 1.2	33 ± 1.4
							I	29 ± 0.8	36 ± 0.6	48 ± 0.6
		7	40	20	7	150	F	34 ± 1.7	66 ± 0.26	57 ± 1.3
							I	46 ± 1.4	56 ± 1.0	72 ± 1.1
		8	40	20	8	150	F	38 ± 0.4	46 ± 0.4	58 ± 0.4
							I	27 ± 0.8	54 ± 0.6	61 ± 0.6

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

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Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	30	20	5	150	F	9 ± 0.5	5 ± 0.5	11 ± 0.5
							I	7 ± 0.02	18 ± 0.2	20 ± 0.1
		2	30	20	6	150	F	6 ± 0.1	9 ± 0.08	11 ± 1.0
							I	10 ± 0.1	12 ± 0.8	21 ± 0.9
		3	30	20	7	150	F	5 ± 0.09	8 ± 0.07	10 ± 0.08
							I	ND	18 ± 1.1	21 ± 1.3
		4	30	25	5	150	F	10 ± 1.2	8 ± 0.9	5 ± 1.1
							I	8 ± 0.1	14 ± 0.2	19 ± 0.1
		5	30	25	6	150	F	3 ± 0.3	5 ± 0.4	9 ± 0.3
							I	12 ± 1.2	17 ± 0.9	21 ± 1.1
		6	30	25	7	150	F	8 ± 0.6	11 ± 0.5	7 ± 0.5
							I	16 ± 0.8	14 ± 0.7	11 ± 0.7
		7	40	20	5	150	F	7 ± 0.4	11 ± 0.4	9 ± 0.4
							I	19 ± 1.0	22 ± 0.07	18 ± 0.9
		8	40	20	6	150	F	12 ± 0.24	10 ± 0.1	7 ± 0.2
							I	2 ± 0.05	5 ± 0.05	15 ± 0.5
		9	40	20	7	150	F	10 ± 0.3	5 ± 0.3	7 ± 0.3
							I	8 ± 0.6	17 ± 0.5	22 ± 0.17
		10	40	25	5	150	F	5 ± 0.5	8 ± 0.5	11 ± 0.5
							I	13 ± 1.0	12 ± 0.7	21 ± 0.9
		11	40	25	6	150	F	1 ± 0.8	5 ± 0.6	9 ± 0.7
							I	6 ± 0.02	12 ± 0.2	20 ± 0.2
		12	40	25	7	150	F	5 ± 0.06	7 ± 0.5	11 ± 0.5
							I	18 ± 1.6	20 ± 1.1	18 ± 1.4

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	30	20	5	150	F	15 ± 1.3	18 ± 0.9	14 ± 1.1
							I	17 ± 1.5	15 ± 1.1	13 ± 1.3
		2	30	20	6	150	F	11 ± 0.5	17 ± 0.5	20 ± 0.18
							I	12 ± 0.6	25 ± 0.5	32 ± 0.5
		3	30	20	7	150	F	10 ± 1.2	15 ± 0.9	17 ± 1.1
							I	26 ± 0.2	30 ± 0.3	27 ± 0.2
		4	30	25	5	150	F	19 ± 1.5	15 ± 1.1	7 ± 1.3
							I	18 ± 0.3	25 ± 0.3	32 ± 0.3
		5	30	25	6	150	F	9 ± 0.1	14 ± 0.8	18 ± 1.0
							I	20 ± 0.7	25 ± 0.6	32 ± 0.7
		6	30	25	7	150	F	5 ± 0.7	15 ± 0.6	17 ± 0.6
							I	12 ± 1.1	15 ± 0.8	25 ± 1.0
		7	40	20	5	150	F	9 ± 1.5	15 ± 1.1	19 ± 1.3
							I	17 ± 0.4	24 ± 0.4	18 ± 0.3
		8	40	20	6	150	F	2 ± 0.6	6 ± 1.1	15 ± 1.4
							I	23 ± 0.3	32 ± 0.3	26 ± 0.3
		9	40	20	7	150	F	9 ± 0.1	16 ± 0.2	17 ± 0.1
							I	11 ± 1.6	22 ± 1.1	34 ± 0.13
		10	40	25	5	150	F	16 ± 1.1	9 ± 0.8	3 ± 0.02
							I	9 ± 1.5	12 ± 1.1	7 ± 1.3
		11	40	25	6	150	F	15 ± 0.5	8 ± 0.5	9 ± 0.5
							I	14 ± 1.3	18 ± 0.9	22 ± 1.1
		12	40	25	7	150	F	17 ± 1.3	15 ± 0.9	19 ± 1.1
							I	19 ± 1.5	21 ± 1.1	33 ± 1.3

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	30	20	5	150	F	1 ± 0.06	2 ± 0.05	1 ± 0.05
							I	2 ± 0.03	4 ± 0.08	3 ± 0.09
		2	30	20	6	150	F	ND	ND	2 ± 0.02
							I	ND	ND	3 ± 0.13
		3	30	20	7	150	F	3 ± 0.14	2 ± 0.1	2 ± 0.11
							I	4 ± 0.15	3 ± 0.11	2 ± 0.14
		4	30	25	5	150	F	2 ± 0.13	2 ± 0.12	1 ±0.12
							I	ND	ND	3 ± 0.09
		5	30	25	6	150	F	ND	1 ± 0.3	2 ± 0.02
							I	3 ± 0.08	4 ± 0.7	2 ± 0.08
		6	30	25	7	150	F	ND	2 ± 1.0	1 ± 0.12
							I	ND	3 ± 0.11	2 ± 0.13
		7	40	20	5	150	F	1 ± 0.07	2 ± 0.06	1 ± 0.06
							I	3 ± 0.1	3 ± 0.08	4 ± 0.9
		8	40	20	6	150	F	1 ± 0.12	1 ± 0.02	2 ± 0.11
							I	5 ± 0.16	2 ± 0.03	3 ± 0.10
		9	40	20	7	150	F	1 ± 0.11	2 ± 0.01	2 ± 0.02
							I	ND	ND	4 ± 0.04
		10	40	25	5	150	F	2 ± 0.13	2 ± 0.01	2 ± 0.016
							I	4 ± 0.07	4 ± 0.02	3 ± 0.06
		11	40	25	6	150	F	ND	2 ± 0.2	1 ± 0.02
							I	ND	2 ± 0.05	4 ± 0.12
		12	40	25	7	150	F	1 ± 0.09	2 ± 0.07	2 ± 0.08
							I	4 ± 0.09	3 ± 0.07	2 ± 0.08

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	30	20	5	150	F	ND	1.2 ± 0.06	1 ± 0.06
							I	ND	5.4 ± 0.3	3 ± 0.02
		2	30	20	6	150	F	0.9 ± 0.01	0.4 ± 0.02	1 ± 0.09
							I	5.2 ± 0.12	6 ± 0.9	4.9 ± 1.0
		3	30	20	7	150	F	1.4 ± 0.2	1 ± 0.02	1.3 ± 12
							I	3.4 ± 1.6	4.1 ±0.3	5.6 ± 0.14
		4	30	25	5	150	F	1 ± 0.04	1.2 ± 0.4	1.4 ± 0.12
							I	3.5 ± 0.7	4.1 ± 0.6	5 ± 0.06
		5	30	25	6	150	F	ND	ND	0.8 ± 0.03
							I	ND	1.5 ± 0.06	3.1 ± 0.7
		6	30	25	7	150	F	1 ± 0.02	0.8 ± 0.02	0.5 ± 0.02
							I	3.5 ± 0.09	5.2 ± 0.7	4.2 ± 0.08
		7	40	20	5	150	F	0.5 ± 0.14	0.3 ± 0.04	1 ± 0.04
							I	5.4 ± 0.8	5 ± 0.06	5.8 ± 0.7
		8	40	20	6	150	F	0.8 ± 0.1	1 ± 0.02	1.2 ± 0.1
							I	3.7 ± 0.5	4 ± 0.04	4.7 ± 0.4
		9	40	20	7	150	F	1.7 ± 0.04	1 ± 0.07	0.9 ± 0.02
							I	6.2 ± 0.19	5.8 ± 0.7	3.6 ± 0.08
		10	40	25	5	150	F	1 ± 0.01	1.2 ± 0.02	0.4 ± 0.02
							I	5.2 ± 0.7	5.8 ± 0.6	6 ± 0.07
		11	40	25	6	150	F	1.3 ± 0.16	0.7 ± 0.02	1 ± 0.14
							I	6 ± 0.13	5.6 ± 0.02	4.1 ± 0.2
		12	40	25	7	150	F	0.8 ± 0.14	1 ± 0.03	1.3 ± 0.04
							I	2.4 ± 0.17	3 ± 0.06	3.4 ± 0.07

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	30	20	5	150	F	25 ± 1.6	31 ± 1.1	27 ± 1.4
							I	10 ± 0.7	35 ± 0.6	19 ± 0.6
		2	30	20	6	150	F	31 ± 1.0	29 ± 0.7	18 ± 0.9
							I	12 ± 0.6	24 ± 0.5	30 ± 0.6
		3	30	20	7	150	F	29 ± 0.6	35 ± 0.5	19 ± 0.5
							I	34 ± 0.8	51 ± 0.6	48 ± 0.7
		4	30	25	5	150	F	36 ± 1.2	32 ± 0.9	29 ± 1.0
							I	48 ± 0.8	51 ± 0.6	58 ± 0.7
		5	30	25	6	150	F	25 ± 0.9	31 ± 0.7	36 ± 0.8
							I	19 ± 0.1	25 ± 0.2	41 ± 0.1
		6	30	25	7	150	F	25 ± 0.9	32 ± 0.7	38 ± 0.8
							I	61 ± 0.11	59 ± 0.8	48 ± 0.9
		7	40	20	5	150	F	18 ± 0.7	25 ± 0.6	32 ± 0.6
							I	11 ± 1.2	14 ± 0.9	29 ± 1.1
		8	40	20	6	150	F	36 ± 0.4	29 ± 0.4	18 ± 0.3
							I	58 ± 0.7	48 ± 0.6	32 ± 0.6
		9	40	20	7	150	F	25 ± 1.3	36 ± 0.9	21 ± 1.1
							I	34 ± 0.6	41 ± 0.5	59 ± 0.6
		10	40	25	5	150	F	19 ± 1.3	28 ± 1.0	32 ± 1.2
							I	41 ± 0.9	45 ± 0.7	59 ± 0.8
		11	40	25	6	150	F	16 ± 0.5	21 ± 0.5	34 ± 0.5
							I	47 ± 0.4	52 ± 0.4	60 ± 0.4
		12	40	25	7	150	F	39 ± 0.17	36 ± 0.5	29 ± 0.5
							I	52 ± 1.1	60 ± 0.8	47 ± 1.0

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	30	20	5	150	F	12 ± 0.6	22 ± 0.5	19 ± 0.6
							I	14 ± 0.6	19 ± 0.5	24 ± 0.5
		2	30	20	6	150	F	19 ± 0.2	21 ± 0.3	18 ± 0.2
							I	15 ± 1.1	18 ± 0.8	21 ± 1.0
		3	30	20	7	150	F	6 ± 0.2	19 ± 0.9	21 ± 1.1
							I	17 ± 1.6	23 ± 1.1	24 ± 1.4
		4	30	25	5	150	F	11 ± 1.3	21 ± 0.9	23 ± 1.1
							I	19 ± 1.7	24 ± 1.2	21 ± 1.5
		5	30	25	6	150	F	13 ± 1.4	21 ± 1.0	19 ± 1.2
							I	15 ± 0.7	24 ± 0.6	17 ± 0.6
		6	30	25	7	150	F	9 ± 1.2	14 ± 0.9	23 ± 1.1
							I	15 ± 0.7	24 ± 0.6	19 ± 0.7
		7	40	20	5	150	F	17 ± 0.5	9 ± 0.5	20 ± 0.5
							I	20 ± 0.2	16 ± 0.3	21 ± 0.2
		8	40	20	6	150	F	13 ± 1.3	25 ± 0.9	21 ± 1.1
							I	18 ± 0.6	22 ± 0.5	21 ± 0.6
		9	40	20	7	150	F	16 ± 0.3	18 ± 0.3	21 ± 0.2
							I	13 ± 0.3	22 ± 0.3	17 ± 0.2
		10	40	25	5	150	F	12 ± 0.8	18 ± 0.6	24 ± 0.7
							I	11 ± 0.8	14 ± 0.7	20 ± 0.7
		11	40	25	6	150	F	19 ± 0.4	24 ± 0.4	17 ± 0.4
							I	14 ± 0.8	24 ± 0.6	19 ± 0.7
		12	40	25	7	150	F	12 ± 1.5	27 ± 0.3	25 ± 1.3
							I	11 ± 0.5	25 ± 0.23	23 ± 0.4

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

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Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	30	20	6	150	F	20 ± 0.19	14 ± 0.4	18 ± 0.5
							I	14 ± 0.2	20 ± 0.1	25 ± 0.2
		2	30	20	7	150	F	19 ± 1.2	17 ± 0.8	9 ± 0.9
							I	14 ± 1.1	17 ± 0.8	27 ± 0.15
		3	30	25	6	150	F	11 ± 1.0	19 ± 0.7	15 ± 0.8
							I	6 ± 1.6	12 ± 1.1	22 ± 1.2
		4	30	25	7	150	F	9 ± 1.3	14 ± 0.9	18 ± 0.2
							I	21 ± 0.1	23 ± 0.1	24 ± 0.2
		5	40	20	6	150	F	18 ± 0.4	19 ± 0.3	17 ± 0.4
							I	16 ± 1.3	14 ± 0.9	25 ± 1.0
		6	40	20	7	150	F	18 ± 0.6	16 ± 0.4	11 ± 0.5
							I	7 ± 0.9	15 ± 0.6	24 ± 0.7
		7	40	25	6	150	F	11 ± 0.5	19 ± 0.3	10 ± 0.4
							I	14 ± 1.0	17 ± 0.7	25 ± 0.8
		8	40	25	7	150	F	9 ± 1.4	12 ± 0.9	16 ± 1.0
							I	15 ± 0.5	17 ± 0.4	23 ± 0.5

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	30	20	6	150	F	58 ± 0.3	62 ± 0.2	80 ± 0.3
							I	98 ± 0.6	145 ± 0.5	180 ± 0.13
		2	30	20	7	150	F	48 ± 0.5	54 ± 0.4	61 ± 0.5
							I	36 ± 1.0	32 ± 0.7	45 ± 0.8
		3	30	25	6	150	F	25 ± 0.8	62 ± 0.6	71 ± 0.7
							I	75 ± 0.2	131 ± 0.2	159 ± 0.3
		4	30	25	7	150	F	24 ± 0.6	52 ± 0.4	87 ± 0.5
							I	32 ± 1.6	53 ± 1.1	98 ± 1.2
		5	40	20	6	150	F	52 ± 1.3	77 ± 0.9	81 ± 1.0
							I	63 ± 1.5	85 ± 1.0	142 ± 1.1
		6	40	20	7	150	F	77 ± 0.6	85 ± 0.11	62 ± 0.5
							I	96 ± 0.6	85 ± 0.4	72 ± 0.5
		7	40	25	6	150	F	42 ± 1.3	84 ± 0.8	66 ± 0.9
							I	16 ± 0.2	52 ± 0.2	99 ± 0.3
		8	40	25	7	150	F	28 ± 1.6	49 ± 1.1	77 ± 1.2
							I	24 ± 0.3	35 ± 0.3	87 ± 0.4

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	30	20	6	150	F	ND	ND	0.5 ± 0.12
							I	ND	0.4 ± 0.03	1.5 ± 0.02
		2	30	20	7	150	F	0.5 ± 0.01	0.4 ± 0.02	0.6 ± 0.1
							I	ND	1 ± 0.08	2.4 ± 0.09
		3	30	25	6	150	F	0.6 ± 0.012	0.9 ± 0.01	0.7 ± 0.02
							I	2 ± 0.04	2.5 ± 0.03	3.6 ± 0.4
		4	30	25	7	150	F	ND	ND	0.5 ± 0.02
							I	4.1 ± 0.3	2.6 ± 0.03	3 ± 0.04
		5	40	20	6	150	F	1 ± 0.15	0.5 ± 0.01	0.1 ± 0.002
							I	5 ± 0.23	4 ± 0.12	3.8 ± 0.2
		6	40	20	7	150	F	ND	0.7 ± 0.08	0.2 ± 0.01
							I	ND	ND	2 ± 0.1
		7	40	25	6	150	F	0.1 ± 0.01	0.7 ± 0.04	0.3 ± 0.02
							I	4 ± 0.04	3.2 ± 0.09	3.7 ± 0.04
		8	40	25	7	150	F	0.9 ± 0.3	0.7 ± 0.01	0.3 ± 0.02
							I	3.5 ± 0.6	2.9 ±0.1	3 ± 0.04

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	30	20	6	150	F	0.4 ± 0.02	0.3 ± 0.04	0.42 ± 0.015
							I	0.1 ± 0.01	ND	0.19 ± 0.018
		2	30	20	7	150	F	0.3 ± 0.02	0.4 ± 0.01	0.3 ± 0.02
							I	ND	0.2 ± 0.03	0.11 ± 0.03
		3	30	25	6	150	F	ND	0.13 ± 0.003	ND
							I	ND	ND	ND
		4	30	25	7	150	F	0.1 ± 0.02	0.2 ± 0.001	0.41 ± 0.02
							I	ND	ND	ND
		5	40	20	6	150	F	0.5 ± 0.12	0.4 ± 0.02	0.38 ± 0.03
							I	0.24 ± 0.02	0.1 ± 0.01	0.15 ± 0.03
		6	40	20	7	150	F	ND	ND	ND
							I	ND	ND	ND
		7	40	25	6	150	F	0.47 ± 0.07	0.38 ± 0.012	0.34 ± 0.01
							I	0.1 ± 0.01	0.21 ± 0.013	0.1 ± 0.02
		8	40	25	7	150	F	0.3 ± 0.01	0.44 ± 0.03	0.47 ± 0.03
							I	ND	ND	0.17 ± 0.02

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	30	20	6	150	F	24 ± 1.2	19 ± 0.8	7 ± 0.9
							I	31 ± 0.5	45 ± 0.3	51 ± 0.4
		2	30	20	7	150	F	16 ± 1.4	20 ± 0.9	11 ± 0.2
							I	27 ± 0.7	33 ± 0.5	48 ± 0.6
		3	30	25	6	150	F	18 ± 1.8	24 ± 1.2	17 ± 1.3
							I	32 ± 1.4	55 ± 0.9	41 ± 1.0
		4	30	25	7	150	F	18 ± 0.9	9 ± 0.6	13 ± 0.7
							I	48 ± 1.0	52 ± 0.7	37 ± 0.8
		5	40	20	6	150	F	23 ± 0.7	19 ± 0.5	15 ± 0.6
							I	51 ± 0.2	49 ± 0.2	52 ± 0.3
		6	40	20	7	150	F	22 ± 1.1	26 ± 0.7	17 ± 0.8
							I	57 ± 0.1	51 ± 0.8	49 ± 0.9
		7	40	25	6	150	F	27 ± 0.16	25 ± 0.9	19 ± 1.0
							I	55 ± 1.7	53 ± 1.2	47 ± 1.3
		8	40	25	7	150	F	19 ± 0.4	24 ± 0.3	21 ± 0.4
							I	39 ± 0.7	44 ± 0.5	31 ± 0.6

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	30	20	6	150	F	8 ± 0.3	10 ± 0.2	11 ± 0.3
							I	18 ± 0.8	28 ± 0.5	30 ± 0.6
		2	30	20	7	150	F	7 ± 0.2	11 ± 0.1	14 ± 0.2
							I	18 ± 0.9	29 ± 0.6	24 ± 0.7
		3	30	25	6	150	F	2 ± 0.4	9 ± 0.3	11 ± 0.4
							I	19 ± 0.9	27 ± 0.6	24 ± 0.7
		4	30	25	7	150	F	8 ± 0.1	10 ± 0.1	11 ± 0.2
							I	14 ± 0.5	19 ± 0.4	20 ± 0.5
		5	40	20	6	150	F	9 ± 1.0	12 ± 0.2	7 ± 0.8
							I	28 ± 0.9	31 ± 0.16	27 ± 0.7
		6	40	20	7	150	F	9 ± 1.8	11 ± 1.2	10 ± 0.3
							I	24 ± 0.8	29 ± 0.5	24 ± 0.6
		7	40	25	6	150	F	7 ± 1.7	11 ± 1.1	9 ± 0.2
							I	17 ± 1.4	27 ± 0.9	21 ± 1.0
		8	40	25	7	150	F	8 ± 0.4	7 ± 0.3	4 ± 0.4
							I	19 ± 0.8	27 ± 0.5	21 ± 0.6

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

APPENDIX VI

Enzyme activity of consortia developed at different physicochemical conditions

CS 01

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	20	15	5	150	F	14 ± 0.5	21 ± 0.6	17 ± 0.6
							I	18 ± 0.2	21 ± 0.3	24 ± 0.2
		2	20	15	6	150	F	20 ± 1.1	26 ± 1.2	19 ± 1.3
							I	21 ± 1.1	17 ± 1.2	25 ± 1.2
		3	20	15	7	150	F	17 ± 0.9	22 ± 1.0	18 ± 1.0
							I	16 ± 1.5	21 ± 1.6	18 ± 1.7
		4	20	20	5	150	F	9 ± 1.2	24 ± 1.3	19 ± 1.3
							I	20 ± 0.1	25 ± 0.2	18 ± 0.1
		5	20	20	6	150	F	22 ± 0.3	18 ± 0.4	21 ± 0.4
							I	24 ± 1.2	22 ± 1.3	19 ± 1.3
		6	20	20	7	150	F	23 ± 0.6	26 ± 0.7	21 ± 0.6
							I	19 ± 0.8	21 ± 0.9	17 ± 0.9
		7	20	25	5	150	F	17 ± 0.4	19 ± 0.5	11 ± 0.5
							I	15 ± 1.0	11 ± 1.1	7 ± 0.1
		8	20	25	6	150	F	24 ± 1.3	21 ± 1.4	7 ± 0.5
							I	23 ± 0.5	25 ± 0.6	18 ± 0.6
		9	20	25	7	150	F	10 ± 0.3	14 ± 0.4	21 ± 0.3
							I	11 ± 0.6	17 ± 0.7	19 ± 0.7
		10	30	15	5	150	F	9 ± 0.5	12 ± 0.6	17 ± 0.6
							I	8 ± 1.0	14 ± 1.1	26 ± 1.1
		11	30	15	6	150	F	24 ± 0.8	14 ± 0.9	19 ± 0.9
							I	22 ± 0.2	23 ± 0.3	17 ± 0.2
		12	30	15	7	150	F	19 ± 0.6	25 ± 0.7	19 ± 0.7
							I	16 ± 1.6	21 ± 1.7	19 ± 1.7
		13	30	20	5	150	F	17 ± 1.3	20 ± 1.4	17 ± 1.4
							I	25 ± 1.5	23 ± 1.6	22 ± 1.6
		14	30	20	6	150	F	22 ± 0.5	19 ± 0.6	14 ± 0.6
							I	19 ± 0.6	17 ± 0.7	16 ± 0.7
		15	30	20	7	150	F	17 ± 1.2	14 ± 1.3	11 ± 1.3
							I	11 ± 0.2	14 ± 0.3	21 ± 0.2
		16	30	25	5	150	F	9 ± 1.5	18 ± 1.6	22 ± 1.7
							I	15 ± 0.3	12 ± 0.4	17 ± 0.4
		17	30	25	6	150	F	24 ± 1.1	23 ± 1.2	18 ± 1.2
							I	19 ± 0.7	21 ± 0.8	24 ± 0.8
		18	30	25	7	150	F	17 ± 0.7	25 ± 0.8	14 ± 0.8
							I	12 ± 1.1	13 ± 1.2	17 ± 1.3
		19	40	15	5	150	F	11 ± 1.5	15 ± 1.6	18 ± 1.7
							I	19 ± 0.4	29 ± 0.23	25 ± 0.4
		20	40	15	6	150	F	21 ± 1.6	24 ± 1.7	18 ± 1.8
							I	24 ± 0.3	23 ± 0.4	17 ± 0.4
		21	40	15	7	150	F	23 ± 0.1	19 ± 0.2	17 ± 0.1
							I	25 ± 1.6	23 ± 1.7	17 ± 1.8
		22	40	20	5	150	F	17 ± 1.1	19 ± 1.2	14 ± 1.2
							I	11 ± 1.5	24 ± 1.6	18 ± 1.6
		23	40	20	6	150	F	9 ± 0.5	11 ± 0.6	15 ± 0.6
							I	8 ± 1.3	19 ± 1.4	22 ± 1.4
		24	40	20	7	150	F	26 ± 1.3	23 ± 1.4	18 ± 1.4
							I	27 ± 1.5	25 ± 1.6	17 ± 1.7
		25	40	25	5	150	F	19 ± 0.6	27 ± 0.25	24 ± 0.6
							I	12 ± 1.0	14 ± 1.1	19 ± 1.1
		26	40	25	6	150	F	5 ± 0.2	17 ± 0.3	11 ± 0.2
							I	16 ± 1.4	25 ± 1.5	18 ± 1.6
		27	40	25	7	150	F	13 ± 1.3	23 ± 1.4	17 ± 1.4
							I	18 ± 1.5	24 ± 1.6	11 ± 1.7

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	20	15	5	150	F	125 ± 1.3	256 ± 1.4	311 ± 1.5
							I	210 ± 1.0	301 ± 1.1	245 ± 1.1
		2	20	15	6	150	F	114 ± 0.3	125 ± 0.4	109 ± 0.3
							I	196 ± 0.9	214 ± 1.0	301 ± 1.0
		3	20	15	7	150	F	318 ± 0.28	258 ± 1.4	147 ± 1.5
							I	285 ± 1.5	247 ± 1.6	286 ± 1.7
		4	20	20	5	150	F	240 ± 0.7	312 ± 0.8	186 ± 0.8
							I	247 ± 1.0	186 ± 1.1	147 ± 1.1
		5	20	20	6	150	F	287 ± 1.2	152 ± 1.3	96 ± 1.3
							I	268 ± 0.2	104 ± 0.3	79 ± 0.2
		6	20	20	7	150	F	310 ± 1.1	250 ± 1.2	147 ± 1.3
							I	247 ± 0.5	76 ± 0.6	52 ± 0.5
		7	20	25	5	150	F	301 ± 1.3	269 ± 1.4	145 ± 1.5
							I	248 ± 0.7	321 ± 0.8	258 ± 0.7
		8	20	25	6	150	F	147 ± 1.7	211 ± 1.8	140 ± 1.9
							I	163 ± 1.3	136 ± 1.4	114 ± 1.5
		9	20	25	7	150	F	214 ± 0.9	111 ± 1.0	146 ± 1.0
							I	305 ± 0.9	326 ± 1.0	218 ± 1.0
		10	30	15	5	150	F	219 ± 0.7	310 ± 0.8	249 ± 0.8
							I	308 ± 0.2	123 ± 0.3	142 ± 0.2
		11	30	15	6	150	F	314 ± 1.0	315 ± 1.1	247 ± 1.1
							I	333 ± 1.2	286 ± 1.3	141 ± 1.3
		12	30	15	7	150	F	316 ± 1.3	231 ± 1.4	245 ± 1.5
							I	331 ± 1.6	295 ± 1.7	217 ± 1.8
		13	30	20	5	150	F	285 ± 0.4	214 ± 0.5	193 ± 0.4
							I	274 ± 0.7	265 ± 0.8	247 ± 0.8
		14	30	20	6	150	F	263 ± 0.3	196 ± 0.4	147 ± 0.3
							I	158 ± 0.7	131 ± 0.8	92 ± 0.8
		15	30	20	7	150	F	96 ± 0.2	111 ± 0.3	89 ± 0.2
							I	74 ± 0.9	96 ± 1.0	152 ± 0.9
		16	30	25	5	150	F	157 ± 0.4	142 ± 0.5	153 ± 0.5
							I	153 ± 0.8	96 ± 0.9	89 ± 0.9
		17	30	25	6	150	F	215 ± 0.1	196 ± 0.2	174 ± 0.1
							I	227 ± 0.5	201 ± 0.6	174 ± 0.5
		18	30	25	7	150	F	316 ± 0.9	296 ± 1.0	198 ± 1.0
							I	217 ± 0.9	301 ± 1.0	325 ± 1.0
		19	40	15	5	150	F	305 ± 1.7	258 ± 1.8	211 ± 1.9
							I	318 ± 0.7	264 ± 0.8	211 ± 0.8
		20	40	15	6	150	F	204 ± 1.6	196 ± 1.7	121 ± 1.8
							I	79 ± 1.3	141 ± 1.4	88 ± 1.5
		21	40	15	7	150	F	92 ± 0.4	109 ± 0.5	71 ± 0.5
							I	83 ± 0.7	120 ± 0.8	157 ± 0.8
		22	40	20	5	150	F	82 ± 1.6	96 ± 1.7	175 ± 1.8
							I	110 ± 0.7	121 ± 0.8	215 ± 0.8
		23	40	20	6	150	F	201 ± 1.0	241 ± 1.1	312 ± 1.1
							I	314 ± 0.6	351 ± 0.7	296 ± 0.7
		24	40	20	7	150	F	201 ± 0.6	241 ± 0.7	263 ± 0.6
							I	152 ± 0.8	257 ± 0.9	310 ± 0.9
		25	40	25	5	150	F	143 ± 1.2	212 ± 1.3	325 ± 1.3
							I	189 ± 0.8	212 ± 0.9	287 ± 0.9
		26	40	25	6	150	F	192 ± 0.9	255 ± 1.0	312 ± 1.0
							I	355 ± 0.26	321 ± 0.2	254 ± 0.1
		27	40	25	7	150	F	152 ± 0.9	251 ± 1.0	194 ± 1.0
							I	265 ± 1.0	154 ± 1.1	132 ± 1.1

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	20	15	5	150	F	23 ± 0.7	34 ± 0.8	27 ± 0.7
							I	27 ± 1.2	35 ± 1.3	40 ± 1.4
		2	20	15	6	150	F	22 ± 0.4	30 ± 0.5	28 ± 0.4
							I	36 ± 0.7	41 ± 0.8	39 ± 0.8
		3	20	15	7	150	F	15 ± 1.3	24 ± 1.4	29 ± 1.4
							I	19 ± 0.6	24 ± 0.7	26 ± 0.7
		4	20	20	5	150	F	21 ± 1.3	32 ± 1.4	33 ± 1.5
							I	26 ± 0.9	31 ± 1.0	34 ± 1.0
		5	20	20	6	150	F	19 ± 0.5	32 ± 0.6	24 ± 0.6
							I	11 ± 0.4	19 ± 0.5	26 ± 0.4
		6	20	20	7	150	F	16 ± 0.5	31 ± 0.6	28 ± 0.6
							I	31 ± 1.1	34 ± 1.2	42 ± 1.2
		7	20	25	5	150	F	28 ± 0.6	32 ± 0.7	29 ± 0.7
							I	25 ± 0.6	36 ± 0.7	41 ± 0.6
		8	20	25	6	150	F	27 ± 0.2	32 ± 0.3	25 ± 0.2
							I	33 ± 1.1	44 ± 1.2	19 ± 1.2
		9	20	25	7	150	F	29 ± 1.2	19 ± 0.3	24 ± 1.3
							I	27 ± 1.6	32 ± 0.7	28 ± 1.8
		10	30	15	5	150	F	11 ± 1.3	17 ± 1.4	15 ± 1.4
							I	16 ± 1.7	26 ± 1.8	32 ± 1.9
		11	30	15	6	150	F	18 ± 1.4	24 ± 1.5	19 ± 1.5
							I	17 ± 0.7	24 ± 0.8	35 ± 0.8
		12	30	15	7	150	F	19 ± 1.2	24 ± 1.3	33 ± 1.4
							I	25 ± 0.7	34 ± 0.8	41 ± 0.8
		13	30	20	5	150	F	29 ± 0.5	33 ± 0.6	30 ± 0.6
							I	11 ± 0.2	14 ± 0.3	18 ± 0.3
		14	30	20	6	150	F	19 ± 1.3	21 ± 1.4	27 ± 1.4
							I	26 ± 0.6	30 ± 0.7	34 ± 0.7
		15	30	20	7	150	F	34 ± 0.3	29 ± 0.4	22 ± 0.3
							I	36 ± 0.3	33 ± 0.4	31 ± 0.3
		16	30	25	5	150	F	37 ± 0.8	36 ± 0.9	21 ± 0.8
							I	41 ± 0.8	38 ± 0.9	44 ± 0.9
		17	30	25	6	150	F	43 ± 0.4	38 ± 0.5	27 ± 0.5
							I	42 ± 0.8	39 ± 0.9	29 ± 0.9
		18	30	25	7	150	F	32 ± 1.5	36 ± 1.6	29 ± 1.7
							I	25 ± 0.5	24 ± 0.6	31 ± 0.5
		19	40	15	5	150	F	29 ± 0.2	32 ± 0.3	35 ± 0.2
							I	18 ± 1.0	24 ± 1.1	31 ± 1.1
		20	40	15	6	150	F	15 ± 0.7	19 ± 0.8	24 ± 0.8
							I	16 ± 1.5	25 ± 1.6	27 ± 1.7
		21	40	15	7	150	F	24 ± 0.7	32 ± 0.8	35 ± 0.8
							I	29 ± 0.6	34 ± 0.7	41 ± 0.7
		22	40	20	5	150	F	39 ± 0.09	34 ± 1.3	35 ± 1.4
							I	41 ± 0.3	38 ± 0.4	25 ± 0.4
		23	40	20	6	150	F	36 ± 0.3	29 ± 0.4	25 ± 0.3
							I	42 ± 1.5	38 ± 1.6	41 ± 1.7
		24	40	20	7	150	F	28 ± 1.7	33 ± 1.8	36 ± 1.8
							I	44 ± 0.5	39 ± 0.6	31 ± 0.5
		25	40	25	5	150	F	35 ± 0.5	36 ± 0.6	28 ± 0.5
							I	37 ± 1.3	41 ± 1.4	43 ± 1.5
		26	40	25	6	150	F	34 ± 0.2	36 ± 0.3	27 ± 0.2
							I	39 ± 1.7	46 ± 0.21	32 ± 1.9
		27	40	25	7	150	F	29 ± 0.8	21 ± 0.9	32 ± 0.9
							I	35 ± 0.9	43 ± 1.0	39 ± 1.0

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	20	15	5	150	F	6 ± 0.07	8 ± 0.08	9 ± 0.8
							I	13 ± 1.2	11 ± 0.3	16 ± 1.3
		2	20	15	6	150	F	5 ± 0.06	7 ± 0.07	9 ± 0.6
							I	9 ± 0.07	11 ± 0.08	14 ± 0.8
		3	20	15	7	150	F	10 ± 0.2	7 ± 0.1	9 ± 1.1
							I	9 ± 1.3	14 ± 1.4	12 ± 1.5
		4	20	20	5	150	F	9 ± 0.9	7 ± 0.3	6 ± 1.0
							I	12 ± 0.1	17 ± 0.2	15 ± 0.1
		5	20	20	6	150	F	5 ± 0.4	7 ± 0.5	2 ± 0.4
							I	9 ± 0.5	12 ± 0.6	17 ± 0.6
		6	20	20	7	150	F	6 ± 0.9	8 ± 1.0	9 ± 1.0
							I	9 ± 1.7	12 ± 1.8	11 ± 1.9
		7	20	25	5	150	F	2 ± 1.0	8 ± 1.1	9 ± 1.1
							I	13 ± 0.9	9 ± 1.0	15 ± 1.0
		8	20	25	6	150	F	4 ± 0.6	7 ± 0.7	9 ± 0.6
							I	13 ± 1.3	11 ± 1.4	7 ± 1.4
		9	20	25	7	150	F	3 ± 1.6	7 ± 0.7	5 ± 1.8
							I	15 ± 1.5	17 ± 1.6	11 ± 1.6
		10	30	15	5	150	F	10 ± 0.7	7 ± 0.8	9 ± 0.8
							I	12 ± 1.0	17 ± 1.1	14 ± 1.1
		11	30	15	6	150	F	7 ± 0.2	2 ± 0.3	9 ± 0.2
							I	7 ± 0.02	6 ± 1.1	10 ± 1.1
		12	30	15	7	150	F	11 ± 0.7	9 ± 0.8	5 ± 0.8
							I	12 ± 0.5	15 ± 1.6	13 ± 1.7
		13	30	20	5	150	F	7 ± 0.9	5 ± 1.0	9 ± 1.0
							I	14 ± 0.4	9 ± 1.5	16 ± 1.5
		14	30	20	6	150	F	5 ± 1.5	7 ± 1.6	9 ± 1.6
							I	15 ± 1.0	16 ± 1.1	10 ± 1.1
		15	30	20	7	150	F	10 ± 1.4	9 ± 0.5	8 ± 1.6
							I	11 ± 0.9	17 ± 1.0	15 ± 1.1
		16	30	25	5	150	F	9 ± 0.1	4 ± 1.2	10 ± 1.2
							I	2 ± 0.5	11 ± 0.6	17 ± 0.5
		17	30	25	6	150	F	4 ± 1.2	6 ± 1.3	9 ± 1.3
							I	3 ± 1.1	8 ± 1.2	6 ± 1.3
		18	30	25	7	150	F	4 ± 0.08	10 ± 0.9	7 ± 0.09
							I	9 ± 0.2	16 ± 1.3	12 ± 1.3
		19	40	15	5	150	F	5 ± 0.2	9 ± 0.3	6 ± 0.3
							I	11 ± 0.5	17 ± 0.6	14 ± 0.5
		20	40	15	6	150	F	2 ± 1.3	7 ± 1.4	5 ± 1.4
							I	6 ± 1.0	14 ± 1.1	8 ± 1.1
		21	40	15	7	150	F	5 ± 0.8	7 ± 0.9	10 ± 0.9
							I	12 ± 1.6	15 ± 1.7	18 ± 0.19
		22	40	20	5	150	F	7 ± 0.3	5 ± 0.1	9 ± 1.1
							I	14 ± 1.4	17 ± 1.5	13 ± 1.6
		23	40	20	6	150	F	7 ± 0.03	5 ± 0.4	11 ± 0.17
							I	8 ± 0.03	7 ± 0.4	12 ± 0.3
		24	40	20	7	150	F	4 ± 0.2	10 ± 0.3	6 ± 0.3
							I	16 ± 1.2	17 ± 1.3	14 ± 1.3
		25	40	25	5	150	F	3 ± 0.2	7 ± 0.3	5 ± 0.3
							I	9 ± 0.2	15 ± 0.3	12 ± 0.2
		26	40	25	6	150	F	5 ± 0.5	8 ± 0.6	7 ± 0.05
							I	11 ± 0.6	12 ± 0.7	13 ± 0.6
		27	40	25	7	150	F	7 ± 0.9	9 ± 1.0	9 ± 0.2
							I	14 ± 0.8	12 ± 0.9	16 ± 0.9

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	20	15	5	150	F	92 ± 0.9	77 ± 1.0	98 ± 1.0
							I	91 ± 0.2	111 ± 0.3	144 ± 0.2
		2	20	15	6	150	F	75 ± 1.7	121 ± 1.8	149 ± 1.9
							I	121 ± 0.2	154 ± 0.3	132 ± 0.2
		3	20	15	7	150	F	63 ± 0.4	165 ± 0.5	158 ± 0.4
							I	45 ± 0.7	171 ± 0.8	147 ± 0.8
		4	20	20	5	150	F	86 ± 1.2	66 ± 1.3	125 ± 1.4
							I	125 ± 0.2	78 ± 0.3	117 ± 0.3
		5	20	20	6	150	F	149 ± 0.8	89 ± 0.9	114 ± 0.9
							I	131 ± 1.3	151 ± 1.4	125 ± 1.4
		6	20	20	7	150	F	25 ± 0.3	148 ± 0.4	149 ± 0.3
							I	145 ± 1.1	123 ± 1.2	136 ± 1.2
		7	20	25	5	150	F	111 ± 1.5	144 ± 1.6	157 ± 1.6
							I	136 ± 0.4	156 ± 0.5	141 ± 0.4
		8	20	25	6	150	F	128 ± 0.2	149 ± 0.3	132 ± 0.2
							I	149 ± 1.2	174 ± 1.3	122 ± 1.3
		9	20	25	7	150	F	151 ± 1.1	85 ± 1.2	111 ± 1.2
							I	141 ± 0.6	79 ± 0.7	158 ± 0.7
		10	30	15	5	150	F	165 ± 0.6	111 ± 0.7	117 ± 0.6
							I	119 ± 0.5	133 ± 0.2	133 ± 0.1
		11	30	15	6	150	F	96 ± 1.4	98 ± 1.5	155 ± 1.5
							I	85 ± 0.7	112 ± 0.8	141 ± 0.8
		12	30	15	7	150	F	96 ± 1.2	154 ± 1.3	137 ± 1.4
							I	48 ± 1.0	129 ± 1.1	166 ± 1.2
		13	30	20	5	150	F	56 ± 0.7	134 ± 0.8	144 ± 0.7
							I	75 ± 0.4	129 ± 0.5	151 ± 0.5
		14	30	20	6	150	F	94 ± 0.9	112 ± 1.0	149 ± 1.0
							I	111 ± 0.9	154 ± 1.0	138 ± 0.9
		15	30	20	7	150	F	121 ± 0.7	128 ± 0.8	133 ± 0.7
							I	145 ± 0.1	171 ± 0.2	152 ± 0.1
		16	30	25	5	150	F	168 ± 0.5	129 ± 0.6	169 ± 0.5
							I	124 ± 0.7	133 ± 0.8	148 ± 0.8
		17	30	25	6	150	F	112 ± 0.7	128 ± 0.8	122 ± 0.8
							I	145 ± 0.1	85 ± 0.2	166 ± 0.1
		18	30	25	7	150	F	98 ± 0.2	79 ± 0.3	133 ± 0.2
							I	75 ± 1.4	162 ± 1.5	159 ± 1.6
		19	40	15	5	150	F	71 ± 1.3	74 ± 1.4	130 ± 1.4
							I	62 ± 0.1	92 ± 0.2	159 ± 0.1
		20	40	15	6	150	F	125 ± 0.2	112 ± 0.3	167 ± 0.2
							I	148 ± 1.5	147 ± 1.6	111 ± 1.7
		21	40	15	7	150	F	133 ± 1.5	166 ± 1.6	133 ± 1.7
							I	147 ± 0.8	74 ± 0.9	125 ± 0.9
		22	40	20	5	150	F	124 ± 0.2	152 ± 0.3	147 ± 0.2
							I	135 ± 0.9	110 ± 1.0	171 ± 1.0
		23	40	20	6	150	F	147 ± 1.2	117 ± 1.3	176 ± 0.25
							I	144 ± 1.6	124 ± 1.7	147 ± 1.8
		24	40	20	7	150	F	125 ± 1.6	131 ± 1.7	138 ± 1.7
							I	136 ± 0.1	148 ± 0.2	152 ± 0.1
		25	40	25	5	150	F	159 ± 0.7	168 ± 0.8	147 ± 0.8
							I	148 ± 1.3	171 ± 1.4	178 ± 0.18
		26	40	25	6	150	F	133 ± 1.3	167 ± 1.4	163 ± 1.5
							I	111 ± 1.5	164 ± 1.6	170 ± 1.7
		27	40	25	7	150	F	98 ± 1.2	112 ± 1.3	138 ± 1.3
							I	77 ± 1.5	132 ± 1.6	164 ± 1.7

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	20	15	5	150	F	254 ± 0.2	145 ± 0.3	178 ± 0.2
							I	215 ± 0.4	196 ± 0.5	175 ± 0.5
		2	20	15	6	150	F	185 ± 1.7	200 ± 1.8	234 ± 1.9
							I	231 ± 1.4	241 ± 1.5	270 ± 1.6
		3	20	15	7	150	F	215 ± 0.8	198 ± 0.9	177 ± 0.9
							I	244 ± 0.2	270 ± 0.3	159 ± 0.2
		4	20	20	5	150	F	201 ± 0.5	215 ± 0.6	196 ± 0.6
							I	215 ± 0.6	248 ± 0.7	195 ± 0.7
		5	20	20	6	150	F	158 ± 0.6	171 ± 0.7	156 ± 0.6
							I	133 ± 0.4	148 ± 0.5	201 ± 0.5
		6	20	20	7	150	F	123 ± 0.6	142 ± 0.7	178 ± 0.7
							I	95 ± 0.3	115 ± 0.4	97 ± 0.3
		7	20	25	5	150	F	47 ± 1.2	96 ± 1.3	118 ± 1.3
							I	258 ± 0.4	198 ± 0.5	157 ± 0.5
		8	20	25	6	150	F	214 ± 1.5	222 ± 1.6	254 ± 1.7
							I	269 ± 0.9	251 ± 1.0	216 ± 1.0
		9	20	25	7	150	F	234 ± 0.2	242 ± 0.3	196 ± 0.2
							I	251 ± 0.6	210 ± 0.7	187 ± 0.6
		10	30	15	5	150	F	245 ± 0.5	214 ± 0.6	184 ± 0.6
							I	212 ± 0.3	185 ± 0.4	88 ± 0.3
		11	30	15	6	150	F	114 ± 1.1	87 ± 1.2	96 ± 1.2
							I	99 ± 0.6	74 ± 0.7	57 ± 0.7
		12	30	15	7	150	F	169 ± 0.1	114 ± 0.2	84 ± 0.1
							I	175 ± 0.4	141 ± 0.5	154 ± 0.4
		13	30	20	5	150	F	214 ± 0.9	209 ± 1.0	117 ± 1.0
							I	211 ± 0.6	174 ± 0.7	158 ± 0.7
		14	30	20	6	150	F	179 ± 1.6	111 ± 1.7	154 ± 1.7
							I	256 ± 1.6	201 ± 1.7	199 ± 1.7
		15	30	20	7	150	F	197 ± 1.3	222 ± 1.4	187 ± 1.4
							I	222 ± 1.5	149 ± 1.6	218 ± 1.7
		16	30	25	5	150	F	219 ± 0.9	198 ± 1.0	152 ± 1.0
							I	266 ± 0.4	233 ± 0.5	195 ± 0.5
		17	30	25	6	150	F	214 ± 1.4	216 ± 1.5	149 ± 1.5
							I	251 ± 0.9	232 ± 1.0	196 ± 1.0
		18	30	25	7	150	F	218 ± 0.2	198 ± 0.3	175 ± 0.2
							I	247 ± 0.7	215 ± 0.8	197 ± 0.8
		19	40	15	5	150	F	215 ± 1.3	187 ± 1.4	154 ± 1.5
							I	269 ± 1.5	254 ± 1.6	211 ± 1.7
		20	40	15	6	150	F	216 ± 1.7	198 ± 1.8	175 ± 1.9
							I	212 ± 1.1	199 ± 1.2	210 ± 1.2
		21	40	15	7	150	F	198 ± 0.8	185 ± 0.9	164 ± 0.9
							I	178 ± 1.7	192 ± 1.8	211 ± 1.9
		22	40	20	5	150	F	214 ± 1.2	175 ± 1.3	165 ± 1.3
							I	270 ± 0.2	269 ± 1.5	214 ± 1.5
		23	40	20	6	150	F	210 ± 1.6	232 ± 1.7	176 ± 1.8
							I	268 ± 0.3	197 ± 0.4	126 ± 0.4
		24	40	20	7	150	F	250 ± 0.3	235 ± 0.4	197 ± 0.4
							I	221 ± 0.3	252 ± 0.4	265 ± 0.3
		25	40	25	5	150	F	227 ± 0.22	218 ± 0.6	198 ± 0.6
							I	138 ± 0.3	142 ± 0.4	215 ± 0.4
		26	40	25	6	150	F	147 ± 0.2	211 ± 0.3	235 ± 0.2
							I	159 ± 1.7	178 ± 1.8	211 ± 1.8
		27	40	25	7	150	F	212 ± 0.3	199 ± 0.4	164 ± 0.3
							I	220 ± 0.2	195 ± 0.3	212 ± 0.2

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

CS 02

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	30	15	5	150	F	8 ± 0.6	11 ± 0.4	13 ± 0.5
							I	11 ± 0.2	12 ± 0.1	7 ± 0.1
		2	30	15	6	150	F	9 ± 0.3	7 ± 0.7	5 ± 0.1
							I	5 ± 1.2	8 ± 0.7	11 ± 0.2
		3	30	15	7	150	F	4 ± 0.2	2 ± 0.6	8 ± 0.9
							I	12 ± 1.7	13 ± 1.0	10 ± 1.4
		4	30	20	5	150	F	7 ± 0.3	9 ± 0.8	12 ± 1.1
							I	8 ± 0.1	12 ± 0.1	7 ± 0.1
		5	30	20	6	150	F	11 ± 0.4	13 ± 0.3	9 ± 0.3
							I	13 ± 1.3	15 ± 0.8	14 ± 1.1
		6	30	20	7	150	F	12 ± 0.6	9 ± 0.4	4 ± 0.5
							I	5 ± 0.9	12 ± 0.6	10 ± 0.8
		7	30	25	5	150	F	7 ± 0.5	8 ± 0.3	3 ± 0.4
							I	9 ± 0.3	11 ± 0.6	12 ± 0.9
		8	30	25	6	150	F	10 ± 1.5	8 ± 0.9	5 ± 0.2
							I	13 ± 0.6	9 ± 0.4	10 ± 0.5
		9	30	25	7	150	F	11 ± 0.3	8 ± 0.2	12 ± 0.3
							I	12 ± 0.7	9 ± 0.4	5 ± 0.6
		10	40	15	5	150	F	2 ± 0.6	3 ± 0.4	7 ± 0.5
							I	6 ± 0.3	9 ± 0.6	11 ± 0.9
		11	40	15	6	150	F	9 ± 0.9	13 ± 0.5	4 ± 0.07
							I	10 ± 0.2	9 ± 0.1	4 ± 0.02
		12	40	15	7	150	F	12 ± 0.7	14 ± 0.4	11 ± 0.6
							I	8 ± 0.7	6 ± 1.0	9 ± 1.5
		13	40	20	5	150	F	12 ± 1.4	13 ± 0.8	4 ± 1.2
							I	2 ± 0.6	7 ± 1.0	11 ± 1.4
		14	40	20	6	150	F	9 ± 0.6	12 ± 0.4	8 ± 0.5
							I	14 ± 0.19	11 ± 0.4	9 ± 0.6
		15	40	20	7	150	F	10 ± 1.3	8 ± 0.8	4 ± 1.1
							I	9 ± 0.2	6 ± 0.2	12 ± 0.2
		16	40	25	5	150	F	7 ± 0.7	9 ± 1.0	11 ± 1.4
							I	12 ± 0.4	4 ± 0.2	8 ± 0.3
		17	40	25	6	150	F	15 ± 0.13	12 ± 0.7	8 ± 1.0
							I	8 ± 0.8	11 ± 0.5	7 ± 0.7
		18	40	25	7	150	F	9 ± 0.8	5 ± 0.5	8 ± 0.7
							I	11 ± 0.3	8 ± 0.7	13 ± 1.1

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	30	15	5	150	F	312 ± 1.7	286 ± 0.2	174 ± 1.4
							I	245 ± 0.4	253 ± 0.3	149 ± 0.4
		2	30	15	6	150	F	123 ± 1.8	268 ± 1.0	178 ± 1.5
							I	365 ± 0.4	331 ± 0.2	296 ± 0.3
		3	30	15	7	150	F	278 ± 0.1	261 ± 0.1	279 ± 0.1
							I	324 ± 1.8	310 ± 1.0	287 ± 1.5
		4	30	20	5	150	F	247 ± 1.2	313 ± 0.7	281 ± 1.1
							I	312 ± 1.6	248 ± 1.3	314 ± 1.4
		5	30	20	6	150	F	252 ± 0.6	211 ± 0.4	198 ± 0.5
							I	373 ± 0.34	312 ± 0.8	296 ± 1.2
		6	30	20	7	150	F	212 ± 1.4	245 ± 0.8	231 ± 1.2
							I	311 ± 1.7	322 ± 1.0	296 ± 1.4
		7	30	25	5	150	F	126 ± 0.6	145 ± 0.4	211 ± 0.5
							I	333 ± 1.1	313 ± 0.7	299 ± 0.9
		8	30	25	6	150	F	210 ± 0.2	231 ± 0.1	245 ± 0.2
							I	171 ± 1.6	192 ± 0.9	211 ± 1.4
		9	30	25	7	150	F	156 ± 1.4	165 ± 0.8	195 ± 1.2
							I	370 ± 1.7	297 ± 1.0	248 ± 1.5
		10	40	15	5	150	F	258 ± 1.5	264 ± 0.9	231 ± 1.3
							I	366 ± 1.1	344 ± 0.7	257 ± 0.9
		11	40	15	6	150	F	277 ± 0.3	248 ± 0.2	178 ± 0.2
							I	319 ± 1.0	326 ± 0.6	349 ± 0.8
		12	40	15	7	150	F	256 ± 1.5	241 ± 0.9	199 ± 1.2
							I	285 ± 1.7	271 ± 1.0	216 ± 1.4
		13	40	20	5	150	F	251 ± 0.8	268 ± 0.5	197 ± 0.7
							I	313 ± 1.1	325 ± 0.7	291 ± 1.0
		14	40	20	6	150	F	286 ± 0.25	211 ± 0.8	311 ± 1.1
							I	348 ± 0.2	289 ± 0.1	333 ± 0.1
		15	40	20	7	150	F	241 ± 1.3	212 ± 0.8	261 ± 1.1
							I	351 ± 0.5	312 ± 0.3	318 ± 0.4
		16	40	25	5	150	F	258 ± 1.5	261 ± 0.9	285 ± 1.2
							I	371 ± 0.7	324 ± 0.4	312 ± 0.6
		17	40	25	6	150	F	250 ± 1.9	275 ± 1.1	187 ± 1.6
							I	345 ± 1.5	312 ± 0.9	299 ± 1.2
		18	40	25	7	150	F	275 ± 1.0	265 ± 0.6	241 ± 0.8
							I	313 ± 1.0	324 ± 0.6	351 ± 0.9

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	30	15	5	150	F	19 ± 0.8	11 ± 0.5	8 ± 0.7
							I	21 ± 0.2	30 ± 0.2	29 ± 0.2
		2	30	15	6	150	F	18 ± 1.1	14 ± 0.7	16 ± 1.0
							I	5 ± 1.3	9 ± 0.8	11 ± 1.1
		3	30	15	7	150	F	4 ± 1.5	6 ± 0.9	7 ± 0.3
							I	13 ± 1.8	25 ± 1.1	28 ± 1.5
		4	30	20	5	150	F	14 ± 0.4	19 ± 0.3	20 ± 0.4
							I	21 ± 0.8	24 ± 0.5	33 ± 0.7
		5	30	20	6	150	F	9 ± 0.3	12 ± 0.2	17 ± 0.3
							I	25 ± 0.8	30 ± 0.5	25 ± 0.7
		6	30	20	7	150	F	14 ± 0.2	19 ± 0.1	24 ± 0.2
							I	17 ± 0.9	24 ± 0.6	32 ± 0.8
		7	30	25	5	150	F	19 ± 0.5	15 ± 0.3	17 ± 0.4
							I	8 ± 0.9	12 ± 0.5	11 ± 0.8
		8	30	25	6	150	F	8 ± 0.1	15 ± 0.1	18 ± 0.1
							I	5 ± 0.5	7 ± 0.3	11 ± 0.5
		9	30	25	7	150	F	6 ± 1.0	9 ± 0.6	12 ± 0.9
							I	12 ± 1.0	31 ± 0.6	25 ± 0.8
		10	40	15	5	150	F	10 ± 1.9	19 ± 1.1	14 ± 1.6
							I	30 ± 0.8	13 ± 0.5	27 ± 0.7
		11	40	15	6	150	F	19 ± 1.8	24 ± 1.0	28 ± 1.5
							I	15 ± 1.5	17 ± 0.9	25 ± 1.2
		12	40	15	7	150	F	12 ± 0.5	21 ± 0.17	16 ± 0.4
							I	11 ± 0.8	25 ± 0.5	31 ± 0.7
		13	40	20	5	150	F	16 ± 1.8	19 ± 1.0	24 ± 1.5
							I	15 ± 0.8	17 ± 0.5	25 ± 0.7
		14	40	20	6	150	F	9 ± 1.1	17 ± 0.6	20 ± 0.9
							I	34 ± 0.19	31 ± 0.4	24 ± 0.6
		15	40	20	7	150	F	6 ± 0.6	12 ± 0.4	17 ± 0.6
							I	8 ± 0.9	14 ± 0.5	20 ± 0.7
		16	40	25	5	150	F	7 ± 1.3	9 ± 0.8	14 ± 1.1
							I	11 ± 0.9	15 ± 0.5	24 ± 0.8
		17	40	25	6	150	F	4 ± 1.0	9 ± 0.6	14 ± 0.8
							I	6 ± 0.1	8 ± 0.1	17 ± 0.1
		18	40	25	7	150	F	8 ± 1.0	15 ± 0.6	19 ± 0.9
							I	12 ± 1.1	19 ± 0.7	24 ± 0.9

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	30	15	5	150	F	ND	ND	0.12 ± 0.01
							I	9 ± 0.4	15 ± 0.8	12 ± 1.2
		2	30	15	6	150	F	ND	0.2 ± 0.03	0.87 ± 0.3
							I	7 ± 0.8	15 ± 0.5	14 ± 0.7
		3	30	15	7	150	F	ND	0.12 ± 0.02	0.7 ± 0.02
							I	15 ± 0.7	18 ± 0.4	22 ± 0.6
		4	30	20	5	150	F	ND	ND	ND
							I	14 ± 1.0	20 ± 0.6	11 ± 0.8
		5	30	20	6	150	F	ND	ND	0.26 ± 0.01
							I	7 ± 0.4	12 ± 0.3	19 ± 0.4
		6	30	20	7	150	F	ND	0.1 ± 0.02	0.9 ± 0.05
							I	11 ± 1.2	20 ± 0.7	15 ± 1.1
		7	30	25	5	150	F	ND	0.14 ± 0.4	0.78 ± 0.03
							I	12 ± 0.6	8 ± 0.4	14 ± 0.5
		8	30	25	6	150	F	ND	ND	0.1 ± 0.02
							I	11 ± 1.2	7 ± 0.7	17 ± 1.1
		9	30	25	7	150	F	ND	ND	ND
							I	11 ± 1.8	15 ± 1.0	19 ± 1.5
		10	40	15	5	150	F	ND	ND	0.4 ± 0.12
							I	14 ± 1.9	18 ± 1.1	21 ± 1.6
		11	40	15	6	150	F	0.1 ± 0.02	0.2 ± 0.01	0.7 ± 0.3
							I	12 ± 0.8	9 ± 0.5	7 ± 0.7
		12	40	15	7	150	F	0.1 ± 0.04	0.2 ± 0.8	0.56 ± 0.1
							I	7 ± 0.08	5 ± 0.05	9 ± 0.7
		13	40	20	5	150	F	ND	ND	ND
							I	12 ± 0.3	14 ± 0.2	15 ± 0.2
		14	40	20	6	150	F	ND	ND	0.18 ± 0.02
							I	18 ± 0.7	20 ± 0.4	24 ± 0.25
		15	40	20	7	150	F	ND	ND	ND
							I	17 ± 0.3	14 ± 0.2	21 ± 0.2
		16	40	25	5	150	F	ND	ND	ND
							I	15 ± 0.9	21 ± 0.6	11 ± 0.8
		17	40	25	6	150	F	1 ± 0.14	0.2 ± 0.03	0.47 ± 0.01
							I	14 ± 0.9	12 ± 0.5	17 ± 0.7
		18	40	25	7	150	F	ND	ND	0.6 ± 0.14
							I	11 ± 0.5	14 ± 0.3	19 ± 0.5

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	30	15	5	150	F	59 ± 0.2	62 ± 0.1	70 ± 0.2
							I	27 ± 1.1	45 ± 0.7	65 ± 0.9
		2	30	15	6	150	F	32 ± 0.8	41 ± 0.5	47 ± 0.7
							I	47 ± 1.7	52 ± 1.0	75 ± 0.23
		3	30	15	7	150	F	51 ± 0.8	45 ± 0.5	23 ± 0.7
							I	61 ± 0.7	24 ± 0.4	32 ± 0.6
		4	30	20	5	150	F	51 ± 1.4	60 ± 0.8	51 ± 1.2
							I	25 ± 0.4	33 ± 0.2	47 ± 0.3
		5	30	20	6	150	F	35 ± 0.3	40 ± 0.2	49 ± 0.3
							I	47 ± 1.7	51 ± 1.0	58 ± 1.4
		6	30	20	7	150	F	37 ± 1.8	43 ± 1.1	48 ± 1.6
							I	24 ± 0.5	37 ± 0.3	56 ± 0.5
		7	30	25	5	150	F	48 ± 0.5	51 ± 0.3	58 ± 0.4
							I	61 ± 1.5	57 ± 0.9	69 ± 1.3
		8	30	25	6	150	F	28 ± 0.2	32 ± 0.2	48 ± 0.2
							I	57 ± 1.9	41 ± 1.1	61 ± 1.6
		9	30	25	7	150	F	47 ± 0.9	28 ± 0.5	48 ± 0.8
							I	32 ± 1.0	51 ± 0.6	26 ± 0.9
		10	40	15	5	150	F	32 ± 0.8	57 ± 0.5	64 ± 0.7
							I	34 ± 1.3	49 ± 0.8	69 ± 1.1
		11	40	15	6	150	F	41 ± 0.6	37 ± 0.4	45 ± 0.6
							I	18 ± 0.8	48 ± 0.5	63 ± 0.6
		12	40	15	7	150	F	47 ± 1.1	52 ± 0.7	68 ± 1.0
							I	35 ± 1.5	41 ± 0.9	45 ± 1.2
		13	40	20	5	150	F	69 ± 0.11	57 ± 0.6	44 ± 0.8
							I	31 ± 0.1	29 ± 0.1	15 ± 0.1
		14	40	20	6	150	F	44 ± 0.4	52 ± 0.3	47 ± 0.3
							I	33 ± 0.6	47 ± 0.4	65 ± 0.5
		15	40	20	7	150	F	61 ± 1.0	58 ± 0.6	64 ± 0.9
							I	47 ± 1.9	57 ± 1.1	63 ± 1.6
		16	40	25	5	150	F	56 ± 1.1	66 ± 0.6	59 ± 0.9
							I	41 ± 1.0	38 ± 0.6	45 ± 0.9
		17	40	25	6	150	F	44 ± 0.6	29 ± 0.4	60 ± 0.5
							I	32 ± 1.4	29 ± 0.8	48 ± 1.2
		18	40	25	7	150	F	57 ± 1.8	61 ± 1.0	56 ± 1.5
							I	27 ± 1.6	61 ± 0.9	58 ± 1.4

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	30	15	5	150	F	89 ± 0.8	124 ± 0.5	191 ± 0.7
							I	112 ± 1.1	115 ± 0.6	178 ± 0.9
		2	30	15	6	150	F	110 ± 0.2	125 ± 0.2	155 ± 0.2
							I	125 ± 1.1	134 ± 0.6	198 ± 0.32
		3	30	15	7	150	F	154 ± 0.8	142 ± 0.5	161 ± 0.7
							I	134 ± 1.7	135 ± 1.0	185 ± 1.4
		4	30	20	5	150	F	157 ± 1.0	125 ± 0.6	170 ± 0.8
							I	129 ± 1.5	141 ± 0.9	162 ± 1.3
		5	30	20	6	150	F	111 ± 1.6	126 ± 1.0	157 ± 1.4
							I	75 ± 1.1	135 ± 0.7	126 ± 1.0
		6	30	20	7	150	F	13 ± 1.6	131 ± 0.9	148 ± 1.3
							I	61 ± 1.1	124 ± 0.6	123 ± 0.9
		7	30	25	5	150	F	48 ± 1.2	96 ± 0.7	104 ± 1.0
							I	89 ± 0.5	145 ± 0.3	169 ± 0.4
		8	30	25	6	150	F	25 ± 1.3	134 ± 0.8	128 ± 1.1
							I	36 ± 1.3	154 ± 0.8	111 ± 1.1
		9	30	25	7	150	F	75 ± 0.9	98 ± 0.5	162 ± 0.8
							I	95 ± 1.3	147 ± 0.8	184 ± 1.1
		10	40	15	5	150	F	48 ± 0.3	99 ± 0.2	111 ± 0.2
							I	27 ± 0.5	129 ± 0.3	124 ± 0.5
		11	40	15	6	150	F	63 ± 1.4	121 ± 0.8	171 ± 1.2
							I	85 ± 1.1	122 ± 0.6	186 ± 0.9
		12	40	15	7	150	F	149 ± 0.9	134 ± 0.5	157 ± 0.8
							I	25 ± 1.8	134 ± 1.0	125 ± 1.5
		13	40	20	5	150	F	26 ± 1.1	125 ± 0.6	148 ± 0.9
							I	67 ± 1.6	124 ± 0.9	179 ± 1.3
		14	40	20	6	150	F	85 ± 0.3	148 ± 0.2	168 ± 0.3
							I	162 ± 0.3	135 ± 0.2	192 ± 0.2
		15	40	20	7	150	F	152 ± 0.3	168 ± 0.2	167 ± 0.2
							I	148 ± 1.3	142 ± 0.8	162 ± 1.1
		16	40	25	5	150	F	136 ± 0.3	154 ± 0.2	179 ± 0.19
							I	25 ± 0.2	98 ± 0.2	112 ± 0.2
		17	40	25	6	150	F	56 ± 0.5	87 ± 0.3	148 ± 0.5
							I	94 ± 0.6	156 ± 0.4	165 ± 0.5
		18	40	25	7	150	F	97 ± 1.0	163 ± 0.6	177 ± 0.9
							I	18 ± 0.9	125 ± 0.5	189 ± 0.8

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

CS 03

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	20	15	6	150	F	8 ± 0.6	10 ± 0.6	12 ± 0.5
							I	5 ± 0.2	2 ± 0.02	1 ± 0.02
		2	20	15	7	150	F	4 ± 1.3	9 ± 1.1	11 ± 1.1
							I	18 ± 0.17	3 ± 1.0	6 ± 0.1
		3	20	15	8	150	F	11 ± 1.0	13 ± 0.9	15 ± 0.9
							I	2 ± 1.7	4 ± 1.4	5 ± 0.5
		4	20	20	6	150	F	13 ± 1.3	11 ± 1.2	7 ± 1.2
							I	3 ± 0.1	2 ± 0.02	4 ± 0.1
		5	20	20	7	150	F	12 ± 0.4	9 ± 0.4	12 ± 0.3
							I	6 ± 1.3	6 ± 1.2	1 ± 0.2
		6	20	20	8	150	F	4 ± 0.6	3 ± 0.16	9 ± 0.6
							I	5 ± 0.9	1 ± 0.8	3 ± 0.8
		7	20	25	6	150	F	9 ± 0.5	12 ± 0.5	15 ± 0.4
							I	2 ± 1.1	4 ± 1.0	6 ± 0.02
		8	20	25	7	150	F	8 ± 1.5	12 ± 1.3	14 ± 1.3
							I	ND	ND	4 ± 0.5
		9	20	25	8	150	F	11 ± 0.3	9 ± 0.4	15 ± 0.3
							I	ND	6 ± 0.7	4 ± 0.6
		10	30	15	6	150	F	12 ± 0.6	14 ± 0.6	13 ± 0.5
							I	2 ± 0.1	3 ± 1.0	2 ± 1.0
		11	30	15	7	150	F	7 ± 0.9	12 ± 0.8	11 ± 0.8
							I	3 ± 0.2	3 ± 0.3	5 ± 0.02
		12	30	15	8	150	F	4 ± 0.7	9 ± 0.6	14 ± 0.6
							I	5 ± 0.7	4 ± 0.5	6 ± 0.6
		13	30	20	6	150	F	9 ± 1.4	13 ± 1.2	14 ± 1.3
							I	1 ± 0.03	2 ± 0.04	4 ± 0.05
		14	30	20	7	150	F	10 ± 0.6	10 ± 0.6	12 ± 0.5
							I	6 ± 0.7	4 ± 0.6	2 ± 0.06
		15	30	20	8	150	F	11 ± 1.3	14 ± 1.2	13 ± 1.2
							I	1 ± 0.02	ND	2 ± 0.2
		16	30	25	6	150	F	6 ± 1.7	12 ± 1.4	11 ± 1.5
							I	2 ± 0.04	2 ± 0.4	4 ± 0.3
		17	30	25	7	150	F	4 ± 0.2	9 ± 1.1	15 ± 1.1
							I	4 ± 0.8	3 ± 0.8	6 ± 0.7
		18	30	25	8	150	F	8 ± 0.8	13 ± 0.7	9 ± 0.07
							I	6 ± 0.3	7 ± 0.1	2 ± 1.1
		19	40	15	6	150	F	9 ± 1.7	12 ± 1.4	11 ± 1.5
							I	ND	ND	ND
		20	40	15	7	150	F	11 ± 1.8	14 ± 1.5	7 ± 1.6
							I	ND	ND	ND
		21	40	15	8	150	F	16 ± 0.19	15 ± 0.2	11 ± 0.1
							I	2 ±0.2	1 ± 0.05	3 ± 0.3
		22	40	20	6	150	F	12 ± 1.2	11 ± 1.1	9 ± 1.1
							I	2 ± 0.04	2 ± 1.4	4 ± 1.5
		23	40	20	7	150	F	15 ± 0.6	14 ± 0.6	7 ± 0.5
							I	4 ± 1.4	3 ± 0.02	2 ± 0.03
		24	40	20	8	150	F	10 ± 1.4	11 ± 1.2	7 ± 0.3
							I	6 ± 1.7	5 ± 1.4	1 ± 0.03
		25	40	25	6	150	F	11 ± 0.6	6 ± 0.6	10 ± 0.6
							I	4 ± 0.05	1 ± 0.03	3 ± 0.14
		26	40	25	7	150	F	14 ± 0.2	11 ± 0.3	10 ± 0.2
							I	3 ± 0.02	4 ± 1.4	6 ± 0.4
		27	40	25	8	150	F	15 ± 1.4	14 ± 1.2	14 ± 1.3
							I	2 ± 0.07	4 ± 1.5	2 ± 0.2

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	20	15	6	150	F	147 ± 1.5	165 ± 1.3	218 ± 1.3
							I	231 ± 1.1	198 ± 1.0	171 ± 1.0
		2	20	15	7	150	F	210 ± 0.3	211 ± 0.3	241 ± 0.3
							I	219 ± 1.0	231 ± 0.9	228 ± 0.9
		3	20	15	8	150	F	171 ± 1.5	198 ± 1.3	222 ± 1.3
							I	238 ± 1.7	147 ± 1.4	131 ± 1.5
		4	20	20	6	150	F	155 ± 0.8	167 ± 0.7	265 ± 0.7
							I	211 ± 1.1	237 ± 1.0	241 ± 1.0
		5	20	20	7	150	F	233 ± 1.3	215 ± 1.2	247 ± 1.2
							I	198 ± 0.2	222 ± 0.2	213 ± 0.2
		6	20	20	8	150	F	215 ± 1.3	249 ± 1.1	263 ± 1.1
							I	171 ± 0.5	235 ± 0.5	212 ± 0.5
		7	20	25	6	150	F	245 ± 1.5	236 ± 1.3	257 ± 1.3
							I	231 ± 0.7	240 ± 0.7	216 ± 0.7
		8	20	25	7	150	F	201 ± 1.9	231 ± 1.6	268 ± 1.7
							I	228 ± 1.5	227 ± 1.3	205 ± 1.3
		9	20	25	8	150	F	211 ± 1.0	221 ± 0.9	261 ± 0.9
							I	241 ± 0.32	236 ± 0.9	205 ± 0.9
		10	30	15	6	150	F	178 ± 0.8	185 ± 0.7	257 ± 0.7
							I	240 ± 0.2	174 ± 0.3	165 ± 0.2
		11	30	15	7	150	F	174 ± 1.1	165 ± 1.0	244 ± 1.0
							I	236 ± 1.3	212 ± 1.1	144 ± 1.2
		12	30	15	8	150	F	97 ± 1.5	128 ± 1.3	241 ± 1.3
							I	218 ± 1.8	214 ± 1.5	214 ± 1.6
		13	30	20	6	150	F	149 ± 0.4	222 ± 0.4	235 ± 0.4
							I	222 ± 0.8	125 ± 0.7	135 ± 0.7
		14	30	20	7	150	F	128 ± 0.3	148 ± 0.4	246 ± 0.3
							I	187 ± 0.8	149 ± 0.8	74 ± 0.7
		15	30	20	8	150	F	98 ± 0.2	126 ± 0.3	271 ± 0.2
							I	235 ± 0.9	178 ± 0.9	165 ± 0.9
		16	30	25	6	150	F	231 ± 0.5	214 ± 0.5	247 ± 0.4
							I	237 ± 0.9	219 ± 0.8	243 ± 0.8
		17	30	25	7	150	F	221 ± 0.1	224 ± 0.2	253 ± 0.1
							I	227 ± 0.5	226 ± 0.5	239 ± 0.5
		18	30	25	8	150	F	210 ± 1.0	214 ± 0.9	152 ± 0.9
							I	219 ± 1.0	145 ± 0.9	222 ± 0.9
		19	40	15	6	150	F	201 ± 1.9	175 ± 1.6	197 ± 1.7
							I	205 ± 0.8	156 ± 0.8	108 ± 0.7
		20	40	15	7	150	F	214 ± 1.8	251 ± 1.5	216 ± 1.6
							I	209 ± 1.5	234 ± 1.3	204 ± 1.3
		21	40	15	8	150	F	117 ± 0.5	236 ± 0.5	274 ± 0.19
							I	214 ± 0.8	248 ± 0.8	201 ± 0.7
		22	40	20	6	150	F	105 ± 1.8	152 ± 1.5	218 ± 1.6
							I	218 ± 0.8	149 ± 0.7	115 ± 0.7
		23	40	20	7	150	F	124 ± 1.1	210 ± 1.0	258 ± 1.0
							I	223 ± 0.7	221 ± 0.7	234 ± 0.6
		24	40	20	8	150	F	201 ± 0.6	228 ± 0.6	234 ± 0.6
							I	227 ± 0.9	231 ± 0.8	152 ± 0.8
		25	40	25	6	150	F	125 ± 1.3	196 ± 1.1	265 ± 1.2
							I	234 ± 0.9	158 ± 0.8	211 ± 0.8
		26	40	25	7	150	F	124 ± 1.0	147 ± 0.9	267 ± 0.9
							I	238 ± 0.1	125 ± 0.2	141 ± 0.1
		27	40	25	8	150	F	98 ± 1.0	136 ± 0.9	271 ± 0.9
							I	231 ± 1.1	214 ± 1.0	125 ± 1.0

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	20	15	6	150	F	8 ± 0.07	12 ± 0.7	14 ± 0.7
							I	12 ± 1.4	7 ± 0.2	11 ± 1.2
		2	20	15	7	150	F	19 ± 0.4	12 ± 0.4	12 ± 0.4
							I	20 ± 0.8	15 ± 0.7	20 ± 0.7
		3	20	15	8	150	F	24 ± 1.4	18 ± 1.2	29 ± 1.3
							I	12 ± 0.7	17 ± 0.7	18 ± 0.6
		4	20	20	6	150	F	9 ± 1.5	21 ± 1.3	27 ± 1.3
							I	6 ± 1.0	19 ± 0.9	17 ± 0.9
		5	20	20	7	150	F	8 ± 0.6	18 ± 0.6	25 ± 0.5
							I	11 ± 0.4	24 ± 0.5	11 ± 0.4
		6	20	20	8	150	F	15 ± 0.6	26 ± 0.6	7 ± 0.5
							I	17 ± 1.2	18 ± 1.1	14 ± 1.1
		7	20	25	6	150	F	14 ± 0.7	17 ± 0.7	17 ± 0.6
							I	9 ± 0.6	16 ± 0.6	18 ± 0.6
		8	20	25	7	150	F	8 ± 0.02	15 ± 0.3	28 ± 0.2
							I	15 ± 1.2	14 ± 1.1	7 ± 0.1
		9	20	25	8	150	F	16 ± 1.3	12 ± 1.2	22 ± 1.2
							I	17 ± 1.8	11 ± 1.5	6 ± 0.02
		10	30	15	6	150	F	18 ± 1.4	9 ± 1.2	20 ± 1.3
							I	20 ± 1.9	15 ± 1.6	19 ± 1.7
		11	30	15	7	150	F	15 ± 1.5	14 ± 1.3	18 ± 1.4
							I	18 ± 0.8	16 ± 0.7	20 ± 0.7
		12	30	15	8	150	F	19 ± 1.4	24 ± 1.2	9 ± 1.2
							I	17 ± 0.8	25 ± 0.8	17 ± 0.7
		13	30	20	6	150	F	11 ± 0.6	17 ± 0.6	13 ± 0.5
							I	7 ± 0.3	15 ± 0.3	14 ± 0.2
		14	30	20	7	150	F	24 ± 1.4	26 ± 1.2	16 ± 1.3
							I	17 ± 0.7	14 ± 0.7	11 ± 0.6
		15	30	20	8	150	F	9 ± 0.3	13 ± 0.3	15 ± 0.3
							I	8 ± 0.3	14 ± 0.3	15 ± 0.3
		16	30	25	6	150	F	5 ± 0.8	12 ± 0.8	17 ± 0.8
							I	4 ± 0.9	15 ± 0.8	13 ± 0.8
		17	30	25	7	150	F	11 ± 0.5	12 ± 0.5	17 ± 0.4
							I	10 ± 0.9	9 ± 0.8	15 ± 0.8
		18	30	25	8	150	F	8 ± 0.7	4 ± 0.4	14 ± 1.5
							I	7 ± 0.5	8 ± 0.5	18 ± 0.5
		19	40	15	6	150	F	6 ± 0.2	10 ± 0.3	15 ± 0.2
							I	3 ± 0.1	15 ± 1.0	14 ± 1.0
		20	40	15	7	150	F	2 ± 0.8	17 ± 0.7	19 ± 0.7
							I	10 ± 1.7	20 ± 1.4	13 ± 1.5
		21	40	15	8	150	F	14 ± 0.8	29 ± 0.7	14 ± 0.7
							I	12 ± 0.7	17 ± 0.7	11 ± 0.6
		22	40	20	6	150	F	11 ± 1.4	32 ± 1.2	28 ± 1.2
							I	12 ± 0.4	18 ± 0.4	12 ± 0.3
		23	40	20	7	150	F	14 ± 0.3	21 ± 0.4	24 ± 0.3
							I	14 ± 1.7	12 ± 1.4	9 ± 1.5
		24	40	20	8	150	F	12 ± 1.8	21 ± 1.6	34 ± 0.19
							I	17 ± 0.5	19 ± 0.5	22 ± 0.15
		25	40	25	6	150	F	18 ± 0.5	24 ± 0.5	30 ± 0.5
							I	20 ± 1.5	21 ± 1.3	15 ± 1.3
		26	40	25	7	150	F	12 ± 0.2	14 ± 0.3	25 ± 0.2
							I	14 ± 1.9	9 ± 0.6	14 ± 1.7
		27	40	25	8	150	F	11 ± 0.9	17 ± 0.8	21 ± 0.8
							I	9 ± 1.0	24 ± 0.9	16 ± 0.9

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	20	15	6	150	F	1.2 ± 0.8	2.3 ± 0.07	1.5 ± 0.07
							I	5 ± 0.03	3.2 ± 0.01	5 ± 0.12
		2	20	15	7	150	F	0.8 ± 0.6	1.1 ± 0.06	1.4 ± 0.06
							I	6 ± 0.08	1.5 ± 0.07	4.8 ± 0.7
		3	20	15	8	150	F	1.2 ± 0.02	2.5 ± 0.1	1.9 ± 0.2
							I	2.5 ± 0.05	2 ± 0.13	4.5 ± 1.3
		4	20	20	6	150	F	1 ± 0.02	1.2 ± 0.09	0.5 ± 0.03
							I	5.3 ± 0.1	4 ± 0.02	3.7 ± 0.1
		5	20	20	7	150	F	ND	0.5 ± 0.04	1 ± 0.04
							I	4.6 ± 0.06	6 ± 0.06	3.9 ± 0.05
		6	20	20	8	150	F	ND	0.7 ± 0.02	2 ± 0.03
							I	2.9 ± 0.03	1.6 ± 0.12	5.2 ± 0.7
		7	20	25	6	150	F	ND	2.1 ± 0.02	2.1 ± 0.03
							I	6 ± 0.1	2 ± 0.09	4.7 ± 0.9
		8	20	25	7	150	F	ND	ND	2 ± 0.06
							I	3 ± 0.4	4.6 ± 0.12	5.8 ± 0.3
		9	20	25	8	150	F	ND	ND	2.3 ± 0.06
							I	2 ± 0.02	4 ± 0.02	6.3 ± 0.5
		10	30	15	6	150	F	ND	ND	1.7 ± 0.07
							I	4 ± 0.1	3.5 ± 0.02	6.7 ± 1.0
		11	30	15	7	150	F	1.9 ± 0.2	2.4 ± 0.03	2.9 ± 0.2
							I	1 ± 0.01	5 ± 0.09	5.4 ± 0.2
		12	30	15	8	150	F	1.8 ± 0.08	2.3 ± 0.07	1.5 ± 0.07
							I	6.5 ± 0.7	4 ± 0.4	4.5 ± 0.05
		13	30	20	6	150	F	ND	ND	0.9 ± 0.02
							I	1.2 ± 0.03	6 ± 0.13	3.2 ± 0.4
		14	30	20	7	150	F	1.9 ± 0.6	1.7 ± 0.14	2 ± 0.3
							I	5 ± 1.1	5 ± 0.2	2.4 ± 0.05
		15	30	20	8	150	F	0.7 ± 0.06	2.5 ± 0.4	2.3 ± 0.4
							I	4.8 ± 0.1	4.2 ± 0.9	4.8 ± 0.9
		16	30	25	6	150	F	ND	ND	ND
							I	3.6 ± 0.15	5.2 ± 0.5	3.6 ± 0.05
		17	30	25	7	150	F	0.8 ± 0.03	1 ± 0.1	1.5 ± 1.2
							I	4.5 ± 0.3	4 ± 0.1	5.2 ± 1.1
		18	30	25	8	150	F	ND	ND	4.7 ± 0.8
							I	4.2 ± 0.3	4.7 ± 0.2	6.4 ± 1.2
		19	40	15	6	150	F	1.9 ± 0.3	2 ± 0.3	2.6 ± 0.2
							I	3.8 ± 0.5	6.2 ± 0.5	5.2 ± 0.5
		20	40	15	7	150	F	1.2 ± 0.04	2 ± 0.2	1 ± 0.03
							I	6 ± 1.1	5 ± 0.02	2 ± 0.05
		21	40	15	8	150	F	ND	2.6 ± 0.8	0.4 ± 0.8
							I	7 ± 0.22	5.4 ± 1.5	3 ± 1.6
		22	40	20	6	150	F	ND	ND	ND
							I	5 ± 1.6	4 ± 0.4	5 ± 1.4
		23	40	20	7	150	F	ND	1 ± 0.03	1.6 ± 0.3
							I	4 ± 0.3	2 ± 0.03	6 ± 0.3
		24	40	20	8	150	F	1.5 ± 0.3	3 ± 0.21	2.5 ± 0.2
							I	2 ± 0.3	4.9 ± 1.1	7 ± 1.2
		25	40	25	6	150	F	0.9 ± 0.3	2 ± 0.03	1.7 ± 0.2
							I	2 ± 0.2	ND	4 ± 0.2
		26	40	25	7	150	F	1.7 ± 0.05	2.7 ± 0.05	1.7 ± 0.05
							I	1 ± 0.06	1 ± 0.06	3 ± 0.6
		27	40	25	8	150	F	ND	ND	1.5 ± 0.9
							I	4 ± 0.9	2 ± 0.8	5 ± 0.8

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	20	15	6	150	F	98 ± 1.0	98 ± 0.9	110 ± 0.9
							I	84 ± 0.2	97 ± 0.3	18 ± 0.2
		2	20	15	7	150	F	110 ± 1.9	58 ± 1.6	104 ± 1.7
							I	125 ± 0.2	36 ± 0.3	69 ± 0.2
		3	20	15	8	150	F	112 ± 0.4	27 ± 0.4	74 ± 0.4
							I	124 ± 0.8	69 ± 0.7	74 ± 0.7
		4	20	20	6	150	F	98 ± 1.4	48 ± 1.2	98 ± 1.2
							I	75 ± 0.3	57 ± 0.3	89 ± 0.2
		5	20	20	7	150	F	85 ± 0.9	48 ± 0.8	112 ± 0.8
							I	111 ± 1.4	49 ± 1.2	82 ± 1.3
		6	20	20	8	150	F	17 ± 0.3	68 ± 0.4	85 ± 0.3
							I	91 ± 1.2	47 ± 1.1	129 ± 1.1
		7	20	25	6	150	F	116 ± 0.19	52 ± 1.4	32 ± 1.5
							I	87 ± 0.4	96 ± 0.5	134 ± 0.4
		8	20	25	7	150	F	57 ± 0.2	110 ± 0.3	98 ± 0.2
							I	121 ± 1.3	124 ± 1.1	125 ± 1.2
		9	20	25	8	150	F	98 ± 1.2	104 ± 1.0	87 ± 1.1
							I	141 ± 0.7	105 ± 0.7	121 ± 0.6
		10	30	15	6	150	F	93 ± 0.6	101 ± 0.6	55 ± 0.6
							I	134 ± 0.1	105 ± 0.2	111 ± 0.1
		11	30	15	7	150	F	87 ± 1.5	101 ± 1.3	71 ± 1.4
							I	134 ± 0.8	123 ± 0.7	98 ± 0.7
		12	30	15	8	150	F	98 ± 1.4	75 ± 1.2	68 ± 1.2
							I	121 ± 1.2	85 ± 1.0	114 ± 1.0
		13	30	20	6	150	F	93 ± 0.7	96 ± 0.7	74 ± 0.7
							I	118 ± 0.5	47 ± 0.5	125 ± 0.4
		14	30	20	7	150	F	92 ± 1.0	52 ± 0.9	58 ± 0.9
							I	104 ± 0.9	102 ± 0.9	138 ± 0.9
		15	30	20	8	150	F	87 ± 0.7	121 ± 0.7	25 ± 0.7
							I	121 ± 0.1	134 ± 0.2	147 ± 0.1
		16	30	25	6	150	F	88 ± 0.5	110 ± 0.5	65 ± 0.5
							I	105 ± 0.8	114 ± 0.7	117 ± 0.7
		17	30	25	7	150	F	87 ± 0.8	106 ± 0.7	74 ± 0.7
							I	142 ± 0.1	89 ± 0.2	124 ± 0.1
		18	30	25	8	150	F	71 ± 0.2	95 ± 0.3	85 ± 0.2
							I	112 ± 1.6	78 ± 1.3	135 ± 1.4
		19	40	15	6	150	F	96 ± 1.4	95 ± 1.2	111 ± 1.3
							I	74 ± 0.1	74 ± 0.2	140 ± 0.1
		20	40	15	7	150	F	110 ± 0.2	52 ± 0.3	78 ± 0.2
							I	98 ± 1.7	35 ± 1.4	86 ± 1.5
		21	40	15	8	150	F	112 ± 1.7	78 ± 1.4	92 ± 1.5
							I	141 ± 0.9	77 ± 0.8	79 ± 0.8
		22	40	20	6	150	F	108 ± 0.2	66 ± 0.3	91 ± 0.2
							I	131 ± 1.0	61 ± 0.9	138 ± 0.9
		23	40	20	7	150	F	105 ± 1.4	54 ± 1.2	128 ± 1.2
							I	112 ± 1.8	81 ± 1.6	147 ± 1.6
		24	40	20	8	150	F	89 ± 1.7	17 ± 1.5	24 ± 1.6
							I	112 ± 0.1	48 ± 0.2	151 ± 0.24
		25	40	25	6	150	F	85 ± 0.8	96 ± 0.8	84 ± 0.7
							I	58 ± 1.5	85 ± 1.3	125 ± 1.3
		26	40	25	7	150	F	93 ± 1.5	99 ± 1.3	110 ± 1.3
							I	75 ± 1.7	87 ± 1.4	138 ± 1.5
		27	40	25	8	150	F	91 ± 1.3	62 ± 1.1	74 ± 1.2
							I	124 ± 1.7	75 ± 1.4	141 ± 1.5

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	20	15	6	150	F	85 ± 0.2	131 ± 0.3	120 ± 0.2
							I	198 ± 0.5	200 ± 0.5	148 ± 0.4
		2	20	15	7	150	F	56 ± 1.9	112 ± 1.6	101 ± 1.7
							I	201 ± 1.6	108 ± 1.3	98 ± 1.4
		3	20	15	8	150	F	75 ± 0.9	124 ± 0.8	78 ± 0.8
							I	147 ± 0.2	210 ± 0.28	193 ± 0.2
		4	20	20	6	150	F	63 ± 0.6	120 ± 0.6	44 ± 0.5
							I	198 ± 0.7	201 ± 0.6	147 ± 0.6
		5	20	20	7	150	F	71 ± 0.6	57 ± 0.6	58 ± 0.6
							I	201 ± 0.5	148 ± 0.5	73 ± 0.4
		6	20	20	8	150	F	54 ± 0.7	46 ± 0.7	79 ± 0.6
							I	178 ± 0.3	192 ± 0.3	201 ± 0.3
		7	20	25	6	150	F	12 ± 1.3	47 ± 1.2	58 ± 1.2
							I	165 ± 0.5	209 ± 0.5	196 ± 0.4
		8	20	25	7	150	F	121 ± 1.7	92 ± 1.4	112 ± 1.5
							I	207 ± 1.0	148 ± 0.9	204 ± 0.9
		9	20	25	8	150	F	68 ± 0.2	97 ± 0.3	104 ± 0.2
							I	196 ± 0.6	203 ± 0.6	149 ± 0.6
		10	30	15	6	150	F	75 ± 0.6	93 ± 0.6	106 ± 0.5
							I	187 ± 0.3	164 ± 0.4	102 ± 0.3
		11	30	15	7	150	F	78 ± 1.2	108 ± 1.1	103 ± 1.1
							I	199 ± 0.7	158 ± 0.6	142 ± 0.6
		12	30	15	8	150	F	72 ± 0.1	125 ± 0.2	99 ± 0.1
							I	200 ± 0.4	162 ± 0.5	89 ± 0.4
		13	30	20	6	150	F	119 ± 1.0	128 ± 0.9	101 ± 0.9
							I	174 ± 0.7	156 ± 0.6	202 ± 0.6
		14	30	20	7	150	F	124 ± 1.7	116 ± 1.5	111 ± 1.6
							I	185 ± 1.7	105 ± 1.5	203 ± 1.6
		15	30	20	8	150	F	42 ± 1.4	94 ± 1.2	96 ± 1.3
							I	198 ± 1.7	117 ± 1.5	59 ± 1.5
		16	30	25	6	150	F	76 ± 1.0	108 ± 0.9	58 ± 0.9
							I	181 ± 0.5	56 ± 0.5	65 ± 0.4
		17	30	25	7	150	F	89 ± 1.5	129 ± 1.3	62 ± 1.4
							I	209 ± 1.0	148 ± 0.9	120 ± 0.9
		18	30	25	8	150	F	12 ± 0.2	124 ± 0.3	87 ± 0.2
							I	185 ± 0.8	117 ± 0.7	96 ± 0.7
		19	40	15	6	150	F	145 ± 1.5	114 ± 1.3	96 ± 1.3
							I	178 ± 1.7	62 ± 1.4	19 ± 1.5
		20	40	15	7	150	F	52 ± 1.9	112 ± 1.6	82 ± 1.7
							I	165 ± 1.2	35 ± 1.1	19 ± 1.1
		21	40	15	8	150	F	98 ± 0.9	110 ± 0.9	67 ± 0.8
							I	124 ± 1.9	89 ± 1.6	27 ± 1.7
		22	40	20	6	150	F	67 ± 1.3	101 ± 1.1	57 ± 1.2
							I	179 ± 1.5	89 ± 1.3	68 ± 1.4
		23	40	20	7	150	F	56 ± 1.8	98 ± 1.5	69 ± 1.6
							I	201 ± 0.4	152 ± 0.4	210 ± 0.3
		24	40	20	8	150	F	72 ± 0.4	78 ± 0.4	52 ± 0.3
							I	194 ± 0.3	147 ± 0.3	81 ± 0.3
		25	40	25	6	150	F	73 ± 0.6	121 ± 0.6	68 ± 0.5
							I	183 ± 0.4	124 ± 0.4	88 ± 0.3
		26	40	25	7	150	F	89 ± 0.2	134 ± 0.16	96 ± 0.2
							I	124 ± 1.8	112 ± 1.6	76 ± 1.7
		27	40	25	8	150	F	47 ± 0.3	130 ± 0.4	112 ± 0.3
							I	167 ± 0.2	152 ± 0.3	73 ± 0.2

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

CS 04

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	20	15	6	150	F	18 ± 1.24	15 ± 1.43	12 ± 1.11
							I	14 ± 0.21	9 ± 0.40	12 ± 0.31
		2	20	15	7	150	F	19 ± 2.64	11 ± 3.26	8 ± 2.53
							I	9 ± 2.48	8 ± 3.05	11 ± 2.36
		3	20	15	8	150	F	17 ± 2.15	13 ± 2.62	5 ± 2.03
							I	12 ± 3.51	4 ± 4.40	9 ± 3.41
		4	20	20	6	150	F	13 ± 2.77	10 ± 3.42	11 ± 2.66
							I	7 ± 0.38	5 ± 0.30	5 ± 0.24
		5	20	20	7	150	F	12 ± 0.88	8 ± 0.96	17 ± 0.75
							I	6 ± 2.77	4 ± 0.42	8 ± 2.66
		6	20	20	8	150	F	11 ± 1.38	9 ± 1.61	14 ± 1.25
							I	2 ± 0.05	3 ± 2.35	4 ± 1.83
		7	20	25	6	150	F	8 ± 1.08	8 ± 1.22	16 ± 0.95
							I	13 ± 0.25	10 ± 2.74	12 ± 2.13
		8	20	25	7	150	F	5 ± 0.02	7 ± 0.74	14 ± 2.91
							I	7 ± 0.28	9 ± 1.47	13 ± 1.15
		9	20	25	8	150	F	17 ± 0.76	15 ± 0.80	12 ± 0.62
							I	12 ± 1.49	10 ± 1.75	11 ± 1.36
		10	30	15	6	150	F	11 ± 1.25	18 ± 1.43	6 ± 0.12
							I	11 ± 2.25	13 ± 2.75	5 ± 2.13
		11	30	15	7	150	F	2 ± 0.86	11 ± 2.23	4 ± 1.74
							I	9 ± 0.51	2 ± 0.47	4 ± 0.37
		12	30	15	8	150	F	5 ± 1.44	12 ± 1.69	12 ± 1.32
							I	8 ± 0.57	4 ± 0.47	6 ± 0.47
		13	30	20	6	150	F	16 ± 2.96	14 ± 3.67	8 ± 2.85
							I	12 ± 0.37	6 ± 4.21	8 ± 3.27
		14	30	20	7	150	F	18 ± 1.31	16 ± 1.51	10 ± 1.18
							I	6 ± 0.43	7 ± 1.67	2 ± 1.30
		15	30	20	8	150	F	11 ± 2.76	17 ± 3.41	12 ± 2.65
							I	7 ± 0.54	5 ± 0.50	9 ± 0.40
		16	30	25	6	150	F	17 ± 3.45	19 ± 4.31	8 ± 3.34
							I	5 ± 0.84	4 ± 0.90	6 ± 0.070
		17	30	25	7	150	F	14 ± 2.57	9 ± 3.17	6 ± 2.46
							I	13 ± 1.75	11 ± 0.09	12 ± 1.62
		18	30	25	8	150	F	18 ± 1.70	8 ± 0.03	9 ± 0.58
							I	12 ± 2.65	7 ± 0.27	11 ± 2.54
		19	40	15	6	150	F	19 ± 0.52	5 ± 0.40	17 ± 3.41
							I	11 ± 0.94	9 ± 0.03	10 ± 0.81
		20	40	15	7	150	F	15 ± 0.61	4 ± 0.52	13 ± 3.51
							I	8 ± 0.86	5 ± 0.13	13 ± 0.72
		21	40	15	8	150	F	13 ± 0.37	18 ± 0.29	11 ± 0.23
							I	5 ± 3.62	3 ± 4.53	8 ± 0.51
		22	40	20	6	150	F	17 ± 2.60	14 ± 3.20	14 ± 2.48
							I	6 ± 0.41	4 ± 4.25	7 ± 3.30
		23	40	20	7	150	F	11 ± 0.26	11 ± 1.46	14 ± 1.13
							I	7 ± 0.2	2 ± 0.12	11 ± 2.89
		24	40	20	8	150	F	10 ± 2.96	10 ± 3.67	8 ± 2.85
							I	4 ± 3.51	10 ± 4.39	10 ± 3.40
		25	40	25	6	150	F	20 ± 0.21	19 ± 1.63	11 ± 1.27
							I	5 ± 0.35	11 ± 2.88	12 ± 2.23
		26	40	25	7	150	F	19 ± 0.49	15 ± 0.45	4 ± 0.35
							I	3 ± 0.32	12 ± 4.14	9 ± 0.21
		27	40	25	8	150	F	15 ± 0.95	16 ± 3.66	9 ± 0.84
							I	8 ± 0.55	9 ± 0.44	4 ± 0.44

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	20	15	6	150	F	207 ± 3.08	214 ± 3.83	98 ± 2.97
							I	214 ± 2.35	222 ± 2.87	101 ± 2.23
		2	20	15	7	150	F	201 ± 0.69	218 ± 0.71	121 ± 0.56
							I	199 ± 2.10	209 ± 2.55	123 ± 1.98
		3	20	15	8	150	F	196 ± 3.07	228 ± 3.81	85 ± 2.96
							I	229 ± 3.48	234 ± 4.35	96 ± 3.38
		4	20	20	6	150	F	211 ± 1.70	211 ± 2.03	98 ± 1.58
							I	201 ± 2.41	207 ± 2.95	91 ± 2.29
		5	20	20	7	150	F	187 ± 2.79	205 ± 3.45	25 ± 2.68
							I	156 ± 0.45	216 ± 0.40	147 ± 0.32
		6	20	20	8	150	F	147 ± 2.67	158 ± 3.29	112 ± 2.55
							I	227 ± 1.13	196 ± 1.28	123 ± 1.00
		7	20	25	6	150	F	226 ± 3.01	148 ± 3.74	152 ± 2.90
							I	215 ± 1.58	165 ± 1.87	96 ± 1.45
		8	20	25	7	150	F	205 ± 3.87	189 ± 4.86	120 ± 3.77
							I	201 ± 3.02	199 ± 3.76	210 ± 2.91
		9	20	25	8	150	F	174 ± 2.07	201 ± 2.51	201 ± 1.95
							I	185 ± 2.20	211 ± 2.67	209 ± 2.08
		10	30	15	6	150	F	197 ± 1.67	213 ± 1.99	123 ± 1.55
							I	198 ± 0.52	204 ± 0.49	96 ± 0.39
		11	30	15	7	150	F	211 ± 2.38	201 ± 2.91	98 ± 2.26
							I	216 ± 2.75	215 ± 3.40	74 ± 2.64
		12	30	15	8	150	F	215 ± 3.08	216 ± 3.83	99 ± 2.97
							I	210 ± 3.75	218 ± 4.70	48 ± 3.65
		13	30	20	6	150	F	224 ± 0.95	213 ± 1.04	52 ± 0.82
							I	214 ± 1.66	238 ± 1.97	62 ± 1.53
		14	30	20	7	150	F	229 ± 0.75	218 ± 0.78	57 ± 0.61
							I	202 ± 1.76	226 ± 2.11	121 ± 1.64
		15	30	20	8	150	F	196 ± 0.47	228 ± 0.42	118 ± 0.33
							I	187 ± 2.01	187 ± 2.44	126 ± 1.89
		16	30	25	6	150	F	208 ± 1.06	219 ± 1.19	147 ± 0.93
							I	211 ± 1.91	215 ± 2.31	124 ± 1.79
		17	30	25	7	150	F	215 ± 0.39	220 ± 0.32	154 ± 0.25
							I	218 ± 1.21	224 ± 1.38	132 ± 1.08
		18	30	25	8	150	F	226 ± 2.14	214 ± 2.60	146 ± 2.02
							I	210 ± 2.04	211 ± 2.47	154 ± 1.92
		19	40	15	6	150	F	230 ± 0.17	196 ± 4.85	127 ± 3.76
							I	229 ± 1.76	195 ± 2.11	136 ± 1.64
		20	40	15	7	150	F	224 ± 3.66	174 ± 4.59	164 ± 3.56
							I	210 ± 3.05	199 ± 3.80	168 ± 2.94
		21	40	15	8	150	F	211 ± 1.05	210 ± 1.18	124 ± 0.92
							I	204 ± 1.74	214 ± 2.08	216 ± 1.62
		22	40	20	6	150	F	189 ± 3.70	216 ± 4.64	210 ± 3.60
							I	215 ± 1.71	186 ± 2.04	207 ± 1.59
		23	40	20	7	150	F	198 ± 2.28	167 ± 2.79	186 ± 2.17
							I	218 ± 1.54	198 ± 1.82	85 ± 1.41
		24	40	20	8	150	F	214 ± 1.42	127 ± 1.66	71 ± 1.29
							I	232 ± 1.87	201 ± 2.24	132 ± 1.74
		25	40	25	6	150	F	222 ± 2.73	205 ± 3.38	148 ± 2.62
							I	215 ± 1.90	212 ± 2.29	55 ± 1.78
		26	40	25	7	150	F	229 ± 2.11	112 ± 2.57	67 ± 1.99
							I	238 ± 0.38	105 ± 0.31	49 ± 0.24
		27	40	25	8	150	F	222 ± 2.15	85 ± 2.61	85 ± 2.03
							I	241 ± 0.29	205 ± 2.87	198 ± 2.22

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	20	15	6	150	F	32 ± 1.11	28 ± 0.79	9 ± 0.31
							I	39 ± 0.31	35 ± 0.18	21 ± 0.27
		2	20	15	7	150	F	24 ± 2.53	24 ± 1.85	25 ± 3.15
							I	35 ± 2.36	11 ± 0.72	18 ± 2.93
		3	20	15	8	150	F	21 ± 2.03	18 ± 1.47	13 ± 2.50
							I	41 ± 3.41	16 ± 2.51	27 ± 4.29
		4	20	20	6	150	F	18 ± 2.66	24 ± 1.94	25 ± 3.31
							I	36 ± 0.23	18 ± 0.13	21 ± 0.17
		5	20	20	7	150	F	19 ± 0.75	35 ± 0.51	16 ± 0.83
							I	29 ± 2.66	27 ± 1.94	23 ± 3.31
		6	20	20	8	150	F	21 ± 1.25	33 ± 0.89	27 ± 1.49
							I	34 ± 1.83	25 ± 1.32	29 ± 2.23
		7	20	25	6	150	F	14 ± 0.95	18 ± 0.66	14 ± 0.09
							I	40 ± 2.13	16 ± 1.55	17 ± 2.63
		8	20	25	7	150	F	17 ± 2.91	7 ± 2.13	11 ± 3.64
							I	29 ± 1.15	18 ± 0.81	10 ± 0.35
		9	20	25	8	150	F	19 ± 0.62	25 ± 0.42	21 ± 0.67
							I	18 ± 1.36	26 ± 0.97	23 ± 1.63
		10	30	15	6	150	F	7 ± 0.12	24 ± 0.79	21 ± 1.31
							I	21 ± 2.13	33 ± 1.55	14 ± 2.63
		11	30	15	7	150	F	12 ± 1.74	18 ± 1.25	17 ± 2.12
							I	24 ± 0.37	39 ± 0.23	9 ± 0.34
		12	30	15	8	150	F	11 ± 1.32	16 ± 0.94	18 ± 1.57
							I	32 ± 3.47	17 ± 2.55	16 ± 4.37
		13	30	20	6	150	F	15 ± 2.85	15 ± 2.08	17 ± 3.56
							I	19 ± 3.27	29 ± 2.40	11 ± 4.11
		14	30	20	7	150	F	16 ± 1.18	18 ± 0.83	13 ± 1.39
							I	11 ± 1.30	14 ± 0.93	14 ± 1.55
		15	30	20	8	150	F	28 ± 2.65	18 ± 1.93	28 ± 3.30
							I	38 ± 0.40	11 ± 0.25	23 ± 0.38
		16	30	25	6	150	F	32 ± 3.34	9 ± 2.46	26 ± 4.21
							I	35 ± 0.70	7 ± 0.48	19 ± 0.77
		17	30	25	7	150	F	25 ± 2.46	19 ± 1.79	24 ± 3.05
							I	28 ± 1.62	24 ± 1.17	14 ± 1.97
		18	30	25	8	150	F	29 ± 1.58	32 ± 1.13	11 ± 1.91
							I	21 ± 2.54	33 ± 1.85	16 ± 3.16
		19	40	15	6	150	F	31 ± 3.41	24 ± 2.51	18 ± 4.30
							I	22 ± 0.81	39 ± 0.56	24 ± 0.91
		20	40	15	7	150	F	35 ± 3.51	27 ± 2.58	16 ± 4.42
							I	24 ± 0.72	29 ± 0.49	28 ± 0.80
		21	40	15	8	150	F	36 ± 0.23	27 ± 0.12	19 ± 0.16
							I	17 ± 3.51	24 ± 2.59	17 ± 4.43
		22	40	20	6	150	F	34 ± 2.48	31 ± 1.81	11 ± 3.09
							I	42 ± 0.28	34 ± 2.42	10 ± 4.15
		23	40	20	7	150	F	29 ± 1.13	21 ± 0.80	9 ± 1.34
							I	19 ± 2.89	14 ± 2.12	12 ± 3.62
		24	40	20	8	150	F	25 ± 2.85	12 ± 2.08	14 ± 3.56
							I	15 ± 3.40	16 ± 2.50	19 ± 4.29
		25	40	25	6	150	F	27 ± 1.27	17 ± 0.90	11 ± 1.51
							I	11 ± 2.23	11 ± 1.63	18 ± 2.77
		26	40	25	7	150	F	31 ± 0.35	25 ± 0.22	21 ± 0.32
							I	16 ± 3.21	27 ± 2.36	26 ± 4.03
		27	40	25	8	150	F	35 ± 2.84	16 ± 2.08	24 ± 3.55
							I	19 ± 3.44	19 ± 2.53	22 ± 4.34

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	20	15	6	150	F	0	0	0
							I	5.4 ± 0.15	1 ± 0.04	6.2 ± 0.64
		2	20	15	7	150	F	ND	ND	0.2 ± 0.02
							I	3.7 ± 0.6	2 ± 0.02	4.5 ± 0.50
		3	20	15	8	150	F	0.9 ± 0.02	0.7 ± 0.01	0.2 ± 0.030
							I	2.6 ± 0.03	5 ± 0.077	5.6 ± 0.92
		4	20	20	6	150	F	ND	0.1 ± 0.04	0.3 ± 0.01
							I	1.2 ± 0.04	6.8 ± 0.35	6.1 ± 0.28
		5	20	20	7	150	F	ND	0.2 ± 0.01	0.4 ± 0.07
							I	5.2 ± 0.34	6.4 ± 1.56	4.8 ± 0.21
		6	20	20	8	150	F	0.1 ± 0.01	ND	ND
							I	4.6 ± 0.82	5.8 ± 0.079	4.5 ± 0.72
		7	20	25	6	150	F	0.4 ± 0.24	0.2 ± 0.073	0.5 ± 0.012
							I	5.8 ± 0.19	2.9 ± 0.027	3.2 ± 0.07
		8	20	25	7	150	F	0.7 ± 0.040	0.1 ± 0.03	0.8 ± 0.027
							I	6.5 ± 0.91	1.8 ± 0.30	1.8 ± 0.80
		9	20	25	8	150	F	0.5 ± 0.006	0.1 ± 0.01	0.2 ± 0.02
							I	5.7 ± 0.34	2.9 ± 0.17	2 ± 0.024
		10	30	15	6	150	F	ND	ND	ND
							I	3.6 ± 0.29	5.6 ± 0.80	2.3 ± 0.17
		11	30	15	7	150	F	ND	ND	ND
							I	2.5 ± 0.24	1.6 ± 0.073	1.4 ± 0.12
		12	30	15	8	150	F	0.1 ± 0.01	0.3 ± 0.01	0.8 ± 0.055
							I	2.9 ± 0.47	1.8 ± 0.034	5 ± 0.36
		13	30	20	6	150	F	0.7 ± 0.06	0.5 ± 0.049	0.2 ± 0.02
							I	5.4 ± 0.20	2.5 ± 0.98	6.1 ± 0.1
		14	30	20	7	150	F	ND	ND	ND
							I	5.7 ± 0.39	3.4 ± 0.93	4.5 ± 0.28
		15	30	20	8	150	F	ND	ND	ND
							I	6.3 ± 0.23	4.7 ± 0.71	5.4 ± 0.11
		16	30	25	6	150	F	0.4 ± 0.49	ND	ND
							I	2.5 ± 0.012	6 ± 0.28	2.9 ± 0.1
		17	30	25	7	150	F	0.25 ± 0.01	0.1 ± 0.032	0.8 ± 0.057
							I	1.5 ± 0.07	2 ± 0.29	3.5 ± 0.56
		18	30	25	8	150	F	ND	0.7 ± 0.02	0.5 ± 0.078
							I	2 ± 0.081	3 ± 0.14	4 ± 0.70
		19	40	15	6	150	F	ND	ND	ND
							I	3.4 ± 0.23	5 ± 0.041	1 ± 0.10
		20	40	15	7	150	F	0.3 ± 0.01	0.2 ± 0.02	4.5 ± 0.88
							I	5.4 ± 0.28	7 ± 0.16	5 ± 2.17
		21	40	15	8	150	F	0.47 ± 0.01	0.6 ± 0.02	0.7 ± 0.02
							I	5.4 ± 0.068	4 ± 0.061	6.5 ± 3.58
		22	40	20	6	150	F	ND	0.5 ± 0.075	0.8 ± 0.014
							I	4 ± 0.30	1 ± 0.012	5.2 ± 3.19
		23	40	20	7	150	F	ND	ND	ND
							I	2 ± 0.068	2 ± 0.02	2.4 ± 0.54
		24	40	20	8	150	F	ND	ND	ND
							I	1.2 ± 0.07	3 ± 0.036	2.5 ± 0.061
		25	40	25	6	150	F	1 ± 0.15	0.9 ± 0.067	0.2 ± 0.052
							I	3 ± 0.61	6.5 ± 0.61	4.7 ± 0.48
		26	40	25	7	150	F	0.4 ± 0.19	0.7 ± 0.1	ND
							I	4 ± 0.40	5 ± 0.63	3.6 ± 0.27
		27	40	25	8	150	F	0.5 ± 0.01	0.4 ± 0.070	0.2 ± 0.10
							I	5 ± 0.89	6 ± 0.27	5.2 ± 0.77

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	20	15	6	150	F	12 ± 2.19	25 ± 2.67	15 ± 2.07
							I	21 ± 0.58	18 ± 0.56	48 ± 0.44
		2	20	15	7	150	F	54 ± 3.89	36 ± 4.88	58 ± 3.79
							I	72 ± 0.51	48 ± 0.47	74 ± 0.37
		3	20	15	8	150	F	62 ± 0.93	85 ± 1.02	59 ± 0.79
							I	32 ± 1.74	74 ± 2.08	65 ± 1.61
		4	20	20	6	150	F	41 ± 2.88	26 ± 3.57	15 ± 2.77
							I	56 ± 0.64	48 ± 0.65	18 ± 0.51
		5	20	20	7	150	F	28 ± 1.88	52 ± 2.26	13 ± 1.75
							I	41 ± 2.93	71 ± 3.63	14 ± 2.82
		6	20	20	8	150	F	52 ± 0.81	36 ± 0.87	12 ± 0.68
							I	16 ± 2.57	58 ± 3.16	75 ± 2.45
		7	20	25	6	150	F	19 ± 3.36	15 ± 4.20	48 ± 3.26
							I	18 ± 1.00	48 ± 1.11	48 ± 0.87
		8	20	25	7	150	F	12 ± 0.51	63 ± 0.48	52 ± 0.37
							I	25 ± 2.72	24 ± 3.36	53 ± 2.61
		9	20	25	8	150	F	26 ± 2.47	75 ± 3.03	24 ± 2.35
							I	34 ± 1.54	81 ± 1.82	48 ± 1.42
		10	30	15	6	150	F	25 ± 1.37	62 ± 1.60	42 ± 1.25
							I	81 ± 0.41	58 ± 0.34	47 ± 0.27
		11	30	15	7	150	F	53 ± 3.18	47 ± 3.96	75 ± 3.07
							I	65 ± 1.69	19 ± 2.01	71 ± 1.56
		12	30	15	8	150	F	74 ± 2.87	68 ± 3.55	77 ± 2.76
							I	25 ± 2.42	23 ± 2.97	73 ± 2.31
		13	30	20	6	150	F	81 ± 1.62	74 ± 1.93	63 ± 1.50
							I	32 ± 1.04	15 ± 1.17	62 ± 0.91
		14	30	20	7	150	F	65 ± 2.21	36 ± 2.70	64 ± 2.09
							I	45 ± 2.02	80 ± 2.44	42 ± 1.89
		15	30	20	8	150	F	27 ± 1.61	29 ± 1.91	41 ± 1.48
							I	38 ± 0.34	64 ± 0.25	19 ± 0.20
		16	30	25	6	150	F	34 ± 1.20	25 ± 1.38	21 ± 1.07
							I	28 ± 1.67	18 ± 1.99	25 ± 1.54
		17	30	25	7	150	F	26 ± 1.72	17 ± 2.05	22 ± 1.60
							I	53 ± 0.36	47 ± 0.28	55 ± 0.22
		18	30	25	8	150	F	63 ± 0.62	14 ± 0.62	47 ± 0.49
							I	18 ± 3.23	44 ± 4.02	65 ± 3.12
		19	40	15	6	150	F	26 ± 2.99	16 ± 3.71	77 ± 2.88
							I	39 ± 0.40	84 ± 0.20	41 ± 0.26
		20	40	15	7	150	F	48 ± 0.61	26 ± 0.61	42 ± 0.48
							I	68 ± 3.41	59 ± 4.26	46 ± 3.30
		21	40	15	8	150	F	71 ± 3.43	47 ± 4.28	63 ± 3.32
							I	72 ± 1.97	23 ± 2.38	18 ± 1.85
		22	40	20	6	150	F	62 ± 0.56	75 ± 0.54	29 ± 0.42
							I	41 ± 2.04	48 ± 2.47	82 ± 1.92
		23	40	20	7	150	F	45 ± 2.83	16 ± 3.50	81 ± 2.72
							I	82 ± 3.79	25 ± 4.75	18 ± 3.68
		24	40	20	8	150	F	23 ± 3.57	57 ± 4.47	27 ± 3.47
							I	24 ± 0.35	53 ± 0.27	26 ± 0.21
		25	40	25	6	150	F	26 ± 1.79	59 ± 2.14	84 ± 0.14
							I	86 ± 3.02	51 ± 3.75	52 ± 2.91
		26	40	25	7	150	F	58 ± 3.08	52 ± 3.83	24 ± 2.97
							I	57 ± 3.44	48 ± 4.30	63 ± 3.34
		27	40	25	8	150	F	41 ± 2.69	62 ± 3.32	28 ± 2.58
							I	29 ± 3.43	64 ± 4.28	18 ± 3.32

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	20	15	6	150	F	48 ± 0.58	62 ± 0.56	58 ± 0.44
							I	69 ± 1.06	58 ± 1.19	78 ± 0.93
		2	20	15	7	150	F	52 ± 3.84	71 ± 4.82	75 ± 3.74
							I	76 ± 3.24	96 ± 4.04	110 ± 3.14
		3	20	15	8	150	F	80 ± 1.95	54 ± 2.36	47 ± 1.83
							I	74 ± 0.60	98 ± 0.58	78 ± 0.46
		4	20	20	6	150	F	84 ± 1.32	25 ± 1.53	56 ± 1.19
							I	85 ± 1.48	102 ± 1.74	89 ± 1.36
		5	20	20	7	150	F	65 ± 1.38	59 ± 1.61	85 ± 1.25
							I	89 ± 1.11	110 ± 1.26	58 ± 0.98
		6	20	20	8	150	F	77 ± 1.54	53 ± 1.82	96 ± 1.41
							I	112 ± 0.67	121 ± 0.69	69 ± 0.54
		7	20	25	6	150	F	44 ± 2.79	57 ± 3.46	48 ± 2.68
							I	89 ± 1.10	56 ± 1.24	17 ± 0.97
		8	20	25	7	150	F	48 ± 3.44	62 ± 4.30	49 ± 3.33
							I	79 ± 2.10	104 ± 2.55	25 ± 1.98
		9	20	25	8	150	F	52 ± 0.47	71 ± 0.42	52 ± 0.33
							I	104 ± 1.39	89 ± 1.62	78 ± 1.26
		10	30	15	6	150	F	82 ± 1.23	52 ± 1.42	44 ± 1.10
							I	102 ± 0.73	79 ± 0.76	52 ± 0.60
		11	30	15	7	150	F	66 ± 2.57	57 ± 3.16	18 ± 2.45
							I	110 ± 1.48	120 ± 1.74	27 ± 1.35
		12	30	15	8	150	F	48 ± 0.38	49 ± 0.30	69 ± 0.24
							I	89 ± 1.01	115 ± 1.13	102 ± 0.88
		13	30	20	6	150	F	74 ± 2.03	65 ± 2.46	66 ± 1.91
							I	51 ± 1.46	133 ± 1.71	54 ± 1.33
		14	30	20	7	150	F	88 ± 3.58	48 ± 4.49	49 ± 3.48
							I	62 ± 3.58	112 ± 4.48	104 ± 3.47
		15	30	20	8	150	F	69 ± 3.01	96 ± 3.74	53 ± 2.90
							I	75 ± 3.56	120 ± 4.45	98 ± 3.45
		16	30	25	6	150	F	45 ± 2.10	14 ± 2.54	58 ± 1.98
							I	94 ± 1.11	116 ± 1.26	85 ± 0.98
		17	30	25	7	150	F	25 ± 3.14	52 ± 3.91	48 ± 3.03
							I	69 ± 2.06	116 ± 2.50	81 ± 1.94
		18	30	25	8	150	F	98 ± 0.61	16 ± 0.60	88 ± 0.47
							I	78 ± 1.68	110 ± 2.00	102 ± 1.55
		19	40	15	6	150	F	101 ± 0.20	75 ± 3.85	79 ± 2.98
							I	58 ± 3.47	98 ± 4.34	96 ± 3.37
		20	40	15	7	150	F	92 ± 3.86	63 ± 4.85	56 ± 3.76
							I	41 ± 2.56	89 ± 3.15	72 ± 2.45
		21	40	15	8	150	F	45 ± 2.00	55 ± 2.41	55 ± 1.88
							I	63 ± 3.83	124 ± 4.81	62 ± 3.73
		22	40	20	6	150	F	81 ± 2.70	58 ± 3.33	74 ± 2.58
							I	52 ± 3.18	112 ± 3.96	61 ± 3.07
		23	40	20	7	150	F	78 ± 3.63	49 ± 4.55	77 ± 3.53
							I	74 ± 0.89	110 ± 0.96	57 ± 0.75
		24	40	20	8	150	F	99 ± 0.85	47 ± 0.92	96 ± 0.72
							I	96 ± 0.69	98 ± 0.71	55 ± 0.56
		25	40	25	6	150	F	69 ± 1.29	69 ± 1.49	99 ± 1.16
							I	89 ± 0.88	115 ± 0.96	42 ± 0.75
		26	40	25	7	150	F	72 ± 0.60	57 ± 0.59	100 ± 0.47
							I	104 ± 3.80	116 ± 4.76	46 ± 3.69
		27	40	25	8	150	F	99 ± 0.82	85 ± 0.88	89 ± 0.69
							I	112 ± 0.63	127 ± 0.15	78 ± 0.50

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

CS 05

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
1	Protease	1	30	15	5	150	F	9 ± 0.66	11 ± 1.16	11 ± 1.34
							I	27 ± 0.17	21 ± 0.58	18 ± 0.43
		2	30	15	6	150	F	16 ± 1.52	15 ± 2.18	13 ± 2.95
							I	30 ± 1.42	31 ± 2.06	19 ± 2.76
		3	30	15	7	150	F	15 ± 1.22	17 ± 1.82	15 ± 2.39
							I	20 ± 2.06	15 ± 2.82	17 ± 3.96
		4	30	20	5	150	F	12 ± 1.60	15 ± 2.27	18 ± 3.10
							I	17 ± 0.12	11 ± 0.53	11 ± 0.34
		5	30	20	6	150	F	8 ± 0.44	14 ± 0.90	21 ± 0.92
							I	14 ± 1.60	12 ± 2.27	12 ± 3.10
		6	30	20	7	150	F	9 ± 0.74	9 ± 0.26	20 ± 1.50
							I	19 ± 1.09	13 ± 1.67	24 ± 2.15
		7	30	25	5	150	F	7 ± 0.56	10 ± 1.04	16 ± 1.15
							I	11 ± 1.28	7 ± 1.89	27 ± 2.50
		8	30	25	6	150	F	10 ± 1.75	12 ± 2.45	17 ± 3.38
							I	18 ± 0.68	30 ± 1.18	29 ± 1.38
		9	30	25	7	150	F	17 ± 0.36	13 ± 0.81	19 ± 0.78
							I	28 ± 0.81	27 ± 1.34	30 ± 1.62
		10	40	15	5	150	F	19 ± 0.66	12 ± 1.16	12 ± 1.34
							I	30 ± 1.28	21 ± 1.90	11 ± 2.50
		11	40	15	6	150	F	21 ± 1.04	14 ± 1.61	9 ± 0.05
							I	29 ± 0.20	29 ± 0.62	13 ± 0.50
		12	40	15	7	150	F	20 ± 0.78	19 ± 1.31	10 ± 1.57
							I	16 ± 2.10	15 ± 2.86	14 ± 4.02
		13	40	20	5	150	F	16 ± 1.72	20 ± 2.41	11 ± 3.31
							I	25 ± 1.97	11 ± 2.72	19 ± 3.79
		14	40	20	6	150	F	14 ± 0.70	18 ± 1.21	12 ± 1.41
							I	14 ± 0.77	10 ± 1.30	16 ± 1.55
		15	40	20	7	150	F	17 ± 1.59	17 ± 2.27	14 ± 3.08
							I	25 ± 0.22	15 ± 0.64	25 ± 0.52
		16	40	25	5	150	F	22 ± 0.20	14 ± 2.77	17 ± 3.88
							I	32 ± 0.19	19 ± 0.86	21 ± 0.87
		17	40	25	6	150	F	14 ± 1.48	16 ± 2.13	19 ± 2.87
							I	29 ± 0.97	17 ± 1.53	18 ± 1.92
		18	40	25	7	150	F	12 ± 0.94	81 ± 1.50	21 ± 1.87
							I	30 ± 1.53	21 ± 2.19	15 ± 2.96

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
2	Amylase	1	30	15	5	150	F	121 ± 2.06	200 ± 2.82	145 ± 3.96
							I	225 ± 0.47	230 ± 0.94	125 ± 0.99
		2	30	15	6	150	F	145 ± 2.12	201 ± 2.89	157 ± 4.06
							I	215 ± 0.42	208 ± 0.88	185 ± 0.90
		3	30	15	7	150	F	168 ± 0.12	158 ± 0.52	169 ± 0.33
							I	138 ± 2.13	75 ± 2.89	174 ± 4.07
		4	30	20	5	150	F	148 ± 1.50	95 ± 2.15	187 ± 2.90
							I	147 ± 1.99	165 ± 2.74	125 ± 3.83
		5	30	20	6	150	F	132 ± 0.67	96 ± 1.18	178 ± 1.36
							I	154 ± 1.74	178 ± 2.44	169 ± 3.36
		6	30	20	7	150	F	145 ± 1.72	121 ± 2.41	201 ± 3.31
							I	169 ± 2.06	145 ± 2.81	178 ± 3.95
		7	30	25	5	150	F	165 ± 0.75	181 ± 1.27	147 ± 1.51
							I	178 ± 1.34	174 ± 1.97	158 ± 2.62
		8	30	25	6	150	F	196 ± 0.19	158 ± 0.61	121 ± 0.48
							I	198 ± 1.94	168 ± 2.67	164 ± 3.73
		9	30	25	7	150	F	185 ± 1.71	145 ± 2.41	148 ± 3.31
							I	223 ± 2.08	175 ± 2.84	148 ± 4
		10	40	15	5	150	F	148 ± 1.79	202 ± 2.50	145 ± 3.45
							I	221 ± 1.34	189 ± 1.96	125 ± 2.61
		11	40	15	6	150	F	178 ± 0.32	189 ± 0.76	93 ± 0.71
							I	220 ± 1.19	186 ± 1.79	175 ± 2.33
		12	40	15	7	150	F	198 ± 1.79	125 ± 2.49	58 ± 3.44
							I	211 ± 2.04	175 ± 2.79	185 ± 3.92
		13	40	20	5	150	F	201 ± 0.94	124 ± 1.49	142 ± 1.87
							I	239 ± 0.18	186 ± 2.01	152 ± 2.68
		14	40	20	6	150	F	207 ± 0.18	96 ± 2.29	75 ± 3.12
							I	215 ± 0.17	159 ± 0.58	147 ± 0.43
		15	40	20	7	150	F	165 ± 1.54	97 ± 2.20	94 ± 2.98
							I	222 ± 0.59	148 ± 1.08	165 ± 1.21
		16	40	25	5	150	F	174 ± 1.75	85 ± 2.45	89 ± 3.38
							I	214 ± 0.87	216 ± 1.41	148 ± 1.73
		17	40	25	6	150	F	102 ± 2.28	82 ± 3.08	63 ± 4.36
							I	219 ± 1.76	220 ± 2.46	169 ± 3.39
		18	40	25	7	150	F	200 ± 1.17	154 ± 1.77	145 ± 2.30
							I	225 ± 1.25	231 ± 1.85	148 ± 2.44

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
3	Lipase	1	30	15	5	150	F	35 ± 0.92	17 ± 1.47	33 ± 1.84
							I	19 ± 0.21	14 ± 0.63	52 ± 0.51
		2	30	15	6	150	F	25 ± 1.36	39 ± 0.22	35 ± 2.64
							I	17 ± 1.59	26 ± 2.26	26 ± 3.08
		3	30	15	7	150	F	30 ± 1.79	21 ± 2.50	24 ± 3.46
							I	28 ± 2.21	18 ± 2.99	33 ± 4.23
		4	30	20	5	150	F	29 ± 0.48	26 ± 0.94	29 ± 1.00
							I	26 ± 0.91	29 ± 1.46	24 ± 1.81
		5	30	20	6	150	F	14 ± 0.35	24 ± 0.80	27 ± 0.77
							I	27 ± 0.98	23 ± 1.54	45 ± 1.94
		6	30	20	7	150	F	19 ± 0.18	22 ± 0.59	26 ± 0.45
							I	35 ± 1.14	37 ± 1.72	61 ± 2.23
		7	30	25	5	150	F	32 ± 0.55	27 ± 1.03	14 ± 1.13
							I	34 ± 1.07	44 ± 1.65	57 ± 2.11
		8	30	25	6	150	F	25 ± 0.13	24 ± 0.54	19 ± 0.36
							I	25 ± 0.64	47 ± 1.13	51 ± 1.30
		9	30	25	7	150	F	32 ± 1.21	34 ± 1.81	26 ± 2.37
							I	25 ± 1.15	52 ± 1.74	41 ± 2.26
		10	40	15	5	150	F	18 ± 2.27	28 ± 3.07	28 ± 4.35
							I	25 ± 0.98	34 ± 1.54	48 ± 1.94
		11	40	15	6	150	F	25 ± 2.15	31 ± 2.93	18 ± 4.13
							I	42 ± 1.78	63 ± 0.23	51 ± 3.43
		12	40	15	7	150	F	34 ± 0.54	33 ± 1.02	26 ± 1.12
							I	33 ± 0.97	12 ± 1.52	56 ± 1.91
		13	40	20	5	150	F	29 ± 2.18	19 ± 2.95	28 ± 4.17
							I	42 ± 0.95	28 ± 1.50	48 ± 1.88
		14	40	20	6	150	F	27 ± 1.30	21 ± 1.92	27 ± 0.54
							I	45 ± 0.84	35 ± 1.38	38 ± 1.68
		15	40	20	7	150	F	22 ± 0.77	24 ± 1.29	22 ± 1.54
							I	41 ± 1.04	52 ± 1.62	57 ± 2.06
		16	40	25	5	150	F	14 ± 1.58	25 ± 2.25	23 ± 3.06
							I	25 ± 1.06	47 ± 1.64	41 ± 2.10
		17	40	25	6	150	F	11 ± 0.20	33 ± 1.79	18 ± 2.34
							I	26 ± 0.13	52 ± 0.53	35 ± 0.35
		18	40	25	7	150	F	16 ± 1.22	32 ± 1.82	15 ± 0.38
							I	35 ± 1.34	61 ± 1.96	54 ± 0.61

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
4	Chitinase	1	30	15	5	150	F	11 ± 0.89	9 ± 1.44	14 ± 1.78
							I	10 ± 0.66	5 ± 0.34	12 ± 3.21
		2	30	15	6	150	F	8 ± 0.045	12 ± 0.92	14 ± 0.96
							I	12 ± 0.91	14 ± 1.46	11 ± 1.81
		3	30	15	7	150	F	15 ± 1.74	16 ± 2.44	12 ± 3.35
							I	14 ± 0.85	9 ± 0.38	14 ± 1.69
		4	30	20	5	150	F	13 ± 1.79	7 ± 0.50	15 ± 3.46
							I	14 ± 1.18	11 ± 1.78	2 ± 0.031
		5	30	20	6	150	F	18 ± 0.66	12 ± 1.16	9 ± 0.34
							I	11 ± 0.52	14 ± 1.00	8 ± 0.19
		6	30	20	7	150	F	12 ± 0.68	18 ± 0.19	7 ± 0.39
							I	13 ± 1.50	8 ± 0.15	12 ± 2.90
		7	30	25	5	150	F	9 ± 0.81	9 ± 0.34	11 ± 1.63
							I	7 ± 0.074	15 ± 1.25	13 ± 1.49
		8	30	25	6	150	F	4 ± 0.026	12 ± 0.69	14 ± 0.60
							I	14 ± 1.50	7 ± 0.15	11 ± 2.90
		9	30	25	7	150	F	15 ± 1.61	8 ± 0.28	12 ± 3.11
							I	4 ± 0.17	11 ± 2.95	9 ± 0.16
		10	40	15	5	150	F	9 ± 0.073	12 ± 2.43	5 ± 0.34
							I	8 ± 2.28	14 ± 3.07	6 ± 0.36
		11	40	15	6	150	F	11 ± 1.87	15 ± 2.59	4 ± 0.060
							I	10 ± 0.94	12 ± 1.49	16 ± 0.17
		12	40	15	7	150	F	8 ± 1.64	7 ± 0.31	11 ± 3.16
							I	2 ± 0.01	9 ± 0.56	12 ± 1.96
		13	40	20	5	150	F	5 ± 0.067	11 ± 1.18	9 ± 0.37
							I	7 ± 0.29	15 ± 0.72	8 ± 0.65
		14	40	20	6	150	F	8 ± 0.71	14 ± 2.40	16 ± 0.23
							I	11 ± 0.83	2 ± 0.037	11 ± 1.67
		15	40	20	7	150	F	12 ± 0.32	3 ± 0.76	12 ± 0.70
							I	15 ± 0.31	5 ± 0.75	18 ± 0.70
		16	40	25	5	150	F	13 ± 1.01	4 ± 0.58	9 ± 0.30
							I	8 ± 0.12	11 ± 1.70	5 ± 0.19
		17	40	25	6	150	F	9 ± 0.054	12 ± 1.03	15 ± 1.13
							I	12 ± 1.04	11 ± 1.61	11 ± 2.05
		18	40	25	7	150	F	14 ± 2.01	14 ± 2.75	12 ± 3.86
							I	10 ± 0.062	15 ± 1.12	14 ± 1.28

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
5	Cellulase	1	30	15	5	150	F	40 ± 0.19	27 ± 0.60	50 ± 0.46
							I	52 ± 1.32	32 ± 1.94	35 ± 2.57
		2	30	15	6	150	F	25 ± 0.95	39 ± 1.51	48 ± 1.89
							I	48 ± 2.06	25 ± 2.81	27 ± 3.94
		3	30	15	7	150	F	36 ± 0.96	48 ± 1.52	35 ± 1.91
							I	56 ± 0.84	16 ± 1.37	62 ± 1.67
		4	30	20	5	150	F	37 ± 1.64	53 ± 2.32	34 ± 3.17
							I	47 ± 0.41	57 ± 0.87	15 ± 0.88
		5	30	20	6	150	F	36 ± 0.41	52 ± 0.86	29 ± 0.87
							I	56 ± 0.17	62 ± 2.82	47 ± 3.96
		6	30	20	7	150	F	41 ± 2.23	41 ± 3.02	23 ± 4.28
							I	66 ± 0.64	48 ± 1.14	63 ± 1.31
		7	30	25	5	150	F	11 ± 0.61	28 ± 1.10	41 ± 1.25
							I	63 ± 1.80	65 ± 2.51	58 ± 3.47
		8	30	25	6	150	F	14 ± 0.27	27 ± 0.70	45 ± 0.61
							I	52 ± 2.29	52 ± 3.09	24 ± 4.38
		9	30	25	7	150	F	15 ± 1.09	31 ± 1.66	35 ± 2.14
							I	49 ± 1.23	47 ± 1.84	32 ± 2.41
		10	40	15	5	150	F	16 ± 0.95	37 ± 1.50	52 ± 1.88
							I	52 ± 1.59	52 ± 2.26	55 ± 3.07
		11	40	15	6	150	F	18 ± 0.76	35 ± 1.28	49 ± 1.53
							I	48 ± 0.90	52 ± 1.44	65 ± 1.78
		12	40	15	7	150	F	27 ± 1.38	38 ± 2.02	48 ± 2.69
							I	36 ± 1.77	71 ± 2.47	65 ± 3.40
		13	40	20	5	150	F	25 ± 1.16	41 ± 1.75	45 ± 2.28
							I	47 ± 0.15	52 ± 0.56	71 ± 0.39
		14	40	20	6	150	F	33 ± 0.44	48 ± 0.91	52 ± 0.94
							I	45 ± 0.72	41 ± 1.23	73 ± 0.19
		15	40	20	7	150	F	42 ± 1.23	46 ± 1.83	45 ± 2.40
							I	48 ± 2.25	16 ± 3.04	58 ± 4.31
		16	40	25	5	150	F	46 ± 1.27	45 ± 1.88	34 ± 2.48
							I	57 ± 1.24	25 ± 1.85	66 ± 2.43
		17	40	25	6	150	F	56 ± 0.75	48 ± 1.27	31 ± 1.52
							I	62 ± 1.69	56 ± 2.37	54 ± 3.26
		18	40	25	7	150	F	26 ± 2.14	51 ± 2.91	28 ± 4.09
							I	72 ± 1.96	24 ± 2.69	72 ± 3.76

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

Sl.No	Enzyme	Experimental No.	Temperature (°C)	Salinity (ppt)	pH	Agitation (rpm)	State of bacterial cell*	Estimated enzyme concentration (U/ml ± SD) during different hours of incubation		
								24 hours	48 hours	72 hours
6	Ligninase	1	30	15	5	150	F	96 ± 0.93	117 ± 1.48	101 ± 1.85
							I	162 ± 1.30	157 ± 1.92	148 ± 2.54
		2	30	15	6	150	F	111 ± 0.22	96 ± 0.64	45 ± 0.52
							I	132 ± 1.27	158 ± 1.88	162 ± 2.48
		3	30	15	7	150	F	101 ± 0.93	73 ± 1.48	69 ± 1.84
							I	143 ± 2.03	163 ± 2.78	142 ± 3.90
		4	30	20	5	150	F	75 ± 1.16	47 ± 1.75	87 ± 2.28
							I	112 ± 1.87	147 ± 2.59	168 ± 3.59
		5	30	20	6	150	F	92 ± 1.97	88 ± 2.71	95 ± 3.79
							I	102 ± 1.37	135 ± 2.00	171 ± 2.67
		6	30	20	7	150	F	99 ± 1.92	85 ± 2.65	48 ± 3.69
							I	104 ± 1.27	125 ± 1.88	182 ± 2.47
		7	30	25	5	150	F	75 ± 1.43	96 ± 2.07	107 ± 2.78
							I	99 ± 0.59	157 ± 1.07	141 ± 1.20
		8	30	25	6	150	F	104 ± 1.55	57 ± 2.21	95 ± 3.00
							I	93 ± 1.54	165 ± 2.20	120 ± 2.98
		9	30	25	7	150	F	105 ± 1.06	75 ± 1.64	78 ± 2.10
							I	78 ± 1.63	174 ± 2.30	181 ± 3.14
		10	40	15	5	150	F	118 ± 0.30	97 ± 0.73	85 ± 0.67
							I	147 ± 0.65	180 ± 1.15	165 ± 1.32
		11	40	15	6	150	F	112 ± 1.74	98 ± 2.43	104 ± 3.35
							I	98 ± 1.30	157 ± 1.92	135 ± 2.54
		12	40	15	7	150	F	56 ± 1.05	101 ± 1.63	100 ± 2.08
							I	124 ± 2.16	158 ± 2.94	147 ± 4.15
		13	40	20	5	150	F	42 ± 1.28	118 ± 1.90	85 ± 2.50
							I	135 ± 1.93	174 ± 2.66	152 ± 3.71
		14	40	20	6	150	F	62 ± 0.33	175 ± 0.77	98 ± 0.72
							I	147 ± 0.31	168 ± 0.75	145 ± 0.69
		15	40	20	7	150	F	78 ± 0.29	120 ± 0.21	74 ± 0.65
							I	115 ± 1.57	181 ± 2.24	171 ± 3.04
		16	40	25	5	150	F	82 ± 0.30	105 ± 0.73	102 ± 0.67
							I	98 ± 0.27	152 ± 0.70	125 ± 0.62
		17	40	25	6	150	F	69 ± 0.63	117 ± 1.12	114 ± 1.28
							I	136 ± 0.75	185 ± 0.31	154 ± 1.52
		18	40	25	7	150	F	72 ± 1.26	118 ± 1.87	105 ± 2.46
							I	147 ± 1.06	171 ± 1.63	165 ± 2.08

ND- Value below detection limit of assay/ Not detected

***F- Free state consortia; I-Immobilized state consortia**

LIST OF PUBLICATIONS AND PRESENTATIONS

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A. LIST OF PUBLICATIONS

- **Paul, T.,** Kutty, S. N., & Sebastian, C. D. (2020). Distribution and hydrolytic potential of bacteria during monsoon and post monsoon seasons in the mangrove sediments of Northern Kerala. *Bioscience Biotechnology Research Communications*, 13(4), 1914-1919. https://doi.org/10.21786/bbrc/13.4/42

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B. PRESENTATIONS

Sl. No.	Authors	Title	Type of Participation	Name of the Seminar/ Workshop	Organizer	Duration	Year & Date
1.	Thara Paul, Sreedevi, N. Kutty. and Sebastian, C. D.	Distribution and identification of bacteria from mangrove sediments of North Kerala	Oral	International virtual conference on Biological innovations and computational exploration for pandemic challenges (BICPAC'22) Sponsored by Department of Biotechnology, CSIR and Ministry of Science and Technology Government of India	Department of Biotechnology and Bioinformatics, Bishop Heber college (Autonomous), Thiruchirapalli	2 days	24-25 February 2022
2.	Thara Paul, Sreedevi, N. Kutty. and Sebastian, C. D.	Spatial and Seasonal distribution of cultivable bacteria from mangrove sediments of North Kerala	Oral	ASM seminar on Microbial diversity and ecology-tropics to the polar regions	National centre for Polar and Ocean Research American Society for Microbiology CUSAT-NCPOR Centre for polar science Goa University	1 day	9 November 2022
3.	Thara Paul, Sreedevi, N. Kutty. and Sebastian, C. D.	Bacterial consortia derived from mangrove sediments of northern Kerala for bioremediation	Poster	National conference on Marine Pollution and Ecotoxicology (NCMPE24)	Cochin University of Science and Technology, Kerala	3 days	25 – 27, September 2024
4.	Thara Paul, Sreedevi, N. Kutty. and Sebastian, C. D.	Bacterial diversity in the mangrove sediments of northern Kerala: Metagenomic approach	Poster	International Conference on Marine diversity, Genomics and Sustainable development (ICMBGSD'25)	Cochin University of Science and Technology, Kerala	3 days	9- 11 April 2025