Bioprospecting of the endophytes from *Lagenandra toxicaria* Dalzell

Bioprospecting of the endophytes from *Lagenandra toxicaria* Dalzell

Thesis Submitted to
the University of Calicut in partial fulfillment of
the requirement for the degree of

DOCTOR OF PHILOSOPHY IN BOTANY

By

POOJA PUSHKARAN

Research Supervisor

Dr. N. S. PRADEEP

KSCSTE-MALABAR BOTANICAL GARDEN & INSTITUTE FOR PLANT SCIENCES
Kozhikode, Kerala, 673014, India

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KSCSTE - MALABAR BOTANICAL GARDEN AND INSTITUTE FOR PLANT SCIENCES

(An Institution under Kerala State Council for Science, Technology & Environment)

Post Box No. 1, Kozhikode - 673 014, Kerala, India. Phone: +91 495 2430939

Email: malabarbot.garden@gmail.com, www.mbgs.in

CERTIFICATE

This is to certify that the thesis entitled "**Bioprospecting of the endophytes from *Lagenandra toxicaria* Dalzell**" was submitted to the University of Calicut by **Miss. Pooja Pushkaran**, in partial fulfillment for the award of the degree of Doctor of Philosophy in Botany is a bonafide record of the research work carried out by her under my supervision and guidance. No part of the present work has previously formed the basis for the award of any other degree or diploma.

Kozhikode
23.10.2024

Dr. N. S. Pradeep
Guide

DECLARATION

I hereby declare that the work presented in the thesis entitled "**Bioprospecting of the endophytes from *Lagenandra toxicaria* Dalzell**" is based on the original work done by me under the guidance of **Dr. N. S. Pradeep** and has not been included in any other thesis submitted previously for the award of any degree. 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 found within the permissible limit. I also declare that the thesis is free from AI-generated contents.

Pooja Pushkaran

Kozhikode
Date: 23.10.24

Dr. N. S. Pradeep

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LIST OF ABBREVIATIONS

LC	Least Concern
IUCN	International Union for Conservation of Nature
SA	Salicylic Acid
JA	Jasmonic Acid
Et	Ethylene
ABA	Abscisic Acid
PGP	Plant Growth Promotion
IAA	Indole 3-Acetic Acid
ACC	1-Aminocyclopropane-1-Carboxylate
HCN	Hydrogen Cyanide
MDA	Malondialdehyde
ROS	Reactive Oxygen Species
pH	Potential of Hydrogen
AMF	Arbuscular Mycorrhizal Fungi
ALA	Alpha-Linolenic Acid
EPA	Eicosapentaenoic Acid
DHA	Docosahexaenoic Acid
HTS	High-throughput sequencing
NGS	Next-Generation Sequencing
PCR	Polymerase Chain Reaction
GATK	Genome Analysis Toolkit
GC/MS	Gas Chromatography Mass Spectrometry
CCl_4	Carbon tetrachloride
^0C	Centigrade
sq. km.	Square Kilometre
m	Meter
NaOCl	Sodium Hypochlorite
C_2H_5OH	Ethanol
secs	Seconds
mg/ml	Milligram per Millilitre
NA	Nutrient Agar
PDA	Potato Dextrose Agar
PF	Percentage Frequency
IF	Isolation Frequency
CR	Colonization Rate
CF	Colonization Frequency
D	Simpson's Diversity Index
SWDI	Shannon-Weiner Diversity Index
R1	Margalef Richness index
E	Evenness
MANOVA	Multivariate Analysis of Variance
PCA	Principal Component Analysis
%	Percentage
Hrs	Hours
MR-VP	Methyl Red–Voges Proskauer
TSI	Triple Sugar Iron
H_2S	Hydrogen Sulfide
NB	Nutrient Broth
OD	Optical Density
ml	Millilitre
TE	Tris-EDTA
mg	Milligram
µl	Microlitre

M	Molar
Rpm	Revolution per Minute
DNA	Deoxyribonucleic Acid
nm	Nanometre
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool
TES	Triethylammonium Hydroxide
EDTA	Ethylenediaminetetraacetic Acid
CTAB	Cetyltrimethylammonium Bromide
RNA	Ribonucleic Acid
Ng	Nanogram
ITS	Internal Transcribed Spacer
dNTPs	Deoxyribonucleotide Triphosphate
nBLAST	nucleotide-Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
Bps	Base pairs
rRNA	Ribosomal Ribonucleic Acid
B	Bacteria
F	Fungi
L	Leaf
R	Root
Rh	Rhizome
S	Summer
M	Monsoon
W	Winter
±	Plus-or-minus sign
v/v	Volume/Volume
DMSO	Dimethyl Sulfoxide
HCl	Hydrochloric Acid
NaOH	Sodium Hydroxide
AE	Atropine Equivalent
$AlCl_3$	Aluminium Chloride
UV	Ultraviolet
GAE	Gallic Acid Equivalent
LB	Liebermann–Burchard
TAE	Tannin Equivalent
Na_2CO_3	Sodium Carbonate
$CuSO_4$	Copper Sulphate
H_2O	Water
BSA	Bovine Serum Albumin
MTCC	Microbial Type Culture Collection
CFU	Colony-Forming Unit
Mm	Millimetre
HPLC	High-Performance Liquid Chromatography
µg	Microgram
µM	Micro Molar
mM	Millimolar
µmol	Micromole
^{0}	Degree
km	Kilometre
$K_3Fe(CN)6$	Potassium Ferricyanide
et al.	et aliorum (and of others)
$FeCl_3$	Ferric Chloride
Al	Alkaloid

Fl	Flavanoid
Ph	Phenol
Sa	Saponin
St	Sterol
Ta	Tannin
Te	Terpenoid
Cg	Cardiac glycoside
Gl	Glycoside
Ch	Carbohydrate
Co	Coumarin
Qu	Quinone
Pr	Protein
Ppm	Parts per Million
IC50	Half Maximal Inhibitory Concentration
DPPH	1, 1-Diphenyl-2-Picrylhydrazyl
ABTS	2, 2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulphonic Acid)
FRAP	Ferric Reducing Power Assay
Fe 3+	Ferric Ions
Fe 2+	Ferrous Ions
TPTZ	2,4,6-Tripyridyl-S-Triazine
TCA	Trichloro Acetic Acid
CMC	Carboxymethyl Cellulose
DNS	3,5-Dinitrosalicylic Acid
NCBI	National Center for Biotechnology Information
w/v	Weight/Volume
OFAT	One-Factor-At-A-Time
PB	Plackett-Burman
ANOVA	Analysis of Variance
CCD	Central Composite Design
RSM	Response Surface Methodology
kDa	Kilodalton
N	Normality
V	Volt
m^2	Meter Square
km^2	Kilometre Square
&	And
SDS	Sodium Dodecyl Sulphate
PAGE	Polyacrylamide Gel Electrophoresis
TEMED	N, N, N′, N′ -Tetramethylethylenediamine
DMRT	Duncan's Multiple Range Test
SD	Standard Deviation
U/ml	Units per Millilitre
SmF	Sub-merged Fermentation
REA	Relative Enzymatic Index
VOCs	Volatile Organic Compounds
IAA	Indole 3-Acetic Acid
P	Phosphorus
H_2PO_4	Dihydrogen Phosphate
HPO_4^-	Phosphoric Acid
PSI	Phosphate Solubilization Index
TCP	Tri-calcium Phosphate
$(NH_4)_2SO_4$	Ammonium Sulphate
DEP	Diethyl Phthalate
DOP	Dioctyl Phthalate

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Abstract

Lagenandra toxicaria is a medicinal aquatic plant that harbors a diverse array of endophytes with promising applications. This study delved into the endophytic community, their interactions, and their potential for producing phytochemicals, exhibiting antimicrobial and antioxidant activities, producing enzymes, and promoting plant growth. The research successfully identified the diverse range of endophytes present in each plant part, location, and season, revealing that their diversity varies depending on the season and plant part. Multivariate ANOVA analysis showed that seasonal changes and plant parts influence diversity of endophytes. Notably, a fungal isolate exhibited potent antibiotic activity due to a compound with antimicrobial properties. The potent isolates were found to contain significant amounts of flavonoid, phenol, terpenoid, coumarin, quinone, and glycoside. Fungal isolates from leaves demonstrated antimicrobial and antioxidant activities, while bacterial endophytes were identified as efficient producers of enzymes, particularly protease.

Both fungal and bacterial endophytes exhibited plant growth-promoting properties, including producing indole-3-acetic acid, solubilizing phosphate, and exhibiting ACC deaminase activity. Experimental studies confirmed the quality and stability of enzymes and the effect of IAA on seedling development. HPLC analysis identified the production of IAA and DEP by corresponding isolates under pre-optimized and optimized conditions. This study highlights the vast potential of *Lagenandra toxicaria* endophytes for sustainable agriculture, biotechnology, and environmental remediation. Further optimization of endophyte-plant interactions and bioactive compound production can unlock new opportunities for eco-friendly solutions. The research contributes to the understanding of endophyte-plant interactions. It emphasizes the importance of exploring *Lagenandra toxicaria* endophytes for future applications, demonstrating their potential for sustainable agriculture, biotechnology, and environmental sustainability.

Keywords: *Lagenandra toxicaria*, endophytes, diversity, antimicrobial, enzyme

സംഗ്രഹം

ഔഷധ ജലസസ്യമായ ലഗിനാന്ദ്ര ടോക്സിക്കറിയ നിരവധി വൈവിദ്യമാർന്ന ഗുണങ്ങളുള്ള എൻഡോഫൈറ്റുകളുടെ ആവാസ കേന്ദ്രമാണ്. ഈ പഠനം എൻഡോഫൈറ്റിക് കമ്മ്യൂണിറ്റി, ഘടകങ്ങൾ തമ്മിലുള്ള ഇടപെടലുകൾ, ഫൈറ്റോകെമിക്കലുകൾ ഉൽപ്പാദിപ്പിക്കുന്നതിനുള്ള അവയുടെ സാധ്യതകൾ, ആന്റിമൈക്രോബയൽ, ആന്റിഓക്സിഡന്റ് പ്രവർത്തനങ്ങൾ, എൻസൈമുകൾ ഉൽപ്പാദിപ്പിക്കൽ, സസ്യവളർച്ച പ്രോത്സാഹിപ്പിക്കൽ എന്നിവയെ കുറിച്ച് പരിശോധിക്കുന്നതിന് വേണ്ടിയായിരുന്നു. ചെടിയുടെ ഭാഗങ്ങൾ സ്ഥലങ്ങൾ കാലാവസ്ഥ എന്നിവയെ കണക്കാക്കുമ്പോൾ വൈവിധ്യമാർന്ന എൻഡോഫൈറ്റുകളെ തിരിച്ചറിയാൻ ഈ പഠനത്തിലൂടെ കഴിഞ്ഞു. കാലാവസ്ഥയും സസ്യഭാഗവും അനുസരിച്ച് അവയുടെ വൈവിധ്യം വ്യത്യാസപ്പെടുന്നുവെന്ന് വെളിപ്പെടുത്തുന്നു. മൾട്ടി വേരിയേറ്റ് അനോവ വിശകലനം കാണിക്കുന്നത് എൻഡോഫൈറ്റുകളുടെ വൈവിധ്യത്തെ കാലാനുസൃതമായ മാറ്റങ്ങളും സസ്യഭാഗങ്ങളും സ്വാധീനിക്കുന്നു എന്നാണ്. ശക്തമായ ആൻറിബയോട്ടിക് പ്രവർത്തനം പ്രദർശിപ്പിച്ച ഒരു ഫംഗലിനെ തിരഞ്ഞെടുക്കുകയും അതിൽ എച്ച്.പി.എൽ. സി യിലൂടെ ആന്റിമൈക്രോബിയൽ ഗുണങ്ങളുള്ള ഒരു സംയുക്തത്തിനെ കണ്ടെത്താനും കഴിഞ്ഞു. ഗുണങ്ങൾ ഏറെ വെളിപ്പെടുത്തുന്ന ഐസൊലേറ്റുകളിൽ ഗണ്യമായ അളവിൽ ഫ്ലേവനോയിഡ്, ഫിനോൾ, ടെർപെനോയിഡ്, കൊമറിൻ, കിനോൺ, ഗ്ലൈക്കോസൈഡ് എന്നിവ അടങ്ങിയിരിക്കുന്നതായി കണ്ടെത്തി. ഇലകളിൽ നിന്നുള്ള വേർതിരിച്ചെടുത്ത ഫംഗലുകളിൽ ആന്റിമൈക്രോബയൽ, ആന്റിഓക്സിഡന്റ് പ്രവർത്തനങ്ങൾ പ്രകടമാക്കുന്നു, അതേസമയം ബാക്ടീരിയ എൻഡോഫൈറ്റുകൾ എൻസൈമുകളുടെ, പ്രത്യേകിച്ച് പ്രോട്ടീസിന്റെ കാര്യക്ഷമമായ നിർമ്മാതാക്കളായി തിരിച്ചറിഞ്ഞു.

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കീവേഡുകൾ: ലഗിനാന്ദ്ര ടോക്സിക്കറിയ, എൻഡോഫൈറ്റുകൾ, വൈവിധ്യം, ആന്റിമൈക്രോബയൽ, എൻസൈം

CONTENTS

Abstract

Lagenandra toxicaria is a medicinal aquatic plant that harbors a diverse array of endophytes with promising applications. This study delved into the endophytic community, their interactions, and their potential for producing phytochemicals, exhibiting antimicrobial and antioxidant activities, producing enzymes, and promoting plant growth. The research successfully identified the diverse range of endophytes present in each plant part, location, and season, revealing that their diversity varies depending on the season and plant part. Multivariate ANOVA analysis showed that seasonal changes and plant parts influence diversity of endophytes. Notably, a fungal isolate exhibited potent antibiotic activity due to a compound with antimicrobial properties. The potent isolates were found to contain significant amounts of flavonoid, phenol, terpenoid, coumarin, quinone, and glycoside. Fungal isolates from leaves demonstrated antimicrobial and antioxidant activities, while bacterial endophytes were identified as efficient producers of enzymes, particularly protease.

Both fungal and bacterial endophytes exhibited plant growth-promoting properties, including producing indole-3-acetic acid, solubilizing phosphate, and exhibiting ACC deaminase activity. Experimental studies confirmed the quality and stability of enzymes and the effect of IAA on seedling development. HPLC analysis identified the production of IAA and DEP by corresponding isolates under pre-optimized and optimized conditions. This study highlights the vast potential of *Lagenandra toxicaria* endophytes for sustainable agriculture, biotechnology, and environmental remediation. Further optimization of endophyte-plant interactions and bioactive compound production can unlock new opportunities for eco-friendly solutions. The research contributes to the understanding of endophyte-plant interactions. It emphasizes the importance of exploring *Lagenandra toxicaria* endophytes for future applications, demonstrating their potential for sustainable agriculture, biotechnology, and environmental sustainability.

Keywords: *Lagenandra toxicaria*, endophytes, diversity, antimicrobial, enzyme

സംഗ്രഹം

ഔഷധ ജലസസ്യമായ ലഗിനാന്ദ്ര ടോക്സിക്കറിയ നിരവധി വൈവിദ്യമാർന്ന ഗുണങ്ങളുള്ള എൻഡോഫൈറ്റുകളുടെ ആവാസ കേന്ദ്രമാണ്. ഈ പഠനം എൻഡോഫൈറ്റിക് കമ്മ്യൂണിറ്റി, ഘടകങ്ങൾ തമ്മിലുള്ള ഇടപെടലുകൾ, ഫൈറ്റോകെമിക്കലുകൾ ഉൽപ്പാദിപ്പിക്കുന്നതിനുള്ള അവയുടെ സാധ്യതകൾ, ആന്റിമൈക്രോബയൽ, ആന്റിഓക്സിഡന്റ് പ്രവർത്തനങ്ങൾ, എൻസൈമുകൾ ഉൽപ്പാദിപ്പിക്കൽ, സസ്യവളർച്ച പ്രോത്സാഹിപ്പിക്കൽ എന്നിവയെ കുറിച്ച് പരിശോധിക്കുന്നതിന് വേണ്ടിയായിരുന്നു. ചെടിയുടെ ഭാഗങ്ങൾ സ്ഥലങ്ങൾ കാലാവസ്ഥ എന്നിവയെ കണക്കാക്കുമ്പോൾ വൈവിധ്യമാർന്ന എൻഡോഫൈറ്റുകളെ തിരിച്ചറിയാൻ ഈ പഠനത്തിലൂടെ കഴിഞ്ഞു. കാലാവസ്ഥയും സസ്യഭാഗവും അനുസരിച്ച് അവയുടെ വൈവിധ്യം വ്യത്യാസപ്പെടുന്നുവെന്ന് വെളിപ്പെടുത്തുന്നു. മൾട്ടി വേരിയേറ്റ് അനോവ വിശകലനം കാണിക്കുന്നത് എൻഡോഫൈറ്റുകളുടെ വൈവിധ്യത്തെ കാലാനുസൃതമായ മാറ്റങ്ങളും സസ്യഭാഗങ്ങളും സ്വാധീനിക്കുന്നു എന്നാണ്. ശക്തമായ ആൻറിബയോട്ടിക് പ്രവർത്തനം പ്രദർശിപ്പിച്ച ഒരു ഫംഗലിനെ തിരഞ്ഞെടുക്കുകയും അതിൽ എച്ച്.പി.എൽ. സി യിലൂടെ ആന്റിമൈക്രോബിയൽ ഗുണങ്ങളുള്ള ഒരു സംയുക്തത്തിനെ കണ്ടെത്താനും കഴിഞ്ഞു. ഗുണങ്ങൾ ഏറെ വെളിപ്പെടുത്തുന്ന ഐസൊലേറ്റുകളിൽ ഗണ്യമായ അളവിൽ ഫ്ലേവനോയിഡ്, ഫിനോൾ, ടെർപെനോയിഡ്, കൊമറിൻ, കിനോൺ, ഗ്ലൈക്കോസൈഡ് എന്നിവ അടങ്ങിയിരിക്കുന്നതായി കണ്ടെത്തി. ഇലകളിൽ നിന്നുള്ള വേർതിരിച്ചെടുത്ത ഫംഗലുകളിൽ ആന്റിമൈക്രോബയൽ, ആന്റിഓക്സിഡന്റ് പ്രവർത്തനങ്ങൾ പ്രകടമാക്കുന്നു, അതേസമയം ബാക്ടീരിയ എൻഡോഫൈറ്റുകൾ എൻസൈമുകളുടെ, പ്രത്യേകിച്ച് പ്രോട്ടീസിന്റെ കാര്യക്ഷമമായ നിർമ്മാതാക്കളായി തിരിച്ചറിഞ്ഞു.

ഇൻഡോൾ-3-അസറ്റിക് ആസിഡ് ഉൽപ്പാദിപ്പിക്കുക, ഫോസ്ഫേറ്റ് ലയിപ്പിക്കുക, എസിസി ഡീമിനേസ് പ്രവർത്തനം എന്നിവ ഉൾപ്പെടെയുള്ള സസ്യവളർച്ചയെ പ്രോത്സാഹിപ്പിക്കുന്ന ഗുണങ്ങൾ ഫംഗൽ, ബാക്ടീരിയൽ എൻഡോഫൈറ്റുകളിൽ കാണപ്പെട്ടു. പരീക്ഷണാത്മക പഠനങ്ങൾ എൻസൈമുകളുടെ ഗുണനിലവാരവും സ്ഥിരതയും തൈകളുടെ വികസനത്തിൽ IAA യുടെ സ്വാധീനവും സ്ഥിരീകരിച്ചു. എച്ച്പിഎൽസി വിശകലനം, പ്രീ-ഒപ്റ്റിമൈസ് ചെയ്തതും ഒപ്റ്റിമൈസ് ചെയ്തതുമായ അവസ്ഥകളിൽ അനുബന്ധ ഐസൊലേറ്റുകൾ വഴി ഐ.എ.എ, ഡി.ഇ.പി എന്നിവയുടെ ഉത്പാദനം തിരിച്ചറിഞ്ഞു. സുസ്ഥിര കൃഷി, ബയോടെക്നോളജി, പാരിസ്ഥിതിക പ്രതിവിധി എന്നിവയ്ക്കായുള്ള ലഗിനാന്ദ്ര ടോക്സിക്കറിയ എൻഡോഫൈറ്റുകളുടെ വിപുലമായ സാധ്യതകളെ ഈ പഠനം എടുത്തുകാണിക്കുന്നു. എൻഡോഫൈറ്റ്- സസ്യ ഇടപെടലുകളുടെ കൂടുതൽ ഒപ്റ്റിമൈസേഷനും ബയോ ആക്റ്റീവ് സംയുക്ത ഉൽപാദനവും പരിസ്ഥിതി സൗഹൃദ പരിഹാരങ്ങൾക്കുള്ള പുതിയ അവസരങ്ങൾ തുറക്കും. എൻഡോഫൈറ്റ്-പ്ലാന്റ് ഇടപെടലുകളെക്കുറിച്ചുള്ള ഗ്രാഹ്യത്തിന് ഗവേഷണം സംഭാവന നൽകുകയും ഭാവിയിലെ ആപ്ലിക്കേഷനുകൾക്കായി ലഗിനന്ദ്ര ടോക്സിക്കറിയ എൻഡോഫൈറ്റുകൾ പര്യവേക്ഷണം ചെയ്യുകയും സുസ്ഥിര കൃഷി, ജൈവ സാങ്കേതികവിദ്യ, പരിസ്ഥിതി സുസ്ഥിരത എന്നിവയ്ക്കുള്ള അവയുടെ സാധ്യതകൾ പ്രകടമാക്കുകയും ചെയ്യുന്നു.

കീവേഡുകൾ: ലഗിനാന്ദ്ര ടോക്സിക്കറിയ, എൻഡോഫൈറ്റുകൾ, വൈവിധ്യം, ആന്റിമൈക്രോബയൽ, എൻസൈം

Chapter 1

General Introduction

In recent years, there has been a notable increase in the focus on microbial communities coexisting with higher eukaryotic organisms. Although, until the early 1990s, plants were thought to be a single, sessile biological entity, increased scientific understanding and growing interest in the field suggested that plants harbour countless numbers of microorganisms that live on their surfaces (epiphytes) and inside their tissues (endophytes). Endophytic plethora is regarded as one of the most valuable microbial inhabitants due to its primary function in the secretion of biologically active secondary metabolites. De Bary first used the term "endophyte" in 1866, and it has since become widely used in literature. Endophyte (Greek: endon, "within" + phyton, "plant") refers to microorganisms that colonize living internal plant tissues without causing immediate harm. These microbes perform various biological functions (Baker and Satish, 2012). Endophytic plethora occupy distinct voids within their host and release innovative bioactive substances with significant applications in agriculture, medicine, and industry. The discovery of fungal endophytes in grasses, which were able to produce alkaloids that were toxic to herbivores and later repellent to insects, sparked interest in endophytes as biological pest control agents. Since then, scientific communities worldwide have focused on endophyte research for active compounds with potential therapeutic applications (Strobel and Daisy, 2003).

Endophytic microbes, which can be either facultative or obligatory, play a crucial role in shaping plants' ecological and physiological traits. These microbes engage in complex interactions with their host plants, ranging from mutually beneficial relationships to antagonistic ones (Jha et al., 2018). The location and climate of the host plant significantly influence the composition of endophytic communities (Christian et al., 2016). The endosphere, the internal environment of the plant, is shaped by a combination of random events and deterministic processes that govern the colonization and diversity of endophytic

populations. Endophytes have undergone co-evolution with their host plants over time, leading to the development of specialized strategies for survival, adaptation, and refined relationships (Krings et al., 2007; Yu et al., 2010; Selim et al., 2012; Goyal et al., 2017).

Endophytes have evolved diverse strategies to adapt to their environments and thrive within their host plants (Xia et al., 2022). Endophytes exhibit elevated metabolic activity compared to their free-living counterparts, driven by their unique roles in nature and the activation of multiple metabolic pathways to thrive within host tissues (Strobel et al., 2004; Qadri et al., 2013). This heightened activity enables endophytes to mimic their host plant's chemistry, producing bioactive natural products or derivatives that are often identical or even more potent than those generated by the host itself. The production of Taxol (a chemotherapy medication) from Yews (*Taxus brevifolia*) and other plant sources by a variety of endophytes serves as an example of this (Owen and Hundley, 2004). Interestingly, plants growing in soil polluted with xenobiotics tend to harbour endophytes with genes that degrade contaminants. To maintain a stable symbiotic relationship, endophytes produce substances that promote plant growth, environmental protection, and sustainability, ultimately creating a favourable living space within the host. Endophytes offer protection to plants against herbivores and sometimes act as biocontrol agents. Research has highlighted their significant contributions to biodegradation and nutrient cycling (Krishnamurthy and Naik, 2017; Grabka et al., 2022). Moreover, endophytes have shown remarkable tolerance to heavy metals and antimicrobials and can break down complex organic molecules, making them ideal candidates for bioremediation applications (Siciliano et al., 2001; Fadiji and Babalola, 2020). Bioactive metabolites produced by endophytes have shown potential as pharmaceuticals, medicines, and various therapeutic agents, including antibiotics, antioxidants, anti-inflammatory, antiviral, and anti-cancer compounds, making them valuable for research and applications in the food industry (Tenguria et al., 2011; Manganyi and Ateba, 2020). Many endophytes

have been found to possess the ability to break down xenobiotics naturally, and some can even serve as vectors for introducing degradative traits into plants. Endophytes can generate novel chemicals and enhance their host's capabilities through their diverse relationships with plants, including symbiotic, mutualistic, commensal, and trophobiotic interactions. Owing to these features, endophytic fungi and bacteria are frequently the subject of more research than other endophytes. Their importance in plant biology, ecology, and evolution research is attributed to their genetic diversity, adaptability, and non-harmful colonization of plant tissues. Numerous investigations have focused on endophytic bacteria and fungi, the best sources for supplying a chemical factory that supports the growth of diverse industries while limiting the overuse of plant materials.

Endophytic Bacteria

Endophytic bacteria are intriguing microorganisms that inhabit plant tissues, including stems, leaves, roots, seeds, fruits, ovules, tubers, and legume nodules, without causing harm to the host plant (Quadt-Hallmann et al., 1997). Bacterial endophytes play various crucial roles, including nitrogen fixation, phytohormone synthesis for biocontrol, promoting the growth of the host under adverse conditions, defending against invasive pathogens, and generating bioactive compounds as secondary metabolites for biological activity. A wide range of plant species are harboured and colonized by bacterial species; the majority of these isolates are found in the following genera: *Enterobacter, Kocuria, Lysinibacillus, Methylobacterium, Microbacterium, Paneibacillus, Pantoea, Phyllobacterium, Pseudomonas, Rahnella, Rhodanobacter, Stenotrophomonas, Streptomyces*, and *Xanthomonas*. The most common species among these are *Pseudomonas, Streptomyces,* and *Bacillus*. Endophytic bacteria can enter plant tissues through various means, including germinating radicles (Gagne et al., 1987), secondary roots (Agarwal, 1987), stomata (Roos and Hattingh, 1983), or damaged sites (Leben et al., 1968). These bacteria employ a range of strategies to colonise plant tissues, including motility,

attachment, plant polymer degradation, and evasion of plant defence mechanisms (Liu et al., 2017). Generally, bacterial populations are more abundant in roots than stems and leaves, with roots typically harbouring the highest numbers of endophytes in most plant species (Rosenblueth et al., 2006). The internal colonization, in turn, protects endophytic communities from exposure to extreme environmental conditions such as temperature, osmotic potentials and ultraviolet radiation, giving rise to added ecological advantage to them over epiphytes (Menpara and Chanda, 2014).

Research has demonstrated the potential of endophytic bacteria to boost crop yields and promote sustainable agriculture practices (Brader et al., 2014; Reinhold-Hurek and Hurek, 2011). Additionally, these bacteria have shown promise in biotechnology applications (Eid et al., 2021), including the production of various bioactive compounds with commercial potential, such as antibiotics (Kumar et al., 2024), enzymes (Mamangkey et al., 2022), and biopesticides (Maksimov and Cherapanova, 2018). Some endophytic bacteria have a broad host range, making them suitable for use as bioinoculants in developing sustainable agriculture systems. Beyond their agricultural and biotechnological applications, endophytic bacteria are also being explored for their role in environmental remediation. These bacteria have been found to degrade various pollutants, including pesticides, heavy metals, and hydrocarbons, through bioremediation processes. For example, endophytic bacteria isolated from contaminated soils have been used to detoxify heavy metal pollutants through mechanisms like biosorption and biomineralization (Sharma et al., 2018).

Although endophytic bacteria hold great promise, several obstacles hinder their study and utilization. A major challenge lies in detecting and isolating these bacteria from plant tissues, as they often prove difficult to cultivate in laboratory settings. Furthermore, the complexity of endophytic bacteria diversity and their interactions with plant hosts remains poorly understood. The variety of endophytic colonizers is influenced by various factors, including bacterial,

plant, and environmental characteristics, which adds to the complexity of studying these microorganisms.

Endophytic Fungi

Endophytic fungi are fascinating microorganisms that inhabit plant tissues, including roots, stems, and leaves, both above and below ground. These diverse fungi can be classified into various categories, such as fungal endophytes of dicots, systemic fungal endophytes, and endophytic fungi of specific plant structures like tree bark or xylem (Stone et al., 2000). They have evolved to colonize and thrive asymptomatically within healthy plant tissues, often evading the host's defence mechanisms (Saikkonen et al., 1998). The colonization process is gradual, and the diversity of endophytes is remarkable, with a single species potentially associated with multiple plant species, and multiple species present within a single host. Some endophytes remain dormant, while others interact with each other. The presence of endophytic fungi significantly impacts plant growth, development, fitness, diversity, population dynamics, and ecosystem functioning (Saikkonen et al., 1998; Hardoim et al., 2015). Once inside the host plant, endophytic fungi can assume a quiescent state, remaining latent for an extended period or the entire host plant lifetime until environmental conditions favour fungal growth or the host's ontogenetic state changes (Sieber, 2007; Jia et al., 2016). Factors like host type, tissue type, and abiotic factors influence the colonization rate, diversity, and community composition of endophytic fungi within a host species (Guevara-Araya et al., 2020).

Endophytic fungi have evolved sophisticated strategies to colonize and thrive within their host plants, including mechanisms for host recognition, evasion of defence responses, and tailored infection structures. For instance, to counteract the host's secondary metabolites, which can inhibit fungal growth, endophytic fungi produce detoxification enzymes like cellulases, lactase, xylanase, and protease, enabling them to establish a foothold within the host (Aly et al., 2011). Endophytic fungi within host plants enhance their ability to cope with various

biotic and abiotic stress factors, promoting resilience and adaptability (Fountain et al., 2014).

The symbiotic relationship between fungi and plants produces a diverse array of secondary metabolites, which play a crucial role in shaping the interactions between microscopic fungi and their host plants. The type and number of secondary metabolites produced and the plant's phytotoxins influence the fungi's lifestyle and pathogenic potential. Notably, endophytic fungi can produce secondary metabolites that mimic plant hormones like auxins, gibberellins, and abscisic acid, allowing them to manipulate plant growth, suppress defense responses, and acquire nutrients for their growth and colonization (Pusztahelyi et al., 2015). Endophytic fungi have emerged as a treasure trove of novel, bioactive natural products with vast potential in agriculture, medicine, and the food industry (Strobel et al., 2004). The landmark discovery of paclitaxel (taxol) in *Taxomyces andreanae* in 1993 exemplifies this potential (Stierle et al., 1993). Research has since revealed that fungal endophytes produce an impressive array of structurally diverse and novel bioactive compounds, including flavonoids, alkaloids, steroids, polyphenols, terpenoids, and tannins (Gouda et al., 2016).

Fungal secondary metabolites can be classified into four primary chemical categories: polyketides, terpenoids, shikimic acid-derived compounds, and non-ribosomal peptides (Collemare et al., 2008). Certain metabolites, such as pigments, polyols, and mycosporines, contribute to pathogenicity and stress tolerance, including resistance to temperature and UV radiation (Sinha et al., 2007). These compounds exhibit potent antimicrobial activity by altering microbial cell permeability and interacting with membrane proteins, causing structural and functional changes. Moreover, environmental factors like light, temperature, pH, calcium, and nutrient availability significantly influence secondary metabolite production.

Endophyte research encompasses various fields, including population dynamics, community characterization, biological interactions, anatomical

investigations, and phylogenetic analysis (Murphy and Hodkinson, 2018). Numerous studies have explored the beneficial relationships between hosts and their microbial partners, revealing how they promote plant growth and health (Clay and Schardl, 2002; Feng et al., 2006; Xin et al., 2009; Barretti et al., 2008; Pan and Clay, 2004). Despite this progress, the endophytic ecology of only a limited number of plant species has been thoroughly examined. This suggests that there is vast potential for discovering novel and valuable endophytic microbes among the diverse range of plant species found in various ecosystems. Since medicinal plants in biodiversity hotspots have a higher endophytic diversity, they are always possible sources of bioactive compounds (Strobel et al., 1996). An extensive study of endophytic fungal assemblages in Indian medicinal plants was conducted by Golinska et al. (2015). According to Yu et al. (2010), a review, medicinal plants are a significant source of bioactive endophytic isolates. Specifically, 35 per cent of bioactive isolates with maximum symmetry were derived from medicinal plants, with crop plants coming in second with 29 per cent and plants growing in unusual environments with 18 per cent. To combat the threat of drug resistance against human pathogens, one promising strategy is to isolate endophytic fungi for their antimicrobial role (Tan and Zou, 2001; Rao and Satish, 2016). As these microorganisms are essential to the medicinal qualities of their hosts, it is well known that medicinal plants with a proven ethnobotanical value are promising sources to investigate powerful endophytic microbes (Yu et al., 2010; Nath et al., 2015).

Like terrestrial medicinal plants, aquatic plants have evolved unique bioactive compounds that can be used to develop new medicines. Aquatic medicinal plants are equally important as terrestrial plants in the realm of medicine and healthcare. For centuries, these plants have been used in traditional medicine to treat various health conditions, and their significance is becoming increasingly recognized. They have adapted to survive in water, developing specialized characteristics that enable them to thrive in aquatic environments. These

adaptations have led to producing novel compounds with potential medicinal properties. Aquatic medicinal plants offer several advantages over terrestrial plants. They are often more resilient and can thrive in challenging environments, making them a reliable source of medicinal compounds. Aquatic plants can be more easily cultivated and harvested, reducing the risk of over-exploitation and environmental damage.

Examples of aquatic medicinal plants include seaweeds, water lilies, and cattails, which have been used to treat various health conditions, from digestive issues to skin conditions and wounds. These plants have been found to possess anti-inflammatory, antimicrobial, and antioxidant properties, making them valuable resources for drug discovery and development. Furthermore, aquatic medicinal plants are crucial in maintaining ecosystem balance and biodiversity. They provide habitat and food for aquatic animals, and their medicinal properties can be used to develop sustainable and eco-friendly healthcare solutions.

Endophytes from Aquatic Plants

Endophytic microbes from aquatic plants are a fascinating and relatively unexplored area of research. Aquatic plants, such as seagrasses, corals, and algae, provide a unique habitat for endophytic microbes, including bacteria, fungi, and archaea. Endophytic microbes from aquatic plants have been implicated in producing bioactive compounds, such as pigments, toxins, and signaling molecules, which can significantly impact the surrounding ecosystem. Aquatic plants provide a unique environment for endophytes to colonize and thrive. The submerged roots and leaves of these plants create a habitat for endophytes to enter and establish themselves within the plant tissues. Endophytes colonize aquatic plants through various means, including wounds, stomata, or even through the plant's nutrient uptake mechanisms (Srivastava, 2015). Endophytes from aquatic plants exhibit high diversity, with different bacteria and fungi found in different plant species (Khan, 2016).

Endophytes have been found to colonize a wide range of aquatic plants, including freshwater and marine species. The diversity of endophytes in aquatic plants is vast, with different species of bacteria and fungi found in different plant species. For example, water lilies have been found to harbour endophytes such as Pseudomonas and Bacillus, while cattails have been found to harbour endophytes such as *Pseudomonas fluorescens* (Srivastava, 2015; Lodewyckx, 2002). Endophytes from aquatic plants have potential applications in biotechnology and medicine, such as the production of novel antibiotics and antifungals. Additionally, endophytes could be used to improve the growth and productivity of aquatic plants, which could have significant implications for agriculture and aquaculture. (Gupta et al., 2016). The study of endophytic microbes from aquatic plants is still in its infancy, but it has the potential to reveal new insights into the complex interactions between plants and microbes in aquatic ecosystems. Additionally, these microbes may hold the key to developing novel bioproducts, such as biofuels, bioplastics, and pharmaceuticals.

***Lagenandra toxicaria* Dalz.**

Lagenandra toxicaria, a semi-aquatic plant belonging to the Araceae family, is vital in maintaining ecological balance. Native to Sri Lanka and India, it thrives in wetlands and marshy areas (Nicolson, 1987; Sivadasan and Babu, 1995). With its broad, oval-shaped leaves reaching up to 30 cm in length and submerged roots, this plant is well adapted to aquatic environments. *Lagenandra toxicaria* is categorized as Least Concern (LC) on the IUCN red list and is prized for its ease of care and ability to flourish in various water conditions. *Lagenandra toxicaria* exhibits broad, oval-shaped leaves that can grow up to 30 cm long, with submerged roots that facilitate nutrient uptake from the surrounding water. The plant's rhizomes are toxic, containing compounds that deter herbivores and pathogens. One of its notable features is its capacity to purify water by absorbing excess nutrients, toxins, and pollutants, thereby improving water quality and creating a healthier environment for aquatic life

(Gaya et al., 2017). By regulating nutrient levels, *L. toxicaria* prevents eutrophication and provides essential resources, including oxygen, food, shelter, and breeding grounds for fish and other aquatic species. Its striking appearance also makes it a popular choice for aquariums. However, it's important to note that *Lagenandra toxicaria* can be toxic if ingested by animals or humans.

Lagenandra toxicaria is also reputed to possess medicinal properties, with traditional uses including the treatment of inflammation and pain relief. Research suggests that extracts from this plant may exhibit anti-inflammatory and antioxidant effects, underscoring its potential health benefits. In traditional medicine, the rhizomes of *Lagenandra toxicaria* are valued for their carminative, tonic, and diuretic properties, and are used to address bilious complaints. Additionally, studies have found that rhizome extracts possess insecticidal (Selvakumari, 2010) and antimicrobial properties (Mini and Razeena, 2014).

Lagenandra toxicaria has adapted to live in water, providing a unique opportunity to study endophytes that have evolved to thrive in aquatic environments. The plant's high-water content may lead to a higher diversity of aquatic endophytes. *Lagenandra toxicaria* has a complex root system, providing a habitat for diverse endophytic microorganisms. The plant's leaves are adapted for underwater life, providing a distinct environment for colonising endophytes. The plant's slow growth rate may allow for a more stable and consistent endophytic community, making it easier to study. *Lagenandra toxicaria* has a limited geographical range, which may result in a unique and specialized endophytic community. The plant produces toxic compounds, which may be selected for specific endophytes that can tolerate or detoxify these compounds. *Lagenandra toxicaria* may form symbiotic relationships with endophytes, providing a fascinating system to study plant-microbe interactions. These unique characteristics make *Lagenandra toxicaria* an attractive model organism for studying endophytic diversity, offering insights into the evolution, ecology, and applications of endophytes in aquatic environments.

This study aimed to explore and analyze the endophytic bacterial and fungal diversity within *Lagenandra toxicaria*, which exhibits a range of beneficial properties, including antimicrobial activity against pathogens, antioxidant capabilities to protect against oxidative stress, enzyme production for various industrial applications, and plant growth promotion through the production of phytohormones and other growth-regulating substances.

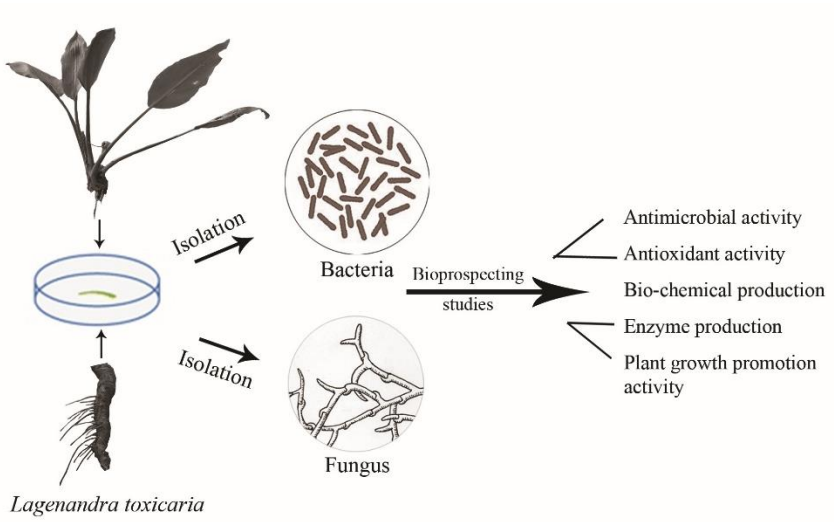


Fig.1 Diagrammatic sketch of the study

Objectives of the Study

- To collect an aquatic medicinal plant, *Lagenandra toxicaria* from the Wayanad district
- To isolate endophytic bacteria and fungi from different parts of the plant
- To identify endophytic isolates by morphological, molecular, and biochemical studies
- To study phytochemical analysis, anti-microbial, and anti-oxidant activity using the crude extract of endophytes and plant materials
- To identify the chemical compound from the extract and study the activity
- To study the enzyme production by endophytes and its activity
- To study the production of plant growth promotion traits and their quantification

Chapter 2

Review of Literature

Plants and microorganisms show various relationships within the ecosystem, and such interactions are essential for regulating biogeochemical cycles. These organisms include bacteria, fungi, algae, and viruses. They can be classified into two based on the location of the host plants, epiphytes, and endophytes based on the microbial interactions where microbial interactions influence the exterior and interior plant surfaces, respectively. Endophytes constitute a vast and fascinating realm within plants, existing in symbiotic harmony with their hosts. Research has consistently shown that nearly all studied plant species harbor one or more endophytes, which provide crucial benefits. These beneficial microorganisms shield their host plants from pathogen invasion, mitigate the impact of adverse environmental conditions, and enhance overall plant resilience.

Endophytes

The term "endophyte" was first introduced by De Bary in 1866 to describe pathogenic microbes residing within plants. Over time, its definition has expanded to encompass various microorganisms, including fungi (Petrini, 1991; Rodriguez et al., 2009), bacteria (Ryan et al., 2008; Griffin and Carson, 2015), and archaea (Muller et al., 2015; Moissl-Eichinger et al., 2018), that inhabit plant tissues without causing harm. Notably, mycorrhizal fungi, which form external mycelium and internal root epidermis connections, are also endophytes (Jumpponen, 2001; Schulz and Boyle, 2006). As per Hallman et al. (1997), the definition of an endophyte is widely recognized and includes any microorganism that lives inside a plant, regardless of the specific tissue it has colonized. The discovery of endophytes dates back to 1898 when Vogl identified mycelium in *Lolium temulentum* grass seeds. Freeman later found endophytic fungi in *Lolium persicum* (annual grass) in 1904. Bacterial endophytes have been recognized for over a century, with the first report of bacteria in healthy plant tissues emerging in 1926 (Hallman et al., 1997). Fungi remain the most commonly isolated endophytic group, but gram-positive and gram-negative

bacteria also inhabit plant tissues as endophytes (Bacon et al., 2002). Berg and Hallmann (2007) noted that bacterial endophytes occupy an ecological niche similar to phytopathogens, making them ideal candidates as biocontrol agents. Within the endophytes, bacteria can colonize internal tissues without posing any threat. The isolation and characterization of the bacterial endophytes reveal their significance to their host plant as well as their applications in the fields of biotechnology, agriculture, pharmaceuticals, and ecology. The study by Suryanarayanan et al. in 2001 clarified that the assemblage of soil microorganisms was the reason why root growth occurs in the soil rather than the air. Microbes found in the rhizosphere are capable of assembling both the internal tissues and the root surface. According to Lynch (1986), bacteria utilize root exudates as their energy source, and some of the substances exchanged between plants and the rhizosphere may improve bacterial population density and attraction. Higher plants also are known to exhibit them (Strobel et al., 2004). The diversity of endophytes on a host plant increases with the age of the plant. A greater number of endophytic fungi can be found in older tissues compared to younger ones (Rajagopal and Suryanarayanan, 2000; Verma et al., 2017).

Diversity and Distribution of Endophytes

Soil microbial communities play a crucial and unique role in ecosystem functions and are among the biosphere's most complex, diverse, and significant assemblages (Zhou, 2003). Endophytic microbes, which can include bacteria, fungi, and other microorganisms, are found ubiquitously across the plant kingdom, colonizing the internal tissues of a vast array of plant species without causing any visible harm to their hosts (Xia et al., 2015; Pirttila and Frank, 2011; Khare et al., 2018). These relationships, which can range from mutualistic to commensal or even latent pathogenic, have evolved in tandem with the emergence of vascular plants, showcasing the deep-rooted and intricate connections that have developed over millions of years between these organisms and the plants they inhabit (Khare et al., 2018). The ecological roles

of these endophytic organisms within their plant hosts are diverse and multifaceted. They can contribute to the host plant's nutrient acquisition, growth promotion, and defense against biotic and abiotic stressors, ultimately enhancing the overall fitness and resilience of the plant. In some of the best-described examples of endophyte-plant symbiosis, such as the rhizobia-legume relationship, the endosymbiotic bacteria can directly fulfill the host plant's need for essential nutrients, like nitrogen, through the fixation of atmospheric nitrogen (Khare et al., 2018). The study by Harrison and Griffin, 2020 reported a sharp increase in studies on endophyte diversity since 2010, with fungi receiving more attention than bacteria (Harrison and Griffin, 2020).

Not all endophytic associations are mutually beneficial, as some may inadvertently increase the susceptibility of the host plant to certain pests or pathogens, or may even exhibit latent pathogenic properties under specific environmental conditions (Deshmukh et al., 2014; Bamisile et al., 2018; Deshmukh et al., 2018; Esmaeel et al., 2018). The diversity of endophytic communities within broad-leaf plants is a subject of great intrigue, as these microscopic inhabitants represent a vast reservoir of untapped metabolic potential and ecological significance. Endophytes have been isolated from a wide range of plant tissues, including roots, stems, leaves, and even reproductive structures, each harboring a unique assemblage of microbial inhabitants tailored to the specific environmental conditions and physiological needs of their host (Deshmukh et al., 2014). The ubiquity of endophytes across the plant kingdom is further exemplified by the estimates that nearly every single one of the approximately 300,000 known plant species may host one or more endophytic microorganisms. This remarkable diversity can be attributed to the evolutionary adaptations and specialized mechanisms that enable endophytes to colonize and persist within their plant hosts successfully. The ability of endophytes to penetrate plant tissues, navigate through complex plant microenvironments, and establish stable associations without causing overt disease symptoms is a testament to their remarkable evolutionary prowess.

Furthermore, the metabolic versatility of endophytes, coupled with their capacity to produce a wide array of bioactive secondary metabolites, has made them a subject of intense interest in natural product discovery and pharmaceutical development (Oliveira et al., 2024).

Porras-Alfaro and Bayman (2011) revealed the emergent properties of endophytes and their role in microbiomes, underlining the importance of characterizing endophytes' diversity and contributions to plant health and ecological processes. A comprehensive review by Hardoim et al., 2015 highlighted the ecological and evolutionary considerations for defining the functioning of microbial endophytes, underscoring the need to explore the intricate relationships between endophytes and their host plants. Furthermore, Carrion et al. (2019) provided insights into the pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome, shedding light on the dynamic interactions between endophytes and plant pathogens. This study underscored the potential of endophytes to confer disease resistance and enhance the resilience of plant ecosystems. Additionally, Afzal et al., 2019 addressed the mechanisms, diversity, host range, and genetic determinants of plant-beneficial endophytic bacteria, elucidating the multifaceted nature of endophytic interactions and their implications for plant-microbe associations.

Moreover, Gottel et al. (2011) studied distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots, revealing the intricate relationships between endophytes and soil types. This research emphasized the need to consider the environmental context in understanding endophytic diversity and its ecological significance. These findings align with the work of Wani et al., 2015, which provided an ecological perspective on plant-endophyte symbiosis, highlighting the broader implications of endophytic interactions for ecosystem functioning and plant adaptation. Despite the valuable insights gained from previous studies, several knowledge gaps remain. First, there is a need to elucidate further the mechanisms through which endophytes influence plant fitness, stress tolerance, and ecosystem functioning, particularly in the

context of rapidly changing environmental conditions (Fox et al., 2019). Additionally, the impact of host genetic variation on endophyte composition and functioning represents an important area for future research (Blekhman et al., 2015).

Endophytes can be broadly categorized into fungal and bacterial endophytes. Fungal endophytes have been extensively studied for their diversity and functional roles. Rodriguez et al., 2009 discuss the three types of fungal endophytes, focusing on Type III endophytes, which grow within plant tissues without causing disease. These endophytes can enhance plant growth, stress tolerance, and resistance to pathogens. On the other hand, bacterial endophytes have been explored for their recent developments and applications. Ryan et al., 2008 noted that as more endophyte genomes are sequenced, our understanding of the mechanisms involved in successful endophyte colonization and interaction with host plants will improve and highlight the biodiversity of bacterial endophytes. These bacteria can promote plant growth through nitrogen fixation, phytohormone production, and disease suppression. Bacterial endophytes are found in nearly every plant species worldwide. Santoyo et al., 2016 highlight the ubiquitous presence of bacterial endophytes and their colonization of internal plant tissues. These endophytes exhibit significant diversity, with different species and strains colonizing various plant parts, including roots, stems, and leaves.

Pundir et al., 2014 explored the bioprospecting potential of endophytic bacteria isolated from indigenous plants in Ambala, India, identifying various fast-growing bacteria with potential applications. The diversity of endophytes varies significantly across different plant species and environments. A study by Suryanarayanan et al., 2003 on tropical forest trees revealed substantial diversity in endophytic fungal communities, with 81 distinct taxa identified from 3600 tissue segments. Similarly, Zheng et al., 2016 emphasized the astounding diversity of endophytic fungi and their ecological distribution in various plant species. Studies have shown that aquatic plants, such as water

lilies and cattails, harbor diverse communities of endophytic bacteria, including species of *Pseudomonas, Bacillus, and Aeromonas* (Shehzadi et al., 2016; Srivastava, 2015). These bacteria have been found to produce plant growth-promoting substances, such as auxins and cytokinins, and to exhibit antimicrobial activity against plant pathogens (Gupta et al., 2016).

Endophyte diversity and distribution have been examined through multiple methodological approaches. Culture-based methods are commonly used to isolate and identify endophytes from plant tissues. Molecular techniques, such as DNA sequencing, have also become increasingly important in identifying endophytes and understanding their phylogenetic relationships. Joshee et al., 2009 explored the diversity and distribution of fungal endophytes in New Zealand Podocarpaceae using culture-based and molecular methods. According to Reinhold-Hurek and Hurek (2011), the mechanisms underlying bacterial endophyte infection and plant establishment involve several known and suspected pathways. These methodologies help identify endophytes and understand their roles within plant systems.

Interactions of Endophytes with Host Plants

Endophytes are distributed across a wide range of biomes and host plants. In marine environments, Venkatachalam et al., 2015 documented the distribution of endophytes in seagrasses, finding a high colonization frequency across different species. The interaction between endophytes and their host plants is a complex and dynamic process. Rosenblueth and Martinez-Romero, 2006 describe how some endophytes are seed-borne, while others colonize plants through various mechanisms. These interactions can lead to various beneficial outcomes for the host plants, including improved growth and resistance to environmental stressors. Frank et al., 2017 discuss the transmission routes of bacterial endophytes, noting that they can be transmitted vertically (from parent to offspring) and horizontally (between plants). Furthermore, the study by Khare et al. (2018) shed light on the multifaceted interactions between

endophytes and plants, emphasizing the developments and prospects in this area.

Jia et al., 2016 examined the relationship between endophytic fungi and medicinal plants. The study showed that host factors and environmental conditions influence endophyte distribution. Endophytes promote plant growth through nitrogen fixation, phytohormone production, nutrient acquisition, and stress tolerance, with colonization being crucial for these benefits.

Kandel et al., 2017 provided further insights into the interactions between endophytes and host plants. They indicated that endophytes could promote plant growth, elicit defence responses against pathogen attacks, act as remediators of abiotic stresses, and have the potential to produce host metabolites for various applications. These findings reinforce the importance of understanding the multifaceted interactions between endophytes and plants. Khare et al., 2018 suggested that there are multifaceted interactions between endophytes and plants, indicating the complexity of this relationship and the need for further research to understand its implications fully. Partida-Martinez and Heil, 2011 also discussed the concept of the "microbe-free plant" and the potential implications of this phenomenon in the context of endophytic interactions with plants. These insights underscore the need for a more comprehensive understanding of endophytic interactions within plant ecosystems.

Competition experiments have revealed that certain endophytes exhibit superior colonization abilities, outcompeting others for host resources. For instance, *Pantoea sp.* displaced *Ochrobactrum sp.* in rice (Verma et al., 2004), while specific *Rhizobium etli*., strains dominated in maize (Rosenblueth and Martinez-Romero, 2004). These findings highlight the complex interactions among endophytes within plant hosts. However, studies on the endophyte host range have uncovered a lack of strict specificity (Zinniel et al., 2002). For example, the diversity of endophytic species in soybeans is influenced by factors such as plant genotype, plant age, tissue type, and time or season of isolation (Kuklinsky-Sobral et al., 2004). This suggests that endophyte communities are

shaped by a combination of host and environmental factors. Notably, soil type plays a significant role in shaping the endophytic population in wheat (Conn and Franco, 2004). This underscores the importance of considering environmental factors when exploring endophyte diversity and plant interactions.

Inoculation with endophytes has been shown to promote tomato plant growth, particularly when endophytic populations are encouraged without stimulating rhizospheric populations (Pillay and Nowak, 1997). However, the population density of endophytic communities varies greatly, depending primarily on bacterial species, host genotypes, and environmental conditions. Factors such as the host's developmental stage and inoculum density also impact endophyte population density (Pillay and Nowak, 1997; Tan et al., 2003). For instance, high-nitrogen fertilization regimes significantly impede sugarcane colonization by *Gluconacetobacter diazotrophicus*, whereas low-nitrogen fertilization promotes colonization.

Similarly, nitrogen fertilization affects the nitrogen-fixing population in rice, with noticeable changes occurring within 15 days of application (Tan et al., 2003). Organic amendments also substantially influence endophytic populations, as demonstrated by Hallmann et al. (1997). Vujkovic-Cvijin et al., 2020 highlighted the confounding variables in microbiota studies of human disease, shedding light on the potential challenges in studying the interactions between endophytes and host plants. This suggests that future research should consider and account for these confounding variables to ensure the validity of findings in this field. Brader et al., 2017 provided ecological and genomic insights into plant-pathogenic and plant-nonpathogenic endophytes, emphasizing the need to distinguish between these categories and understand their specific roles and interactions within plant ecosystems. This highlights a potential future research direction to further explore the genomic and ecological aspects of endophytic interactions with host plants.

The authors discussed the complex interactions between endophytes and their host plants, suggesting that there is a need for further research to elucidate the mechanisms underlying these interactions and their implications for plant health and productivity. On the other hand, the study by Hannula et al., 2017 focused on shifts in the rhizosphere fungal community during secondary succession following abandonment from agriculture. While not directly related to endophytes, this research provides valuable insights into the dynamics of fungal communities in plant ecosystems. Future research could investigate the influence of land-use changes on endophytic fungal communities and their potential implications for plant health and ecosystem functioning. Research has revealed the multifaceted nature of these interactions, the factors influencing endophytic populations, and the potential impacts on host plants. However, there are still knowledge gaps, particularly in understanding the ecological and genomic aspects of endophytic interactions, the confounding variables in microbiota studies, and the implications of the "microbe-free plant" concept. These gaps present potential future research directions for further understanding endophytic interactions with host plants.

Endophytes as a Source of Secondary Metabolites

Endophytes are thought to produce different types of secondary metabolites inside individual plants or microbes, making them an excellent source of medicines for treating a wide range of ailments and having potential applications in the food, cosmetics, pharmaceutical, and agricultural industries (Rajivgandhi et al., 2024). Numerous functional groups were used to categorize these secondary metabolites (Gouda et al., 2016). The location, climate, and season of sample collection all have an impact on how well metabolites are extracted from endophytes. But recently, a revolutionary synthetic technique developed in recent years has made extraction from plants and other natural sources more practicable, efficient, and feasible. Microbial secondary metabolites are primarily derived from a limited number of precursors originating from primary metabolism (Demain and Fang, 2000; Guo et al.,

2008). Endophytes, in particular, produce a range of low-molecular-weight secondary metabolites, including antimicrobial compounds, phytohormones, and precursor vitamins, such as vitamin B12 (Singh et al., 2017) and vitamin B1 (Mercado and Bakker, 2007). These metabolites, including terpenoids, alkaloids, phenylpropanoids, aliphatic compounds, polyketides, and peptides, have shown great potential for various industrial and pharmaceutical applications (Ludwig-Muller, 2015; Tan and Zou, 2001; Strobel et al., 2002, Strobel et al., 2004) with antibacterial, antifungal and cytotoxic properties (Tan and Zou, 2001; Owen and Hundley, 2004).). The diversity of antimicrobial secondary metabolites produced by fungal endophytes has garnered significant interdisciplinary attention due to their potential for drug discovery and development (Mousa and Raizada, 2013). These compounds are synthesized through various pathways, including polyketide, isoprenoid, and amino acid derivation (Jalgaonwala, 2013). Researchers have successfully isolated and evaluated bioactive secondary metabolites from endophytic fungi. For instance, Sharma et al. (2016) examined metabolites from *Pestalotiopsis neglecta* BAB-5510, isolated from *Cupressus torulosa* D. Don leaves. However, challenges remain in scaling up production, prompting questions about the readiness for industrial production of bioactive plant secondary metabolites utilizing endophytes (Kusari and Spiteller, 2011). Despite these challenges, endophytes hold promise as in vitro production platforms for high-value plant secondary metabolites, highlighting their potential for biotechnological applications (Venugopalan and Srivastava, 2015).

Bacterial endophytes have emerged as valuable sources of secondary metabolites with therapeutic potential, as acknowledged by Tan and Zou (2001). For instance, certain root-colonizing bacteria have been found to produce volatile compounds that enhance plant resilience. Specifically, *Pseudomonas chlororapis*, isolated from *Arabidopsis thaliana*, produces 2R and 3R Butenidol, conferring drought tolerance (Cho et al., 2008). Fermentation with endophytic bacteria can also enhance the bioactive compound content in plant

extracts. Limon et al. (2015) demonstrated that *Bacillus subtilis* and *Lactobacillus plantarum* increase soluble phenolic compound content and antioxidant activity in kidney bean extracts. Endophytes are also known to produce anti-inflammatory compounds. Taechowisan et al. (2005) isolated *Streptomyces sp.* from *Alpinia galanga* roots, which produces 3-methylcarbazoles that suppress inflammatory mediators, including NO, PGE2, TNF-α, IL-1β, IL-6, and IL-10. Furthermore, novel peptide antibiotics have been discovered in endophytes. Castillo et al. (2003) identified S*treptomyces sp.* NRRL30562 from the Snake Vine plant as a producer of munumbicins A-D40, exhibiting broad-spectrum antimicrobial activity.

Terpenes are compounds derived from isoprene units through biosynthesis. Approximately 50,000 terpenoid metabolites, including monoterpenes, sesquiterpenes, and diterpenes, have been isolated from various plants, fungi, and bacteria, representing around 400 distinct structural families. However, a relatively small fraction of these widespread metabolites have been identified in prokaryotes (Yamada et al., 2015). Among the diverse volatile compounds, certain Streptomyces terpenoids stand out. Geosmin, 2-methylisoborneol, tricyclic α,β-unsaturated ketone, and albaflavenone are well-known volatile microbial metabolites. Notably, the sesquiterpenoid antibiotic pentalenolactone has been isolated from over 30 Streptomyces species (Takahashi et al., 1983), highlighting the significance of Streptomyces as a rich source of terpenoid compounds. Endophytic bacteria, such as *Pseudomonas fluorescens* ALEB7B, have been shown to enhance the production of valuable compounds in plants. For instance, when ALEB7B colonizes the Chinese medicinal plant *Atractylodes lancea*, it triggers the generation of reactive oxygen species (ROS), leading to increased oxygenous sesquiterpenoid content (Zhou et al., 2015). These terpenoids have various beneficial properties. Terpenoids, in general, exhibit a range of biological activities, including stimulation of the digestive system, and antineoplastic, antibacterial, and antiviral effects (Omojate et al., 2014). Endophytic fungi, such as *Cytonaema sp*., also produce valuable

terpenoids. For example, *Cytonaema sp*. generates triterpenoid helvolic acid, which demonstrates potent antibacterial activity (Kumar et al., 2014). Certain endophytic fungi have been found to produce compounds with antioxidant and antifungal properties. The fungus *Pestalotiopsis microspora*, which inhabits the tree *Taxus wallichiana*, produces 1,3-dihydro-isobenzofuran and isopestacin. These compounds exhibit notable bioactivities, including antioxidant and mild antifungal effects, comparable to those of pestacin (Harper et al., 2003).

Taxol (paclitaxel), a complex diterpene alkaloid from the Taxus tree endophyte Metarhizium anisopliae, is a highly effective anticancer medication (Jalgaonwala et al., 2011). Similarly, camptothecin's S enantiomer exhibits potent anticancer properties as a plant metabolite, antineoplastic agent, genotoxin, and DNA topoisomerase inhibitor, showcasing its therapeutic potential. It is a delta-lactone, pyranoindolizinoquinoline, tertiary alcohol, and quinoline alkaloid. Cytotoxic and antifungal effects are well-known for *Nothapodytes foetida*'s camptothecin (Zhang et al., 2012). Huperzine A is a sesquiterpene alkaloid compound from the *Huperzia serrata* plant, Huperzine A (hupA) can inhibit cholinesterase. The endophytic fungus *Penicillium sp*. LDL4.4 obtained from *Huperzia serrata* has been shown to have similar hupA (Thi et al., 2019). Endophytes isolated from *Pinellia ternata*, such as *Bacillus cereus*, *Aranicola proteolyticus*, *Bacillus thuringiensis*, *Serratia liquefaciens*, and *Bacillus licheniformis*, have been found to produce alkaloids like guanosine and inosine in fermentation broth, mirroring their host plant's production (Liu et al., 2015). This suggests that these endophytes play a significant role in the plant's alkaloid biosynthesis. Other endophytes have been shown to influence alkaloid production in their host plants. For instance, *Acinetobacter* SB1B, an endophyte of *Opium poppy*, upregulates genes responsible for benzylisoquinoline alkaloid (BIA) biosynthesis. In contrast, *Marmoricola sp*. SM3B, another *Opium poppy* endophyte, enhances codeine biosynthesis (Pandey et al., 2016). These findings demonstrate that endophytes can modulate alkaloid-producing genes in their host plants. Notably, *Acinetobacter* and

Marmoricola sp. can be used as microbial inoculants to regulate alkaloid production in *Opium poppy*, highlighting the potential of endophytes in plant-based pharmaceutical production.

Lignans, which include cathartics, emetics, and cholagogue, are a large group of polyphenols from the endophytic *Podophyllum hexandrum* that are thought to have anticancer activities. Resins produced from *P. emodi*, such as etoposide and teniposide, exhibit strong anticancer activity. ampicillin, catechin, cefalexin, oxacillin, and gallic acid are a few substances known to have bactericidal properties (Konuklugil, 1995). *Pestalotiopsis microspore*, an endophyte from *Fagraea bodenii* generates ambuic acid, an organic acid that is highly functionalized cyclohexenone. The substance has ineffective antifungal activities. This one is one of the greatest substances for preventing bacteria from quorum sensing. The manufacture of the cyclic peptide hormones produced by *Listeria innocua* and *Staphylococcus aureus* is inhibited by ambuic acid. In the hunt for anti-pathogenic medications that target Gram-positive bacteria's production of virulence via quorum sensing, ambuic acid is a leading candidate (Nakayama et al., 2009). Due to the Florida Torreya's rarity and extinction threat, samples were taken to check for unique endophytes' existence. *Pestalotiopsis microspora* from *Torreya taxifolia* has the potential to produce Torreyanic acid, a cytotoxic dimeric quinone, which was extracted and identified. When tested on 25 distinct human cancer cell lines, the substance proved cytotoxic. Torreyanic acid has been demonstrated to induce apoptosis and cause cell death in cell lines susceptible to protein kinase C (PKC) agonists, such as 12-Otetradecanoyl phorbol-13-acetate (TPA), and also enhanced G1 arrest of G0 synchronized cells at concentrations of 1–5 g/mL (Lee et al., 1996). An endophytic fungus called *Colletotrichum dematium* was discovered in a *Pteromischum sps*. which is active against *Botrytis cinerea* and *Sclerotinia sclerotiorum*, because this fungus produces the new immunosuppressive peptide antimycotic known as Colutellin A. The nominal molar ratios and mass of the residues of Ile, Val, Ser, N-methyl-Val, and β-amino-isobutryic acid in this

peptide are 3:2:1:1:1 and 1127.70, respectively. Colutellin A prevented the generation of IL-2 from CD4-T cell activation. Colutellin A inhibits the synthesis of IL-2 at such low concentrations, making this a useful indicator of the substance's possible immunosuppressive properties. When tested on human peripheral blood mononuclear cells, cyclosporin A showed extreme cytotoxicity. Colutellin A could be a cutting-edge immunosuppressive medication (Ren et al., 2008). The herb *Tripterygium wilfordii* has potent immunosuppressive qualities. The same or similar physiologically active chemicals may also be produced by its endophytes. The isolated endophytic fungus was a rare kind of Cryptosporiopsis. It was distinctive because, in contrast to the other individuals in this group, its sizable conidiospore was segmented. Because of two unique chemicals, cryptocin, an uncommon tetramic acid, and cryptocandin, a novel lipopeptide, this organism exhibited extraordinary antifungal properties. The substance proved effective against fungi that are harmful to many plants. *Pyricularia oryzae*, the cause of rice blasts and one of the most significant infections in the world about food production, was the most susceptible fungus (Li et al., 2000).

There is a need to explore the biosynthetic pathways of these secondary metabolites to understand their production mechanisms and potentially enhance their yield (Ludwig-Muller, 2015). Additionally, further research is required to optimize the conditions for industrial-scale production of bioactive secondary metabolites utilizing endophytes, addressing the concerns raised by Kusari and Spiteller (2011). Moreover, the ecological role of these secondary metabolites in the plant-endophyte interaction and their potential impact on plant defense mechanisms also merit attention. Understanding these aspects could provide valuable insights into the ecological significance of secondary metabolites produced by endophytes and their potential applications in agriculture and environmental management. Furthermore, exploring the structural diversity and bioactivity of secondary metabolites from different endophytic sources can open new avenues for drug discovery and development.

Endophytes as a Source of Bioactive Compounds

The production of bioactive compounds by endophytes is closely linked to their co-evolution with host microbes, which have potentially acquired genetic information from higher plants through horizontal gene transfer. As a result, the host microorganisms have been better able to adapt to the host plant and carry out particular tasks, such as providing defense against different viruses, insects, and grazing animals. According to reports, senescence, abrupt, extreme climatic changes, or environmental pressures may facilitate the transformation of endophytes into pathogens in the host to accommodate these ecological shifts (Alam et al., 2021). Endophytes make the same bioactive compounds originally of their host plants and can produce more novel chemicals with potent bioactivities (Zhang et al., 2014; Strobel et al., 2004).

Recent studies have underscored the biotechnological potential of endophytes as sources of bioactive compounds. For instance, Singh et al. (2017) highlighted the promise of tyrosol produced by *Diaporthe helianthi*, an endophytic bacterium. Similarly, Specian et al. (2012) emphasized the diverse bioactive compounds produced by the endophytic fungus *Diaporthe helianthi*, which have been utilized in disease treatment. Further research has demonstrated the production of bioactive compounds with bactericidal and antioxidant properties by endophytic fungi. Chatterjee et al. (2019) reported the production of such compounds by *Alternaria alternata* AE1. Marine-derived endophytes have also garnered significant attention as a treasure trove of bioactive compounds. Chaouachi et al. (2021) noted that China-based research groups have focused on exploring these organisms. Sun et al. (2019) specifically reported that marine-derived endophytic fungi, such as *Aspergillus unguis* (AG 1.2), are a rich source of novel pharmaceutically active compounds.

Bacterial endophytes have immense potential in pharmaceutical and drug discovery applications (Strobel, 2006; Guo et al., 2008). Specifically, endophytes associated with medicinal plants serve as rich sources of natural products, which can be utilized to combat oxidative stress and developed into

novel bioactive agents (Nongkhlaw and Joshi, 2015). Endophytes produce diverse metabolites, including amines and amides, which exhibit insecticidal properties without harming mammals. Additionally, they secrete extracellular hydrolyses, such as cellulases, proteinases, lipases, and esterases, enabling plants to resist invasions (Tan and Zou, 2001). Research has identified various endophytic bacteria associated with medicinal plants, including *Hypericum perforatum* and *Ziziphora capitata*. These bacteria belong to genera such as Arthrobacter, Achromobacter, Bacillus, Enterobacter, Pseudomonas, Pantoea, Erwinia, Serratia, and Stenotrophomonas. Notably, *H. perforatum*, which exhibits antibacterial activity, supports the colonization of bacteria with antagonistic properties, surpassing *Z. capitata* (Egamberdieva et al., 2017). These isolates have been found to effectively control tomato root rot caused by *F. oxysporum*.

In the context of antibacterial activity, Kamat et al., 2020 found that *Trichoderma longibrachiatum* MD33 produced dendrobine and other compounds with antibacterial activity. Furthermore, Alsultan et al., 2019 highlighted the production of bioactive compounds with antibacterial properties by endophytic fungal strains isolated from indigenous South African medicinal plants. The research by Sarsaiya et al., 2020 also emphasized the bioactivity of bioactive compounds from endophytic fungi isolated from mangrove plant species against protozoa, bacteria, and pathogenic viruses. Moreover, Hashem et al., 2023 reported that *Bacillus velezensis* Lle-9, isolated from the bulbs of *Lilium leucanthum*, produced bioactive compounds with antifungal activities. The research findings also highlighted the importance of endophytic bacteria in producing bioactive compounds with antimicrobial activities. Khan et al., 2020 demonstrated that endophytic bacteria isolated from *Cordia dichotoma* and *Lilium leucanthum* produced bioactive compounds with antimicrobial activities. Cadamuro et al., 2021 further emphasized the bioactive compounds produced by endophytic bacteria from *Metasequoia glyptostroboides* and *Ginkgo biloba*, which confer resistance to phytopathogens. Furthermore, endophytic bacteria

have been identified as a potential source of bioactive compounds, highlighting their role as a novel source for drug discovery (Cui et al., 2015).

These endophytic microbes play a crucial role in the production of various bioactive compounds, including anticancer, antimicrobial, and antioxidant compounds (Hamilton et al., 2012). Moreover, the bioavailability of bioactive compounds produced by endophytes has been highlighted as a key area for future research. Understanding the bioavailability of these compounds is essential for assessing their potential therapeutic effects on human health (Tena et al., 2020).

Bioprospecting of Endophytes

Bioprospecting of endophytes has gained significant attention due to the potential of these microorganisms in producing bioactive compounds and enhancing plant growth due to their remarkable ability to produce a wide array of secondary metabolites with potential applications in various fields, including agriculture, medicine, and industry (Sharma et al., 2019; Parasuraman et al., 2019; Deshmukh et al., 2018; Rajamanikyam et al., 2017). Endophytes are microorganisms that reside within the tissues of plants without causing any apparent harm to the host. Research on the rhizosphere microbiome and its significance in plant development and human health has shed light on the potential benefits of endophytes. Endophytes can significantly influence plant phenotype in various ways. One key aspect is enhanced disease resistance, where endophytes reduce plant susceptibility to pathogens (Arnold et al., 2003; Compant et al., 2005; Herre et al., 2007; Busby et al., 2016; Christian et al., 2017). Endophytes also contribute to plant resilience against abiotic stressors, such as drought, temperature, and salinity, by enhancing resistance mechanisms (Redman et al., 2002; Marquez et al., 2007; Rodriguez et al., 2008). Furthermore, endophytes can modify phytochemical profiles, leading to changes in plant secondary metabolism (Kusari et al., 2012; Panaccione et al., 2014). Additionally, endophytes can modulate the expression of plant functional

traits, influencing plant growth, development, and ecological interactions (Friesen et al., 2013; Griffin et al., 2016).

These endophytic microorganisms, primarily comprising members of the Ascomycota, Basidiomycota, Zygomycota, and Oomycota phyla, have evolved to establish symbiotic relationships with their host plants, providing a plethora of benefits such as enhanced biotic and abiotic stress tolerance, and the production of bioactive compounds that aid in the survival and adaptation of their hosts (Suryanarayanan et al., 2009). The advent of genome sequencing technology has further highlighted the untapped potential of these "hidden members of the microbial world", as it has enabled us to uncover the vast genetic diversity and biosynthetic capabilities of endophytes (Parasuraman et al., 2019). Numerous studies have reported the isolation of novel bioactive metabolites from endophytic fungi, including antifungal, antioxidant, and anticancer compounds, which have garnered significant interest in the development of potential therapeutic and agricultural applications (Parasuraman et al., 2019; Deshmukh et al., 2018; Xu et al., 2021). The bioprospecting of endophytes has revealed an impressive chemical diversity, with a wide range of secondary metabolites, such as alkaloids, terpenoids, steroids, peptides, benzopyranones, quinones, and isocoumarins, being identified from these microorganisms (Xu et al., 2021).

Research on endophytes has gained significant attention due to their potential to manipulate plant phenotype (Doty, 2011) and prevent pathogen colonization in crops (Busby et al., 2017). Additionally, scientists exploring natural products have become increasingly interested in endophytes as a rich source of beneficial compounds (Strobel and Daisy, 2003; Aly et al., 2010). Endophytes have been found to synthesize a remarkable range of bioactive small molecules, sparking interest in their potential applications (Newman et al., 2003; Strobel et al., 2004; Verma et al., 2009). Notably, certain compounds produced by endophytes have demonstrated therapeutic properties, making them valuable for medical research (Strobel et al., 1996; Kharwar et al., 2011). Endophytes are now

recognized as a treasure trove of bioactive compounds with medicinal importance (Gouda et al., 2016). As highlighted by Nair and Padmavathy (2014), endophytes have emerged as a vital component of plant microbiology, offering promising solutions for pharmaceutical innovation, environmental sustainability, and agricultural development. Their unique properties and diverse applications make endophytes an exciting area of research with significant potential for improving human health and the environment.

Endophytic bacteria have been found to exert numerous beneficial effects on their host plants. These benefits include nitrogen fixation (Kirchhorf et al., 1997; Stolzfus et al., 1997; Reinhold-Hurek and Hurek, 1998), plant growth stimulation (Sturz and Nowak, 2000), and induction of resistance to phytopathogens (Liu, 1995; Sturz and Matheson, 1996). Additionally, these bacteria can degrade organic pollutants in soil contaminated with heavy metals (Sheng et al., 2008). Endophytic bacteria are also known to produce bioactive metabolites with diverse biological activities. These metabolites have been identified as having antiviral, anticancer, anti-inflammatory, antioxidant, and antibiotic properties (Strobel and Daisy, 2003). The wide range of beneficial effects and bioactive compounds produced by endophytic bacteria underscores their potential for applications in sustainable agriculture, environmental remediation, and pharmaceutical development.

Applications of Endophytes

Endophytes have great potential in various applications, particularly in agriculture and biotechnology. Schulz and Boyle (2006) provide an overview of the diverse uses of endophytes, including their role in enhancing plant growth and resistance to pathogens. Additionally, Zhang et al. (2007) focus on the biology and chemistry of endophytes, summarizing the various metabolites isolated from these microorganisms and their potential applications. The potential applications of endophytic bacteria in agriculture are vast. Gayathri et al. (2010) investigated the bioprospecting aspects of endophytic bacteria from mangrove and salt marsh plants, highlighting their potential in

sustainable agriculture. Sharma et al. (2021) identified endophytic bacterial communities associated with *Rosmarinus officinalis*, emphasizing their role in sustaining plant health and productivity. Ayilara et al. (2023) reviewed the challenges of plant microbiome research for sustainable agriculture, mainly focusing on soybean endophytic bacteria. They discussed the complexities of isolating and characterizing endophytic bacteria and the need for advanced techniques to overcome these challenges.

Rana et al. (2019) provided insights into the biodiversity of endophytic fungi from diverse niches and their biotechnological applications. This finding suggests that future research could explore the untapped diversity of endophytic fungi and their potential biotechnological applications in various fields, such as agriculture, bioremediation, and pharmaceuticals. In the context of medical applications, cannabinoids have been the subject of systematic review and meta-analysis for medical use (Whiting et al., 2015). However, the potential interaction between cannabinoids and endophytes, particularly in the context of therapeutic applications, has not been elucidated. Brodin et al. (2015) highlighted the significant non-heritable influences on the variation in the human immune system. Considering the potential role of endophytes in modulating immune responses, it is vital to consider the impact of endophytes in the context of personalized medicine and individual immune system variation.

Fungal entomopathogens, typically susceptible to UV light and low moisture, have been explored as eco-friendly alternatives to chemical pest control. However, a novel approach involves utilizing these fungi as endophytes, potentially overcoming previous limitations. This innovative method offers a promising solution for managing insect pests and plant pathogens (Chen et al., 2021; Vega, 2018). Endophytic fungal entomopathogens may also serve as biofertilizers, providing additional benefits. Endophytic microbes have shown potential to reduce agrochemical reliance in crop cultivation and biofortify micronutrients in cereal crops. Research by Lata et al. (2018) highlights their

ability to induce abiotic stress tolerance in plants, further decreasing dependence on agrochemicals. The biofortification of micronutrients in various cereal crops using endophytic microbes has been explored, demonstrating the potential to enhance food crop nutritional quality. By leveraging endophytic microbes, agricultural practices can become more sustainable, reducing chemical usage while promoting crop resilience and nutritional value. Additionally, White et al. (2019) discussed the bioremediation potential of endophytic microbes, particularly in cleaning contaminated sites. This indicates their ability to contribute to environmental cleanup efforts by remediating soil and water contaminated with various pollutants.

Antagonistic Properties of Endophytes

Endophytes have emerged as a promising source of antimicrobial substances, with a wide range of strains exhibiting antimicrobial properties (Strobel, 2003). Certain endophytes have developed symbiotic relationships with their host plants, enhancing their antimicrobial activity (Yang et al., 1994; Strobel, 2003). To protect their hosts, endophytes produce secondary metabolites that resist pathogenic intrusion (Tan and Zou, 2001). The antimicrobial compounds produced by endophytes have gained attention as alternatives to overcome growing drug resistance. Research has isolated numerous endophytic actinomycetes from medicinal plants, demonstrating broad-spectrum antimicrobial activity and potential as reservoirs of novel bioactive compounds (Zhao et al., 2011). For instance, a study isolated 86 bacterial strains from aquatic environments, with 19 strains showing significant inhibitory activity against *Bacillus cereus* and *Listeria monocytogenes* (Motta et al., 2004). Research has demonstrated that antimicrobial substances produced by endophytes exhibit remarkable thermal resistance, remaining effective up to 100°C. A study by Jalgaonwala et al. (2011) isolated 23 bacterial strains from medicinal plants, showcasing their antimicrobial potential, with 3 isolates exhibiting antibacterial activity, 6 displaying antioxidant properties, and 12 demonstrating antifungal activity.

Medicinal plants have been found to harbor antimicrobial endophytes, including *Cinnamomum tonkinense*, *C. zeylanicum*, *Lippia sidoides*, *Astragalus membranacus*, *Opuntia ficus-indica*, *Ginkgo biloba*, *Torreya grandis*, and *Tripterygium wilfordii*. These plants have been studied by various researchers (Kharwar et al., 2012; de Siqueria et al., 2011). Research on endophytic microorganisms has revealed their potential as bioactive agents against various pathogens. Kumar et al. (2011) investigated the bioactivity of endophytes isolated from *Tylophora indica*, including *Alternaria sp*., *Alternaria tenuissima*, and *Thielavia subthermophila*, against *Sclerotinia sclerotiorum* and *Fusarium oxysporum*. These endophytes demonstrated growth inhibition against the pathogens. Further studies by El-Deeb et al. (2013) isolated 28 endophytes from *Plectranthus tenuiflorus*, with 8 strains (28.5%) exhibiting antimicrobial activity against Staphylococcus, Salmonella, *Escherichia coli*, Klebsiella, Streptococcus, Proteus, and Candida. Notably, 14.2% of the potent strains displayed both antibacterial and antifungal properties.

Selim et al. (2011) isolated ninety-nine bacterial endophytes; fifty-five of them exhibited inhibitory activity against a wide range of pathogenic microorganisms, with diameters of inhibition zones ranging from 9 to 27mm for the test bacteria and from 8 to 31mm for test Candida. *Pseudomonas poae* strain RE-1-1-14, which was isolated from sugar beet roots, was able to suppress the fungal pathogen *Rhizoctonia solani* (Zachow et al., 2011). Long et al., (2003) reported that endophytic bacteria isolated from the roots of Solanum species have potent antibacterial activity. Bacterial isolates HB3 from rhizomes of *Curcuma longa*, KB4 from roots of *Pinus glabra*, and stem isolates of *Eucalyptus globulas* show strong antifungal activity. Endophytic actinomycetes isolated from medicinal plants are valuable reservoirs of novel bioactive compounds with broad-spectrum antimicrobial activity. Zhao et al. (2011) isolated numerous endophytic actinomycetes from 26 medicinal plants, demonstrating their potential as sources of new antimicrobial agents. Further research has identified specific endophytes with antimicrobial properties. For

example, *Streptomyces sp.* isolated from *Citrus nobilis* fruit inhibited pathogens such as *Colletotrichum truncatum*, *Geotrichum candidum*, *F. oxysporum*, and *F. udum*, showcasing its antimicrobial activity (Hong-Thao et al., 2016). Additionally, endophytes isolated from *Tectona grandis* and *Samanea saman* produced inhibitory substances against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*. Endophytes have also been found to confer resistance to plant diseases. Maize plants inoculated with endophytic *Enterobacter aerogenes*, which produces volatile organic compounds (VOCs) such as 2,3-butanediol, showed resistance against *Setosphaeria turcica*, the causal agent of corn leaf blight (D'Alessandro et al., 2014).

Studies have proved that endophytes can control plant pathogens and nematodes (Sturz and Matheson, 1996; Hallmann et al., 1997; Krishnamurthy and Gnanamanickam, 1997). Some bacterial endophytes isolated from *Abelmoschus esculentus* belonging to the group *Pseudomonas sp*, *Bacillus sp*., and *Methylobacterium sp*., enhance plant growth by producing indole acetic acid-like substances and also protect the plants by suppressing the nematode population. Adhikari et al. (2001) specifically reported the effectiveness of endophytic bacterial strains in controlling seedling disease in rice, while also enhancing plant growth. Seo et al., (2010) proposed that endophytic bacteria produced antibiotics, which can act against pathogenic bacteria. The endophytes appear to be involved in plant growth promotional activities including defense against herbivore attack, plant health, growth, and yield (Conn and Franco, 2004; Lucy et al., 2004; Bloemberg and Lugtenberg, 2001; Hallmann et al., 1997; Chanway, 1997; Glick, 1995; Kloepper and Beauchamp, 1992). Endophytic bacteria have a multiplex of applications that enhance agricultural production by enriching wheat growth through phytohormone production (Barbieri, 1986), increasing cotton disease resistance (Chen et al., 1995), increasing rice production through mineral availability (Murty, 1988), contributing to corn pest management (Fahey, 1991), fix nitrogen in rice and wheat (Webster et., 1997), increase potato tuber formation under heat stress

conditions (Bensalim et al., 1998) and decrease susceptibility to frost damage (Xu et al., 1998). Parekh and Chanda, 2007 suggested the emergence of antibiotic-resistant strains and increasing failure of the available chemotherapeutics searched for microbiologically active medicinal plants a necessity. Research on rice plants has consistently shown that plant growth-promoting rhizobacteria (PGPR) can effectively protect and promote growth, providing a viable alternative to chemical nitrogen, phosphorus, and pesticides (Alam et al., 2001; Van and Ferrero, 2006; Chinnusamy et al., 2006; Cong et al., 2009). These beneficial microorganisms offer a sustainable solution for rice cultivation, reducing reliance on chemical inputs while enhancing plant health and productivity.

Endophytes have been found to produce bioactive compounds with anti-cancer properties. Research has identified several endophytic compounds with anti-cancer potential (Firakova et al., 2007). For instance, the endophytic strain EML-CAP3, isolated from *Capsicum annuum* L. leaves, demonstrated potent anti-angiogenic activity. This strain produced lipophilic peptides that inhibited endothelial cell proliferation and exhibited anti-angiogenic potential in tumor progression (Jung et al., 2015). Ginseng (*Panax ginseng*) is renowned for its anti-cancerous ginsenosides. Notably, *Paenibacillus polymyxa*, an endophytic bacterium of Ginseng leaves, has been found to have high ginsenoside concentrations. When inoculated with Ginseng plants under stress conditions, this endophytic strain enhanced plant growth and ginsenoside production (Gao et al., 2015). This synergistic relationship between endophytes and host plants highlights the potential for developing novel cancer therapies and improving crop yields. Research has identified exopolysaccharides (EPS) produced by certain microorganisms as potential anti-tumor agents. The anti-tumoral mechanism of action involves inducing morphological abnormalities in cancer cells, leading to mitochondria dysfunction. Notably, Bacillus was found to be a source of the first discovered anti-tumoral EPS, a natural product with high therapeutic value for cancer treatment. L-asparaginase, an enzyme that catalyzes

the conversion of L-asparagine, has shown significant promise in cancer therapy. L-asparagine is essential for the survival of certain neoplastic cells, such as lymphoblasts. When combined with multi-drug chemotherapy, L-asparaginase has resulted in remarkable improvements and complete remission in most patients, particularly those with acute lymphoblastic leukemia (Holowiecki et al., 2008). Endophytes have emerged as efficient producers of L-asparaginase. Specifically, endophytic strains of *Bacillus licheniformis*, *B. pseudomycoides*, and *Paenibacillus denitriformis* have demonstrated high secretion levels of this enzyme (Joshi and Kulkarni, 2016). Streptomyces are renowned for their ability to synthesize a wide range of bioactive compounds, including antibiotics, fungicides, immune response modulators, and plant growth promoters (Hopwood, 2007). These beneficial microorganisms have been found to inhabit plant roots, where they produce compounds with potential therapeutic applications. A notable example is the endophytic *Streptomyces sp*. LJK 109, isolated from the roots of *Alpinia galangal*. This strain produces 3-methylcarbazoles, a vital anti-inflammatory component that suppresses the production of inflammatory mediators, including nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10), in a dose-dependent manner (Taechowisan et al., 2019).

Endophytic Enzymes and their Production

Enzymes are biocatalysts that are created by living cells to promote certain biochemical processes and provide a lower-energy route between reactants and products. These biomolecules, which are necessary for both synthesis and breakdown events, serve as the main tools for the expression of gene activity. Enzymes are quite particular in the substrates they work on, and they frequently cooperate to catalyze the series of metabolic processes carried out by the living cell. Food manufacturing, animal nutrition, cosmetics, research and development, and principally medicines against many diseases, to name a few, are just a few of the industries that have an increasing demand for enzymes. As

a result, the enzyme market is booming at the moment. These biomolecules are found in all living things animals, plants, and particularly bacteria, which are the source of the vast majority of commercial enzymes. The fact that microbial enzymes are biochemically varied and have a wide range of actions built into them due to their tolerance against variation is another reason why they are chosen in many sectors (Singh et al., 2019).

Microbes with beneficial enzymes, antibiotics, proteins, and salt-tolerant genes have potential biotechnological importance (Thatoi et al., 2013). Soriano et al., (2005); Carrim et al., (2006); Quecine et al., (2008), and Ferreira-Filho et al., (2012) suggested that there is strong attention to microbial enzymes in numerous areas including food processing, detergents, agriculture, textile, pharmaceutical research, medical therapy, and molecular biology. Co-cultivation of microbes or microbial consortium denotes a potentially valuable strategy for discovering novel metabolites (Zhou et al., 2006). Generally, hydrolytic enzymes produced by endophytes are used for their colonization in plant roots. Various enzymatic studies were carried out about endophytes. Thatoi et al., (2013) and Dias et al., (2009) reported that the endophytes from the mangroves exhibit a diverse range of enzymatic activities and can catalyze various biochemical reactions by novel enzymes. Endophytes from aquatic plants have been found to produce a range of enzymes, including amylases, proteases, lipases, and cellulases, which have potential applications in industries such as food, feed, and biofuels. For example, a study on the endophytes of the freshwater plant, *Potamogeton crispus*, found that they produced amylases and proteases with potential applications in the food industry (Gupta et al., 2016).

Halophilic microorganisms possess many hydrolytic enzymes and can function in various conditions (Ventosa and Nieto, 1995). Reports suggest that *Pseudomonas stutzeri*, *Bacillus megaterium*, and *Bacillus licheniformis* produce extracellular amylase (Schmidt and John 1979; Rivera et al., 2003). Thermo-tolerant isoamylase biosynthesized by thermophilic bacterium *Bacillus sp.* has been recently isolated and characterized by Liu et al., (2010). Cellulase is an

important hydrolytic enzyme of commercial use in several industries such as agriculture, pulp and paper, animal feed, food, fermentation, and textile (Kuhad et al., 2011). The presence of varying levels of pectinase and cellulase affects the ability of cellular colonization. Many cellulase-degrading bacteria have been reported from Sundarban mangroves (Ramanathan et al., 2008). Benner et al., (1984) have reported cellulose-degrading bacteria isolated from decaying *Spartina alterniflora*. Endophytic bacteria associated with Brazilian mangrove plants were reported by Castro et al., 2014. Of them, 62% endophytic bacterium showed endoglucanase activity with species belonging to the genus *Bacillus* exhibiting the highest activity. Endophytic bacteria, including Bacillus, Micrococcus, Paenibacillus, Pseudomonas, and Acinetobacter species, have been found to possess strong hydrolytic enzyme activities. These enzymes include amylase, cellulase, esterase, protease, pectinase, lipase, and xylanase (El-Deeb et al., 2013). Specifically, *Bacillus licheniformis*, *B. megaterium*, *B. pumilus*, and *Paenibacillus sp.* exhibited maximum enzyme activities. The distribution of enzyme activities among these bacterial strains varies. Amylolytic activity was observed in *Bacillus licheniformis*, *B. pumilus*, B. megaterium, and *Pseudomonas sp*. Lipolytic activity was detected in *B. pumilus*, *B. megaterium*, and *Pseudomonas sp*. Furthermore, Bacillus, Paenibacillus, and Pseudomonas species were found to produce protease. Previous studies have identified specific endophytic bacteria as producers of extracellular enzymes. For example, *B. megaterium*, *Pseudomonas stutzeri*, and *B. licheniformis* have been reported to produce amylase (Schmidt and John, 1979). Additionally, xylanase enzymes isolated from *Bacillus sp.* TAR-1, C-125, and NCL-86-6-10 demonstrated optimal activity at pH 9-10 (Nakamura et al., 1994; Balakrishnan et al., 1992). More recent studies have focused on the thermal and alkaline stability of these enzymes. Leelavathi et al. (2003) reported the discovery of alkali and thermo-stable xylanase genes from *Bacillus sp.* NG-27 in tobacco plants, active up to 42°C.

Cho et al., (2008) reported two cellulase-producing genes that are, cel5A and cel5B from *Paenibacillus polymyxa* of ginseng root. The antibiotics and catalase are produced by *Serratia marcescens* to resist the plant pathogens (Wei et al., 1996) and large quantities of 2-KDG and chitinase were obtained (Zhang et al., 2011). Vieira and Schenberg (1999) suggested that the zone precipitation of paracasein around the colonies in the 48hrs after incubation could be taken as evidence of proteolytic activity. Some lytic enzymes secreted by endophytic bacteria play an important role in pathogen suppression (Buchenauer, 1998). From *Bacillus sp*., the production of thermophilic as well as alkalophilic extracellular protease enzymes could be optimized and characterized in cultural conditions (Vijayalakshmi et al., 2013). El-Deeb et al., (2012) isolated thirty-eight bacterial endophytes from *Rosa damascena* trigintipeta, among them six isolates could produce cellulase, xylanase, protease, pectinase, amylase, lipase, and chitinase. *Micrococcus sp*., *Acinetobacter sp.*, and *Acetobacter sp.* (TUB6) possessed chitinase, cellulase, xylanase, pectinase, and protease activity, whereas *Micrococcus sp.* (TUB2) showed only lipase activity. Prasad and Sunayana (2014) isolated six bacteria from Avocado and black grapes, in which except one strain from the Avocado all the isolates were found to be lipase producers. Polygalacturonases, a heterogenous group of pectin have been reported in *Aspergillus sp*., and *Bacillus sp*. (Kashyap et al., 2001; Murad and Azzaz, 2011). Pectin esterase is reported in *Aspergillus niger*, *Pseudomonas solanacearum*, *Penicillium occitanis*, *Lactobacillus lactis,* and *A. japonicas* (Jayani et al., 2005; Murad and Azzaz, 2011; Arunachalam and Asha, 2010). Pectin lyases have been reported to be produced by *Aspergillus japonicus*, *Penicillium sp*., *Aspergillus sp*., *Erwinia carotovora*, *Pseudomonas syringae*, and *Bacillus sp*. (Kashyap et al., 2000, 2001; Murad and Azzaz, 2011; Chaudhri, 2012).

Fungi have acquired the capacity to produce enzymes and natural compounds as new metabolites. They may create antibacterial compounds, industrial enzymes, and microbial biomass. Amylases, cellulases, and lipases are some of

the enzymes seen in fungi that can function either directly as antimicrobials or by producing compounds that have antimicrobial action. Additionally, fungal enzymes are utilized to preserve food without compromising nutritional value and food processing to extend shelf life. The beverage and food industry, and also the leather, paper, textiles, and other sectors, rely heavily on fungi as a source of enzymes. Scientific interest in endophytic microorganisms that can endure unfriendly and extremely harsh circumstances has lately increased significantly. Endophytes, which spend at least part of their lives asymptomatically inside plants, have become essential sources of new compounds and industrially significant enzymes. The most reliable source of enzymes with a variety of functions and an untapped sector is the commercial synthesis of enzymes from endophytic fungi (Sathish et al., 2012). By creating certain toxic compounds that render the host inedible to insects and animals, endophytic fungi can help to defend their hosts against attack by both insects and animals. A large array of enzymes with various biological functions are produced by endophytic fungi in plant tissues.

According to several studies, the extracellular enzymes amylase, asparaginase, cellulases, chitinase, gelatinase, hemicellulases, laccase, oxidoreductases, pectinase, phytases, protease, transferases, tyrosinase, and xylanases are generated by endophytic fungi as a defense against infections and as a means of obtaining nutrients from the host and also the prospective uses of endophytic fungi-produced enzymes in the medical, agricultural, food, and biofuel industries. The endophytic fungus can be used for bioremediation of other toxic compounds, and industrial and agricultural waste, as well as for detoxification. These extracellular enzymes break down several macromolecules, including organic phosphate, carbohydrates, proteins, lignin, and sugar-based polymers, into transportable by-products that may be used by the cells for heterotopic metabolism (Wingernder et al., 2012). The host symbiosis process may be aided by the endophyte-producing enzymes. These begin extracellular hydrolyses in order to prevent plant pathogenic infection in addition to forming a relationship

with the host. Because endophytic resources provide a fresh source of enzymes, secondary metabolites, and genes, these fungi have attracted a lot of interest since they rely on the enzyme systems of microorganisms, including other microbial species (Sathish et al., 2012). Exploring endophytes to produce amylolytic enzymes that are active in alkaline and low-temperature environments is becoming more and more popular. Among the amylases produced by diverse bacteria, those from *Bacillus sp.* have garnered the greatest scientific attention due to their outstanding thermostability; nonetheless, *Rhizopus sp.* and *Aspergillus sp.* are widely used as resources for glucoamylase synthesis in the industry (Pandey et al., 2000). *Gibberella pulicaris*, *Acremonium sp*., *Nodilusporium sp*., and *Synnematous sp*. endophytic isolates' capacity to produce enzymes that break down raw starch during fermentation. While *Acremonium sp*. showed broad activity toward both small and large granule-sized raw starches, the enzyme from the other isolates only showed robust activity toward raw starches with smaller granule sizes. Additionally, raw sago starch was broken down by the enzymes *Acremonium sp*. and *Nodilusporium sp.* to produce just glucose whereas the amylase from *Synnematous sp*. produced glucose and maltose (Marlida et al., 2000).

Cellulases are used in the paper industry to recycle waste paper and also used to increase the digestibility of animal feed. To find novel microbial species which, have high productivity and specificity for producing cellulases, research has been done. In addition, while exo-cellobiohydrolase targets the crystalline ends of cellulosic substrates to make cellobiose, endoglucanase hydrolyzes the glycosidic bonds of the same substrates to produce glucose molecules. The use of inexpensive substrates, primarily cellulose, and hemicelluloses, makes these enzyme-based transformations advantageous. In reality, as a source of raw materials for the production of biofuels, namely bioethanol for the transportation sector, cellulosic biomass generated from non-food sources like trees and grasses is being studied. The main class of enzymes used in this process is cellulases (Yoon et al., 2014). *Pichia pastoris* KM71 was discovered

to be the endophyte *Fusiccoccum sp.* BCC4124 is a potent producer of cellulase enzymes. Avicel, filter paper, and methyl 4-(trifluoromethyl) umbelliferyl ether could all be broken down by the recombinant enzyme produced, however, carboxymethylcellulose could not be hydrolyzed (Kanokratana et al., 2008).

The most sought-after microbial lipases are those that are primarily derived from specific bacteria and fungi because of their exceptional properties, such as extreme constancy in organic solvents, the lack of a requirement for cofactors, and the capacity to act on a wide range of substrates. Their industrial uses range from the processing of detergents to flavor enhancement through fatty acid synthesis and alcohol esters, racemic mixture resolution, and the creation of biosensors that may be used as diagnostic aids to identify various ailments (Hasan et al., 2006). When enantioseparating racemic mixtures, lipases are helpful because they catalyze the deacylation and acylation processes of significant pharmaceutical substances. *Candida Antarctica* or *Candida rugosa* are the usual sources of lipases used in organic synthesis; however recently, it was revealed that the endophytic fungus *Cercospora Kikuchi*, which was isolated from *Tithonia diversifolia* (Borges et al., 2009).

Proteases are frequently used in bioremediation processes, wastewater treatment, peptide synthesis, fruit juice fortification, the preparation of diets high in protein, the processing of leather, the production of laundry detergents, the preparation of meat tenderization and cheese, the production of methionine-enriched protein, and the treatment of diseases like cancer and inflammation. Microbiological proteases have a relatively high commercial value compared to animal and plant proteases (Kucerova and Cervinkova, 2016). When cultivated on skim milk medium supplemented with ammonium sulphate and fructose, the endophyte *Acremonium sp.* isolated from leaves of *Saraca asoca* displayed the maximum protease activity (Jain et al., 2012). The greatest keratinase activity is shown by *Penicillium sp.* Morsy1 is an endophytic fungus from *Dendronephthya hemprichi* widely used on various agricultural and domestic wastes (El-Gendy, 2010). Since polyester polyurethane can be broken down by

the serine protease generated by endophytic fungus from *Pestalotiopsis microspora*, these enzymes can be manufactured commercially for bioremediation applications (Basha et al., 2009).

Microorganisms manufacture xylanases to break xylans, a significant component of hemicellulose. Xylanases have drawn a lot of interest due to their biotechnological potential in numerous industrial areas. In addition to being used to clarify vegetable and fruit juices, enhance the digestibility of ruminant feed, bleach wood pulp, produce ethanol and xylitol, and degum flax, hemp, jute, and ramie, xylanases are also utilized in the bleaching of wood pulp (Dushyant et al., 2017). The endophytic fungus PTRa9, which was isolated from the leaves of *C. oblongifolius*, was given the optimal conditions for xylanase production by incubating it for 96 hours in a medium containing rice bran and ammonium sulfate (Wipusaree et al., 2011). A further investigation discovered that utilizing wheat bran as a carbon source enhanced *Aspergillus terreus*'s isolation of thermotolerant xylanase from *Memora peregrina* (Sorgatto et al., 2012).

Mannanase and Laccase are used by the food and beverage, paper, cosmetics, textile, pharmaceutical, petrochemical, and textile industries to synthesise polymers and other products. The laccase-producing endophytic fungus *Myrothecium verrucaria*, which was isolated from pigeon peas, produced laccase in the presence of the redox mediator ABTS (2,2′-casino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), demonstrated effective decolorization capability towards several synthetic dyes, including Congo red, Methyl orange, Methyl red, and Crystal violet (Sun et al., 2017). *Ulocladium sp*. and *Hormonema sp.,* two laccase-producing isolates from *Eucalyptus globulus*, exhibit larger improvements in the saccharification of *E. globulus* wood (Ren et al., 2017).

Because it breaks down the chitin in the pathogen's cell wall, chitinase has been used to manage phytopathogens biologically and employed in the production of antifungal medications, morphogenesis of insects and yeasts, and as fungicide

and insecticide agents. Additionally, they are employed in the synthesis of Chito oligosaccharides, glucosamines, and proteins from leftover shellfish, all of which have extensive uses in the pharmaceutical sector. Neotyphodium sp. and *Colletotrichum musae*, two endophytic fungi, can create chitinase, an enzyme with biochemical characteristics tailored to the environment in which the fungi reside (Khare and Prakash, 2017).

Plant Growth Promotion

The rhizosphere microbiome plays a crucial role in plant development, with research highlighting the dynamic interaction between plants and their associated microorganisms (Chaparro et al., 2014). The composition of rhizosphere microorganisms changes throughout plant development stages, emphasizing the importance of understanding this relationship. Endophytes inhabit plant internal tissues and are key contributors to plant growth promotion. These beneficial microorganisms employ various mechanisms to enhance plant growth, including nitrogen fixation, phytohormone production, nutrient acquisition, and conferring tolerance to abiotic and biotic stresses (Kandel et al., 2017). The complex interactions between fungi and plants involve significant secondary metabolites, which are crucial in inducing systemic and systemic acquired resistance processes (Pusztahelyi et al., 2015). Plant hormones also regulate defense responses, with key regulators including salicylic acid (SA), jasmonic acid (JA), ethylene (Et), and abscisic acid (ABA). Plant roots engage in chemical signaling with microbes in the rhizosphere, facilitating the biofilm formation of beneficial microbes and priming defense in the host plant (Alazem et al., 2014). This intricate communication network enhances plant resilience against pathogens.

Endophytic bacteria possess plant growth-promoting (PGP) attributes, including nitrogen fixation, phosphate solubilization, and phytohormone production. Research has demonstrated the potential of these bacteria to enhance plant growth. For instance, Rana et al. (2021) isolated endophytic bacteria from the Indian Himalayas and found that several strains exhibited

significant PGP traits, promoting maize (*Zea mays* L.) growth. *Streptomyces sp.* has also been identified as promising endophytes, displaying plant-growth-promoting and biocontrol activities (Vurukonda et al., 2018). This highlights the potential of endophytic *Streptomyces sp*. in sustainable agriculture practices, including biocontrol of plant pathogens and plant growth promotion. Endophytic bacteria facilitate plant growth promotion through various mechanisms, including phosphate solubilization and iron chelation to enhance mineral uptake, prevention of pathogen entry through antibacterial and antifungal agents, nitrogen fixation, siderophore production to outcompete pathogens for nutrients, production of phytohormones (auxin, cytokinin), and production of a 1-aminocyclopropane-1-carboxylate deaminase, reducing ethylene levels (Dudeja and Giri, 2014).

The interaction between bacterial endophytes and plants leads to plant hormone production and modulation, as Lopez et al. (2010) and Glick (2012) suggested. Indole 3-acetic acid (IAA), a naturally occurring auxin, plays a significant role in plant growth. Research has identified IAA-producing endophytes in various plant species, including sweet potatoes (Doty et al., 2009). Specifically, endophytic bacteria such as *Bacillus thuringiensis*, *B. pumilis*, *B. cereus*, *Pseudomonas putida*, and *Clavibacter michiganensis*, isolated from *Curcuma longa* L., have been found to produce IAA (Javid et al., 2011; Kumar et al., 2016). Similarly, *Bacillus* EZB-8 and *Artherobacter* EZB-4, isolated from *Capsicum annum* L., produced IAA and enhanced plant biomass while regulating stress-inducible genes. Endophytes have also been shown to confer abiotic stress tolerance. Sziderics et al. (2007) reported reduced osmotic and drought stresses. Furthermore, *Pseudomonas aeruginosa*, a wheat endophyte, demonstrated plant growth promotion, pathogen resistance, and salt tolerance in cucumber seedlings (Pandey et al., 2012).

Kumar et al., (2015) reported that *Bacillus, Agrobacterium*, and *Pseudomonas* produced phytohormones and solubilized tricalcium phosphate from the root of *Cassia tora*. *Achromobacter piechaudi* E6S, an endophytic bacterium produced

IAA, solubilizing phosphate and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Ma et al., 2016). Research has demonstrated the potential of endophytic bacteria in promoting plant growth and mitigating stress. Qin et al., (2014) found that bacteria isolated from the halophyte *Limonium sinense* exhibited efficient ACC deaminase activity, enhancing root and shoot length, seed germination, leaf area, and seedling number under salinity stress. Six endophytic bacteria isolated from *Zea mays* roots, identified as Bacillus and Enterobacter species, displayed beneficial traits (Szilagyi-Zecchin et al., 2014). Four strains (CNPSo 2476, 2477, 2478, and 2480) showed nitrogen fixation abilities, while two Bacillus strains (CNPSo 2477 and 2478) produced IAA, siderophores, and lytic enzymes. *Enterobacter sp*. CNPSo 2480 and *Bacillus sp.* CNPSo 2481 increased root volume and demonstrated antifungal activity against pathogens. A study on cucumber plants investigated endophytic bacteria's potential to control Fusarial wilt disease caused by *Fusarium oxysporum* f. sp. *cucumerinum* and promote plant growth (Ozaktan et al., 2015). Out of 112 isolated endophytic bacterial strains, 30% produced IAA, 46% produced siderophores, 16% produced HCN, 29% solubilized phosphate, and over 53% inhibited *Fusarium oxysporum* f. sp. *cucumerinum* mycelial growth.

Endophytic bacteria have been explored for their phosphate solubilization potential, enhancing plant growth. Researchers isolated endophytic bacteria from three rice cultivars (RD6, Chainat 1, and Black glutinous rice) and evaluated their phosphate solubilization abilities in Pikovskaya's Agar medium (Long et al., 2008). Similarly, 77 endophytic bacteria were isolated from *Solanum nigrum*, with six isolates demonstrating phosphate solubilization. Other studies have also reported phosphate solubilization by endophytic bacteria. Vendan et al. (2010) found that 9 out of 18 endophytic isolates from ginseng plants exhibited phosphate solubilizing ability. Vitorino et al. (2012) evaluated 42 endophytic bacteria from *Hyptis marubiodies* roots, with 20% solubilizing calcium phosphate and iron phosphate, thereby enhancing vegetation and plant growth. Further research confirmed these findings. Patel et

al. (2012) reported that 8 out of 18 endophytic isolates from tomato showed phosphate solubilization activity.

Phosphate-solubilizing bacterial endophytes have been isolated from strawberry meristematic tissues, demonstrating enhanced biomass accumulation and plant development (Dias et al., 2009). Specific isolates, namely CHR2I02, CHR3I01, CHR4I07, BRR1I04, and BRR3I01, showed tricalcium phosphate solubilization in Pikovskaya's Agar medium. To quantify phosphate solubilization, Duangpaeng et al. (2013) employed Pikovskaya's broth culture method. The solubilized phosphate was estimated by measuring absorbance using a spectrophotometer, referencing a standard curve. This approach effectively assessed the phosphate-solubilizing capabilities of the isolated endophytes.

Abscisic acid (ABA) is a plant stress hormone that is crucial in responding to various stresses, including water, salt, and cold temperature. Interestingly, certain bacteria can produce ABA and other beneficial compounds to alleviate plant stress. For instance, *Azospirillum lipoferum* produces ABA and gibberellic acid, which helps mitigate drought stress in maize (Cohen et al., 2009). Other beneficial bacteria have been found to produce compounds that promote plant stress tolerance. *Bacillus amyloliquefaciens* SB-9, isolated from the grapevine, secretes melatonin and its biosynthetic intermediates, including 5-hydroxytryptophan, serotonin, and N-acetylserotonin. Similarly, *Serratia marcescens* UPM39B3 induces the production of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, total soluble phenols, and lignothioglycolic acid in banana plantlets. These beneficial bacteria can also counteract adverse environmental effects. *Bacillus amyloliquefaciens* SB-9 colonization reduces malondialdehyde (MDA) and reactive oxygen species (ROS) production in grapevine roots, thereby alleviating salt and drought-induced stresses (Jiao et al., 2016). Besides phytohormones, salicylic acid (SA) is a critical plant hormone involved in various processes, such as seed germination, root initiation, stomatal closure, floral induction, and

thermogenesis, besides the tolerance of plants to biotic and abiotic stresses. Endophytic bacteria enhanced the growth of sunflower seedlings under water stress through the production of SA, which also inhibited the growth of pathogenic fungi (Forchetti et al., 2010; Klessig et al., 2016).

Siderophores play a significant role in microbial virulence, affecting humans, animals, and plants, as suggested by Neiland (1981). In plant-microbe interactions, siderophores facilitate iron uptake, enhancing plant growth. The rice root microbiome, for instance, supports iron acquisition through siderophore biosynthesis, reception, and iron storage (Sessitsch et al., 2012). Specifically, *Streptomyces sp.* GMKU 3100, isolated from rice, exhibits a wide range of siderophore production (Rungin et al., 2012). Siderophores also contribute to plant protection by competing with phytopathogens for essential nutrients (Verma et al., 2011; Aznar et al., 2015). Furthermore, siderophore-producing bacteria aid in the uptake of metals like iron, zinc, and copper by plants (Carrillo-Castaneda et al., 2002; Gururani et al., 2012). Research on *Phialocephala fortinii* has shed light on siderophore diversity. Bartholdy and Haselwandter (2001) found that five strains of this fungus produced three types of siderophores: ferricrocin, ferrirubin, and ferrichrome C. Notably, their production was influenced by pH and iron (III) concentration in the culture medium.

Antioxidant Properties of Endophytes

Antioxidants are important for the human body as they help in preventing cell damage caused by free radicals. Endophytic microbes have been identified as potential sources of antioxidant compounds, which may significantly reduce the use of agrochemicals in the cultivation of crop plants (Sarker and Oba, 2018). The compounds produced by endophytic microbes with antioxidant capacity include phenolics, mannitol, flavonoids, and other carbohydrates (White et al., 2019; Feng and Wang, 2020). Lactic acid bacteria, commonly used as probiotics, have also been found to possess antioxidant properties (White and Torres, 2010). Endophytic microbes have been found to play a significant role

in reducing oxidative stress in host plants. This reduction in oxidative stress is achieved through the mediation of reactive oxygen species and the production of antioxidant compounds by endophytes. Furthermore, endophytes have been shown to enhance stress tolerance to oxidative stress in plants. In a study by Cui et al., 2015, the endophytic fungi associated with Rhodiola spp. were found to exhibit high antioxidant activities, with some isolates showing significant potential for scavenging free radicals and chelating metal ions. This finding is consistent with the work of Gutierrez et al., 2012, where the endophytic fungal strain AE1 isolated from *Azadirachta indica* exhibited strong antioxidant potential, as evidenced by its ability to scavenge free radicals and its high antioxidant activity.

The arbuscular mycorrhizal fungi (AMF) inoculation led to increases in ascorbic acid and antioxidant activity in shallot bulbs, indicating improved antioxidant properties (Gutierrez et al., 2012). In a similar vein, AMF inoculation was found to improve berry antioxidant properties under elevated temperatures, demonstrating a protective role of AMF inoculation against warming effects on berry quality (Jasim et al., 2016).

The association between neem and endophytic microorganisms has been extensively explored, revealing intriguing connections. Neem, renowned for its complex compounds with diverse biological activities, harbors endophytic microorganisms that produce "mimetic" compounds, mirroring neem's properties. Where neem-associated endophytes were found to produce over 30 compounds, including neem-like molecules (Golubkina et al., 2019). Beyond neem, researchers have investigated the antioxidant capacities of various plant-based materials. Studies on grapefruit peel and old apple cultivars have demonstrated high radical scavenging capacities and robust antioxidant properties, respectively (Ilyasov et al., 2020; Suleria et al., 2020). Endophytes play a pivotal role in regulating the synthesis of secondary metabolites, including antioxidants, within host plants (Salari et al., 2019). This endophyte-

host interaction underscores the potential for plant-based materials to serve as rich sources of antioxidants and other beneficial compounds.

Endophytic microbes play a crucial role in mitigating oxidative stress in plants by regulating reactive oxygen species and producing antioxidant compounds (Hamilton et al., 2012). This concept is further supported by White et al. (2019), who highlight the potential applications of endophytic microbes in crop management through antioxidant compound production. Pimentel et al. (2011) also demonstrate that endophytes enhance plant tolerance to oxidative stress. Endophytic fungi produce bioactive metabolites with antioxidant and antimicrobial properties, promoting plant growth and nutrient uptake (Rana et al., 2020; Hamilton et al., 2012). The genetic basis of plant-endophytic bacteria interactions has been explored, revealing the potential for utilizing endophytic bacteria as a sustainable resource for natural antioxidants (Pinski et al., 2019). Beyond antioxidant properties, endophytes exhibit therapeutic effects. Endophytic bacteria promote host plant growth and alleviate salt stress, enhancing overall plant health (Fan et al., 2020).

Endophytes and Human Health

Beyond their role in plant-microbe interactions, endophytes have significant implications for human health. Recent years have seen a surge in interest in the health benefits of various compounds and microorganisms, particularly endophytes. Certain plants, like turmeric, have been found to possess medicinal properties, primarily due to their rich content of bioactive compounds like curcumin. Turmeric has been shown to aid in managing oxidative and inflammatory conditions, metabolic syndrome, arthritis, anxiety, and hyperlipidemia (Tilman and Clark, 2014). Other beneficial compounds for human health include omega-3 polyunsaturated fatty acids (PUFAs), such as alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These fatty acids have been extensively studied for their numerous health benefits, including reducing inflammation and improving cardiovascular health (Hewlings and Kalman, 2017). Probiotic bacteria have also gained

popularity due to the growing evidence of their beneficial effects on human health. These beneficial microorganisms have been shown to support gut health, boost immunity, and even aid in disease prevention (Shahidi and Ambigaipalan, 2018).

In addition to these findings, plant bioactive compounds have also demonstrated functional activities that suggest they could significantly prevent a wide range of chronic diseases (Frumkin et al., 2017). However, knowledge gaps still need to be addressed despite the known benefits of these compounds and microorganisms. For example, while the therapeutic potential of various phenolic compounds, such as apigenin and hydroxytyrosol, has been highlighted, there is a need for further research to fully understand their effects on human health (Salehi et al., 2019). Similarly, the potential effects of neonicotinoids on human health remain poorly understood, despite the adverse effects observed in other species. Moreover, the emergence and spread of antibiotic resistance and the evolution of new strains of disease-causing agents are significant concerns for global health. The role of the environment in the risk of antibiotic resistance to clinical outcomes has been acknowledged. However, there is still a lack of documented approaches to formally assess these risks (Kechagia et al., 2013).

The natural environment and its ecosystem services have been prominently featured in contemporary ecological models of health, emphasizing its fundamental role in supporting human life, health, and well-being (Mauri and Menon, 2017). Furthermore, the Mediterranean diet, which includes olive oil as a quintessential part, has been associated with a healthy way of eating and living (Barkia et al., 2019). Additionally, microalgae have been recognized as a potential source of renewable nutrition, with growing interest in algae-based dietary supplements and their potential health benefits (Durazzo et al., 2019). The gut microbiome is crucial in modulating the human immune system (Kau et al., 2011). As part of the plant microbiome, endophytes may interact with the human gut microbiome and influence immune responses. Furthermore,

FOXP3+ regulatory T cells are essential in maintaining immune homeostasis (Nishikawa and Sakaguchi, 2010), and it is plausible that endophytes may have a role in regulating T cell function. Zimmermann et al., 2019 discussed the mapping of human microbiome drug metabolism by gut bacteria and their genes. This study provides insights into the potential of endophytic microorganisms to contribute to human health through drug metabolism and related processes. Further research on the bioactive compounds produced by endophytes could lead to developing new drugs and therapeutic agents (Singh et al., 2016).

Ecological Function and Impact of Environmental Factors on Endophytes

The root microbiome plays a vital role in plant health and productivity, and its ecological function has been explored across various plant species (Fitzpatrick et al., 2018). Unraveling the specific contributions of endophytes to the root microbiome's ecological function is a crucial area for future research, holding great promise for enhancing plant resilience and performance. Endophytes also have broader implications, influencing both environmental and health outcomes. For instance, air pollution has significant environmental and health impacts (Manisalidis et al., 2020). However, the potential of endophytes to mitigate these effects remains largely unexplored, presenting an exciting avenue for investigation. Additionally, the health impacts of wildfire smoke exposure have been critically reviewed (Reid et al., 2016), but the potential contribution of endophytes in ameliorating the effects of smoke exposure warrants investigation.

Endophytes significantly impact ecosystem-level processes, influencing their environments in complex ways (Clay and Holah, 1999; Kivlin et al., 2013; Griffin et al., 2017; Laforest-Lapointe et al., 2017; Christian et al., 2019). Contrary to the common assumption of solely mutualistic relationships between endophytes and their hosts, interactions range from mutualism to commensalism and even latent or mild antagonism (Carroll, 1988; Rodriguez et al., 2009; Saikkonen et al., 1998; Schulz and Boyle, 2005; Hardoim et al., 2008).

Fungal endophytes, in particular, have been identified as a rich source of phytochemicals and bioactive natural products (Aly et al., 2010). These microorganisms produce valuable compounds with potential applications in medicine, agriculture, and various industries. This highlights the importance of bioprospecting endophytes for novel bioactive compounds.

Hardoim et al. (2015) emphasized the role of environmental factors in shaping the diversity and composition of endophytic microbial communities within plants. They highlighted that factors such as soil type, climate, and plant genotype can significantly influence the abundance and diversity of endophytes. For example, the composition of endophytic communities was found to vary between different plant species and geographic locations, indicating the influence of environmental factors on endophyte diversity. Environmental factors, such as drought, can influence the development and composition of the plant root microbiome, including endophytic microorganisms (Xu et al., 2018). This finding emphasizes the need to consider the effects of environmental stress on the bioprospecting of endophytes and their potential applications in sustainable agriculture and environmental management. In the study by Grabrucker (2012), the focus was on environmental factors in autism, but it provided valuable insights into the broader impact of environmental factors on biological systems. The author discussed the influence of environmental factors on neurological development and function, highlighting the potential relevance of environmental conditions on the symbiotic relationship between endophytes and plants. This insight suggests that environmental factors may be crucial in shaping the interactions between endophytes and their host plants.

Advances in Technology

Modern techniques like metagenomics, next-generation sequencing, and high-throughput screening enhance the ability to identify and characterize endophytes and their metabolites. These are very powerful technique that allows for the comprehensive analysis of microbial communities by sequencing their collective genomes. This approach has been particularly useful in studying

endophytes, non-pathogenic microorganisms living inside plant tissues. The integration of metagenomics into endophyte research has enabled the identification of microbial diversity, functional capabilities, and the ecological roles of these microorganisms. A substantial body of research has focused on endophytic microorganisms' diversity and community structure. For instance, a study on the root-associated bacterial endophytes of *Panax ginseng* utilized shotgun metagenomic analysis to reveal age-related shifts in microbial populations (Hong et al., 2019). Similarly, metagenomic profiling has been employed to analyze the community structure and diversity of bacterial endophytes in maize plants, demonstrating the influence of different fertilization practices (Fadiji et al., 2020).

A comparative metagenomics approach has been used to study functional diversity among endophytes of different host plants, revealing significant variations in metabolic pathways and ecological functions (Kaul et al., 2016). Another study assessed the metagenomic data of endophytes in *Aloe vera*, highlighting their roles in plant growth promotion and stress resistance (Akinsanya et al., 2015). Advanced methodologies such as high-throughput sequencing (HTS) and next-generation sequencing (NGS) have revolutionized the study of endophytes. For example, 16S rRNA gene sequencing has been prevalent in characterizing endophytic bacterial communities (Alsaedi et al., 2022). Additionally, metagenomic sequencing of PCR amplicons has been used to assess the bacterial endophytes in *Aloe vera*, providing insights into their taxonomic composition and functional potential (Akinsanya et al., 2015).

McKenna et al. (2010) introduced the Genome Analysis Toolkit (GATK), a MapReduce framework designed to analyse NGS data. This framework provides a scalable and efficient platform for processing and interpreting large-scale DNA sequencing data. The authors demonstrated the utility of GATK in analyzing next-generation DNA sequencing data, showcasing its potential for identifying genetic variants and elucidating complex genomic features within endophyte populations. DePristo et al. (2011) proposed a variation discovery

and genotyping framework using NGS data, which offers valuable insights into the genetic diversity and polymorphisms present in endophytic populations. Metzker (2010) provided an overview of sequencing technologies, highlighting the advancements and capabilities of next-generation sequencing platforms. The author discussed the transformative impact of NGS on genomic research and emphasized its potential for studying diverse microbial communities, including endophytic microorganisms. By leveraging the latest NGS technologies, researchers can gain deeper insights into endophytes' genetic makeup and functional attributes, thereby enhancing our understanding of their ecological roles and biotechnological potential. The findings presented by McKenna et al., 2010 and DePristo et al., 2011 underscore the significance of NGS data analysis frameworks in elucidating the genetic landscape of endophytic communities. These frameworks enable the identification of genetic variations, population diversity, and evolutionary dynamics within endophytes, laying the groundwork for comprehensive genomic studies. Moreover, the insights shared by Metzker (2010) shed light on the technological advancements driving NGS, emphasizing its applicability to studying endophytic microorganisms and uncovering novel genetic features.

***Lagenandra toxicaria* Dalz.**

Lagenandra is the rhizomatic aroid plant genera that are widely diverse in Kerala. There are various species reported in *Lagenandra*. Arends and Van der Laan, 1978 reported the somatic chromosome numbers of *Lagenandra* Dalzell. Sivadasan and Babu, 1995 studied on the rare and endemic variety of *Lagenandra toxicaria* var. *barnesii* Fischer. GC-MS analysis of oil extracted from the rhizome of *L. toxicaria* and reported the chemical constituents by Selvakumari and Britto, 2008. The methanol and benzene extract of the rhizome of *L. toxicaria* have insecticidal potential against *Tribolium castaneum* (Selvakumari; 2010). Sereena et al., 2011 worked on the histochemical and phytochemical characterizations of both plant's rhizomes. They reported that *L. toxicaria* is not the genuine source of hallakam and adulterant of *Kaempferia*

rotunda. Kurdekar et al., 2012 studied the antimicrobial activity of the rhizome of *L. toxicaria* against human pathogens. From this study, maximum zone of inhibition showed against chloroform and acetone extracts of *Candida albicans* in cold percolation and soxhlet methods, respectively.

Selvakumari (2014) focused on the pharmacognostical standardization of *Lagenandra toxicaria*. This study provided insights into the botanical and pharmacognostical characteristics of *Lagenandra toxicaria*, contributing to its standardization for medicinal and commercial purposes. Selvakumari (2014) also conducted an X-ray diffraction study of a crystal obtained from *Lagenandra toxicaria*, providing valuable insights into its crystallographic properties and structural characteristics. The ISSR markers were used to analyze molecular variations of *Lagenandra spp*. of Kerala and reported a diverse genomic variability between each species in Kerala by Prakashkumar et al., 2015. *L. toxicaria* is very efficient in the removal of iron and coliform content in water and also as a turbidity reducer at all concentrations of plant material (Snisha and Harilal, 2017). Senarathne et al. (2017) investigated the feasibility of using problematic aquatic weeds, including *Lagenandra toxicaria*, for vermicomposting in the Coconut Triangle Area of Sri Lanka. The study highlighted the potential of *Lagenandra toxicaria* in generating vermicompost, thus addressing environmental and agricultural challenges. Gaya *et al*., 2017 studied the heavy metal assessment and biochemical response of *L. toxicaria,* revealing moderate arsenic accumulation with the highest carbohydrate and soluble protein content. Instead, they also reported the maximum protein level. Krishnakumar et al., 2020 conducted a study to identify and explore the bioactivities of endophytic fungi from *Lagenandra toxicaria*. The findings of this study shed light on the potential bioactive compounds present in *Lagenandra toxicaria* and the role of endophytic fungi in contributing to its bioactivities. In a study by Akarsha and Gulimane (2021), the genotoxic and antigenotoxic potential of *Lagenandra toxicaria* rhizome methanol extract was evaluated using an *Allium cepa* assay. The study provides insights into the

potential genotoxic and antigenotoxic properties of *Lagenandra toxicaria*, contributing to its safety assessment and potential pharmaceutical applications. Krishnakumar and Varghese (2022) explored the nematicidal activity of *Lagenandra toxicaria* rhizome extracts against root-knot nematode and burrowing nematode. The findings indicated the potential of *Lagenandra toxicaria* as a natural nematicidal agent, with implications for agricultural pest management. The study investigates the hepatoprotective effects of *Lagenandra toxicaria* against carbon tetrachloride (CCl_4)-induced liver fibrosis in Wistar rats. The methanol extract of *L. toxicaria* has demonstrated a significant amelioration of carbon tetrachloride (CCl_4)-induced collagen accumulation and inflammation, indicating its potential therapeutic value for liver fibrosis. This is supported by bioactive compounds such as octadecenamide, palmitic acid, and stigma steroids in the methanol extract of *L. toxicaria*, which have been reported to possess anti-inflammatory and antifibrotic activities. These findings suggest that the bioactive compounds present in the *L. toxicaria* extract may contribute to its observed anti-inflammatory and antifibrotic effects (Akarsha et al., 2022). Another study also conducted by Akshara et al., 2022a evaluated the morphology, physicochemical, and digestibility of starch isolated from *L. toxicaria.* It revealed that it contains low amylose content, which renders it suitable as a food thickening agent. Pooja et al. (2023) analyzed the antimicrobial properties of *Lagenandra toxicaria* and its fungal endophytes. The study revealed the antimicrobial potential of *Lagenandra toxicaria* and the bioactive compounds produced by its fungal endophytes.

Chapter 3

Isolation, Diversity Analysis and Identification of Endophytic Bacteria and Fungi from *Lagenandra toxicaria* Dalzell

Introduction

The exploration of endophytic microbes has gained significant attention in the scientific community, as these elusive organisms hold the potential to unveil a plethora of untapped natural resources. These organisms, which reside within the internal tissues of plants, possess the remarkable ability to establish symbiotic relationships with their host, often conferring beneficial effects. The isolation and characterization of these elusive microbes have become a growing interest among researchers, as they can unveil new avenues for biotechnological applications and environmental stewardship. The process of isolating endophytic microbes involves a meticulous and systematic approach. It typically begins with carefully selecting plant samples and implementing surface sterilization techniques to eliminate epiphytic (surface-dwelling) microorganisms. The internal plant tissues are then aseptically dissected and subjected to culture-dependent or culture-independent methods, depending on the specific research objectives. The potential of these microbes for biotechnological applications is immense, making this research particularly exciting and promising.

Culture-dependent approaches rely on the cultivation of endophytic microbes on specialized growth media, allowing for the identification and characterization of diverse bacterial and fungal species. These methods provide valuable insights into the physiological and metabolic properties of the isolated endophytes, paving the way for potential biotechnological applications. Alternatively, culture-independent techniques, such as metagenomic analyses, have revolutionized the field by enabling the exploration of the entire microbial community within a plant, including those that are recalcitrant to cultivation. These approaches, which leverage advanced molecular biology tools, have revealed the remarkable diversity and complexity of endophytic microbiomes, shedding light on their ecological roles and potential functional contributions.

The isolation and study of endophytic microbes have profound implications for various domains, including agriculture, environmental remediation, and pharmaceutical development. Endophytes have been known to enhance plant growth, increase resistance to biotic and abiotic stresses, and produce a myriad of bioactive compounds with therapeutic potential. By unlocking the secrets of these enigmatic microorganisms, researchers can potentially harness their capabilities to develop innovative solutions for pressing global challenges.

The isolation of endophytic microbes represents a captivating frontier in microbiology. Furthermore, the isolation of endophytic microbes provides valuable insights into the complex interplay between plants and their associated microbial communities. The application of rigorous methodologies and the integration of cutting-edge technologies to unravel the complexities of these intriguing microbes, pave the way for groundbreaking discoveries and sustainable advancements in various domains. This chapter represents the collection of plant materials, isolation of endophytic bacteria and fungi, morphological, molecular, and biochemical studies for identifying isolates, and diversity studies for analyzing the endophytic distributions.

Materials and Methods

Selection of Plant Species

Lagenandra toxicaria Dalzell is an aquatic herb used for the current study based on some particular features. Being a rhizome plant, it can remain healthy even if separated from its habitat. Therefore, it is a suitable plant for endophytic studies in aquatic species. *L. toxicaria* (Araceae) is traditionally used in medicine for kidney disorders, heart diseases, and swellings; water purifies in dug wells. *L. toxicaria* comprises antimicrobial activity due to the presence of esters and fatty acids. The most prominent chemicals are diethyl phthalate, oleic acid, palmitic acid, and dioctyl phthalate.

Collection Sites/Locations

Three different sites of *Lagenandra toxicaria* Dalzell in Wayanad district were selected for the collection of plant samples from June 2019 to June 2022 in three

seasons summer, monsoon, and winter (Fig.2). Wayanad district is situated on the southern top of the Deccan plateau with a total area of 2132 sq. km. Wayanad District, located in Kerala, boasts a tropical climate characterized by average temperatures ranging from 15°C to 35°C, with heavy monsoon rainfall occurring from June to September. As the only plateau in Kerala, it forms part of the Mysore Plateau, connecting the Western Ghats to the Eastern Ghats, with elevations spanning 700 to 2,100 meters above sea level. The district comprises three municipal towns: Kalpetta, Sulthan Bathery, and Mananthavady, making it a unique and fascinating region. The study sites, are Kottavayal located in Kalpetta, Pakkom in Sultan Bathery, and Mattilayam in Mananthavady. The details of each sampling site are presented in Table 1.

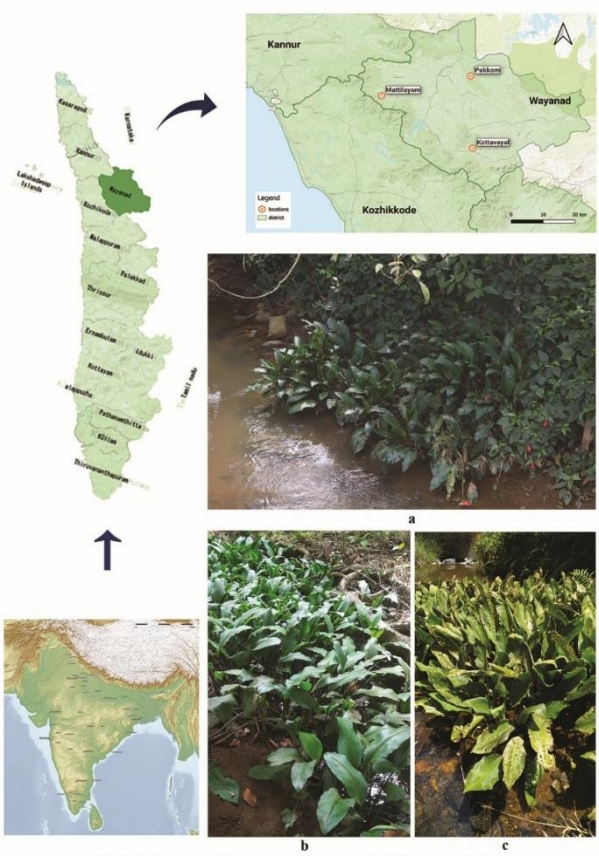


Fig.2 Study area and locations a. Kottavayal, b.Pakkom, c. Mattilayam

Sl No.	Locations	Sites	Geographical coordinates	
			Latitude	Longitude
1	Kottavayal	I	11°34’52.2” N	76°06’22.7” E
2	Pakkom	II	11°48’03.3” N	76°06’01.5” E
3	Mattilayam	III	11°44’26.3” N	75°50’43.7” E

Table 1: Details of the collection sites

Collection of Plant Samples

Healthy and mature plants were selected randomly from each site, and their plant parts, i.e. leaves, roots, and rhizomes, were collected in triplicates in sterile bags. To ensure the endophytic nature of the isolates, plant tissues (leaf, root, and rhizome) were sealed with paraffin, stored in sterile polythene bags, and

transported in an ice box to the laboratory for storage at 4°C. After morphological characterization, the plant specimens were identified properly and voucher herbaria specimens were prepared according to standard procedures (Jain and Rao, 1977) and maintained at Malabar Botanical Garden and Institute for Plant Sciences, Kozhikode, Kerala, India (Voucher numbers: MBGH 7763, MBGH 7762, and MBGH 7761). The plant samples were processed to isolate endophytes and bioactive metabolites.

Isolation of Endophytic Bacteria and Fungi

Surface Sterilization of Plant Materials

The most commonly employed approach for detecting and quantifying endophytes involves isolating them from surface-sterilized host plant tissues, effectively distinguishing internal microorganisms from external contaminants. Surface sterilization of host plant tissues/samples is an integral component of endophytic work and is used to remove epiphytes and other microorganisms from the surface of plant tissues. Under aseptic conditions, endophytes were isolated from diverse asymptomatic regions of the selected plant, building on previous techniques (Bacon, 1988; Ahmed et al., 2012). Surface sterilization of the plant material was performed using an optimized protocol derived from established guidelines (Hallmann et al., 2006). Wash the materials several times under running tap water followed by surface sterilization with different sterilizing agents or sterilants. The sterilants used to remove the surface microorganisms are dilute sodium hypochlorite (NaOCl), and ethanol (C_2H_5OH). The time required in each sterilant is crucial and usually varies with the host plant, tissue thickness, texture, and sensitivity. To eliminate surface microorganisms, the plant material was subjected to a series of sterilization rinses, including a 70% ethanol (C2H5OH) treatment for 1 minute, then with 2% sodium hypochlorite (NaOCl) for 7 minutes used for leaf; 4% for root and rhizome followed by 70% ethanol for 30 secs, and finally with sterile distilled water (5-6) times. Plant material was then dried between the folds of sterile filter papers and subjected to isolate resident endophytic bacteria and fungi.

Isolation of Bacterial and Fungal Isolates

Following drying, surface-sterilized plant materials (leaf, root, and rhizome) were segmented into smaller explants to expose internal tissues. Each explant was then placed on nutrient-rich media: Nutrient Agar (NA) with nystatin (0.5 mg/ml) and Potato Dextrose Agar (PDA) with chloramphenicol (0.5 mg/ml). Seven explants were positioned per plate, and sealed with parafilm to prevent contamination. 420 healthy, surface-sterilized explants were inoculated across 60 agar-poured petriplates. Incubation occurred at 36±2°C for bacterial growth and 28±2°C for fungal growth, with regular monitoring for microbial/mycelial development. Upon observing growth, subculturing ensured purity. Endophytic cultures were transferred to freshly prepared NA and PDA plates until pure cultures were obtained. Control setups involved placing water droplets from the final surface sterilization rinse on agar plates without plant tissue.

Maintenance of Endophytes

Following purification, the endophytic isolates were individually transferred to NA and PDA slants. The preserved bacterial and fungal endophytes were refrigerated at 4°C, awaiting further analysis.

Diversity Analysis

The percentage frequency (PF) of different endophytic bacterial and fungal isolates was calculated as the number of endophytes isolated from a particular tissue of a particular plant divided by the total number of endophytes isolated from that very plant multiplied by 100.

Percentage frequency = No. of endophytes isolated from a plant tissue / Total no. of endophytes isolated from that plant × 100

The diversity of endophytic assemblages from the host plants was estimated based on Isolation Frequency (IF) and colonization rate (CR) using the formula (Biswas et al., 2013). Isolation frequency is calculated by the number of isolates obtained from the isolation process divided by the total number of each plant tissue segment placed for isolation multiplied by 100.

Isolation frequency (IF) = No. of isolates obtained / Total no. of tissue segments placed × 100

The colonization rate was quantified as the percentage of tissue segments harboring one or more endophytic isolates relative to the total number of segments subjected to isolation. The diversity of the endophytes was assessed using various statistical indices. The proportionality of individual taxa in the total endophytic isolates obtained was assessed by Colonization Frequency (CF) using the formula (Hata and Futai, 1995). Simpson's Diversity Index (D) was used to measure species diversity within the endophytic community, calculated as follows:

$$D = \Sigma ni(ni-1) / N(N-1)$$

The Simpson's Diversity Index (D) formula utilizes species-specific abundance (ni) and total population size (N). The resulting values ranged from 0 to 1, with higher values corresponding to reduced diversity and lower values indicating increased diversity and species heterogeneity.

The Shannon-Weiner Diversity Index, also known as the Shannon-Weiner Index (SWDI) (Maheshwari and Rajagopal, 2011), measures the diversity of species in a community.

The formulae were as follows:

$$H' = - \sum [(ni / N) \times \ln (ni / N)]$$

N = total population size, ln = natural logarithm, n = number of individuals.

The diversity of endophytes in the host plants was assessed using the Margalef Richness Index (R1), which quantitatively measures species richness (Yuan et al. 2010).

$$R1 = \frac{S - 1}{ln\, N}$$

S represents the overall species count, and N denotes the cumulative total of isolates across all species.

The evenness index (E) quantifies the spread of populations among various species present.

$$E = \frac{H'}{\ln S}$$

Where (S) is the total population size.

Seasonality, geographical location, and tissue type effects on endophytic distribution and diversity were examined using multivariate linear model analysis (MANOVA) and bi-plot analysis using SPSS v16 and R v2.15.1 (R Development Core Team 2009) software, respectively. MANOVA analysis assessed the effects of site, season, and tissue on endophyte diversity metrics (CR, Shannon-Wiener, Simpson's, Species Evenness, and Margalef Richness). Principal Component Analysis, conducted using R software, revealed interactions between endophytes and sampling variables while also visualizing multivariate patterns through correlation matrix analysis of z-scores.

Identification of Isolates

Morphological Characterization of Bacterial Endophytes

Morphological analysis, comprising macroscopic and microscopic evaluations, served as the initial step in identifying the endophytic bacterial isolates. Macro characters include different colony characteristics like colony color, colony texture, margin, shape, and elevation. The microscopic analysis was done by gram staining, motility test, and endospore to identify cell characteristics like cell shape, gram-positive or negative, motile or non-motile, and endospore-forming or not.

Gram Staining

A bacterial smear was prepared on a clean glass slide and gently heat-fixed. The slide was then subjected to Gram staining, involving sequential treatments with crystal violet for 1 minute, Gram's iodine solution, and rinses with tap water. Decolorization with 95% alcohol was followed by counterstaining with safranin for 45 seconds and a final tap water rinse. The stained preparation was examined under a compound microscope to determine the Gram reaction of the bacterial strains.

Motility Test

The isolates were transferred into the center of the coverslip, which was coated with Vaseline on its edges. The hanging drop technique was employed, in which the cavity slide was inverted over the cover slip. Microscopic examination

revealed the isolates' distinctive swarming motility, aiding in their identification.

Endospore Staining

The strains were subjected to bacterial endospore staining to identify the presence of endospore. The bacterial strains were inoculated in Nutrient agar medium and incubated for 72 hours for the staining study. Prepare a 48-hour broth culture and make a thin smear of bacteria on a separate clean glass slide. The bacterial smear was air-dried and heat-fixed. Next, the smear was flooded with malachite green solution and steamed for 5 minutes, with additional stain added as needed. After steaming, the slide was rinsed under gently running tap water. A 30-second counterstaining with safranin followed, then a final rinse with distilled water. The slide was blotted dry with blotting paper, completing the Albert staining procedure. Observe it under an oil immersion microscope.

Biochemical Characterization of Bacterial Endophytes

The biochemical characteristics of bacterial endophytes were determined using various tests, namely, indole, MRVP, citrate, urease, litmus milk, carbohydrate catabolism, carbohydrate fermentation, TSI, and catalase.

Indole Production Test

An indole production test was conducted to determine an organism's ability to convert tryptophan to indole. The bacteria strains were inoculated with tryptone broth and incubated for 48 hours at 37°C.

MRVP Test

The MRVP test was conducted to identify the organism capable of performing mixed acid fermentation. The bacteria strains were inoculated in MRVP broth and incubated for 48 hours at 37°C.

Citrate Utilization Test

To assess citrate utilization, endophytic bacterial isolates were inoculated onto Simmons citrate agar media and incubated at 37°C for 48 hours. A positive reaction was indicated by a colour change from green to blue, reflecting a pH shift due to citrate metabolism. In this medium, citrate served as the sole carbon

and energy source, allowing bacteria capable of citrate utilization to alter the pH and change the medium's colour.

Urease Production Test

The urease test is a valuable diagnostic tool for identifying bacteria, particularly those capable of hydrolyzing urea. This test involves inoculating the test organism onto urea broth or agar medium and incubating it at 37°C for 48 hours.

Litmus Milk Test

A litmus milk test was conducted to distinguish between different species of bacteria. The lactose, litmus, and casein in the medium and also be metabolized by different types of bacteria. The strains of bacteria were inoculated in glucose agar medium slants and incubated for 48 hours at 37°C.

Carbohydrate Catabolism by Microorganisms

Carbohydrate reduction test was conducted to identify the organism capable of reducing carbohydrate present in the media. The strains of bacteria are inoculated in glucose agar medium slants and incubated for 48 hours at 37°C.

Fermentation of Carbohydrates

A carbohydrate fermentation test was conducted to identify the organism capable of utilizing the carbohydrates present in the media. The bacteria strains were inoculated in fermentation medium slants and incubated for 48 hours at 37°C.

Triple Sugar Iron Test

A fermentation test was conducted to determine the organism's ability to metabolize specific carbohydrates. The basal growth medium was supplemented with the target carbohydrate, and its composition was prepared accordingly. This test assessed carbohydrate utilization, gas production, and hydrogen sulfide (H_2S) production, providing insight into the organism's metabolic capabilities.

Catalase

The catalase test was performed to detect the production of catalase enzyme in aerobic organisms. Catalase, a ubiquitous enzyme in oxygen-tolerant life forms,

catalyzes the decomposition of hydrogen peroxide into water and oxygen. To assess catalase activity, bacterial strains were inoculated onto trypticase soy agar plates and incubated at 37°C for 48 hours. The test results indicated the presence or absence of catalase enzyme in the organisms.

Molecular Characterization of Bacterial Endophytes

Genomic DNA isolation

Bioactive endophytic bacterial strains were cultured in 2 mL sterile nutrient broth (NB) medium overnight at 37°C ± 2°C. The resulting bacterial suspension (OD 0.6) was centrifuged at 13,000 rpm for 3 minutes to pellet the cells. Total cellular DNA extraction followed the SDS method outlined by Wilson (1990). For DNA extraction, the cell pellet was resuspended in 875 µL TE buffer using vortex mixing. Then, 100 µL of 10% SDS and 5 µL of Proteinase K (10 mg/mL) were added, and the mixture was thoroughly inverted and incubated at 37°C for 60 minutes, until the suspension became clear and viscous. Next, an equal ratio of phenol: chloroform: isoamyl alcohol (25:24:1) solution was added, gently mixed by inverting, and incubated at room temperature for 5 minutes. The mixture was centrifuged at 10,000×g for 10 minutes at 4°C. The viscous upper phase containing nucleic acids was transferred to a new 2.0 mL Eppendorf tube, and this step was repeated twice. The collected supernatant then underwent a chloroform wash by mixing with chloroform: isoamyl alcohol (24:1) and centrifuging at 10,000×g for 10 minutes. To precipitate nucleic acids, 100 µL of 5M sodium acetate and 500 µL of isopropanol were added to the aqueous phase. The mixture was gently inverted several times, resulting in a white, viscous DNA precipitate. The sample was then stored overnight at -20°C. The DNA pellet was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant isopropanol was carefully removed without disturbing the pellet. The pellet was then washed with 500 µL of 70% ethanol by inverting the tube several times, followed by centrifugation at 12,000 rpm for 15 minutes. The ethanol was carefully removed, and the tube rim was blot-dried with a paper towel to remove excess liquid. The pellet was air-dried and re-suspended in 50

µL TE buffer. The resulting DNA solution was stored at -20°C. To assess the quality of the extracted genomic DNA, agarose gel electrophoresis was performed. A 2 µL sample of the total DNA solution was loaded onto a 0.8% agarose gel containing 0.5 µg/mL ethidium bromide. Electrophoresis separated the DNA, which was visualized using a Bio-Rad gel documentation system, following the protocol outlined by Wilson (1990).

Quantification of DNA

DNA quantification was performed using two methods. Initially, 0.8% agarose gel electrophoresis with 0.5 µg/mL ethidium bromide was employed, comparing band intensities of bacterial DNA isolates to a standard lambda DNA marker (300 ng/µL). Additionally, spectrophotometric quantification was conducted using NanoDrop. A standard DNA sample of known concentration was prepared, and UV spectrophotometer readings were taken at 260 nm. The ratio of absorbance at 260 nm and 280 nm (A260/A280) was calculated to assess DNA purity and concentration. A ratio of 1.8 was considered indicative of pure DNA.

PCR Amplification of 16S Region

Extracted DNA was amplified using universal primers: 8-27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1495 R (5'-CTACGGCTACCTTGTTACGA-3'). The 25 µL PCR reaction mixture consisted of 1 µL DNA, 12.5 µL Master mix, 1 µL of 0.2 µM primers, and 9 µL Milli-Q water. PCR amplification was performed on an Eppendorf Mastercycler Gradient with the following program: initial denaturation at 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, concluding with a final extension at 72°C for 5 minutes, as outlined by Mirza et al. (2006). The PCR products were then electrophoresed on 1% agarose gel with ethidium bromide in 1X-TAE buffer. Amplicon size was determined by comparison to a 1Kb molecular weight DNA ladder, and visualization was conducted under a gel documentation system.

Sequencing of Amplified PCR Product and Sequence Analysis for Bacterial Identification

The amplified 16S rDNA products underwent Sanger sequencing. Sequence quality was assessed using Applied Biosystems' Sequence Scanner software v5.2. BIOEDIT v7.2.5 was employed for sequence assembly. To ensure sequence accuracy, DECIPHER GPL v3.0 detected potential chimeric artifacts, following Wright et al. (2012) guidelines. Phylogenetic neighbors were identified via the EzBioCloud server (http://www.ezbiocloud.net/identify), and homology searches were conducted using the BLAST search algorithm, as described by Yoon et al. (2017).

Phylogenetic Analysis

Phylogenetic analysis was conducted using MEGA 11 (Kumar et al., 2018). Sequence alignment was performed with CLUSTAL MUSCLE (Edgar, 2004), and nucleotide similarity values were calculated. The evolutionary history was inferred using the Maximum Likelihood (ML) method (Saitou and Nei, 1987), while evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). The robustness of tree topologies was evaluated through bootstrap analysis with 1000 replications, following Felsentein's (1985) methodology. This comprehensive analysis enabled the reconstruction of a reliable phylogenetic tree.

Morphological Characterization of Fungal Endophytes

Endophytic fungal isolates were initially identified through morphological characterization, examining both macroscopic and microscopic features. This involved studying colony characteristics, including colour from the front and reverse, texture, shape, margin, elevation, and optical density. Most of the endophytic fungal isolates showed variations in these features whereas some showed similarities/resemblances. The fungal isolates showing absolute resemblance for these characteristics were considered as repeats. Endophytic fungal isolates were stained with lactophenol cotton blue and observed under the microscope at different magnifications (40X, and 100X). Characterization was done based on their microscopic features like the presence or absence of

septa, type of conidiophores, shape and colour of conidia, etc. Relevant fungal identification keys were also used to aid the identification process (St-Germain and Summerbell, 2011).

Molecular Characterization of Fungal Endophytes

Genomic DNA Isolation

Genomic DNA was isolated from selected endophytic fungal isolates using a modified protocol from Moller et al. (1992). Fungal samples were grown in potato dextrose broth at 28°C ± 2°C for 6-7 days to obtain mature cultures. The mycelial mass was harvested by filtering through autoclaved muslin cloth. Approximately 50 mg of dried mycelial mass was ground into a fine powder using liquid nitrogen in an autoclaved, pre-cooled mortar. Pre-warmed TES lysis buffer (500 µL, 60°C) was added, comprising 100 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), and 2% SDS. The mixture was transferred to a 2 mL sterilized microcentrifuge tube, and 5 µL proteinase K was added. Incubation followed at 60°C for 60 minutes to facilitate DNA release. After incubation, 140 µL of 5M NaCl and 64 µL of 10% CTAB (w/v) were added to the suspension. The mixture was incubated at 65°C for 10 minutes. DNA extraction was performed by adding an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), followed by centrifugation at 14,000g for 10 minutes. This step was repeated twice. The supernatant was collected, and an equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by centrifugation. RNA was digested by adding 7 µL RNAase and incubating at 37°C for 60 minutes. An additional chloroform wash was performed to further purify the DNA. DNA precipitation was achieved by adding 3M sodium acetate (pH 5.2) and storing at -20°C overnight. The DNA pellet was obtained by centrifugation at 12,000 rpm for 10 minutes at 4°C, washed twice with 70% ethanol, and air-dried. The DNA pellet was resuspended in 50 µL TE buffer.

Quantification of DNA

DNA was quantified on 0.8% agarose gel by comparing the band intensities of fungal DNA isolates with standard DNA (lambda DNA marker) of known

concentration (300ng/µl). DNA quantification was cross-checked spectrophotometrically using NanoDrop. DNA concentration and purity were assessed using UV spectrophotometry. A standard DNA sample of known concentration was prepared, and absorbance readings were taken at 260 nm. The ratio of absorbance at 260 nm and 280 nm (A260/A280) was calculated to evaluate DNA purity and concentration. A ratio of 1.8 was considered indicative of pure DNA.

PCR Amplification of ITS Region

DNA samples were diluted with TE buffer to achieve a uniform concentration of 50 ng/µl for PCR analysis. PCR-based amplification of specific fungal DNA regions was performed using ITS-based primers. The eukaryote-specific ITS primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were employed as described by White et al. (1990). PCR reactions were conducted in a BioRad Thermal Cycler using a 25 µl reaction mixture for each DNA sample. PCR reaction involves the following main steps: Denaturation of DNA strands, annealing of primers to complementary sequences of the template DNA, and finally extension or elongation which is facilitated by polymerases and complementary dNTPs to synthesize new strands.

Sequencing of Amplified PCR Product for Fungal Identification

PCR products after amplification were analyzed electrophoretically on 1% agarose gel in 1X-TAE buffer and detected by ethidium bromide. The amplicon size was determined by comparing it with the 1Kb molecular weight DNA ladder. The amplified products were used for Sanger's sequencing method. The results were obtained in FASTA format which were further analyzed by using nBLAST (nucleotide- Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information). The obtained data was compared with the GenBank database to identify the isolated fungal endophytes. All the sequences were submitted to NCBI GenBank to get the

NCBI GenBank accession numbers, and the phylogenetic tree was constructed using the same method for bacterial isolates.

Results

Three to five plant samples were collected from each Kottavayal, Pakkom, and Mattilayam of Wayanad district, Kerala site. A total of 11340 tissue segments (420 segments of each tissue from each site) were incubated in petri plates with nutrient agar (NA) for bacteria and potato dextrose agar (PDA) for fungi.

Isolation of Endophytic Microorganisms

Endophytic Bacteria

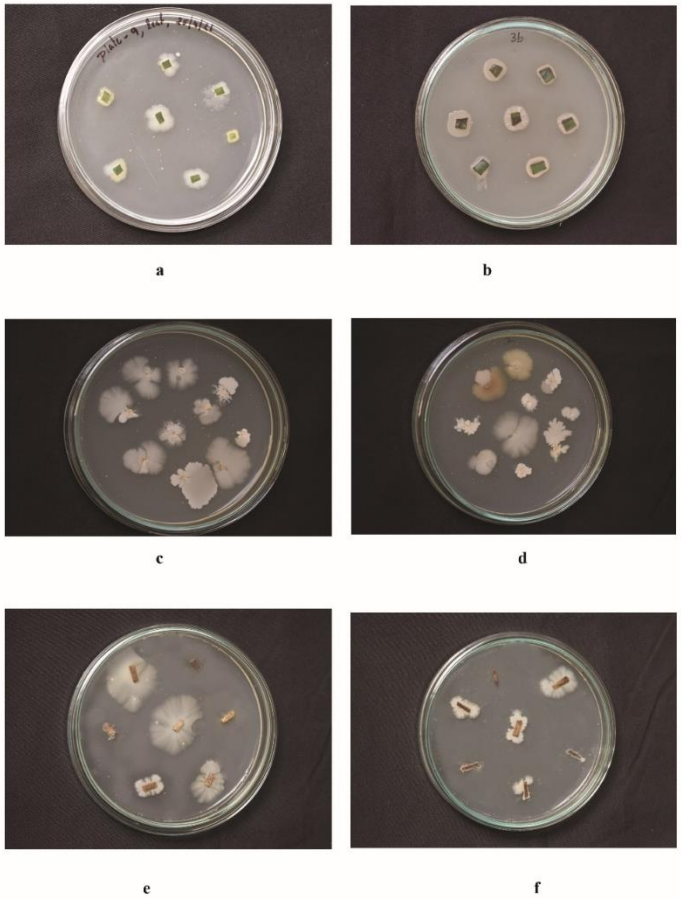


Fig.3 Isolation plates of endophytic bacteria. a,b - Leaf; c,d - Root; e,f - Rhizome

461 endophytic bacteria were isolated from the leaf segments in the summer season, 170 were isolated in the winter season, and 69 isolates were in the monsoon season. From the root segments 701 bacteria in summer, 200 in winter, and 58 in monsoon. 507 isolates in summer, 149 in winter, and 47 in monsoon from the rhizome segments. A total of 2362 bacterial isolates were obtained from the fragmentation method. 1669 endophytic bacteria were isolated from all tissues in the summer season, 519 in the winter, and 174 in the monsoon season. Kottavayal holds 591 isolates from all plant samples in the summer, 184 in winter, and 54 in monsoon. In the case of Pakkom 573 isolates in summer, 174

in winter, and 74 in monsoon season. 505 endophytic bacteria from Mattilayam in summer, 161 in winter, and 46 isolates in monsoon. The data of isolated endophytic bacteria are detailed in Table 2. The represented isolation plates are figured in Fig.3.

		Locations											
		Kottavayal				Pakkom				Mattilayam			
		S	W	M	T	S	W	M	T	S	W	M	T
Plant parts	Leaf	161	63	16	240	143	51	32	226	157	56	21	234
	Root	267	72	18	357	235	70	26	331	199	58	14	271
	Rhizome	163	49	20	232	195	53	16	264	149	47	11	207
	Total	591	184	54	829	573	174	74	821	505	161	46	712

Table 2: Endophytic bacteria isolated from *Lagenandra toxicaria* Dalzell
*S: Summer, W: Winter, M: Monsoon

Diversity Analysis

The isolates obtained from *L. toxicaria* were used to calculate percentage frequency (PF), isolation frequency (IF), colonization rate (CR), colonization frequency (CF), and different diversity indices. The highest percentage frequency of endophytic bacteria was observed in root samples of 45.17% from Kottavayal in the summer season followed by Pakkom (41.01%) and Mattilayam (39.40%) (Table 3). In the winter season, the highest percentage frequency was also recorded in root samples of 40.22% at Pakkom followed by Kottavayal (39.13%) and Mattilayam (36.02%) (Table 4). 45.65% frequency was observed in leaf samples from Mattilayam followed by Pakkom (43.24%) and Kottavayal (37.03%) in the monsoon season (Table 5). In the summer season, the isolation frequency observed was highest in the root samples of all sites. i.e., 63.57% from Kottavayal followed by Pakkom (55.95%) and Mattilayam (47.38%) (Table 3). In the winter season, the highest isolation frequency was shown by the root samples from Kottavayal (17.14%) followed by the root samples from Pakkom (16.66%) (Table 4). Whereas, Mattilayam shows the lowest isolation frequency for all root, leaf, and rhizome samples. In addition to this, the leaf samples from Kottavayal show a 15% isolation frequency. In the monsoon season, the highest isolation frequency was observed

in leaf samples from Pakkom (7.61%) followed by root (6.19%) from Pakkom and 4.76% of rhizome from Kottavayal (Table 5).

	Summer								
	Kottavayal			Pakkom			Mattilayam		
	Leaf	Root	Rhizome	Leaf	Root	Rhizome	Leaf	Root	Rhizome
PF	27.24	45.17	27.58	24.95	41.01	34.03	31.08	39.40	29.50
IF	38.33	63.57	38.80	34.04	55.95	46.42	37.38	47.38	35.47

Table 3: Percentage frequency and Isolation frequency in summer season of endophytic bacteria

	Winter								
	Kottavayal			Pakkom			Mattilayam		
	Leaf	Root	Rhizome	Leaf	Root	Rhizome	Leaf	Root	Rhizome
PF	34.23	39.13	26.63	29.31	40.22	30.45	34.78	36.02	29.19
IF	15	17.14	11.66	12.14	16.66	12.61	13.33	13.80	11.19

Table 4: Percentage frequency and Isolation frequency in winter season of endophytic bacteria

	Monsoon								
	Kottavayal			Pakkom			Mattilayam		
	Leaf	Root	Rhizome	Leaf	Root	Rhizome	Leaf	Root	Rhizome
PF	29.62	33.33	37.03	43.24	35.13	21.62	45.65	30.43	23.91
IF	3.80	4.28	4.76	7.61	6.19	3.80	5	3.33	2.61

Table 5: Percentage frequency and Isolation frequency in monsoon season of endophytic bacteria

The highest colonization rate of 60% was observed in root samples from Kottavayal followed by 53.33% from Pakkom and 46.42% in rhizome samples from Pakkom in summer. The colonization rate in winter and monsoon is similar to the isolation frequency. That 7.62% and 5.95% are the highest colonization rate represented by root and leaf samples from Pakkom, respectively. The lowest rate was observed in rhizome samples from Mattilayam at 2.62% in monsoon season. In winter, 17.14% was observed as the highest colonization rate from root samples of Kottavayal and the lowest in rhizome from Mattilayam at 11.19%. Regarding diversity indices, the summer season showed

the highest SWDI of 2.46 in the root samples from Kottavayal followed by the Pakkom (2.45) root samples and leaf samples from Mattilayam (2.42). In the monsoon, leaf samples from Pakkom showed the highest SWDI of 1.13 followed by root samples from Kottavayal and minimum diversity represented by rhizome and root samples from Mattilayam of 0.22 and 0.51, respectively. The root and rhizome samples of 1.65 and 1.62 are the highest H' shown in the winter season and 1.23 and 1.29 from the rhizome and root of Mattilayam showed the lowest H', respectively.

Simpson's diversity index (D) showed that 0.9 is the highest diversity index for root samples from Pakkom and leaf samples from Mattilayam followed by root samples from Kottavayal (0.89) in the summer season. 0.64 is the highest index shown by root samples from Kottavayal and 0.62 by leaf samples from Pakkom in monsoon and rhizome samples from Mattilayam represented the lowest index of 0.16. In the winter 0.78 is the highest diversity index showed by root samples from Kottavayal followed by 0.77 from rhizome samples of Kottavayal and root samples from root. The lowest diversity index was represented by rhizome from Mattilayam (0.67) and leaf samples from Kottavayal (0.69). Margalef Richness Index 2.90 of root samples from Kottavayal showed the highest richness in the summer season followed by root samples from Pakkom (2.77). The lowest richness was observed in leaf samples from Kottavayal (2.09). In the monsoon, 0.17 of the rhizome samples from Mattilayam represented the lowest richness index, and 1.27 of root samples from Kottavayal was the highest. The lowest richness index of 1.02, 1.10, and 1.17 were shown by root, rhizome, and leaf samples from Mattilayam, and the highest index of 1.79 was shown by rhizome samples from Kottavayal in winter.

The highest evenness index in the summer season was observed in rhizome samples from Kottavayal and root samples from Pakkom. The lowest was observed in 0.86 in leaf samples from Kottavayal. In the monsoon, the highest evenness was represented by 0.96 and 0.90 in root samples from Kottavayal and leaf samples from Pakkom, respectively, and the lowest of 0.63 was observed

in leaf samples from Mattilayam and rhizome samples from Kottavayal. 0.96 and 0.95 evenness indices were observed as the highest in the winter season from leaf and root samples from Mattilayam and the lowest was represented by leaf samples from Kottavayal of 0.86. Table 6 represents the colonization rate, SWDI, SDI, Margalef richness index, and evenness.

Location	Season	Samples	CR	SWDI	SDI	Margalef Richness Index	Evenness
Kottavayal	Summer	Leaf	36.43±9.12	1.91±-0.11	0.81±0.01	2.09±0.17	0.86±0.01
		Root	60.00±3.78	2.45±0.05	0.89±0.00	2.90±0.22	0.93±0.10
		Rhizome	38.33±6.48	2.22±0.15	0.88±0.02	2.26±0.29	0.96±0.03
	Monsoon	Leaf	5.71±1.01	0.90±0.07	0.53±0.05	0.97±0.08	0.82±0.07
		Root	4.28±0.71	1.11±0.38	0.64±0.14	1.27±0.57	0.96±0.00
		Rhizome	4.76±5.16	0.65±0.62	0.39±0.35	0.85±0.75	0.63±0.55
	Winter	Leaf	15.00±0.00	1.32±0.09	0.69±0.01	1.20±0.18	0.86±0.01
		Root	17.14±4.46	1.65±0.17	0.78±0.03	1.57±0.23	0.93±0.01
		Rhizome	11.66±7.22	1.62±0.35	0.77±0.07	1.79±0.55	0.94±0.04
Pakkom	Summer	Leaf	34.04±6.15	2.28±0.38	0.88±0.05	2.74±0.91	0.94±0.02
		Root	53.33±15.97	2.46±0.13	0.90±0.01	2.77±0.31	0.96±0.00
		Rhizome	46.42±3.97	2.22±0.06	0.88±0.01	2.31±0.18	0.94±0.02
	Monsoon	Leaf	7.62±1.80	1.13±0.49	0.62±0.21	1.09±0.55	0.90±0.09
		Root	5.95±1.09	0.93±0.49	0.53±0.26	0.92±0.47	0.83±0.21
		Rhizome	3.81±0.41	0.69±0.23	0.44±0.12	0.80±0.37	0.85±0.13
	Winter	Leaf	11.90±4.12	1.57±0.32	0.75±0.08	1.75±0.39	0.89±0.05
		Root	16.66±4.85	1.58±0.22	0.77±0.04	1.49±0.46	0.93±0.06
		Rhizome	12.61±2.88	1.39±0.13	0.72±0.04	1.28±0.13	0.91±0.02
Mattilayam	Summer	Leaf	37.38±11.30	2.42±0.26	0.90±0.02	2.62±0.43	0.93±0.01
		Root	41.90±10.01	2.25±0.02	0.88±0.00	2.38±0.12	0.94±0.02
		Rhizome	35.47±5.35	2.25±0.21	0.88±0.02	2.64±0.44	0.93±0.03
	Monsoon	Leaf	5.00±2.57	0.56±0.50	0.36±0.32	0.46±0.42	0.63±0.55
		Root	5.00±1.00	0.51±0.18	0.33±0.16	0.52±0.05	0.73±0.26
		Rhizome	2.62±2.06	0.22±0.39	0.16±0.28	0.17±0.29	0.32±0.56
	Winter	Leaf	13.33±6.07	1.40±0.14	0.74±0.03	1.17±0.14	0.96±0.02
		Root	13.81±5.06	1.29±0.28	0.70±0.08	1.02±0.28	0.95±0.03
		Rhizome	11.19±5.16	1.23±0.26	0.67±0.08	1.10±0.23	0.90±0.03

Table 6: Detailed diversity indices of endophytic bacteria

The effects of location, season, and plant parts on the concerned factors were tested by Multivariate ANOVA (MANOVA), and the pairwise comparisons of each factor were done using the Tukey test. Multivariate analysis of variance (MANOVA) was performed on community metrics, including CR, SWDI, SDI, Margalef richness index, and evenness data, using the Pillai trace statistic. Individual factors showed that the season effects were highly significant i.e., P<0.01. Indeed, the interaction effect on diversity is significantly less i.e. *P>0.01 and 0.05* on the location*season, location*plant, season*plant, and location*season*plant. From the observations, the endophytic bacterial diversity is statistically significant at a 1% level of significance location-wise, season-wise, and also plant-wise but in the paired condition, there is no statistical difference was observed (Table 7).

Effect	*df*	Pillai's trace	F	P
Location	2	0.310	1.8	0.070
Season	2	1.470	27.183	0.000
Plant	2	0.312	1.810	0.069
Location*Season	4	0.453	1.303	0.180
Location*Plant	4	0.418	1.189	0.267
Season*Plant	4	0.467	1.347	0.153
Location*Season*Plant	6	0.630	0.937	0.584

Table 7: Results of multivariate ANOVA for endophytic bacterial diversity

Table 8 shows the effect of location, seasons, and plant parts on the dependent characteristics. The location effect on SDI, SWDI, and Margalef richness is statistically more or less significant. The effect of seasons and the dependent variables showed greater significance than the other factors. Statistical analysis yielded significant inter-group differences ($p < 0.05$), suggesting that the variables had distinct effects. A p-value above 0.05 would have indicated no significant differences.

Source	Dependent Variable	*df*	F	P
Location	CR	2	2.120	0.130
	SDI	2	3.679	0.032

	SWDI	2	4.514	0.016
	Evenness	2	1.463	0.241
	Margalef Richness	2	5.607	0.006
Season	CR	2	274.675	0.000
	SDI	2	62.190	0.000
	SWDI	2	166.490	0.000
	Evenness	2	7.090	0.002
	Margalef Richness	2	121.916	0.000
Plant	CR	2	7.612	0.001
	SDI	2	1.801	0.175
	SWDI	2	2.712	0.076
	Evenness	2	1.272	0.289
	Margalef Richness	2	1.339	0.271
Location * Season	CR	4	0.769	0.551
	SDI	4	2.437	0.059
	SWDI	4	2.186	0.083
	Evenness	4	1.821	0.139
	Margalef Richness	4	2.110	0.093
Location * Plant	CR	4	1.087	0.373
	SDI	4	0.548	0.701
	SWDI	4	1.604	0.187
	Evenness	4	0.434	0.783
	Margalef Richness	4	2.043	0.102
Season * Plant	CR	4	4.671	0.003
	SDI	4	1.324	0.274
	SWDI	4	1.030	0.401
	Evenness	4	1.195	0.324
	Margalef Richness	4	0.537	0.709
Location * Season * Plant	CR	6	1.072	0.397
	SDI	6	0.128	0.998
	SWDI	6	0.241	0.981
	Evenness	6	0.319	0.955
	Margalef Richness	6	0.599	0.775

Table 8: Effect of factors on dependent characteristics

A pairwise comparison was done using Tukey's test. For SDI, SWDI, and Margalef Richness, Kottavayal and Pakkom show no significant difference. CR and Evenness show no significant difference concerning all locations i.e., CR and evenness do not show effective location changes (Table 9).

Dependent Variable	(I) Location	(J) Location	Mean Difference (I-J)	Std. Error	P
CR	Kottavayal	Pakkom	0.106	1.676	0.950
		Mattilayam	3.068	1.698	0.077
	Pakkom	Mattilayam	2.962	1.676	0.083
SDI	Kottavayal	Mattilayam	0.087*	0.039	0.032
	Pakkom	Kottavayal	0.009	0.039	0.813
		Mattilayam	0.096*	0.039	0.017
SWDI	Kottavayal	Mattilayam	0.189*	0.084	0.029
	Pakkom	Kottavayal	0.048	0.083	0.566
		Mattilayam	0.237*	0.083	0.006
Evenness	Kottavayal	Mattilayam	0.065	0.056	0.251
	Pakkom	Kottavayal	0.027	0.056	0.624
		Mattilayam	0.093	0.056	0.101
Margalef Richness	Kottavayal	Mattilayam	0.314*	0.114	0.008
	Pakkom	Kottavayal	0.029	0.113	0.801
		Mattilayam	0.342*	0.113	0.004

Table 9: Location-wise comparison of dependent variables
* Shows statistical significance

In the case of season, all the dependable variables are statistically significant, since the p-value <0.01 except in condition evenness of summer to winter. The diversity of bacteria shows significant differences concerning seasons (Table 10).

Dependent Variable	(I) Season	(J) Season	Mean Difference (I-J)	Std. Error	P
CR	Summer	Monsoon	37.619*	1.698	0.000
		Winter	28.889*	1.653	0.000

	Winter	Monsoon	8.730*	1.698	0.000
SDI	Summer	Monsoon	0.434*	0.039	0.000
		Winter	0.145*	0.038	0.000
	Winter	Monsoon	0.289*	0.039	0.000
SWDI	Summer	Monsoon	1.530*	0.084	0.000
		Winter	0.823*	0.082	0.000
	Winter	Monsoon	0.707*	0.084	0.000
Evenness	Summer	Monsoon	0.192*	0.056	0.001
		Winter	0.014	0.055	0.798
	Winter	Monsoon	0.178*	0.056	0.003
Margalef Richness	Summer	Monsoon	1.740*	0.114	0.000
		Winter	1.149*	0.111	0.000
	Winter	Monsoon	0.591*	0.114	0.000

Table 10: Season-wise comparison of dependent variables
* Shows statistical significance

The CR of root samples with leaf and rhizome showed the significance of $p<0.01$ and also SWDI on the root with rhizome samples. Only root differs significantly in CR. All the other pair-wise comparisons do not show any significant difference concerning plant parts i.e., Margalef Richness, Evenness SWDI, and SDI have no effect concerning plant parts since the p-value is greater than 0.05 (Table 11).

Dependent Variable	(I) Plant part	(J) Plant part	Mean Difference (I-J)	Std. Error	P
CR	Root	Leaf	5.740*	1.698	0.001
		Rhizome	5.688*	1.676	0.001
	Rhizome	Leaf	0.052	1.676	0.975
SDI	Leaf	Rhizome	0.054	0.039	0.167
	Root	Leaf	0.016	0.039	0.695
		Rhizome	0.070	0.039	0.078
SWDI	Leaf	Rhizome	0.111	0.083	0.188
	Root	Leaf	0.082	0.084	0.336
		Rhizome	0.192*	0.083	0.024
Evenness	Leaf	Rhizome	0.048	0.056	0.390
	Root	Leaf	0.040	0.056	0.478

		Rhizome	0.088	0.056	0.118
Margalef Richness	Leaf	Rhizome	0.101	0.113	0.373
	Root	Leaf	0.083	0.114	0.472
		Rhizome	0.184	0.113	0.109

Table 11: Plant part-wise comparison of dependent variables
* Shows statistical significance

The principal component (PC) analysis on the standardized data sets of bacteria reduces dimensionality and identifies the key components explaining the variants in the data. It was found that the first PC explains 85.6% of the total variance, while the second PC explains around 12.2%. A biplot was generated to visualize the results of the PCA performed in each data set. The biplot simultaneously displays the observations' scores and the original variables' loadings on the first to PC. Each point on the biplot represents an individual observation, colored by specific characteristics. As shown in the picture (Fig.4), each vector is of similar length, pointing in the same directions. However, CR and evenness are not correlated. The standard deviation and variance for the PCA are represented in Table 12. Eigenvectors for the principal component analysis are shown in Table 13.

Importance of components	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**
Standard Deviation	2.068	0.781	0.265	0.192	0.055
Proportion of Variance	0.855	0.122	0.014	0.007	0.001
Cumulative Proportion	0.855	0.977	0.991	0.999	1.000

Table 12: Standard deviation and variance of PCA

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
CR	-0.430	0.530	-0.711	-0.088	-0.139
SDI	-0.470	-0.218	0.297	-0.636	-0.487
SWDI	-0.479	0.123	0.246	-0.193	0.810
Evenness	-0.380	-0.774	-0.401	0.294	0.085
Margalef Richness	-0.467	0.234	0.428	0.680	-0.280

Table 13: Eigenvectors for principal component analysis

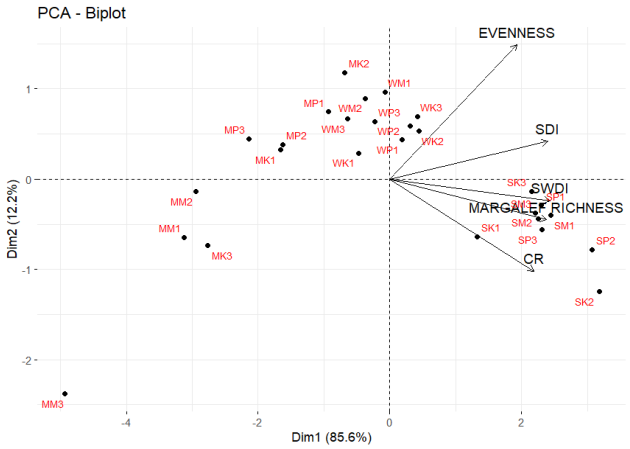


Fig.4: Biplot illustrates the PCA results performed on endophytic bacteria

The plot shows the projection of the dependent variables (arrows) and observations (points) onto the principal component space. Variables are scaled to unit variance, and observations are centered. The arrows' lengths and directions indicate each variable's contribution to the principal components, while the positions of the points reflect the relative similarity of observations.

Identification of Bacterial Endophytes

Morphological Characteristics of Bacterial Endophytes

Thirty-five bacterial endophytes from *Lagenandra toxicaria* were selected for further studies based on the maximum frequency of occurrence, growth rate, and differences in appearance. The selected bacterial endophytes were analyzed by studying their macromorphological, micromorphological, and biochemical characteristics. The macromorphological characteristics like colony shape, size, elevation, margin, surface, optical density, and colour, and micromorphological characteristics like cell shape, gram staining, endospore, and motility were studied. The isolates exhibit a range of colony shapes, including irregular, filamentous, rhizoid, circular, and spiral forms. E.g. BRWS3, BRWS13 (colonies have an irregular shape, not uniform or circular); BRWW6 (colonies have a thread-like or filamentous shape); BRWW12 (colonies have a root-like or rhizoid shape); BRWM2, BLWS1 (colonies are circular).

The sizes vary from punctiform (very small) to large - BRWS3, BRWW6 (colonies are of moderate size, not too small or too large); BRWW12, BLWS7 (colonies are very small, pinpoint in size); BRWM2, BLWW1 (colonies are large). The elevation of the colonies ranges from flat to raised, with some exhibiting a slightly raised or convex shape. BRWS3, BLWS1 (colonies are flat, not raised or convex); BRWW6, BRWM2 (colonies are raised, convex, or domed); BRWW12, BLWS7 (colonies are slightly raised, not flat but not highly convex). The colony margins vary from entire (smooth), lobate (having rounded projections), or irregular. BRWS3, BRWW12 (colonies have a lobate or wavy margin); BRWW6, BRWS13 (colonies have an irregular margin, not uniform or smooth); BLWS1, BLWS7 (colonies have a smooth, entire margin). The surfaces of the colonies are described as mucoid (slimy), rough, smooth, or dry. BRWS3, BRWW12 (colonies have a mucoid or slimy surface); BRWW6, BRWS13 (colonies have a rough or uneven surface); BLWS1, BLWS7 (colonies have a smooth surface). The optical density is primarily opaque, with a few isolates being translucent. BRWS3, BRWW6 (colonies are opaque, not transparent); BRWM2, BLWW1 (colonies are translucent, partially transparent). Most isolates have a cream or white color, with a few exhibiting a pale yellow or creamy white hue. BRWS13, BRWW6 (colonies are cream-coloured), and BLWS1, BLWS7 (colonies are white) (Table 14).

The 35 bacterial isolates exhibited various characteristics, including cell shape, Gram staining, motility, and endospore presence. Cell shape varied among the isolates, with the majority being rod-shaped (e.g., BRWS3, BRWW6, BRWW12). Some isolates had a long rod shape (e.g., BRWS1, BRWM2), while others had a helical rod shape (e.g., BLWW5) or a spiral shape (e.g., BLWW10). One isolate, BRhWW10, had a coccus (spherical) shape. Gram staining revealed that most isolates were Gram-positive (e.g., BRWS3, BRWW6, BRWW12), while a few were Gram-negative (e.g., BRWM3, BLWW5, BLWW10). Motility was observed in most isolates (e.g., BRWS3, BRWW6, BRWW12), with some being non-motile (e.g., BRWM3, BLWS1).

Endospore presence was observed in most isolates (e.g., BRWS3, BRWW6, BRWW12), while a few did not produce endospores (e.g., BRWM3, BLWS1). Some peculiar features were observed in BRWS1, and BRWM2, which had a long rod shape and was motile; BLWW5, which had a helical rod shape and was Gram-negative; BLWW10, which had a spiral shape and was motile; and BRhWW10, which had a coccus shape and was non-motile (Table 15). The macroscopic morphology and microscopic details are represented in Fig.5.

The biochemical test helps identify the bacterial isolates' metabolic capabilities and characteristics. BRWS3 exhibits a wide range of metabolic capabilities, including indole production, acid production, and carbohydrate utilization. The isolate, BRWW6, has limited metabolic capabilities but still utilizes carbohydrates and produces catalase enzymes. BLWW1 exhibits a range of metabolic capabilities, including acid production, carbohydrate utilization, and catalase production (Table 16). 65.71% of isolates produce catalase enzyme; 57.14% utilize citrate as a carbon source; 42.85% produce acid from glucose; 34.28% produce acetoin from glucose; 14.28% of few isolates produce indole from tryptophan and some isolates of 28.57% hydrolyze urea.

Bacterial isolates BRWS3, BRWW6, BRWW12, BRWS1, BRWM2, BLWW1, BLWW4, BLWW2, BLWS7, BLWW8, BLWW7, BLWM4, BLWW9, BLWS10, BRhWS1, BRhWM3, and BRhWS22 were identified as species of *Bacillus*. BRhWW7 and BRhWM27 as species of *Enterobacter*; BRWS15 and BLWS11 as species of *Lysinibacillus* based on their phenotypic characters. All the isolated endophytic bacteria belong to the families Bacillaceae (77.14%), Paenibacillaceae (2.85%), Lysinibacillaceae (5.71%), Rhizobiaceae (2.85%), Pseudomonadaceae (2.85%), Enterobacteriaceae (5.71%), and Planococcaceae (2.85%). The members of Bacillaceae are the predominant species in the endophytic bacterial isolates. Figure 5 represents the colonies and cell morphology

Sl No.	Bacterial Isolates	Shape	Size	Elevation	Margin	Surface	Optical density	Color
1	BRWS3	Irregular	Moderate	Flat	Lobate	Mucoid	Opaque	Cream
2	BRWW6	Filamentous	Moderate	Raised	Irregular	Rough	Opaque	Cream
3	BRWW12	Rhizoid	Punctiform	Slightly raised	Lobate	Mucoid	Opaque	Creams
4	BRWS13	Irregular	Small	Raised	Sightly irregular	Slightly rough	Opaque	Cream
5	BRWS15	Irregular	Punctiform	Slightly raised	Slightly irregular	Mucoid	Opaque	Cream
6	BRWS1	Irregular	Moderate	Slightly raised	Irregular	Mucoid	Opaque	Cream
7	BRWM2	Circular	Large	Raised	Undulate	Rough	Translucent	Cream
8	BRWM3	Irregular	Large	Raised	Irregular	Slightly rough	Translucent	Creamy white
9	BLWW1	Irregular	Large	Flat	Lobate	Dry	Translucent	White
10	BLWW4	Circular	Moderate	Slightly raised	Slightly irregular	Slightly rough	Opaque	White
11	BLWW2	Rhizoid	Large	Raised	Fimbricate	Matte	Opaque	Cream
12	BLWS7	Circular	Small	Slightly raised	Entire	Smooth	Opaque	White
13	BLWW5	Spiral	Punctiform	Flat	Wavy	Wrinkled	Opaque	White
14	BLWW8	Irregular	Large	Slightly raised	Irregular	Dry	Opaque	Cream

15	BLWW7	Irregular	Small	Flat	Slightly irregular	Slightly rough	Opaque	White
16	BLWM4	Irregular	Large	Slightly raised	Lobate	Rough	Opaque	Cream
17	BLWM1	Circular	Small	Slightly raised	Entire	Smooth	Opaque	White
18	BLWW10	Spiral	Punctiform	Flat	Irregular	Rough	Opaque	White
19	BLWW9	Irregular	Small	Flat	Lobate	Dry	Opaque	Cream
20	BLWS10	Circular	Small	Slightly raised	Slightly rough	Smooth	Opaque	White
21	BLWS9	Rhizoid	Large	Slightly raised	Irregular	Dry	Opaque	White
22	BLWS4	Circular	Medium	Flat	Irregular	Rough	Opaque	Cream
23	BLWS2	Circular	Small	Flat	Entire	Rough	Opaque	Cream
24	BLWS1	Circular	Small	Slightly raised	Entire	Smooth	Opaque	White
25	BLWS3	Circular	Small	Flat	Slightly irregular	Slightly rough	Opaque	White
26	BLWS6	Circular	Medium	Slightly raised	Entire	Rough	Opaque	Cream
27	BLWS11	Circular	Small	Convex	Entire	Slightly rough	Opaque	Cream
28	BRhWS1	Circular	Small	Flat	Entire	Rough	Opaque	Cream
29	BRhWS8	Irregular	Medium	Slightly raised	Irregular	Dry	Opaque	Cream
30	BRhWW11	Circular	Small	Flat	Slightly irregular	Rough	Opaque	Cream

31	BRhWW7	Circular	Small	Flat	Slightly irregular	Slightly Rough	Opaque	Cream
32	BRhWM3	Irregular	Small	Flat	Slightly irregular	Slightly rough	Opaque	White
33	BRhWW10	Circular	Small	Slightly raised	Entire	Smooth	Translucent	White
34	BRhWM27	Irregular	Medium	Flat	Entire	Smooth	Translucent	Pale yellow
35	BRhWS22	Circular	Small	Slightly raised	Entire	Slightly rough	Opaque	Cream

Table 14: Macro-morphological characters of endophytic bacterial isolates

Sl No.	Bacterial Isolates	Cell shape	Gram staining	Motility	Endospore
1	BRWS3	Rod	Positive	Motile	Present
2	BRWW6	Rod	Positive	Motile	Present
3	BRWW12	Rod	Positive	Motile	Present
4	BRWS13	Rod	Positive	Motile	Present
5	BRWS15	Rod	Positive	Motile	Present
6	BRWS1	Long rod	Positive	Motile	Present
7	BRWM2	Long Rod	Positive	Motile	Present
8	BRWM3	Rod	Negative	Non-motile	Absent
9	BLWW1	Rod	Positive	Non-motile	Present
10	BLWW4	Rod	Positive	Motile	Present
11	BLWW2	Rod	Positive	Motile	Present

12	BLWS7	Rod	Positive	Motile	Present
13	BLWW5	Spiral	Negative	Motile	Absent
14	BLWW8	Rod	Positive	Motile	Present
15	BLWW7	Rod	Positive	Motile	Present
16	BLWM4	Rod	Positive	Motile	Present
17	BLWM1	Rod	Negative	Motile	Absent
18	BLWW10	Spiral	Negative	Motile	Absent
19	BLWW9	Rod	Positive	Motile	Present
20	BLWS10	Rod	Positive	Motile	Present
21	BLWS9	Rod	Positive	Motile	Present
22	BLWS4	Rod	Positive	Motile	Present
23	BLWS2	Rod	Positive	Motile	Present
24	BLWS1	Rod	Positive	Non-motile	Absent
25	BLWS3	Rod	Positive	Motile	Present
26	BLWS6	Rod	Positive	Motile	Present
27	BLWS11	Rod	Positive	Motile	Present
28	BRhWS1	Rod	Positive	Motile	Present
29	BRhWS8	Rod	Positive	Motile	Present
30	BRhWW11	Rod	Negative	Motile	Absent
31	BRhWW7	Rod	Negative	Motile	Absent
32	BRhWM3	Rod	Positive	Motile	Present

33	BRhWW10	Coccus	Positive	Non-motile	Present
34	BRhWM27	Rod	Negative	Motile	Absent
35	BRhWS22	Rod	Positive	Motile	Present

Table 15: Micro-morphological characters of endophytic bacterial isolates

Sl No.	Bacterial isolates	Indole	MR	VP	Citrate	Urease	Litmus milk	CHO Catabolism	CHO Fermentation	TSI	Catalase
1	BRWS3	+	+	+	+	+	+	+	+	+	+
2	BRWW6	-	-	-	-	-	-	+	+	-	+
3	BRWW12	-	-	-	-	-	-	-	-	-	+
4	BRWS13	-	-	-	+	-	-	+	-	-	+
5	BRWS15	-	-	-	+	-	-	-	+	-	+
6	BRWS1	-	+	+	+	-	+	-	-	+	+
7	BRWM2	-	+	+	+	-	+	+	+	+	+
8	BRWM3	-	-	-	+	-	-	-	-	-	+
9	BLWW1	-	+	+	+	-	+	+	+	+	+
10	BLWW4	-	+	+	+	-	+	-	+	+	+
11	BLWW2	-	+	+	-	-	+	-	-	-	+
12	BLWS7	-	+	+	+	-	+	-	-	+	+
13	BLWW5	+	-	-	+	+	+	+	+	+	+
14	BLWW8	-	+	+	+	-	+	-	-	+	+
15	BLWW7	-	+	+	+	-	+	+	+	+	+

16	BLWM4	-	+	+	+	-	+	-	-	+	+
17	BLWM1	-	+	-	+	-	+	-	-	+	+
18	BLWW10	+	-	-	+	+	+	+	-	+	+
19	BLWW9	-	+	+	+	-	+	-	-	+	+
20	BLWS10	-	+	+	+	-	+	+	+	+	+
21	BLWS9	-	+	+	+	-	+	-	-	+	+
22	BLWS4	-	+	+	-	-	+	-	-	-	+
23	BLWS2	-	+	+	-	-	+	-	-	-	+
24	BLWS1	-	+	+	+	+	-	+	-	+	+
25	BLWS3	-	+	+	+	-	+	+	+	+	+
26	BLWS6	-	+	+	-	-	+	-	-	-	+
27	BLWS11	-	+	+	+	-	+	+	+	+	+
28	BRhWS1	-	+	+	+	-	+	-	-	+	+
29	BRhWS8	-	+	+	+	-	+	+	+	+	+
30	BRhWW11	+	-	-	+	-	+	-	-	+	+
31	BRhWW7	+	+	+	+	-	+	-	-	+	+
32	BRhWM3	-	+	+	+	-	+	+	+	+	+
33	BRhWW10	-	+	+	+	-	+	+	+	+	+
34	BRhWM27	+	+	+	+	-	+	-	-	+	+
35	BRhWS22	-	+	+	+	-	+	+	-	+	+

Table 16: Biochemical test of endophytic bacterial isolates

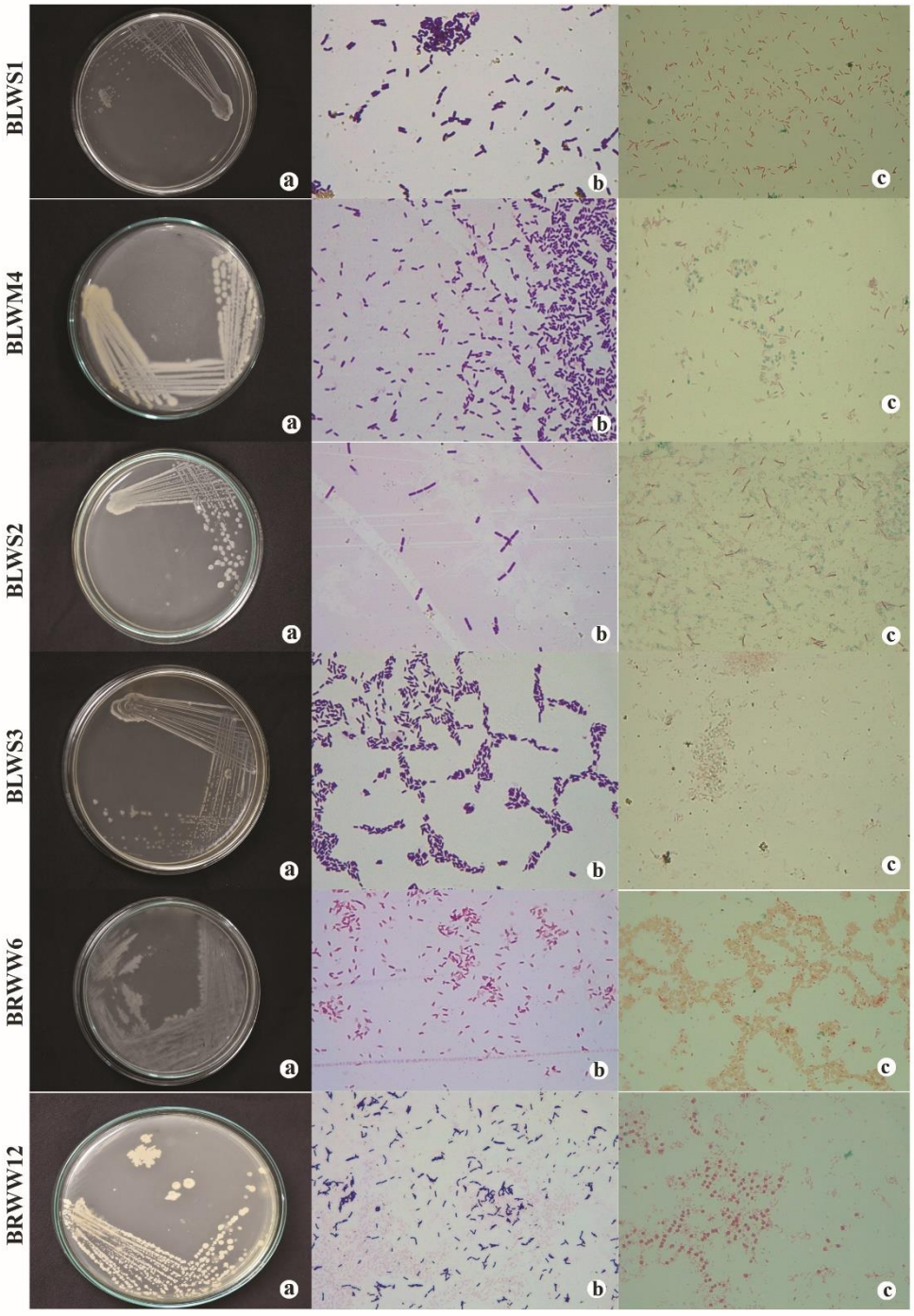


Fig.5 Endophytic bacterial isolates. a - Colonies; b - Cells; c - Endospore staining

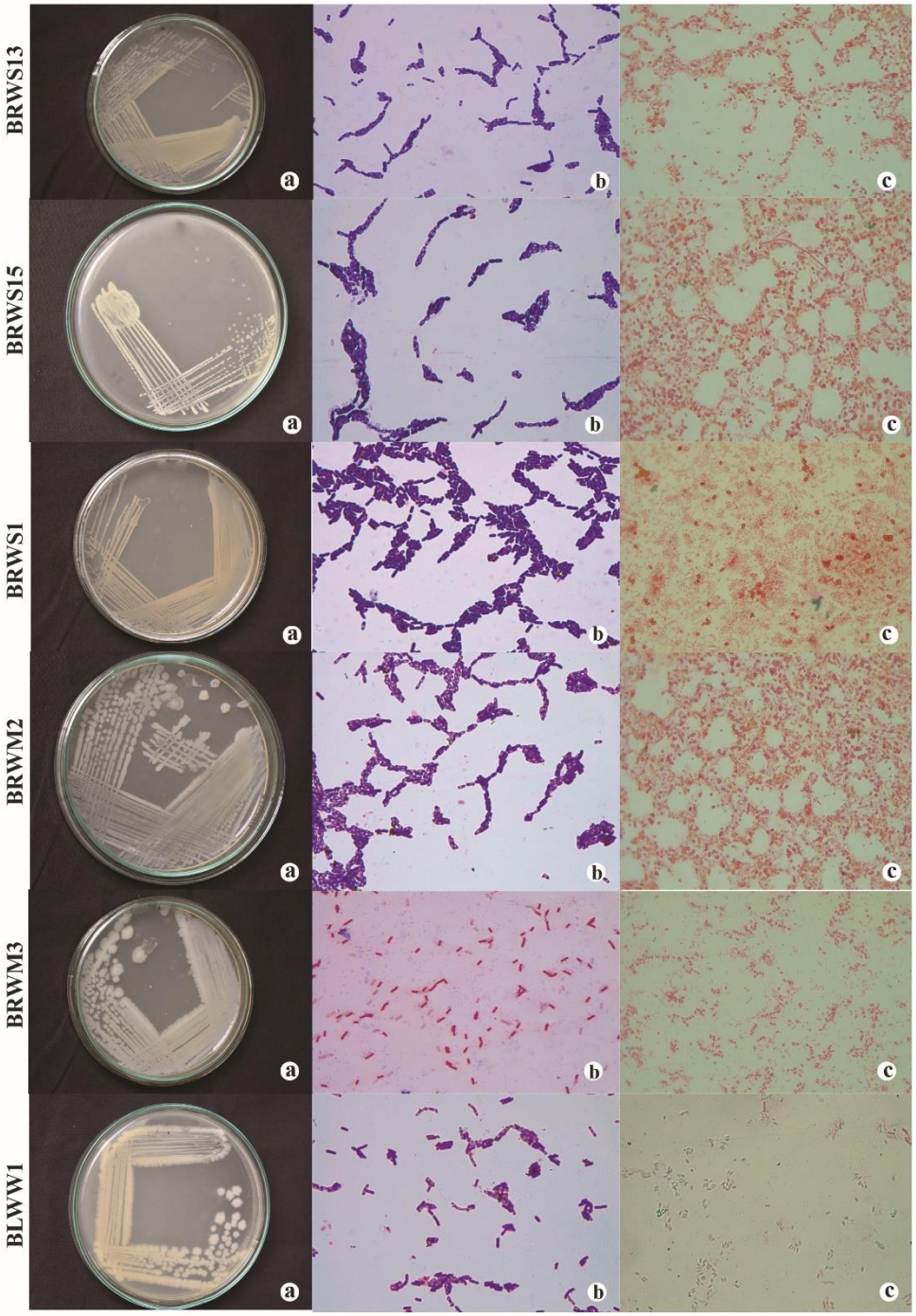


Fig.5a Endophytic bacterial isolates. a - Colonies; b - Cells; c - Endospore staining

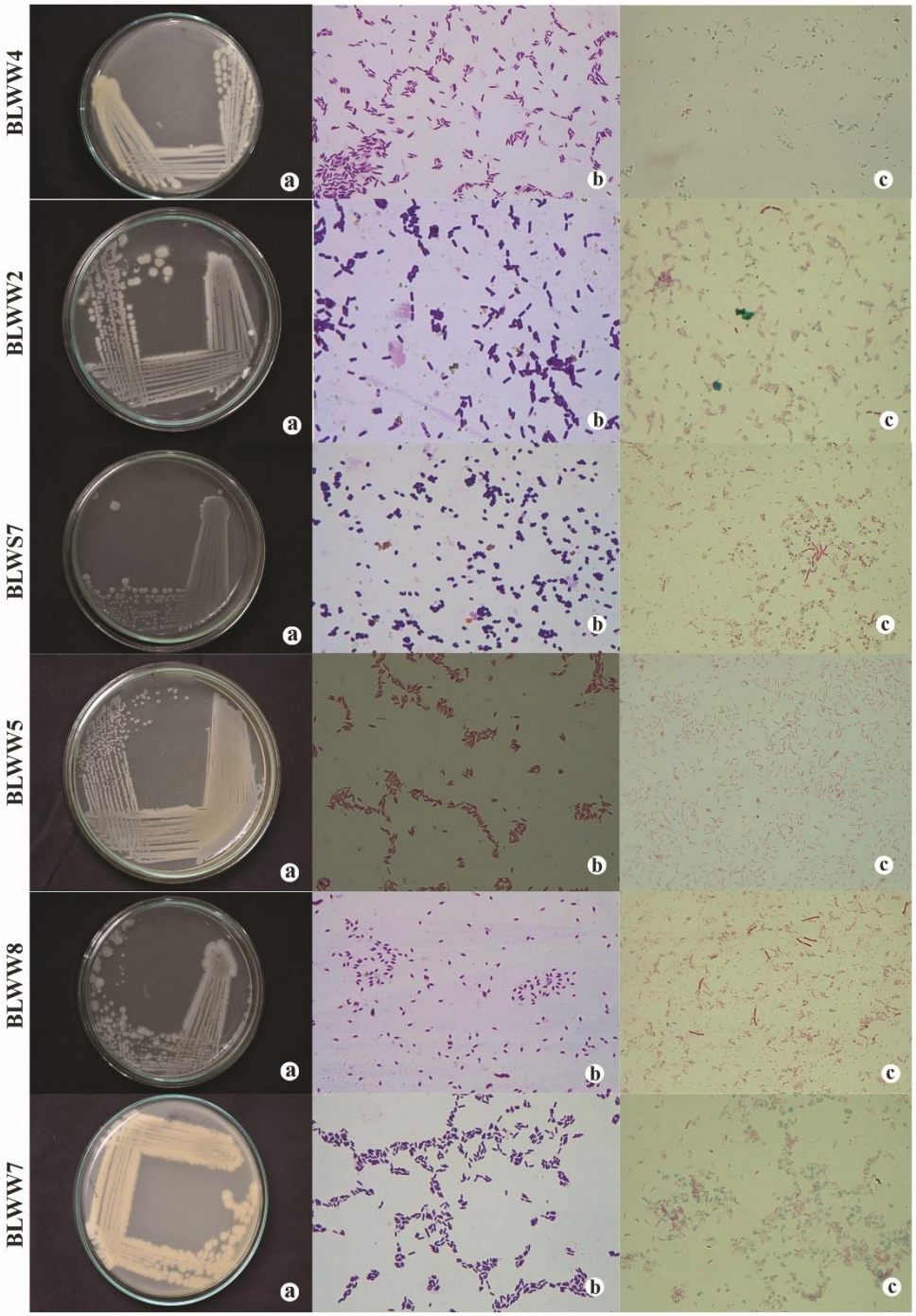


Fig.5b Endophytic bacterial isolates. a - Colonies; b - Cells; c - Endospore staining

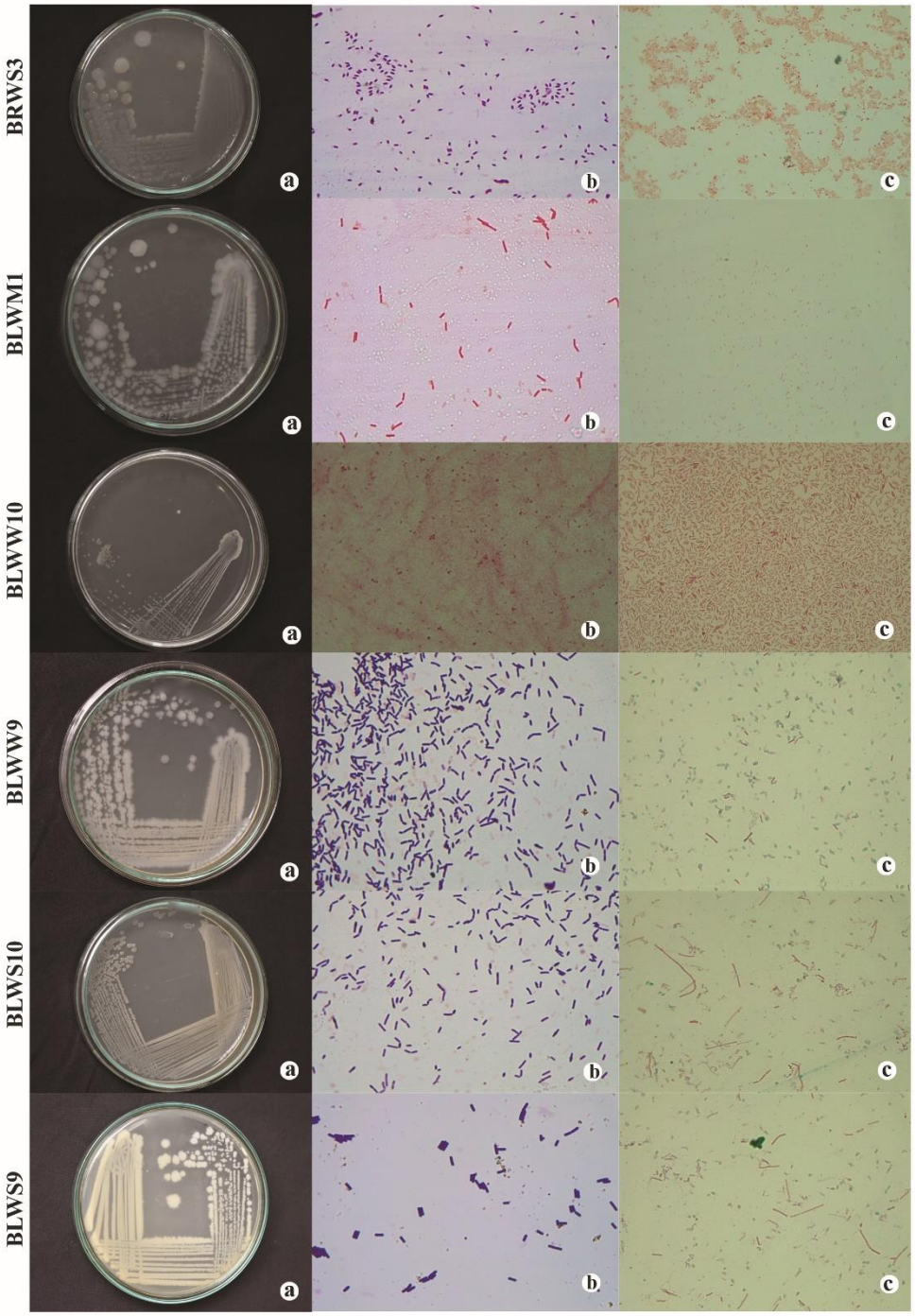


Fig.5c Endophytic bacterial isolates. a - Colonies; b - Cells; c - Endospore staining

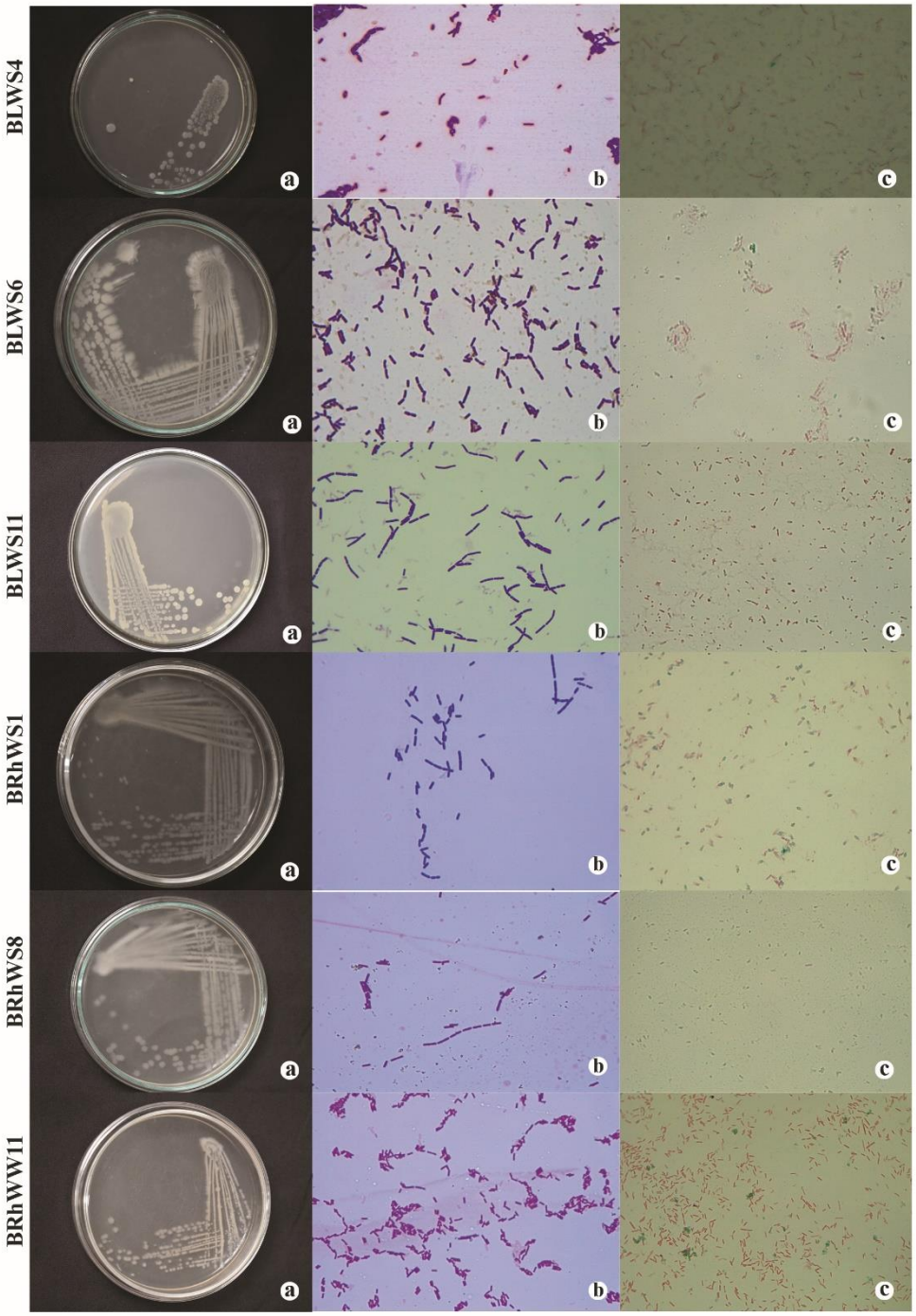


Fig.5d Endophytic bacterial isolates. a - Colonies; b - Cells; c - Endospore staining

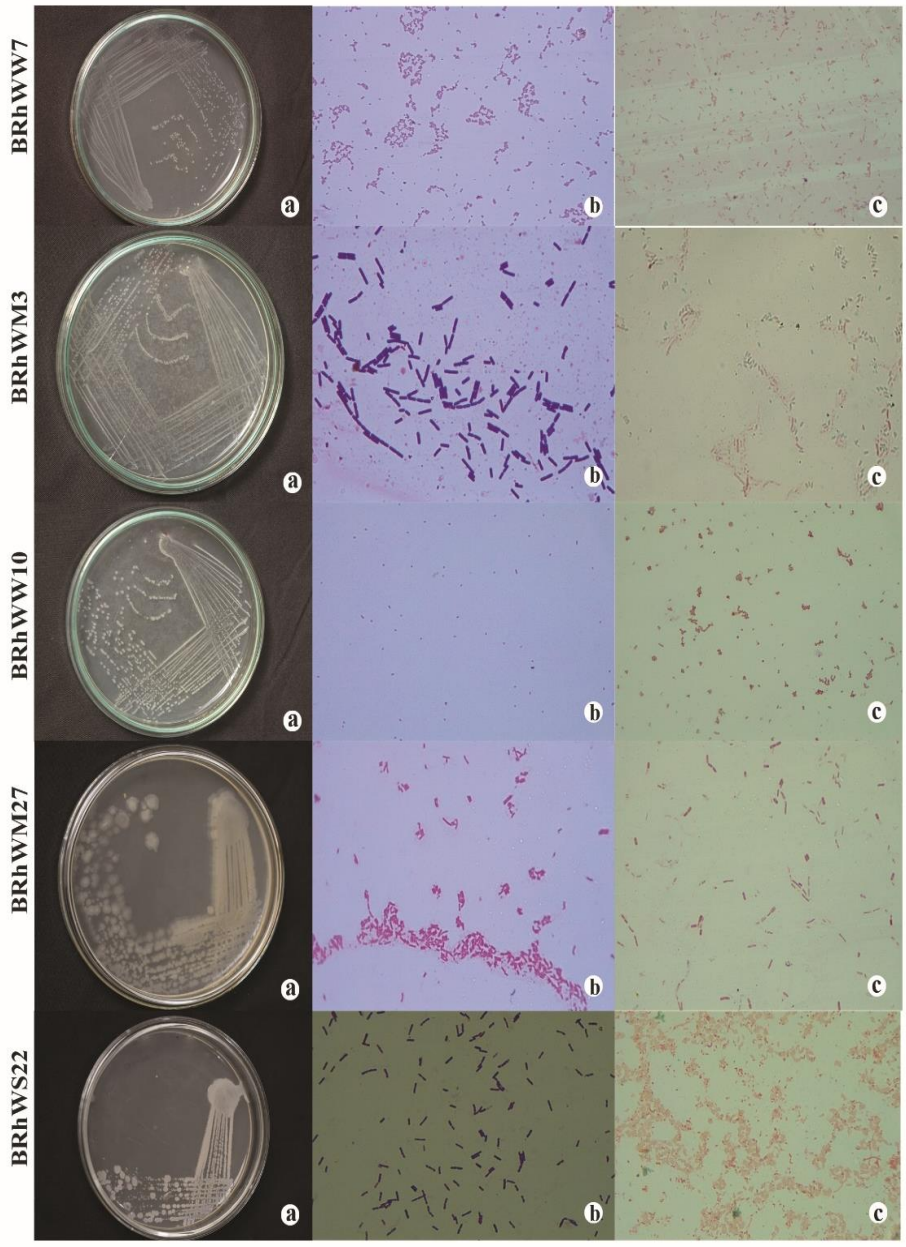


Fig. 5e Endophytic bacterial isolates.
a – Colonies; b – Cells; c – Endospore staining

Molecular Taxonomical Identification

Molecular taxonomic characterization of the isolates involved genomic DNA extraction, PCR amplification of the 16S rRNA region, and sequence analysis. DNA quality assessment yielded A260/A280 ratios between 1.6 and 1.8. Genomic DNA was resolved on 1.0% agarose gels containing 10 µg/mL ethidium bromide. PCR amplification using universal primers 8-27F and 1495R produced approximately 1600 bp fragments (Fig. 6). Sequence assembly was performed using BIOEDIT v7.2.5's contig assembly program, generating nearly full-length sequences. DECIPHER GPL v3.0 analysis confirmed the absence of chimeric artifacts. Phylogenetic neighbors were identified through similarity-based searches on the EzBioCloud server, utilizing quality-controlled 16S rRNA sequence databases, a maximum likelihood analysis was conducted with 1000 bootstrap replications, and phylogenetic trees were constructed using MEGA 11 for each isolate (Fig.8 – Fig.42) and a tree constructed for showing the relationship of all isolated endophytic bacteria (Fig.43). In the tree, each isolate is clustered in a strongly supported clade with a reference strain. Based on the similarity results, comparing the sequences and morphological characteristics concluded the identity of isolates. Among the identified isolates, *Bacillus amyloliquefaciens*, *B. mycoides*, *Bacillus albus*, *Bacillus cereus,* and *Bacillus ferrooxidans* are the isolates that are most frequently colonized in the various segments in different seasons of 11.96%, 8.4%, 8.1%, 7.7%, and 6.9% respectively. The lowest percentage of colonization frequency was observed in the isolates *Robertmurraya crescens* (0.04%), *Enterobacter sp.* (0.065%), and *Pseudomonas indoloxydans* (0.06%) The colonization frequency (CF%) of identified bacterial isolates are represented in Fig.7. The details of the isolates with strain code, similarity index, identity, family, and accession number are shown in Table 17.

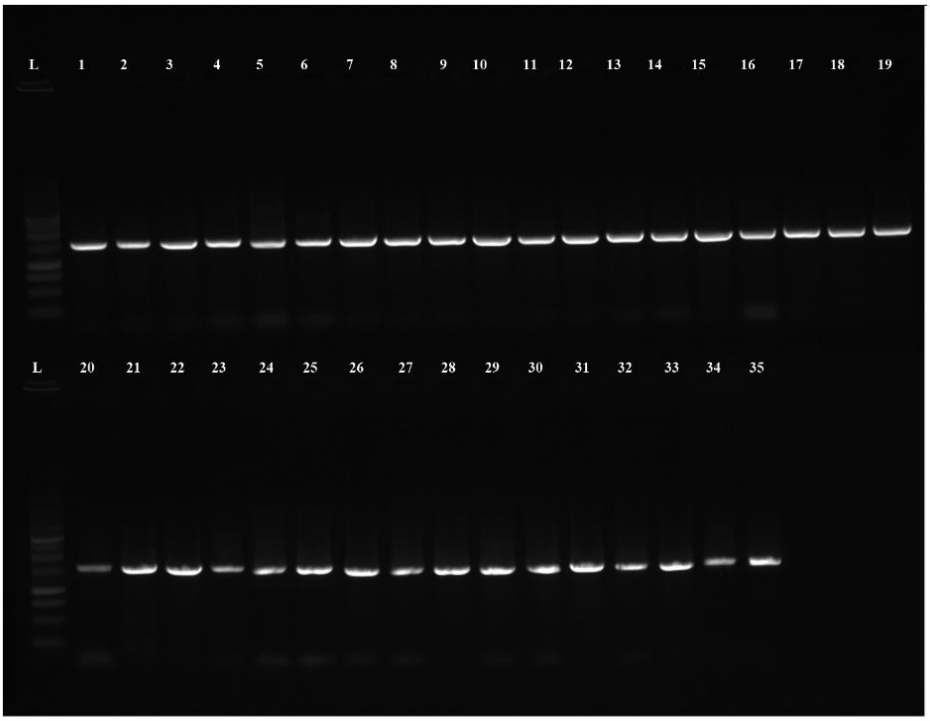


Fig.6 16S rRNA amplification of endophytic bacterial DNA

Sl No.	Strain Code	% of Similarity	Identity	Family	Accession No.
1	BRWS3	96.58	*Bacillus amyloliquefaciens*	Bacillaceae	PQ157941
2	BRWW6	95.10	*Bacillus mycoides*	Bacillaceae	PQ157949
3	BRWW12	87.11	*Bacillus ferrooxidans*	Bacillaceae	PQ157957
4	BRWS13	86.69	*Brevibacillus brevis*	Paenibacillaceae	PQ158264
5	BRWS15	96.80	*Lysnibacillus pakistanensis*	Lysinibacillaceae	PQ158268
6	BRWS1	96.10	*Bacillus licheniformis*	Bacillaceae	PQ158460
7	BRWM2	96.86	*Bacillus siamensis*	Bacillaceae	PQ158462
8	BRWM3	93.99	Sutcliffiella sp.	Bacillaceae	PQ161090
9	BLWW1	87.54	*Bacillus anthracis*	Bacillaceae	PQ161091
10	BLWW4	82.95	*Bacillus albus*	Bacillaceae	PQ160479
11	BLWW2	97.34	*Bacillus sp.* 1	Bacillaceae	PQ158545
12	BLWS7	96.73	*Bacillus toyonenesis*	Bacillaceae	PQ158546
13	BLWW5	94.37	*Cytobacillus oceanisediminis*	Bacillaceae	PQ161093
14	BLWW8	88.75	*Bacillus cereus*	Bacillaceae	PQ158561
15	BLWW7	97.38	*Bacillus hominis*	Bacillaceae	PQ158565
16	BLWM4	96.33	*Bacillus thuringiensis*	Bacillaceae	PQ158566
17	BLWM1	91.72	*Rhizobium rhizophilum*	Rhizobiaceae	PQ161096
18	BLWW10	92.56	*Robertmurraya crescens*	Bacillaceae	PQ161098
19	BLWW9	97.25	*Bacillus arachidis*	Bacillaceae	PQ159144
20	BLWS10	92.47	*Bacillus altitudinis*	Bacillaceae	PQ161099
21	BLWS9	84.92	*Priestia megaterium*	Bacillaceae	PQ161055
22	BLWS4	98.71	*Bacillus sp.* 2	Bacillaceae	PQ159906
23	BLWS2	94.37	*Bacillus sp.* 3	Bacillaceae	PQ160865
24	BLWS1	89.41	*Gottfriedia luciferensis*	Bacillaceae	PQ161117
25	BLWS3	93.52	*Priestia taiwanensis*	Bacillaceae	PQ161118
26	BLWS6	97.89	*Bacillus sp.* 4	Bacillaceae	PQ160486
27	BLWS11	93.01	*Lysinibacillus sphaericus*	Lysinibacillaceae	PQ160690
28	BRhWS1	94.12	*Bacillus acidicola*	Bacillaceae	PQ161119
29	BRhWS8	98.63	*Bacillus paramobilis*	Bacillaceae	PQ160473

30	BRhWW11	98.61	*Pseudomonas indoloxydans*	Pseudomonadaceae	PQ162377
31	BRhWW7	98.58	*Enterobacter sp.*	Enterobacteriaceae	PQ160358
32	BRhWM3	95.89	*Bacillus aerius*	Bacillaceae	PQ160428
33	BRhWW10	93.80	*Paenisporosarcina quisquiliarum*	Planococcaceae	PQ160489
34	BRhWM27	93.70	*Enterobacter asburiae*	Enterobacteriaceae	PQ160436
35	BRhWS22	98.86	*Bacillus velezensis*	Bacillaceae	PQ160453

Table 17: Identification of selected endophytic bacterial isolates

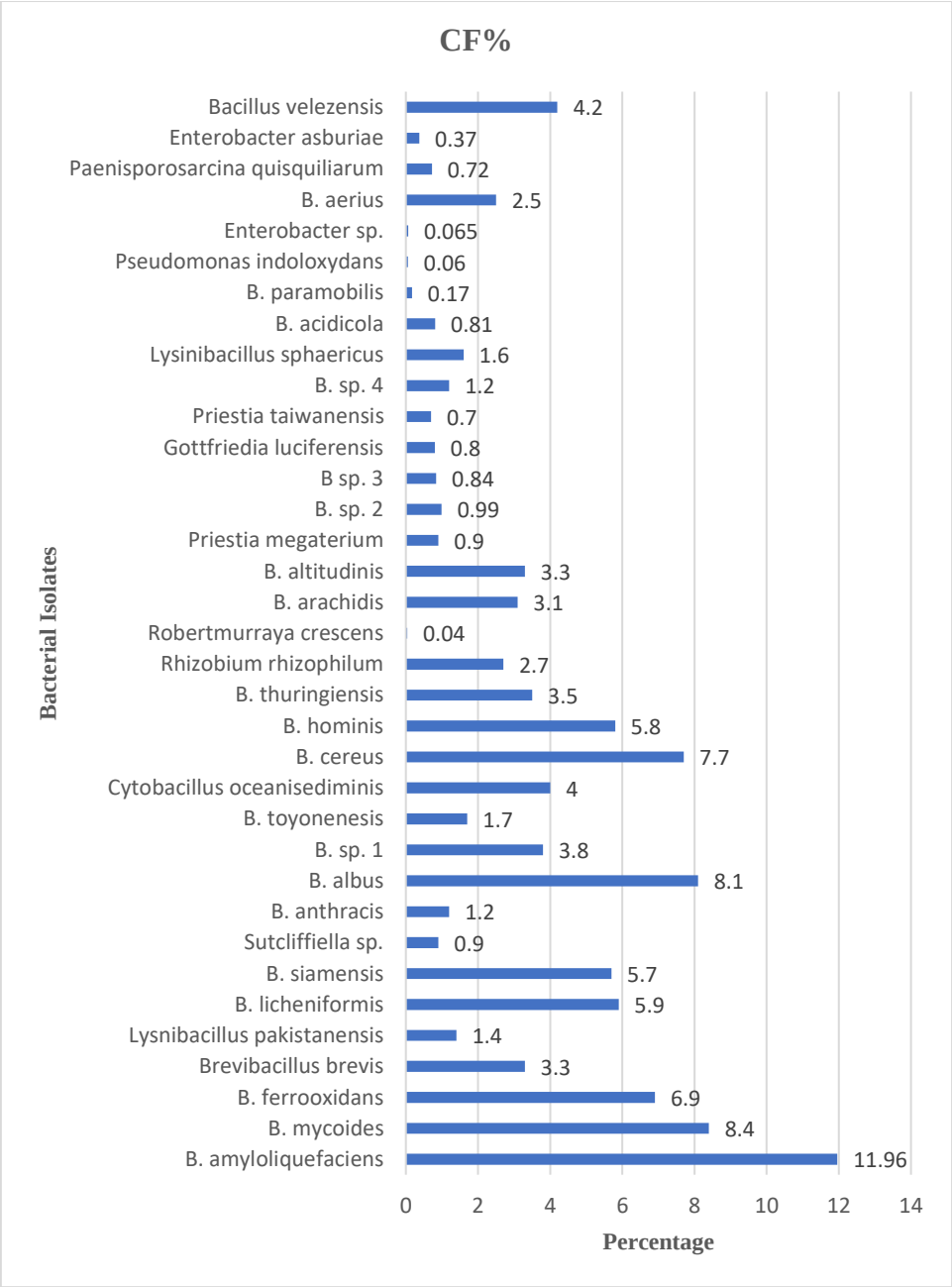


Fig.7 Colonization frequency of selected endophytic bacterial isolates

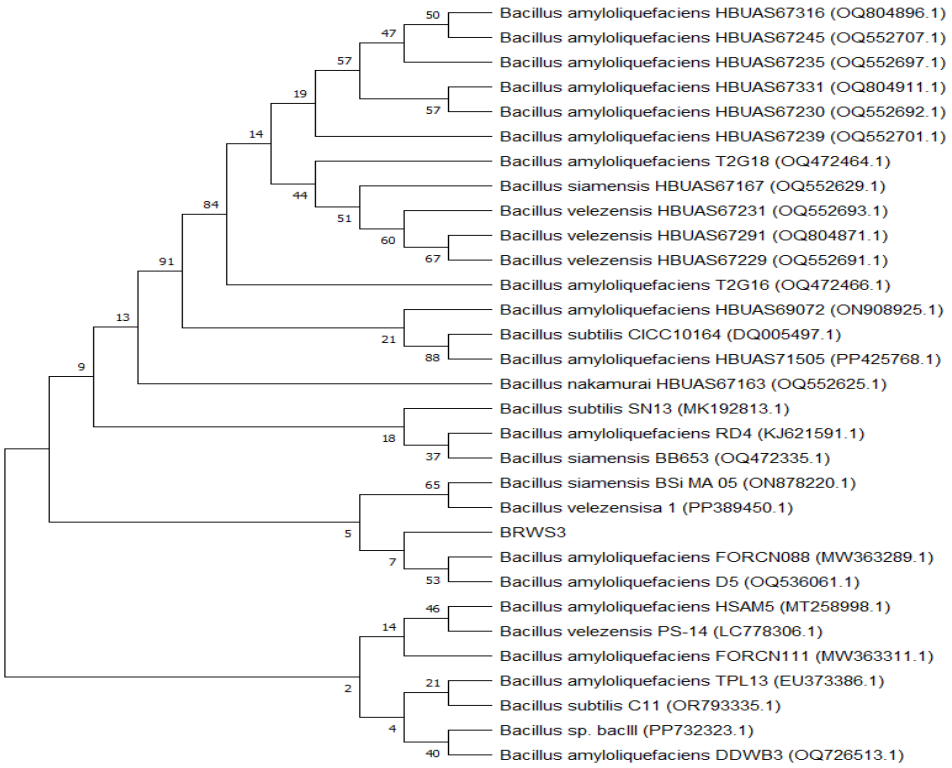


Fig.8 Maximum Likelihood phylogenetic tree of BRWS3

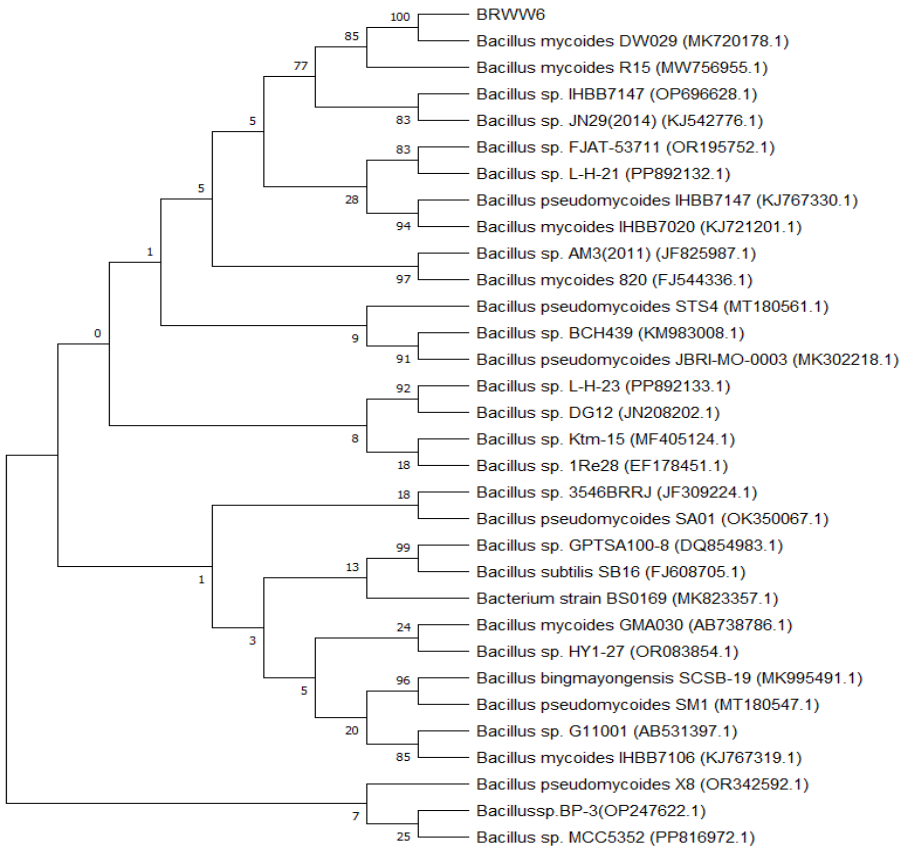


Fig.9 Maximum Likelihood phylogenetic tree of BRWW6

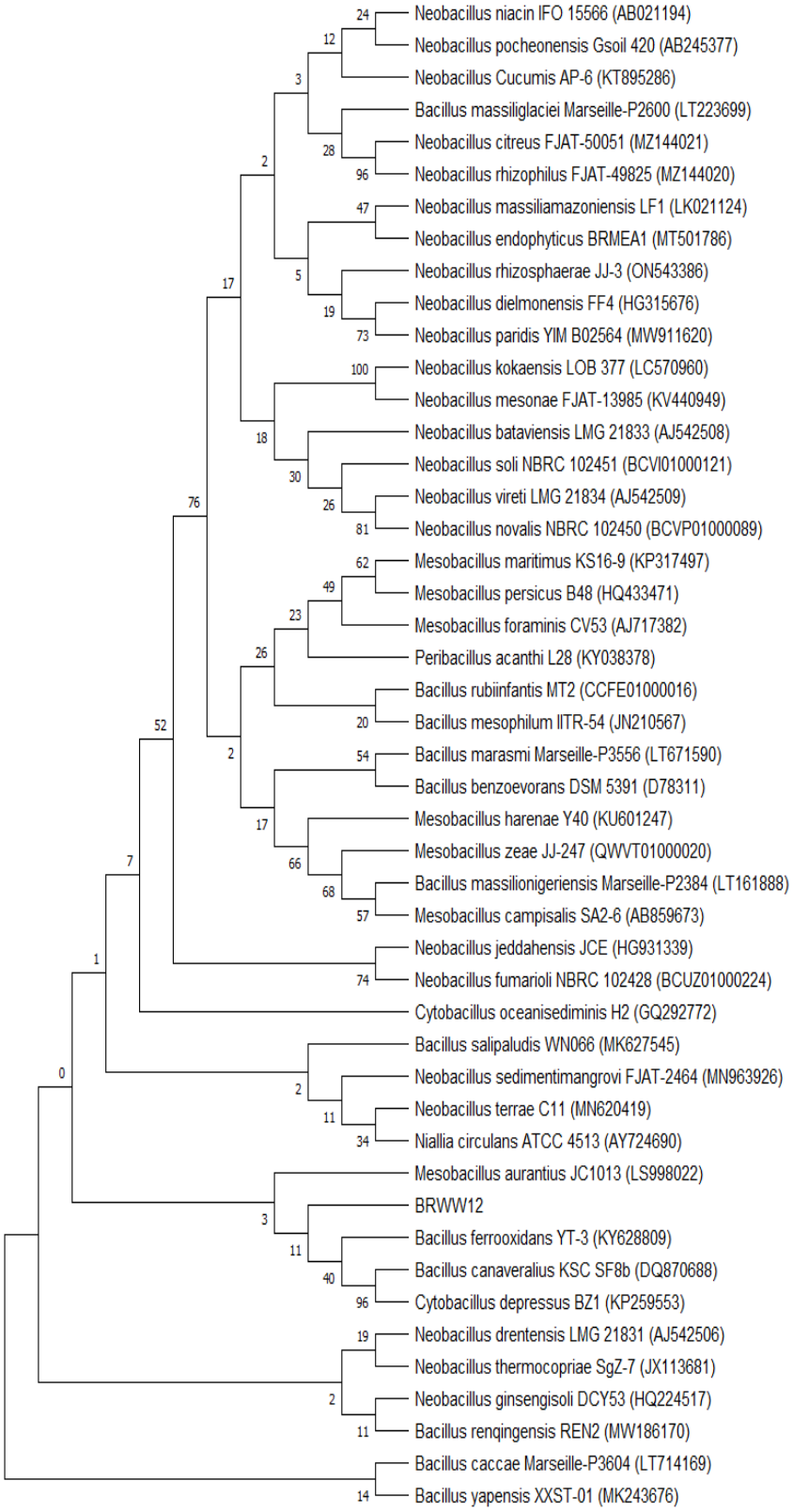


Fig.10 Maximum Likelihood phylogenetic tree of BRWW12

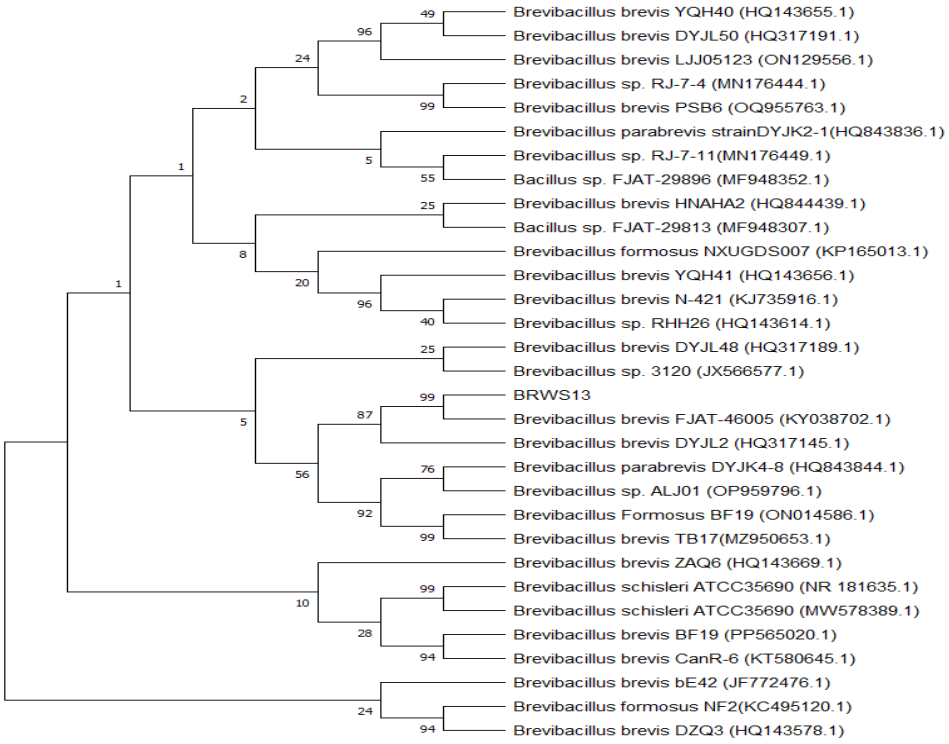


Fig.11 Maximum Likelihood phylogenetic tree of BRWS13

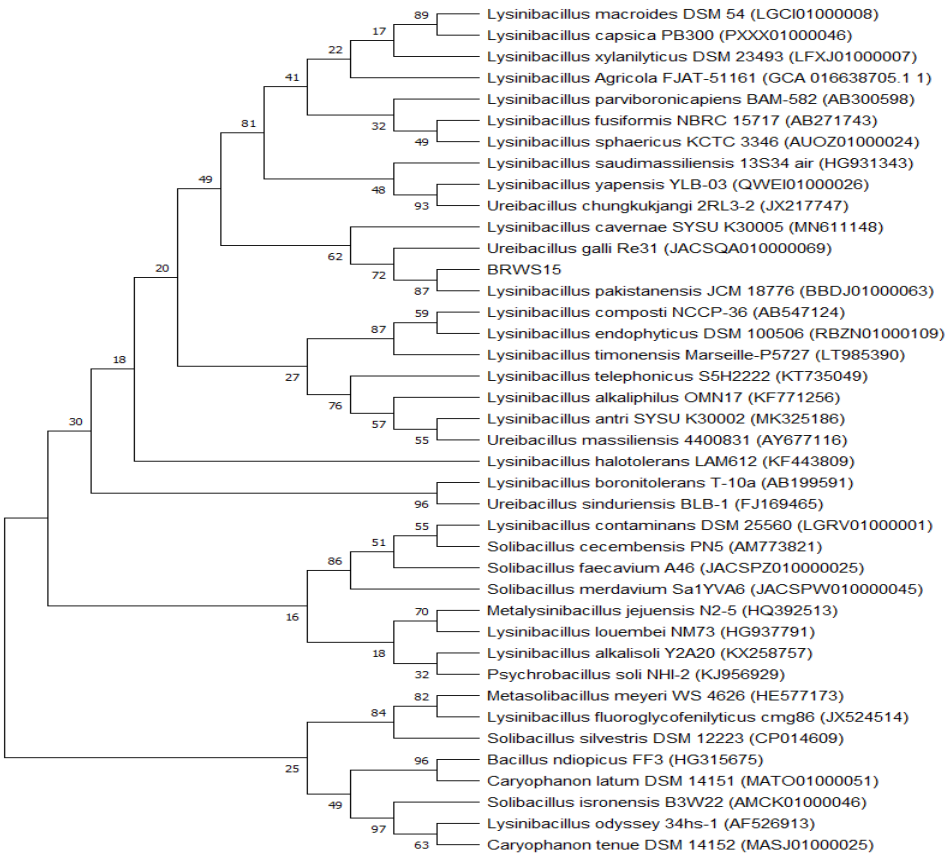


Fig.12 Maximum Likelihood phylogenetic tree of BRWS15

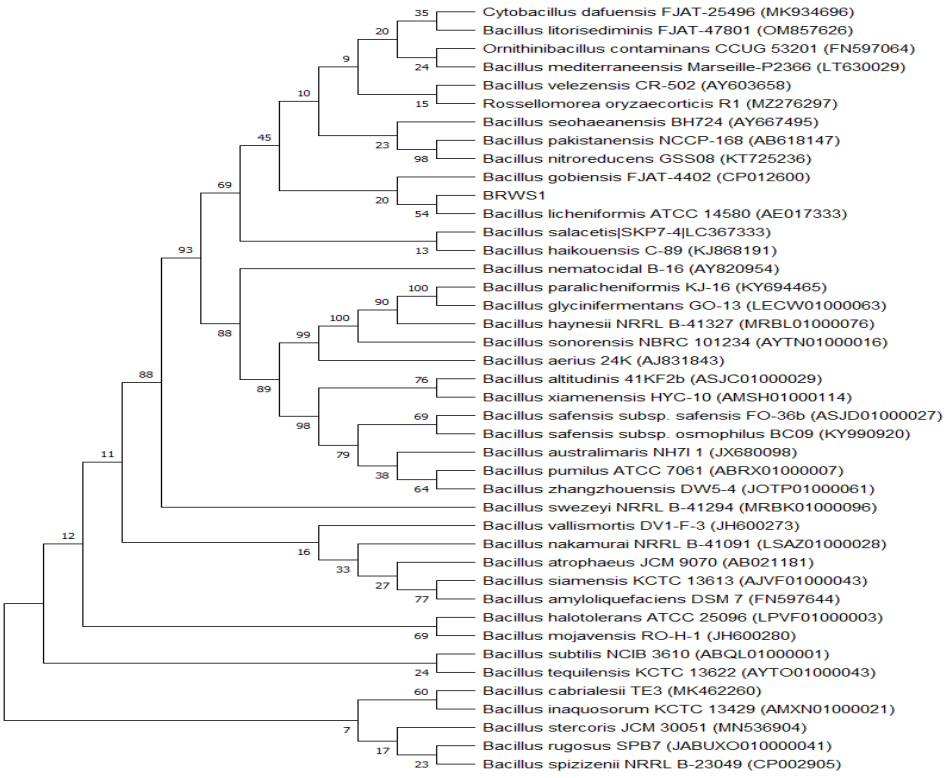


Fig.13 Maximum Likelihood phylogenetic tree of BRWS1

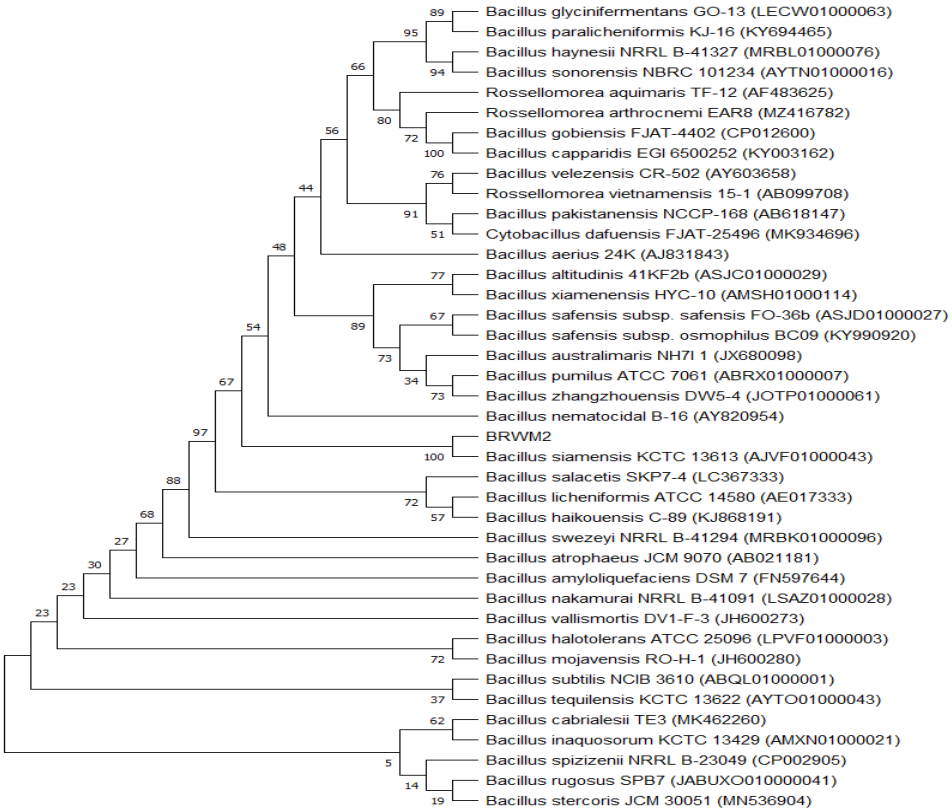


Fig.14 Maximum Likelihood phylogenetic tree of BRWM2

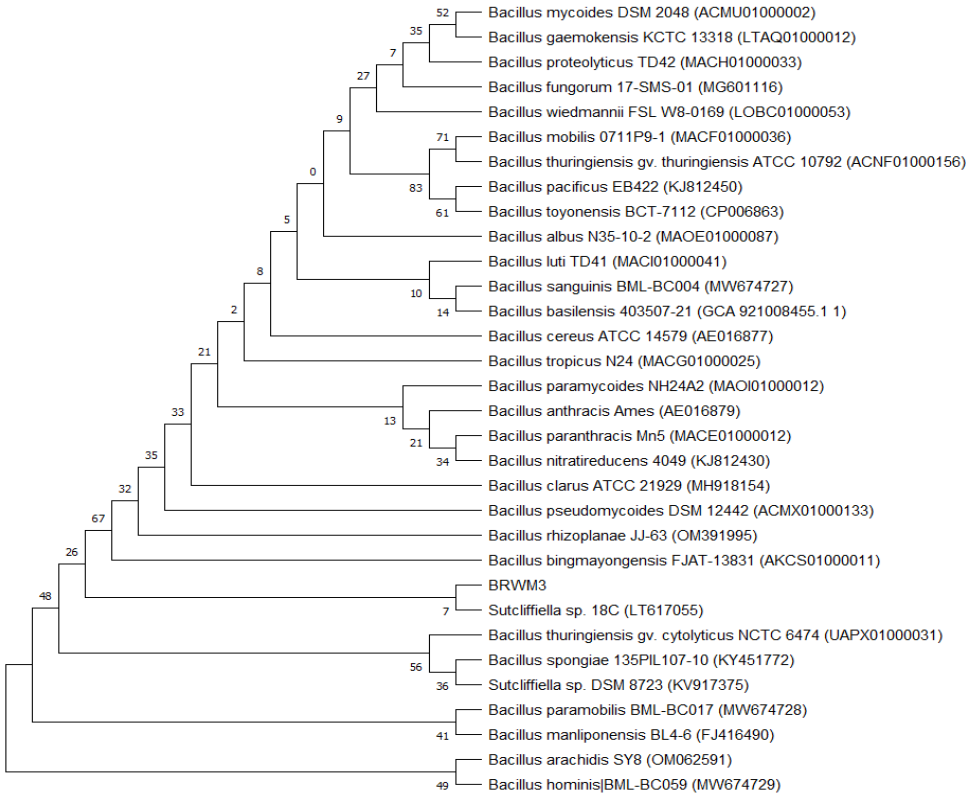


Fig.15 Maximum Likelihood phylogenetic tree of BRWM3

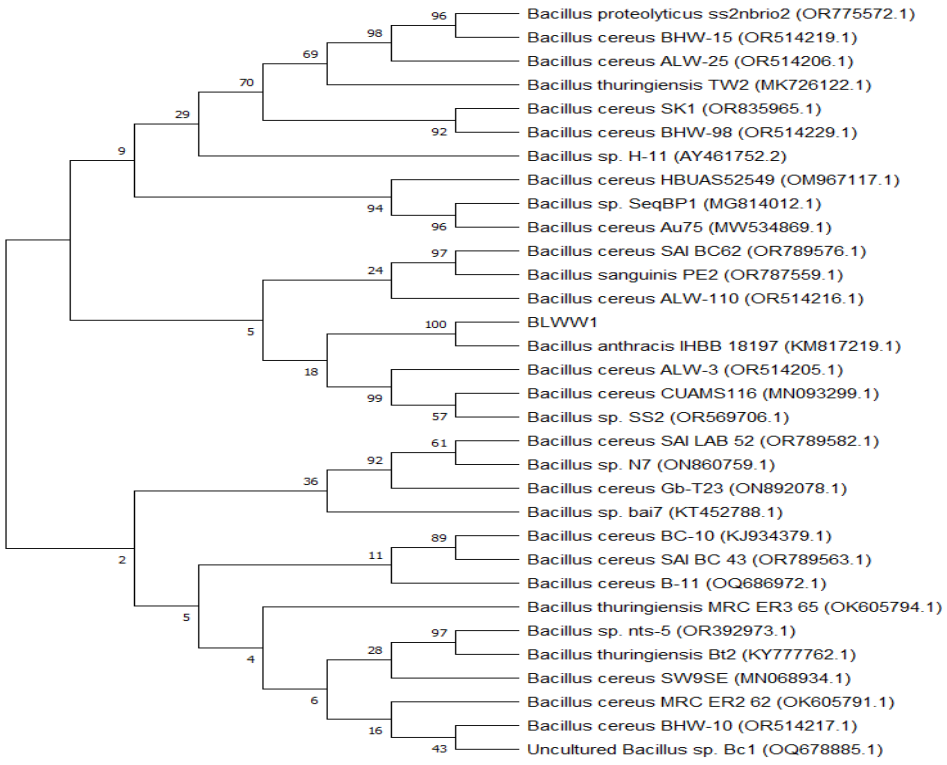


Fig.16 Maximum Likelihood phylogenetic tree of BLWW1

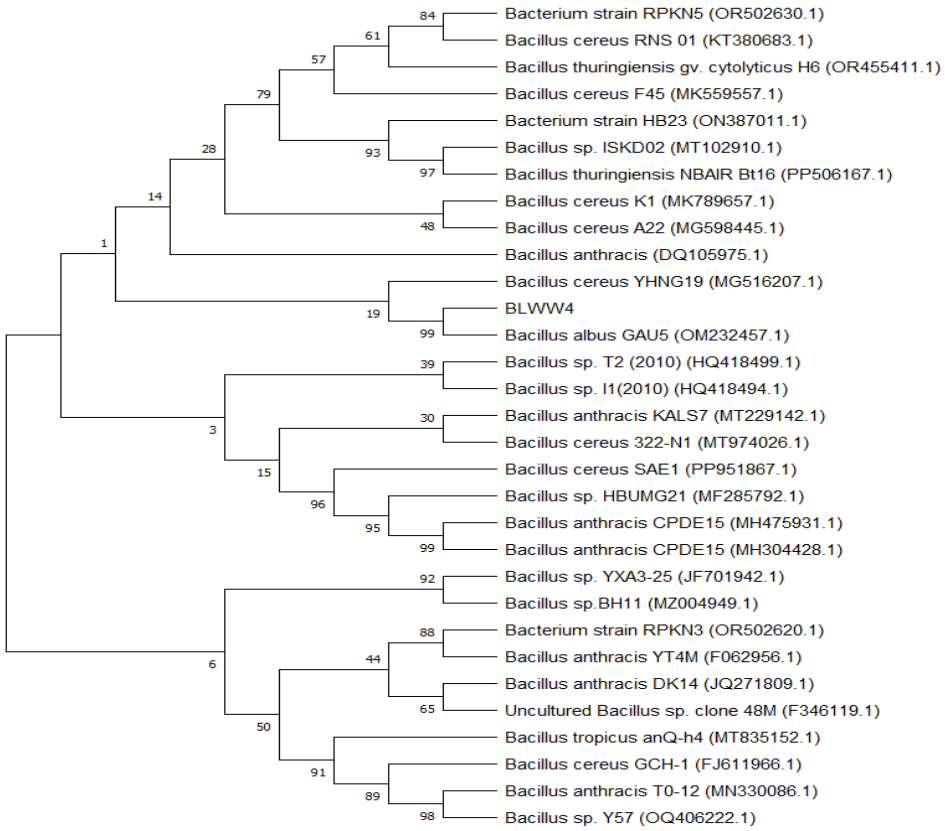


Fig.17 Maximum Likelihood phylogenetic tree of BLWW4

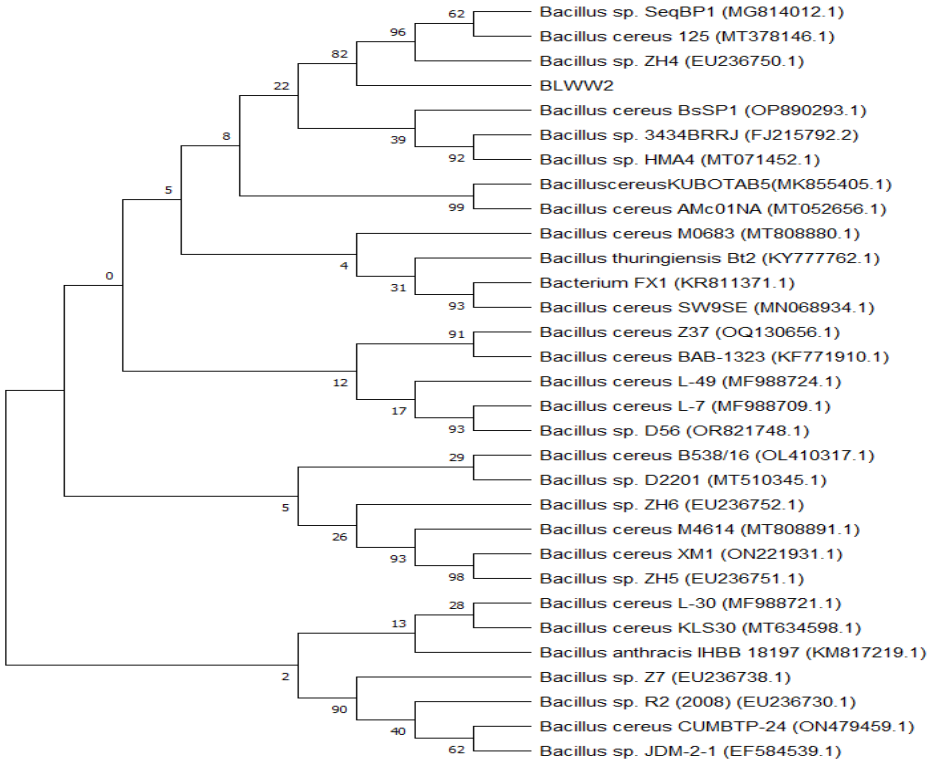


Fig.18 Maximum Likelihood phylogenetic tree of BLWW2

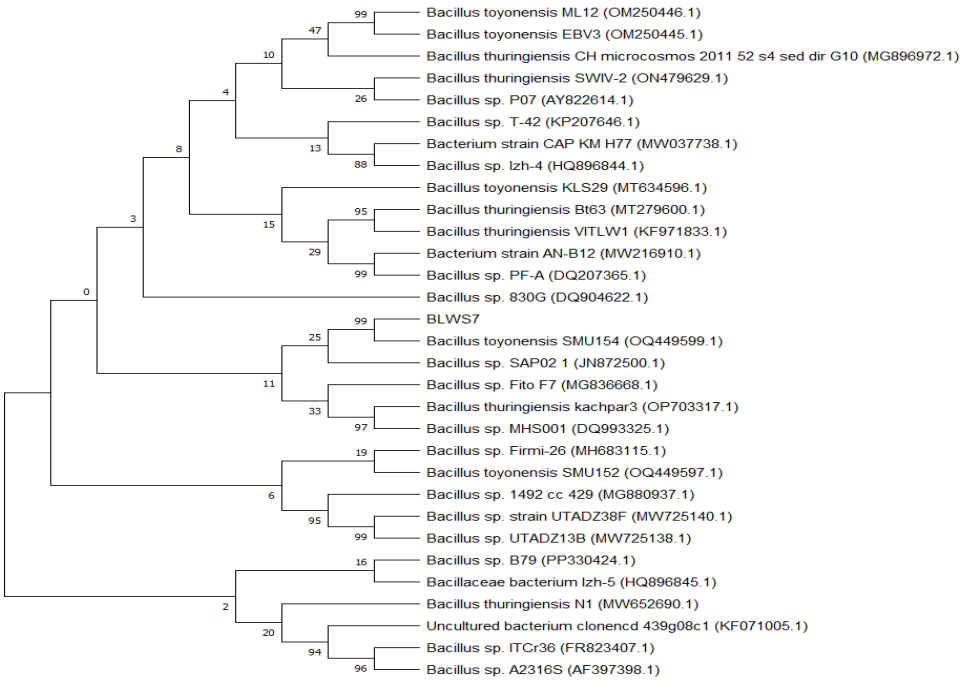


Fig.19 Maximum Likelihood phylogenetic tree of BLWS7

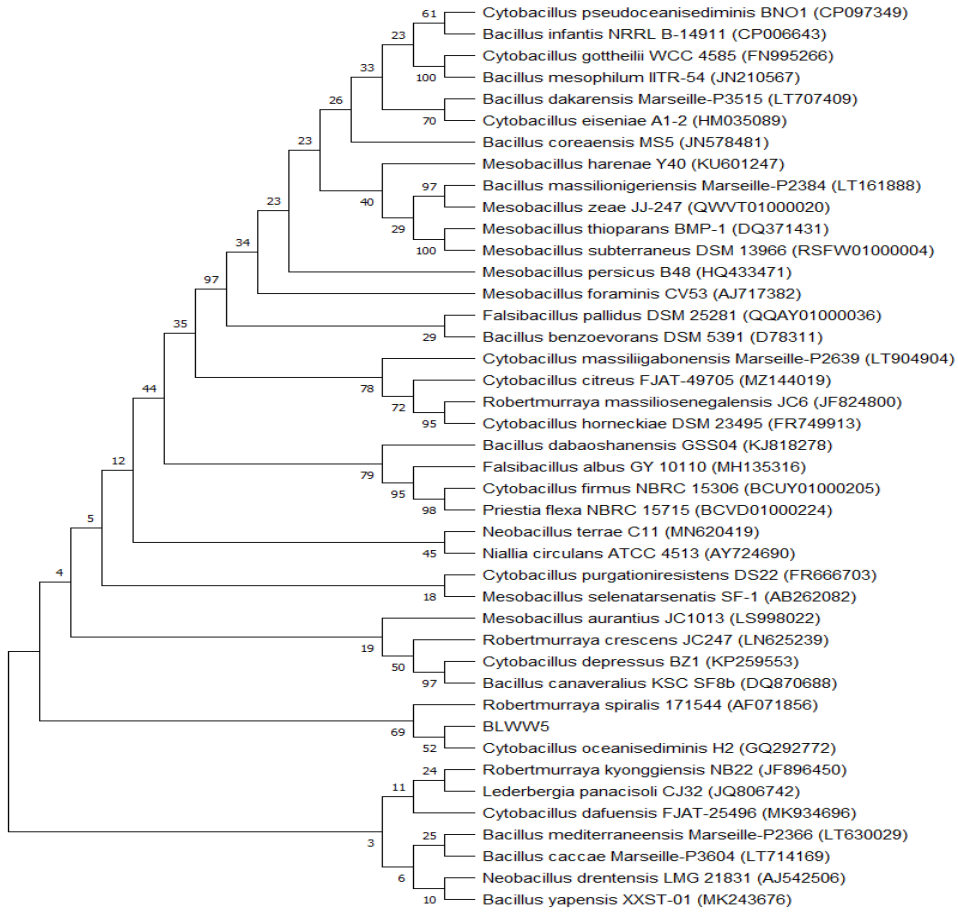


Fig.20 Maximum Likelihood phylogenetic tree of BLWW5

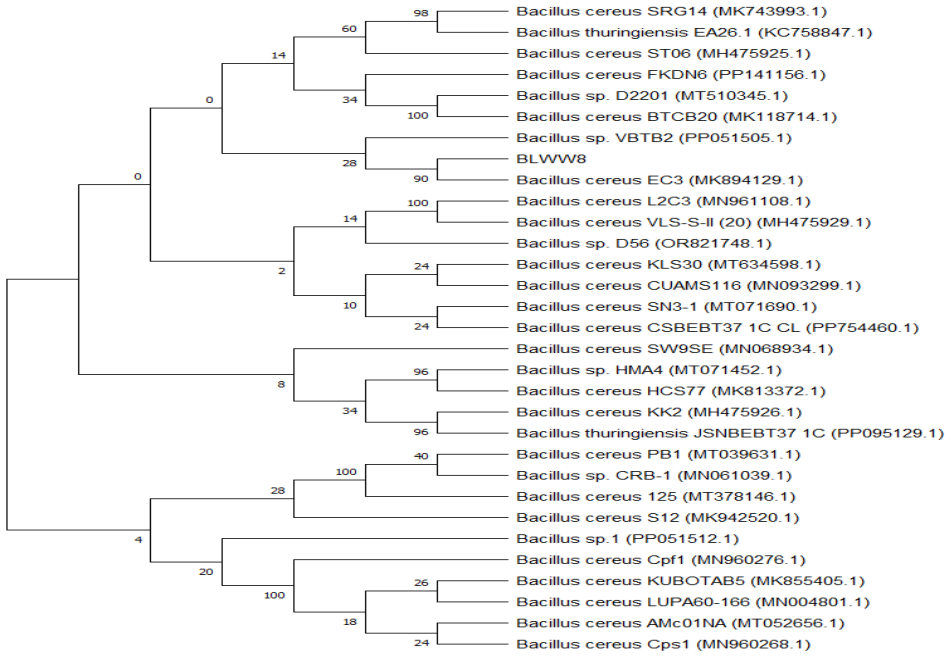


Fig.21 Maximum Likelihood phylogenetic tree of BLWW8

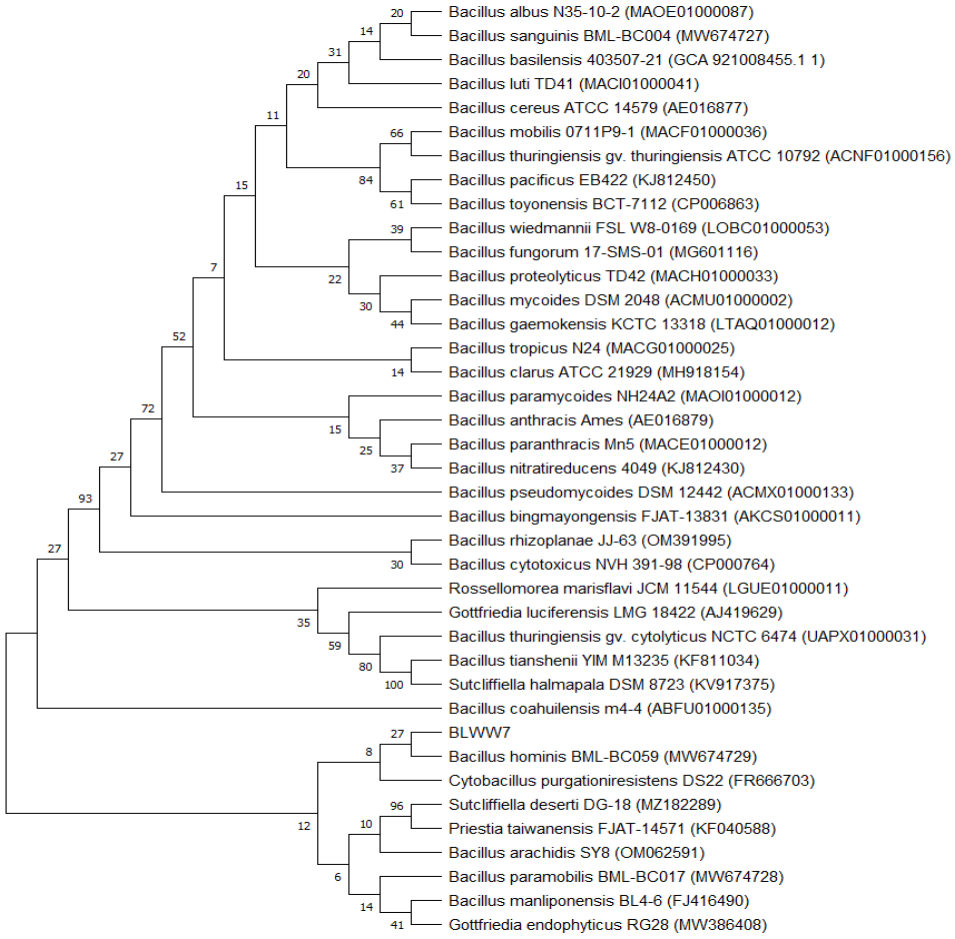


Fig.22 Maximum Likelihood phylogenetic tree of BLWW7

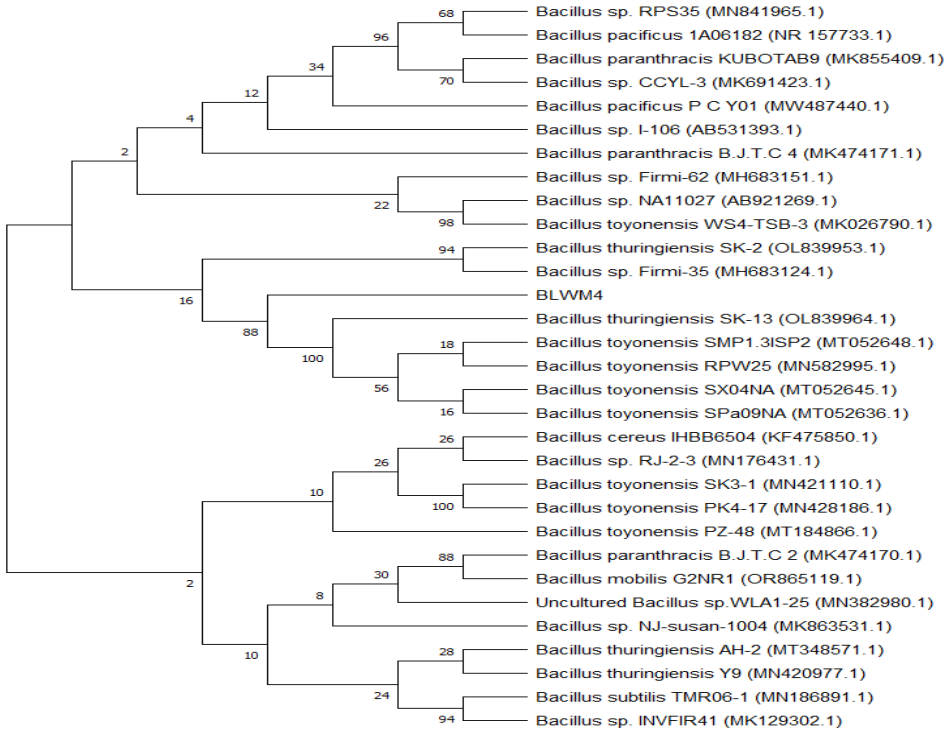


Fig.23 Maximum Likelihood phylogenetic tree of BLWM4

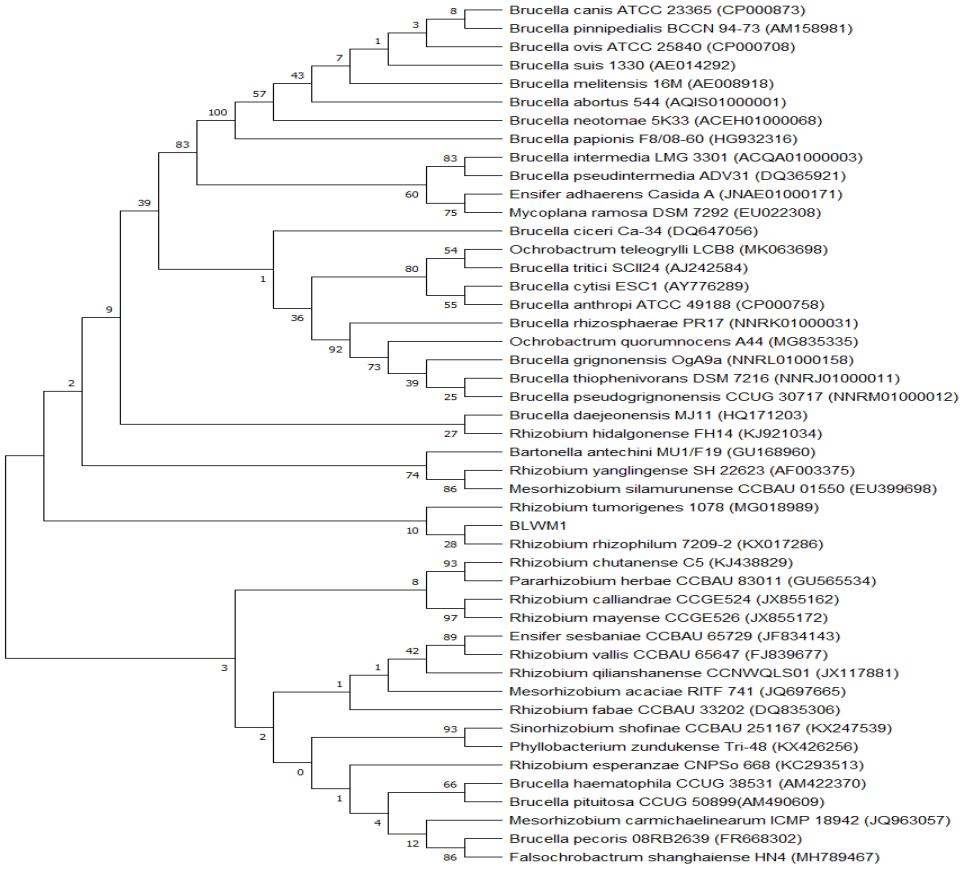


Fig.24 Maximum Likelihood phylogenetic tree of BLWM1

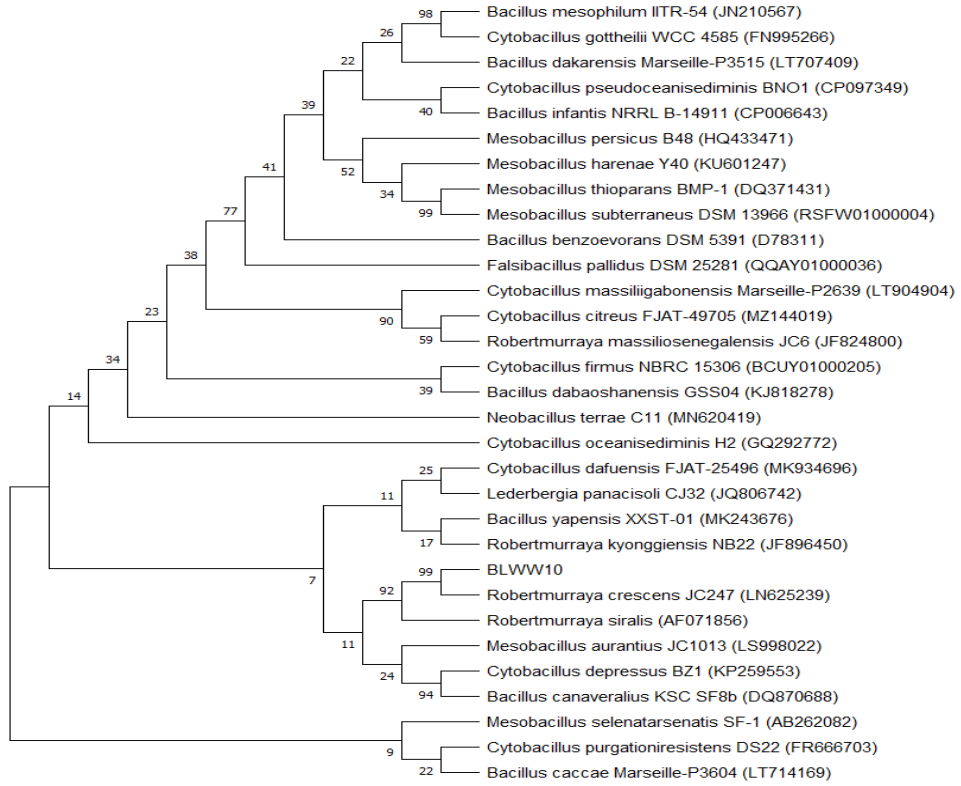


Fig.25 Maximum Likelihood phylogenetic tree of BLWW10

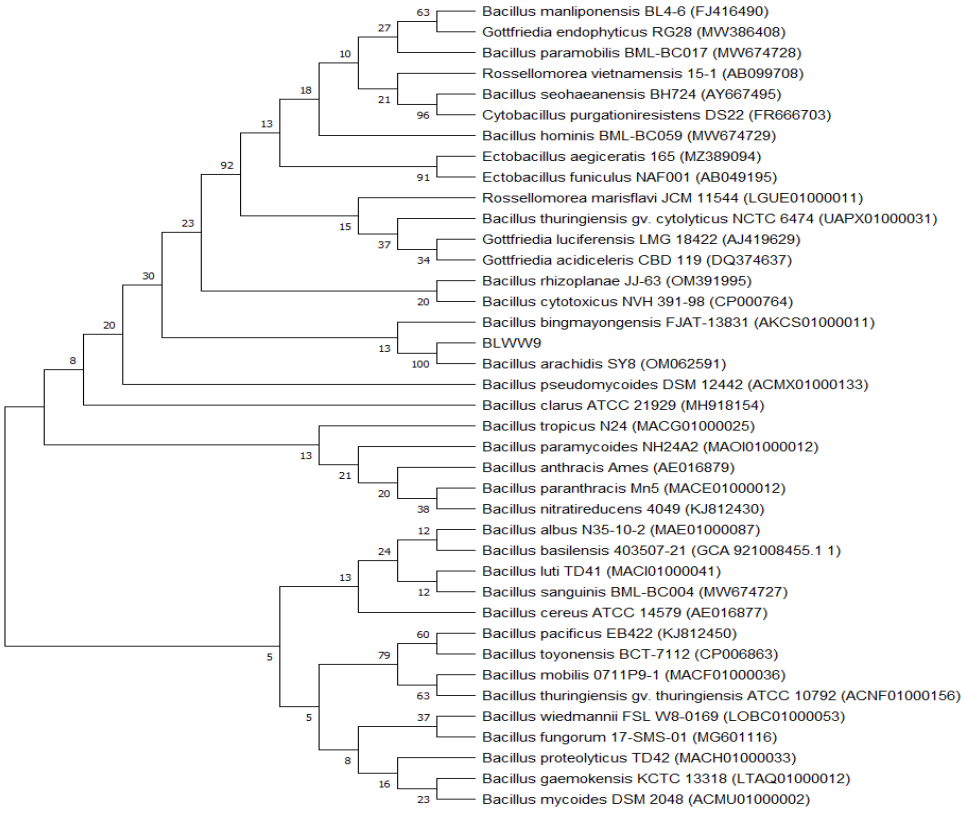


Fig.26 Maximum Likelihood phylogenetic tree of BLWW9

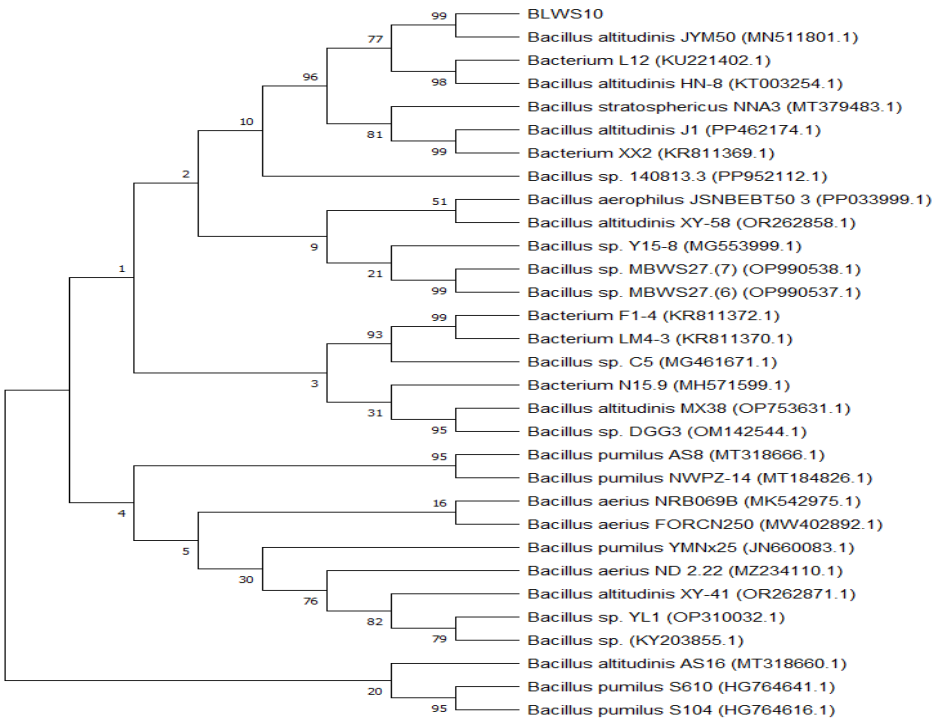


Fig.27 Maximum Likelihood phylogenetic tree of BLWS10

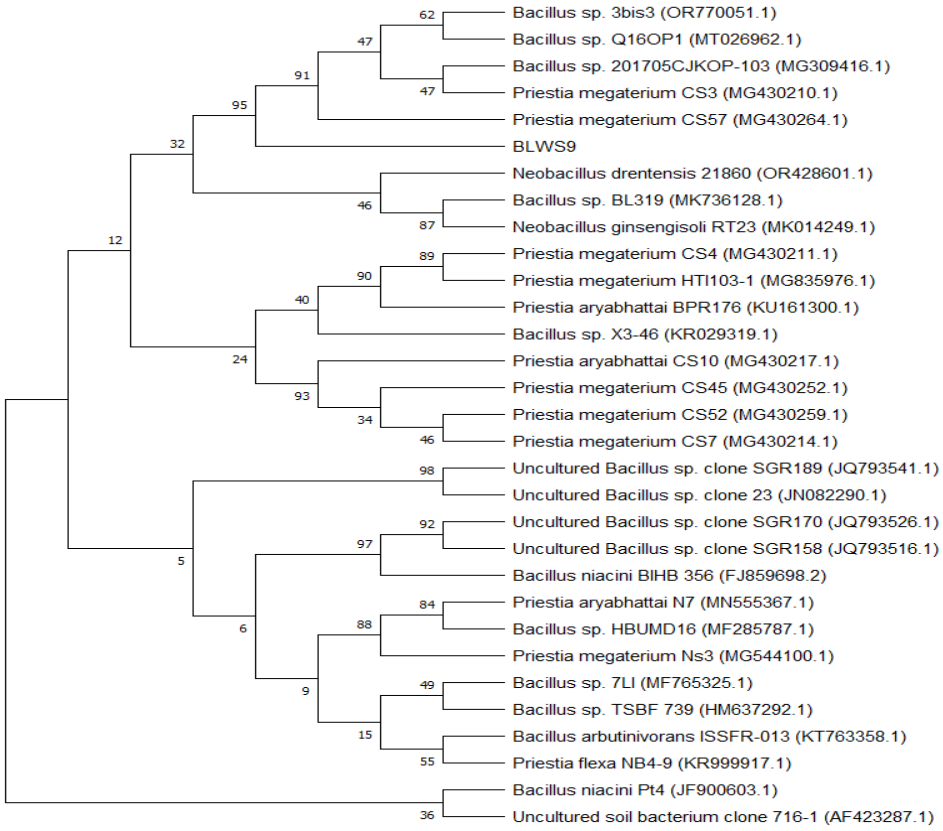


Fig.28 Maximum Likelihood phylogenetic tree of BLWS9

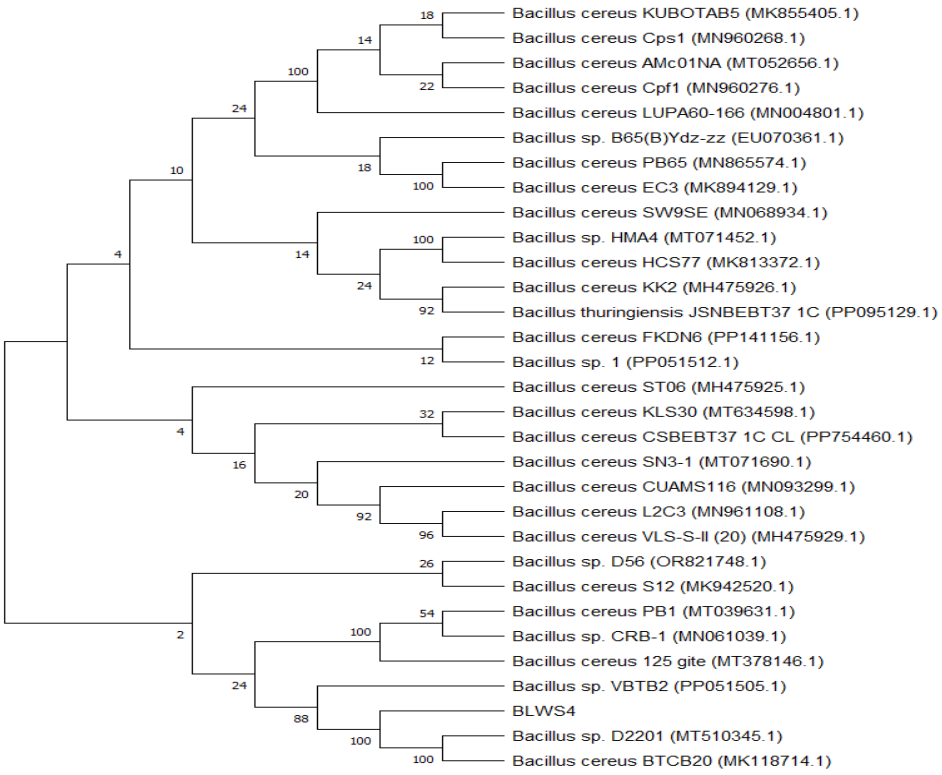


Fig.29 Maximum Likelihood phylogenetic tree of BLWS4

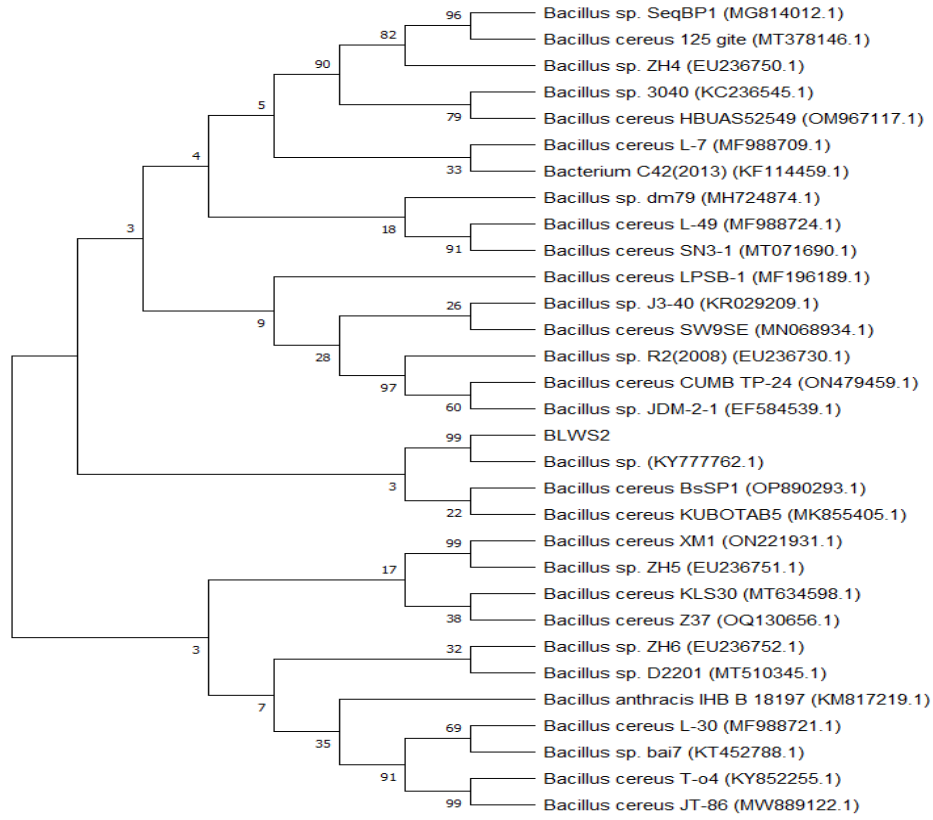


Fig.30 Maximum Likelihood phylogenetic tree of BLWS2

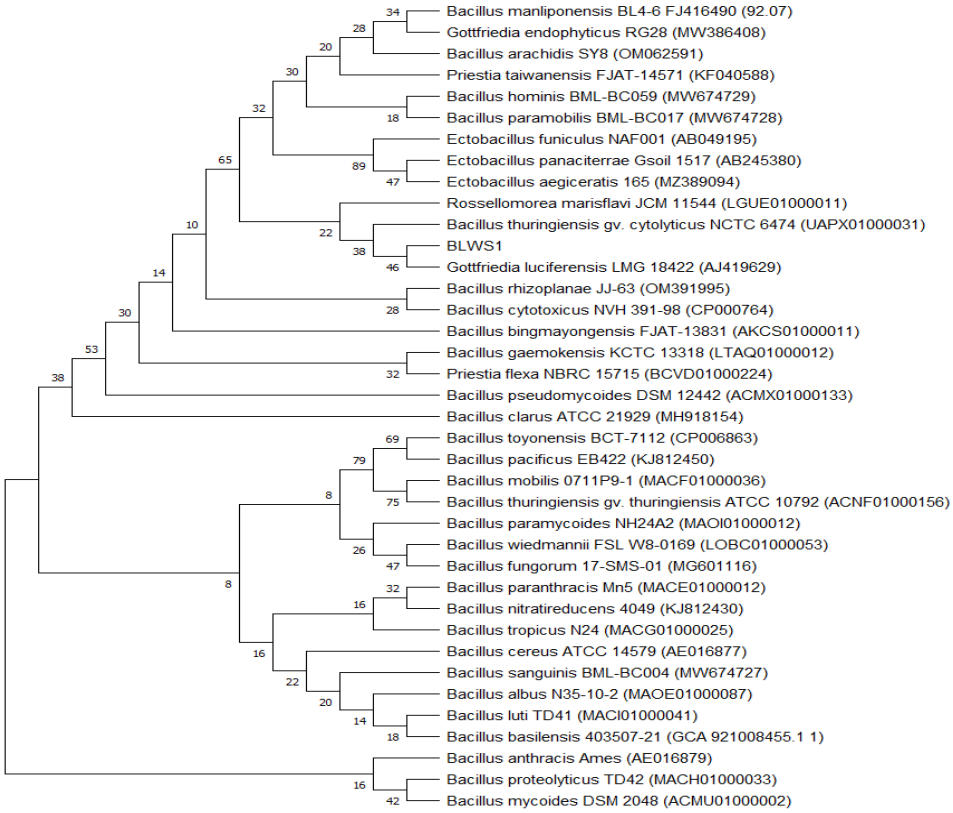


Fig.31 Maximum Likelihood phylogenetic tree of BLWS1

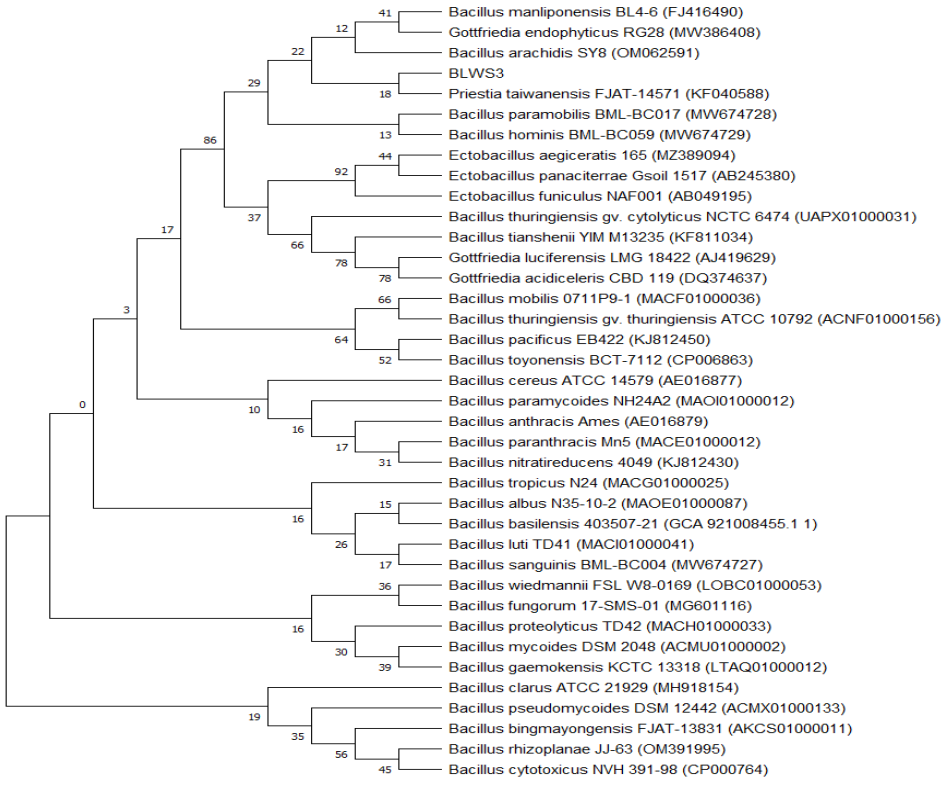


Fig.32 Maximum Likelihood phylogenetic tree of BLWS3

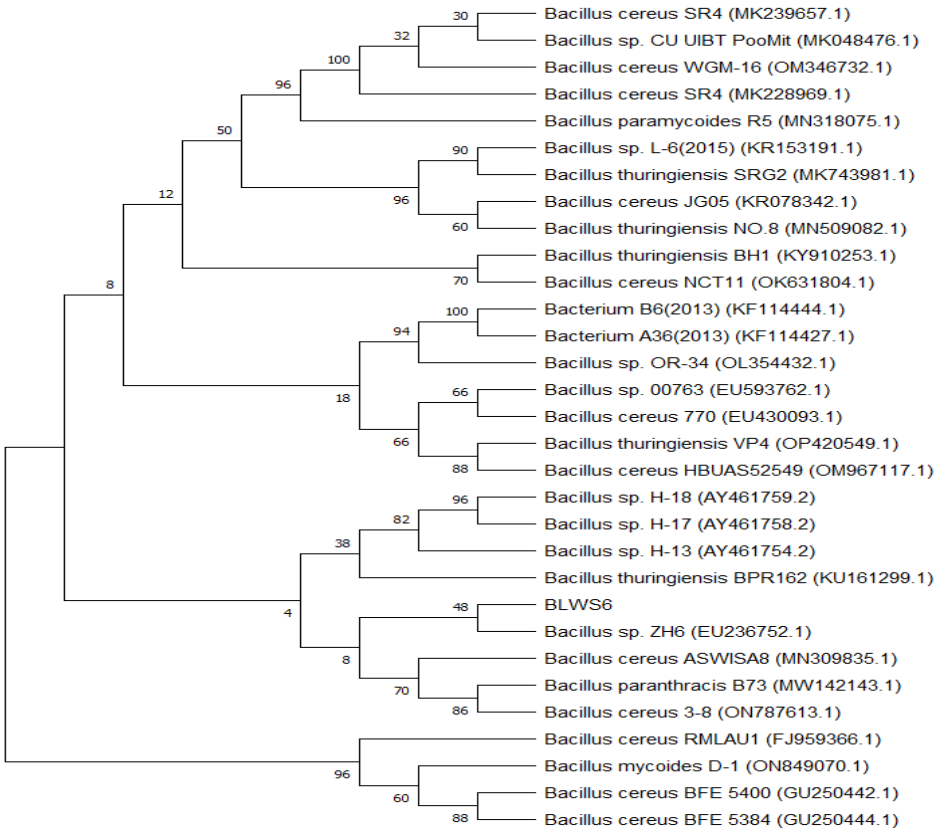


Fig.33 Maximum Likelihood phylogenetic tree of BLWS6

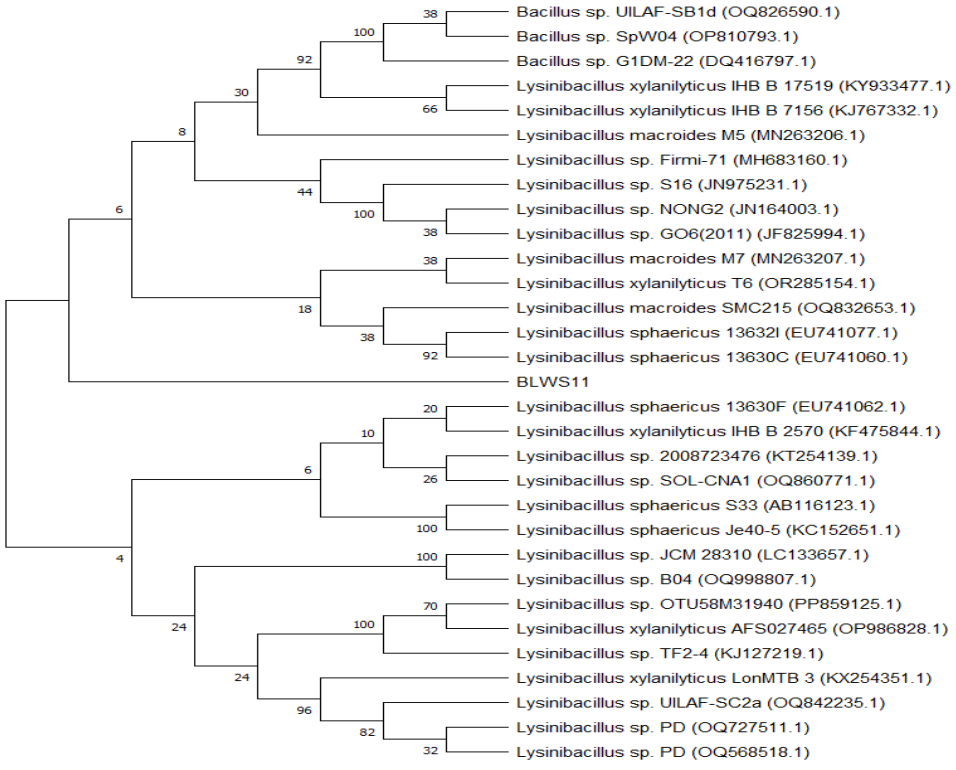


Fig.34 Maximum Likelihood phylogenetic tree of BLWS11

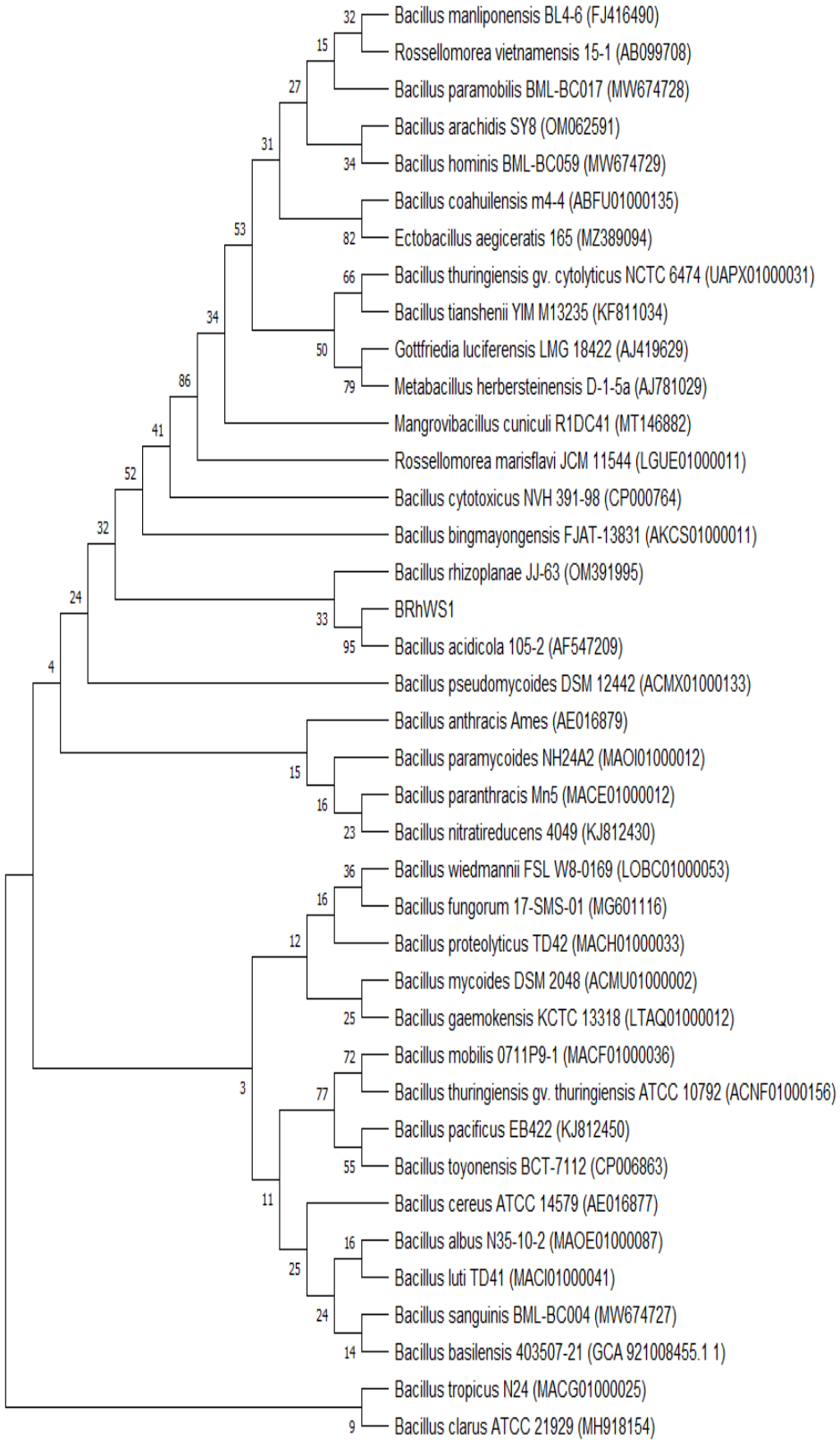


Fig.35 Maximum Likelihood phylogenetic tree of BRhWS1

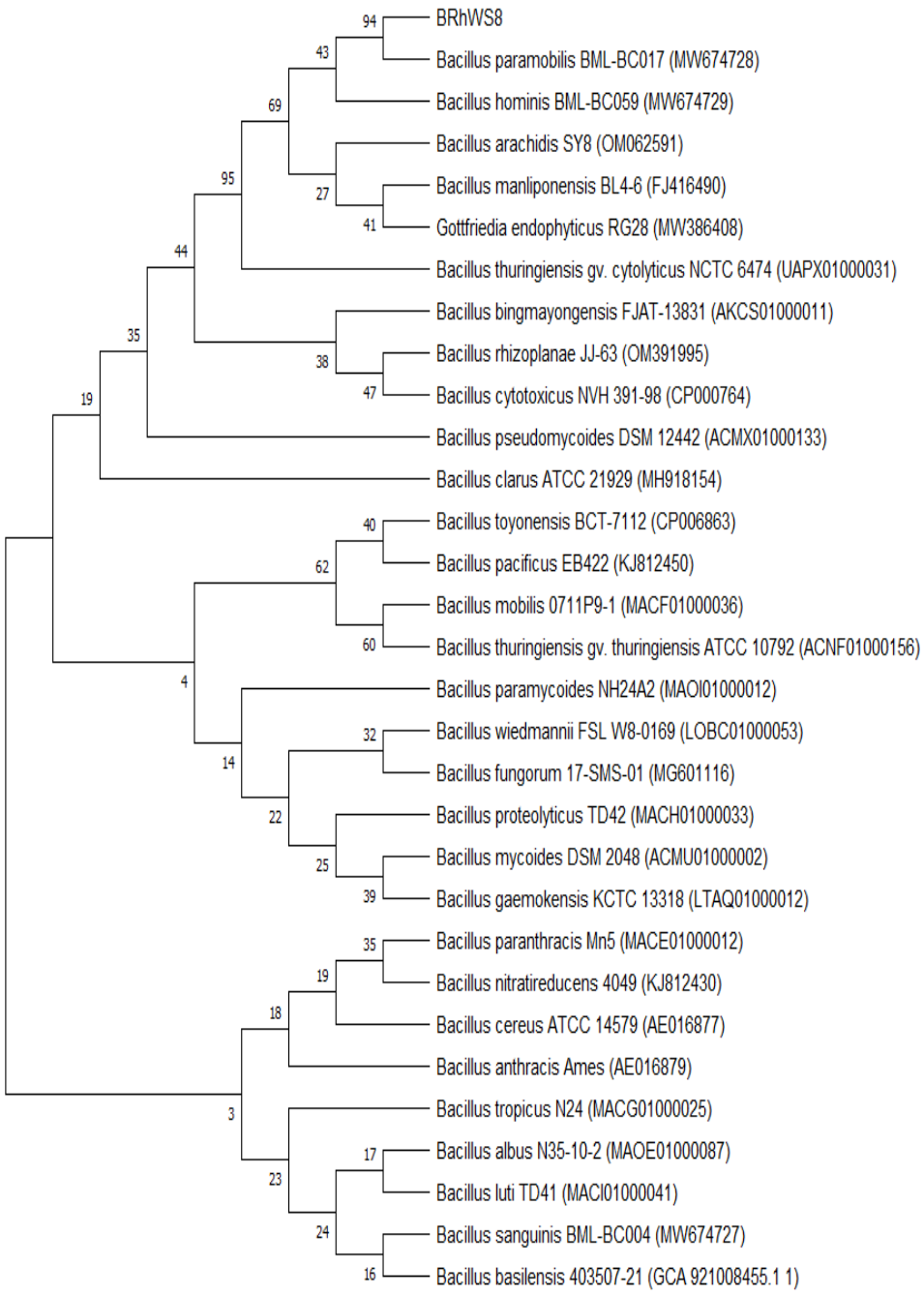


Fig.36 Maximum Likelihood phylogenetic tree of BRhWS8

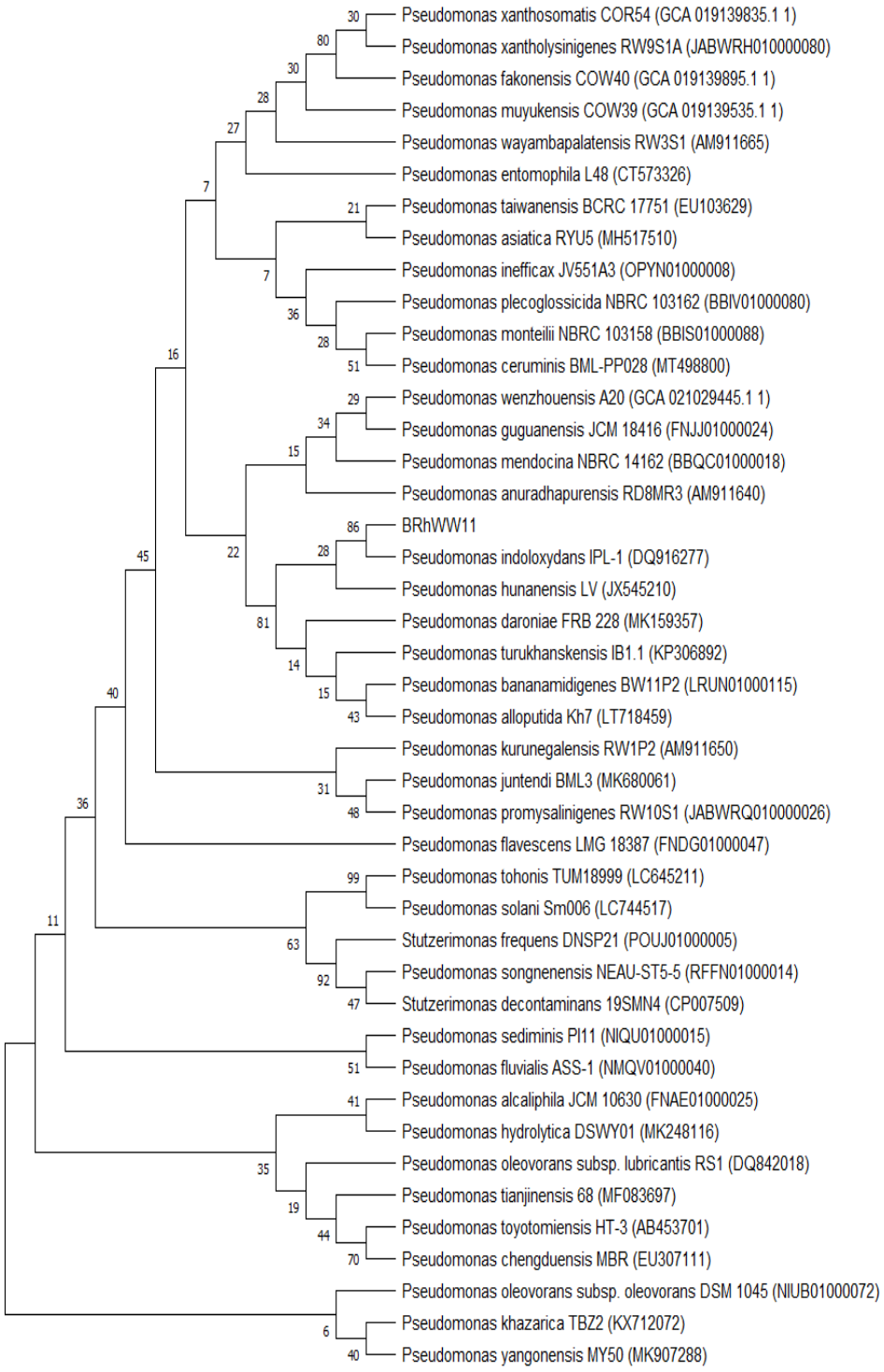


Fig.37 Maximum Likelihood phylogenetic tree of BRhWW11

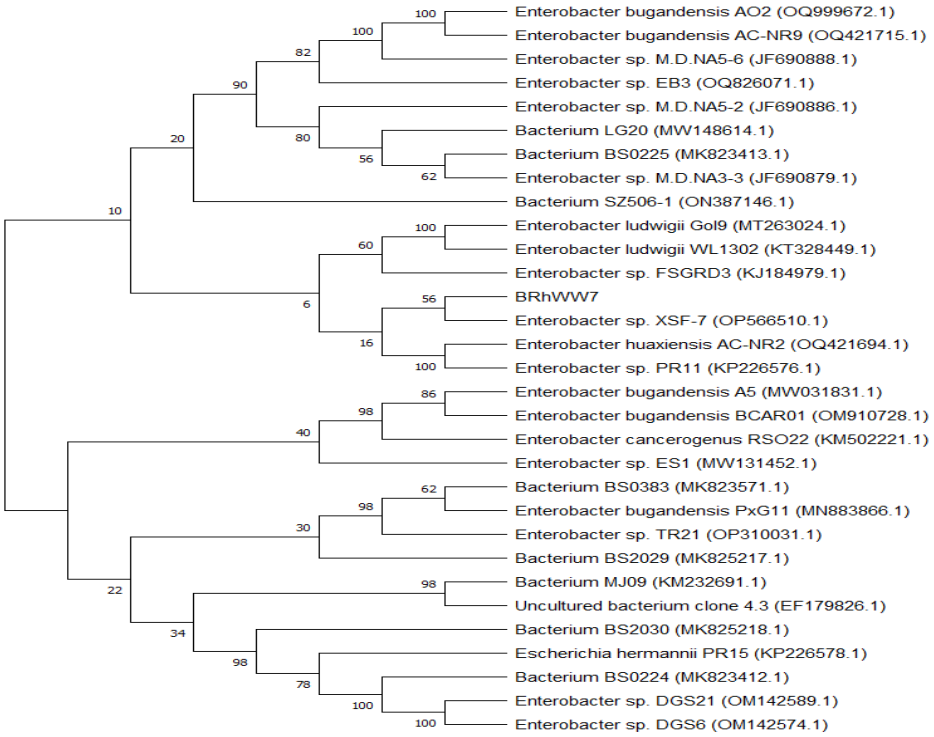


Fig.38 Maximum Likelihood phylogenetic tree of BRhWW7

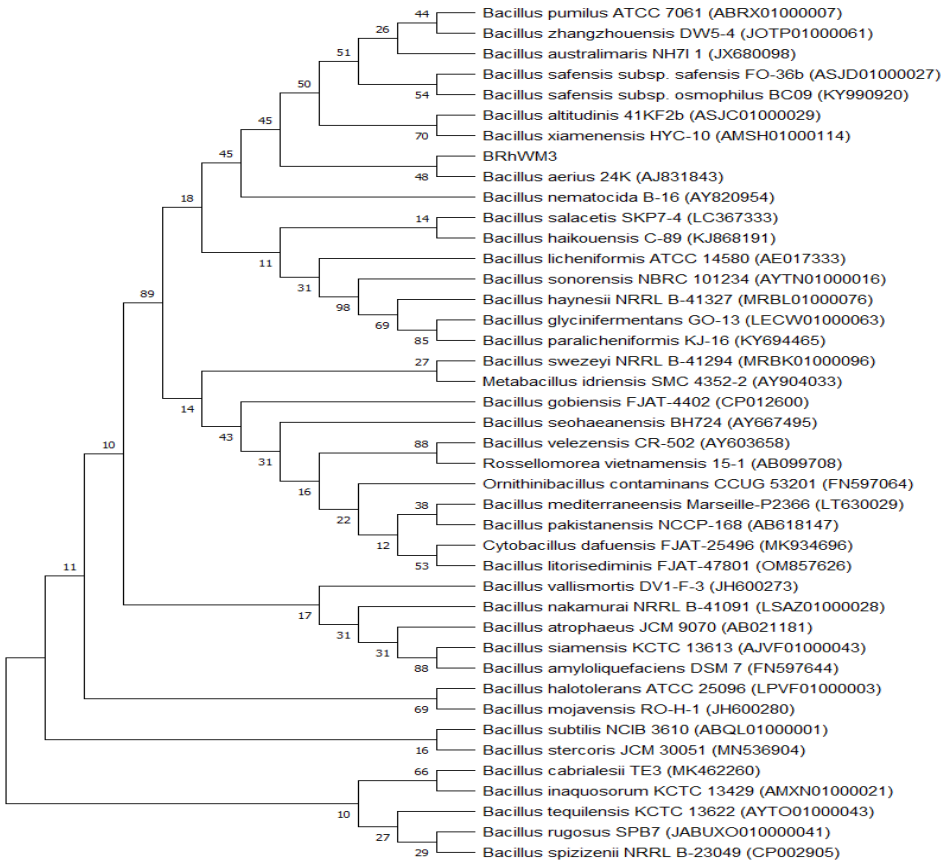


Fig.39 Maximum Likelihood phylogenetic tree of BRhWM3

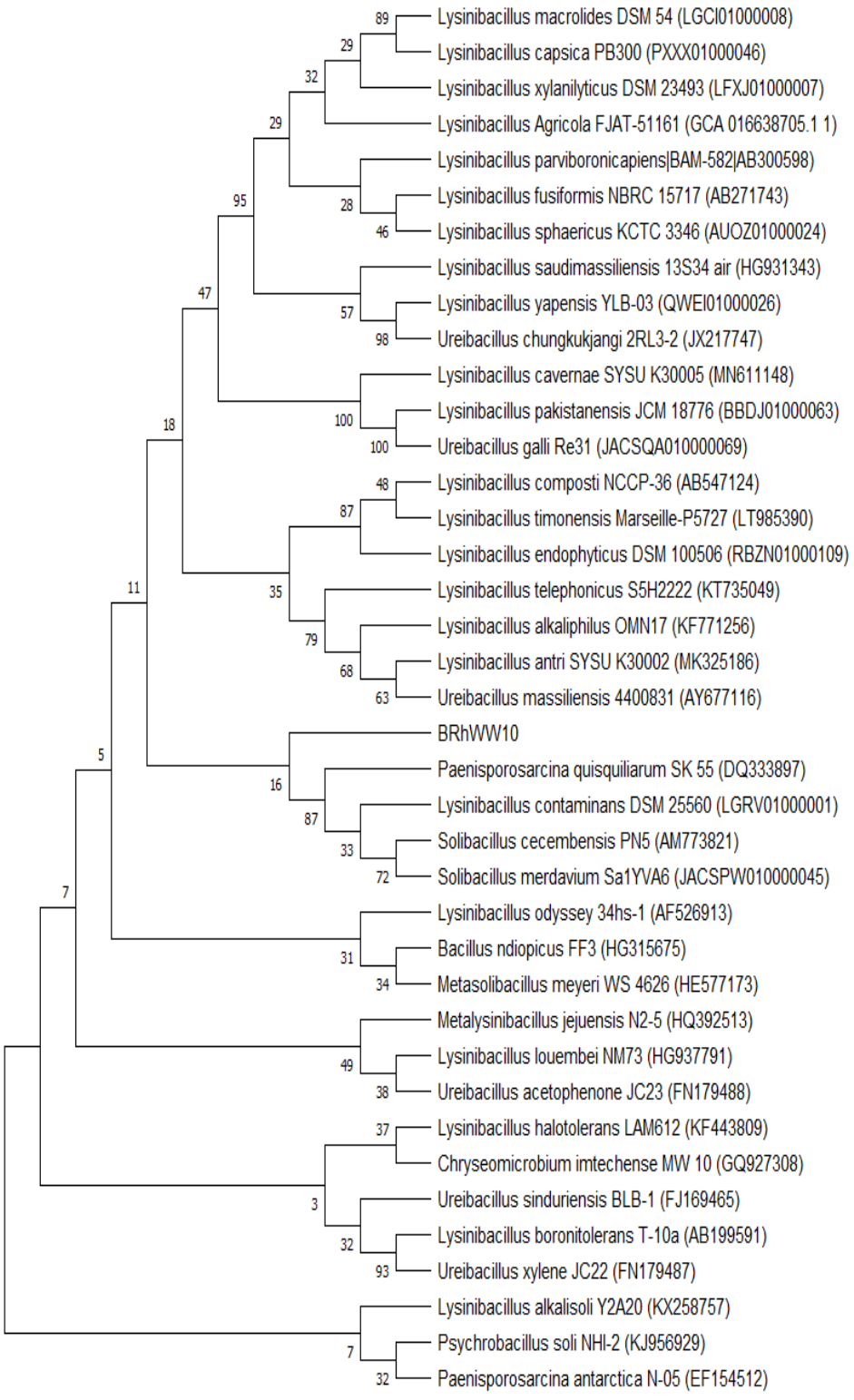


Fig.40 Maximum Likelihood phylogenetic tree of BRhWW10

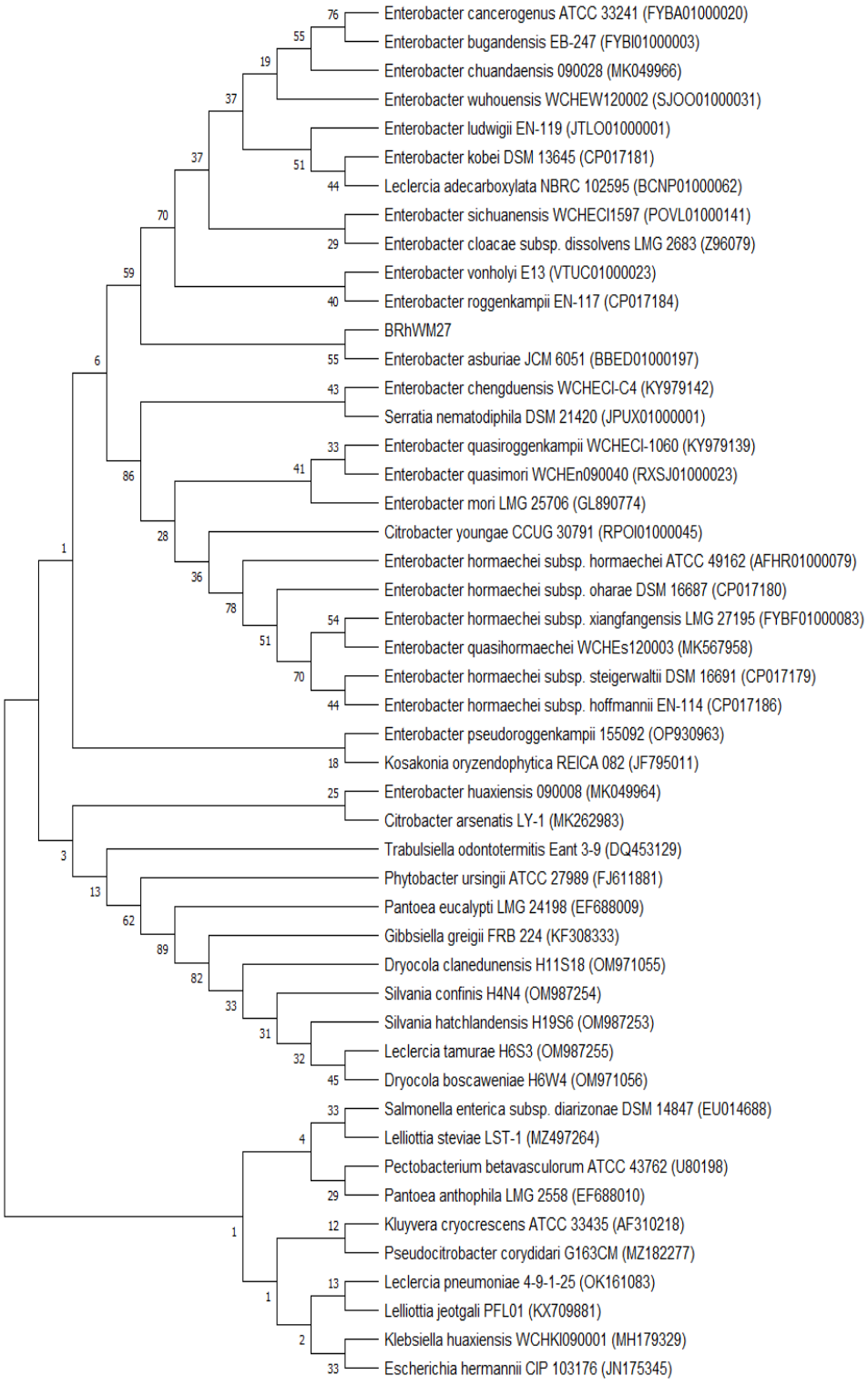


Fig.41 Maximum Likelihood phylogenetic tree of BRhWM27

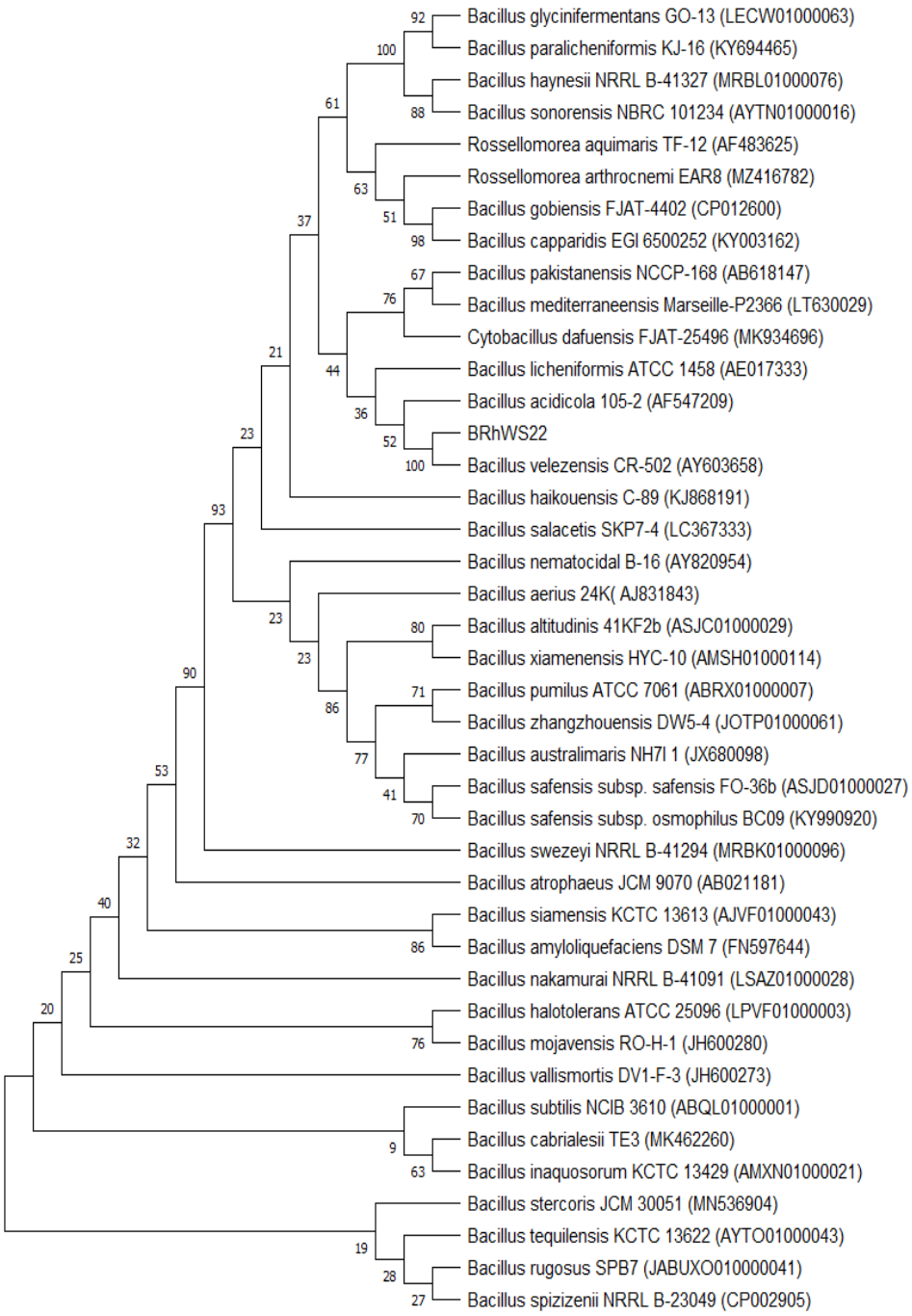


Fig.42 Maximum Likelihood phylogenetic tree of BRhWS22

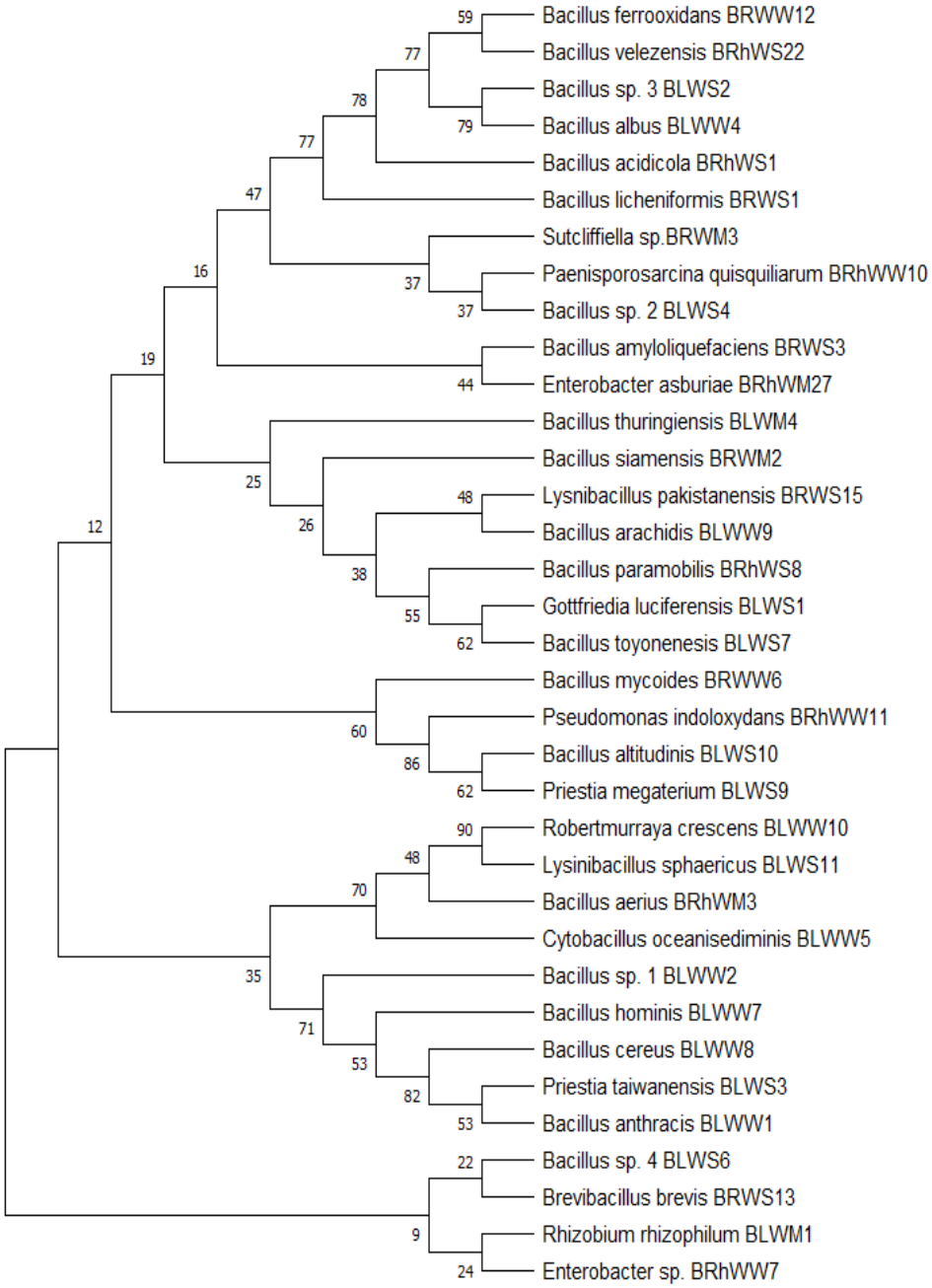


Fig.43 Maximum Likelihood phylogenetic tree showing the relationship of isolated endophytic bacteria and their nearest relatives of all bacterial isolates

Endophytic Fungi

304 endophytic fungi were isolated from the leaf segments in the summer season, 196 were isolated in the winter season, and 108 isolates were in the monsoon season. From the root segments, 499 fungi were in summer, 168 in winter, and 115 in monsoon. 7 isolates in summer, 7 in winter, and 4 in monsoon

from the rhizome segments. A total of 1408 fungal isolates were obtained by using the fragmentation method. 810 endophytic fungi were isolated from all tissues in the summer season, 371 in the winter, and 227 in the monsoon season. Kottavayal holds 373 isolates from all plant parts in the summer season, 175 in winter, and 93 in monsoon. In the case of Pakkom 420 isolates in summer, 154 in winter, and 94 in the monsoon season. 17 endophytic fungi from Mattilayam in summer, 42 in winter, and 40 isolates in monsoon. There were no isolates were obtained from rhizome samples from Pakkom in the monsoon season. The data of isolated endophytic fungi are detailed in Table 18. The represented isolation plates are figured in Fig.44.

	Locations											
	Kottavayal				**Pakkom**				**Mattilayam**			
	S	**W**	**M**	**T**	**S**	**W**	**M**	**T**	**S**	**W**	**M**	**T**
Leaf	119	80	40	239	179	85	46	310	6	31	22	59
Root	250	93	51	394	239	66	48	353	10	9	16	35
Rhizome	4	2	2	8	2	3	0	5	1	2	2	5
Total	373	175	93	641	420	154	94	668	17	42	40	99

Table 18: Endophytic fungi isolated from *Lagenandra toxicaria* Dalzell
*S: Summer, W: Winter, M: Monsoon

Diversity Analysis

Percentage Frequency of Endophytic Fungal Isolates

A percentage frequency of 67.02% was observed in root isolates from Kottavayal in the summer, followed by root (58.82%) from Mattilayam and 56.90% from Pakkom (Table 19). The highest percentage frequency in winter was observed in the leaf (73.80%) from Mattilayam followed by 55.19% from the leaf of Pakkom and 53.14% from the root of Kottavayal (Table 20). In the monsoon, 55% was observed as the highest percentage frequency in leaf from Mattilayam and then in root from Kottavayal

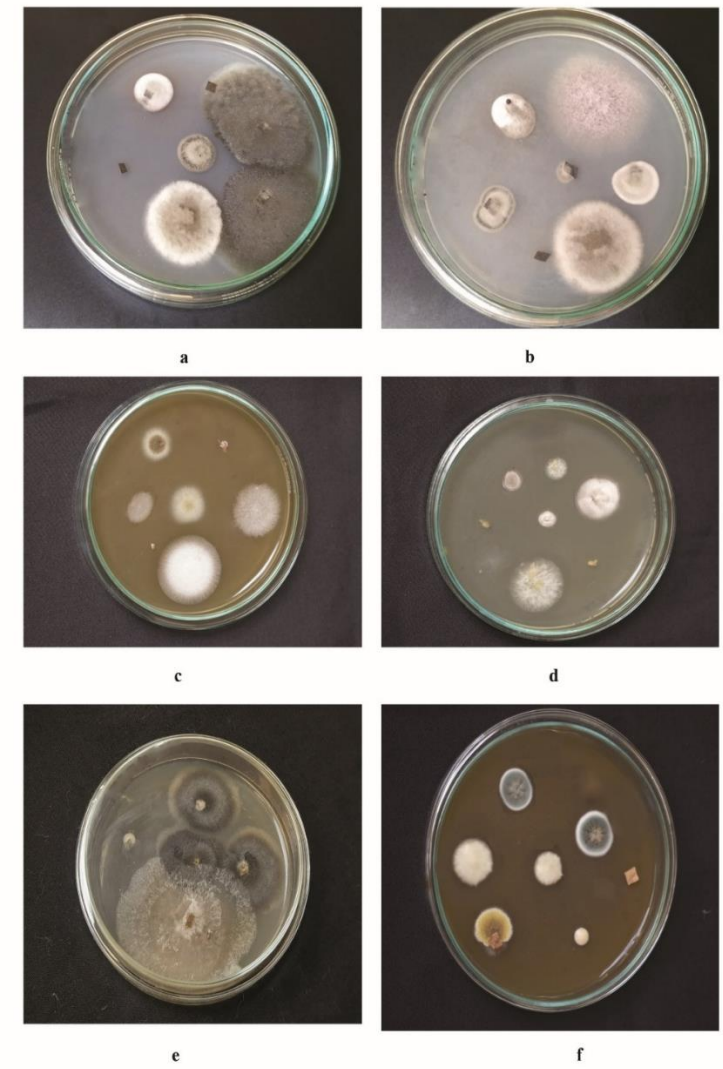


Fig.44 Isolation plates of endophytic fungus. a,b - Leaf; c,d - Root; e,f - Rhizome

(54.83%) and in root from Pakkom (51.06%) (Table 21). An isolation frequency of 59.52% was observed in root samples from Kottavayal, 56.90%, and 42.61% in root and leaf from Pakkom, respectively in summer (Table 19). The highest isolation frequency was observed in the root of Kottavayal (22.14%), 20.23% in the leaf from Pakkom, and 19.04% in the leaf from Kottavayal (Table 20). In the monsoon season, root samples from Kottavayal and Pakkom showed the highest isolation frequency i.e., 12.14% and 11.42%, respectively (Table 21).

	Summer								
	Kottavayal			Pakkom			Mattilayam		
	Leaf	Root	Rhizome	Leaf	Root	Rhizome	Leaf	Root	Rhizome
PF	31.90	67.02	1.07	42.61	56.90	0.47	35.29	58.82	5.88

IF	28.33	59.52	0.95	42.61	56.90	0.47	1.42	2.38	0.23

Table 19: Percentage frequency and Isolation frequency in summer season of endophytic fungi

	Winter								
	Kottavayal			**Pakkom**			**Mattilayam**		
	Leaf	**Root**	**Rhizome**	**Leaf**	**Root**	**Rhizome**	**Leaf**	**Root**	**Rhizome**
PF	45.71	53.14	1.14	55.19	42.85	1.94	73.80	21.42	4.76
IF	19.04	22.14	0.47	20.23	15.71	0.71	7.38	2.14	0.47

Table 20: Percentage frequency and Isolation frequency in winter season of endophytic fungi

	Monsoon								
	Kottavayal			**Pakkom**			**Mattilayam**		
	Leaf	**Root**	**Rhizome**	**Leaf**	**Root**	**Rhizome**	**Leaf**	**Root**	**Rhizome**
PF	43.01	54.83	2.15	48.93	51.06	0	55	40	5
IF	9.25	12.14	0.47	10.95	11.42	0	5.23	3.80	0.47

Table 21: Percentage frequency and Isolation frequency in monsoon season of endophytic fungi

In the case of the colonization rate, the highest rate was observed in 59.04% and 56.66% of the root samples from Kottavayal and Pakkom in the summer season. Similarly, root samples from Kottavayal and leaves from Pakkom showed the highest colonization rate at 22.14% and 20% in winter. The lowest colonization rate was observed in rhizome samples from Mattilayam at 0.71% in summer and winter. The rhizome samples from Pakkom in summer have the same rate of colonization. 12.14% and 11.19% are the highest colonization rate observed in root samples from Kottavayal and Pakkom in monsoon. The highest SWDI was observed in root samples from Pakkom in the summer season of 3.44, followed by root samples from Kottavayal (3.41). The diversity index values of 0.23 and 0.59 were observed as the lowest in summer by rhizome samples from Kottavayal and leaf samples from Mattilayam. In the monsoon season, 2.17 is the highest SWDI observed from root samples of Pakkom, followed by root samples from Kottavayal (1.94), and the lowest value was observed of 0.54 from leaf samples of Mattilayam. The SWDI of 2.58 followed by 2.36 and 2.23 are the highest diversity indices observed from leaf samples from Pakkom,

Kottavayal, and root samples from Pakkom in the winter season. The rhizome samples from Pakkom represented the lowest SWDI (0.34) in winter followed by 0.67 from root samples of Mattilayam.

The Simpson's diversity index of root samples from Kottavayal and Pakkom (0.96) showed the highest diversity in summer, followed by leaf samples from Pakkom (0.86) and Kottavayal (0.84), and the lowest index was shown by rhizome samples from Kottavayal of 0.16. In the case of monsoons, root samples from Pakkom and Kottavayal have the highest SDIs, 0.87 and 0.82, followed by leaf samples from Pakkom (0.81). The lowest diversity was observed in leaf (0.31) and root (0.49) samples from Mattilayam. The leaf samples from Pakkom showed the highest diversity index of 0.92 in winter, followed by 0.89 and 0.87 in leaf samples from Kottavayal and root samples from Kottavayal and Pakkom. The Margalef richness index showed that the summer season showed the highest richness index from the root samples of Pakkom (8.16) and Kottavayal (7.91). In the summer, leaf samples from Kottavayal and Pakkom also showed an average richness of 3.76 and 3.62. The leaf and root samples from Mattilayam had less richness of 1.08 and 1.10, respectively. The lowest richness index was observed in rhizome samples from Kottavayal.

The highest richness in monsoon was observed in root samples of Pakkom and Kottavayal at 3.15 and 2.59, respectively, followed by leaf samples of Pakkom (2.36) and Kottavayal (2.17). Comparatively, samples from Mattilayam have less richness. In the case of species evenness, complete evenness was observed in rhizome samples from Kottavayal in winter. Also, root samples from Mattilayam in the monsoon season showed the highest value of 0.99 followed by the root and leaf samples from Pakkom at 0.95 and 0.93. The root samples from Pakkom and Kottavayal showed the highest evenness in the summer season of 0.95 and the lowest 0.33 from the rhizome samples from Kottavayal. Other than the complete evenness in the winter season, the highest value was observed in the leaf samples from Pakkom at 0.96 followed by leaf samples

from Kottavayal and Mattilayam at 0.93. The lowest richness was observed in rhizome samples from Pakkom of 0.50. The SWDI, SDI, Margalef richness index, and evenness of rhizome samples from Kottavayal in monsoon, Pakkom in summer, and Mattilayam in all seasons are zero. Table 22 represents the colonization rate, SWDI, SDI, Margalef richness index, and evenness.

Location	Season	Samples	CR	SWDI	SDI	Margalef Richness Index	Evenness
Kottavayal	Summer	Leaf	25.71±3.57	2.19±0.46	0.84±0.07	3.76±1.26	0.82±0.08
		Root	59.04±3.66	3.41±0.12	0.96±0.00	7.91±0.86	0.95±0.00
		Rhizome	0.95±0.41	0.23±0.39	0.16±0.28	0.48±0.83	0.33±0.57
	Monsoon	Leaf	9.52±6.07	1.54±0.64	0.71±0.23	2.17±0.99	0.84±0.10
		Root	12.14±4.28	1.94±0.32	0.82±0.06	2.59±0.43	0.92±0.03
		Rhizome	0.71±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Winter	Leaf	19.04±3.93	2.36±0.08	0.89±0.00	3.55±0.41	0.93±0.02
		Root	22.14±5.66	2.20±0.04	0.87±0.01	2.94±0.20	0.91±0.02
		Rhizome	1.43±0.00	0.69±0.00	0.50±0.00	1.44±0.00	1.00±0.00
Pakkom	Summer	Leaf	41.66±8.64	2.31±0.41	0.86±0.07	3.62±0.75	0.84±0.10
		Root	56.66±5.26	3.44±0.22	0.96±0.01	8.16±1.61	0.95±0.01
		Rhizome	0.71±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Monsoon	Leaf	10.95±2.97	1.83±0.36	0.81±0.07	2.36±0.85	0.93±0.04
		Root	11.19±2.29	2.17±0.05	0.87±0.01	3.15±0.08	0.95±0.02
		Rhizome	0	0	0	0	0
	Winter	Leaf	20.00±1.43	2.58±0.10	0.92±0.01	4.09±0.52	0.96±0.00
		Root	15.71±4.28	2.23±0.17	0.87±0.03	3.59±0.29	0.90±0.04
		Rhizome	1.07±0.50	0.34±0.48	0.25±0.35	0.72±1.01	0.50±0.70
Mattilayam	Summer	Leaf	1.42±0.71	0.59±0.55	0.39±0.34	1.08±0.96	0.66±0.57
		Root	2.38±1.48	0.79±0.69	0.44±0.39	1.10±0.97	0.63±0.55
		Rhizome	0.71±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Monsoon	Leaf	5.00±4.46	0.54±0.57	0.31±0.31	0.57±0.55	0.51±0.44
		Root	3.81±2.50	0.68±0.01	0.49±0.01	0.84±0.52	0.99±0.01
		Rhizome	1.43±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Winter	Leaf	7.38±2.97	1.57±0.32	0.76±0.07	1.98±0.53	0.93±0.01
		Root	2.14±0.71	0.67±0.02	0.48±0.03	1.02±0.37	0.9±0.04
		Rhizome	0.71±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 22: Detailed diversity indices of endophytic fungi

MANOVA on individual factors and interaction effects showed a high significance i.e., $p<0.01$ on the location, season, plant, location*season,

location*plant, season*plant, and location*season*plant. From the observation, the endophytic fungal diversity is statistically significant at a 1% level of significance (Table 23).

Effect	*df*	Pillai's trace	F	P
Location	2	0.935	6.673	0.000
Season	2	1.084	8.998	0.000
Plant	2	1.484	21.847	0.000
Location*Season	4	1.004	2.681	0.000
Location*Plant	4	1.267	3.710	0.000
Season*Plant	4	1.382	4.223	0.000
Location*Season*Plant	6	1.322	2.106	0.001

Table 23: Results of multivariate ANOVA for endophytic fungal diversity

Table 24 shows the analysis of the effect of location, seasons, and plant parts on the dependent characteristics. The location effect on SDI, SWDI, evenness, and Margalef richness is statistically significant from 1% to 5%. The CR and Margalef richness effect of all factors showed statistical significance. Instead of this other dependent such as SDI and evenness are not very significant in their effects. In the case of SWDI, location*plant and season*plant effects showed good significance.

Source	Dependent Variable	*df*	F	P
Location	CR	2	78.560	0.000
	SDI	2	19.518	0.000
	SWDI	2	54.343	0.000
	Evenness	2	3.277	0.048
	Margalef Richness	2	42.131	0.000
Season	CR	2	84.737	0.000
	SDI	2	3.944	0.027
	SWDI	2	9.431	0.000
	Evenness	2	3.727	0.033
	Margalef Richness	2	21.418	0.000
Plant	CR	2	112.806	0.000
	SDI	2	60.506	0.000
	SWDI	2	101.22	0.000

	Evenness	2	27.586	0.000
	Margalef Richness	2	68.659	0.000
Location * Season	CR	4	29.269	0.000
	SDI	4	0.354	0.840
	SWDI	4	1.949	0.121
	Evenness	4	0.354	0.840
	Margalef Richness	4	4.452	0.004
Location * Plant	CR	4	25.806	0.000
	SDI	4	1.215	0.319
	SWDI	4	8.769	0.000
	Evenness	4	0.935	0.435
	Margalef Richness	4	9.174	0.000
Season * Plant	CR	4	26.644	0.000
	SDI	4	1.572	0.200
	SWDI	4	8.505	0.000
	Evenness	4	1.128	0.357
	Margalef Richness	4	16.234	0.000
Location * Season * Plant	CR	6	8.114	0.000
	SDI	6	0.880	0.530
	SWDI	6	0.945	0.483
	Evenness	6	1.105	0.378
	Margalef Richness	6	3.630	0.004

Table 24: Effect of factors on dependent characteristics

For pairwise comparison, CR shows a significant difference between the three locations at a 5% level of significance. No significant difference is shown between Kottavayal and Pakkom. In the case of SDI and SWDI, significance was observed in Mattilayam compared to Kottavayal and Pakkom. Also, Evenness doesn't show any significant difference concerning location. Location-wise, all characteristics except CR don't show any significant difference between Kottavayal and Pakkom (Table 25).

Dependent Variable	(I) Location	(J) Location	Mean Difference (I-J)	Std. Error	P
CR	Kottavayal	Mattilayam	15.3910*	1.15179	.000
	Pakkom	Kottavayal	2.7704	1.13774	.050
		Mattilayam	18.1614*	1.17597	.000

SDI	Kottavayal	Mattilayam	0.2676*	.05104	.000
	Pakkom	Kottavayal	0.0664	0.05042	0.394
		Mattilayam	0.3340	0.05211	0.000
SWDI	Kottavayal	Mattilayam	1.0693*	0.10802	0.000
	Pakkom	Kottavayal	0.2559	0.10670	0.054
		Mattilayam	1.3252*	0.11028	0.000
Evenness	Kottavayal	Mattilayam	0.0842	0.07977	0.547
	Pakkom	Kottavayal	0.0452	0.07880	0.835
		Mattilayam	0.1294	0.08144	0.262
Margalef Richness	Kottavayal	Mattilayam	2.0416*	0.2344	0.000
	Pakkom	Kottavayal	0.4882	0.22679	0.092
		Mattilayam	2.5298	0.23441	0.000

Table 25: Location-wise comparison of dependent variables
* Shows statistical significance

In the case of season, SDI shows no significant difference, as the p-value is greater than 0.05. For evenness, only significance is shown between summer and winter. CR and Margalef richness vary significantly concerning seasons. (Table 26).

Dependent Variable	(I) Season	(J) Season	Mean Difference (I-J)	Std. Error	P
CR	Summer	Monsoon	15.9167*	1.15179	0.000
		Winter	11.5896*	1.13774	0.000
	Winter	Monsoon	4.3271*	1.17597	0.002
SDI	Summer	Monsoon	0.0030	0.05104	0.998
	Winter	Summer	0.1208	0.05042	0.054
		Monsoon	0.1238	0.05211	0.057
SWDI	Summer	Monsoon	0.3763*	0.10802	0.003
	Winter	Summer	0.0270	0.10670	0.965
		Monsoon	0.4034*	0.11028	0.002
Evenness	Monsoon	Summer	0.0854*	0.07977	0.537
	Winter	Summer	0.2056*	0.07880	0.033
		Monsoon	0.1202	0.08144	0.313
Margalef Richness	Summer	Monsoon	1.5954*	0.22959	0.000
		Winter	0.7917*	0.22679	0.003
	Winter	Monsoon	0.8036*	0.23441	0.004

Table 26: Season-wise comparison of dependent variables
* Shows statistical significance

Among plant parts, CR and Margalef richness vary significantly concerning plant parts. The leaf and root show no significant difference in SDI, SWDI, and

Evenness. But rhizome differs significantly from leaf and root. The CR and other characteristics in all samples showed the significance of P<0.01 and also SWDI on root with rhizome samples (Table 27).

Dependent Variable	(I) Plant part	(J) Plant part	Mean Difference (I-J)	Std. Error	P
CR	Leaf	Rhizome	14.7029*	1.04910	0.000
	Root	Leaf	4.9470*	1.04910	0.000
		Rhizome	19.6499*	1.30124	0.000
SDI	Leaf	Rhizome	0.6083*	0.05767	0.000
	Root	Leaf	0.0304	0.04649	0.792
		Rhizome	0.6387*	0.05767	0.000
SWDI	Leaf	Rhizome	1.5693*	0.12203	0.000
	Root	Leaf	0.2219	0.09838	0.074
		Rhizome	1.7911*	0.12203	0.000
Evenness	Leaf	Rhizome	0.5963*	0.09012	0.000
	Root	Leaf	0.0848	0.07266	0.479
		Rhizome	0.6811*	0.09012	0.000
Margalef Richness	Leaf	Rhizome	2.2455*	0.25938	0.000
	Root	Leaf	0.9026*	0.20912	0.000
		Rhizome	3.1481*	0.25938	0.000

Table 27: Plant part-wise comparison of dependent variables
* Shows statistical significance

The principal component (PC) analysis was performed on the standardized data set of fungi to reduce dimensionality and identify the key components explaining the variants in the data. The first two principal components explain more than 99% of the total variance. The loading for the first principle component is quite uniform across all original variables. Each variable contributes similarly and positively to the variance captured by the first PC. In considering the second PC, CR contributes highly positively followed by SDI and evenness with a significant negative contribution. A biplot was generated to visualize the results of the PCA performed in each data set (Fig. 45). The biplot simultaneously displays the scores of the observations and the loadings of the original variables on the first to PC. The principal component analysis (PCA) plot displays the first principal component (PC1) on the x-axis and the second principal component (PC2) on the y-axis. PC1 accounts for 95% of the variance, while PC2 explains 4% of the variance, cumulatively capturing around 99% of

the total variance in the dataset. Each point on the biplot represents an individual observation, colored by specific characteristics. The vectors represent the original variables. CR has a longer vector, indicating a significant influence on PC1. The remaining factors are highly positively correlated, as they are so close to each other. The similarity in vector length indicates uniformity in their influence. The direction of the vectors suggests that these variables are positively correlated, as they point in similar directions. The standard deviation and variance for the PCA are represented in Table 28. Eigenvectors for the principal component analysis are shown in Table 29.

Importance of components	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**
Standard Deviation	2.180	0.494	0.017	0.006	0.002
Proportion of Variance	0.951	0.048	0.000	0.000	0.000
Cumulative Proportion	0.951	0.999	0.999	1.000	1.000

Table 28: Standard deviation and variance of PCA

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
CR	0.409	0.907	0.876	0.000	-0.000
SDI	0.455	-0.241	0.372	-0.091	0.766
SWDI	0.456	-0.201	-0.054	0.839	-0.208
Evenness	0.455	-0.246	0.425	-0.429	-0.605
Margalef Richness	0.457	-0.127	-0.818	-0.320	0.047

Table 29: Eigenvectors for the principal component analysis

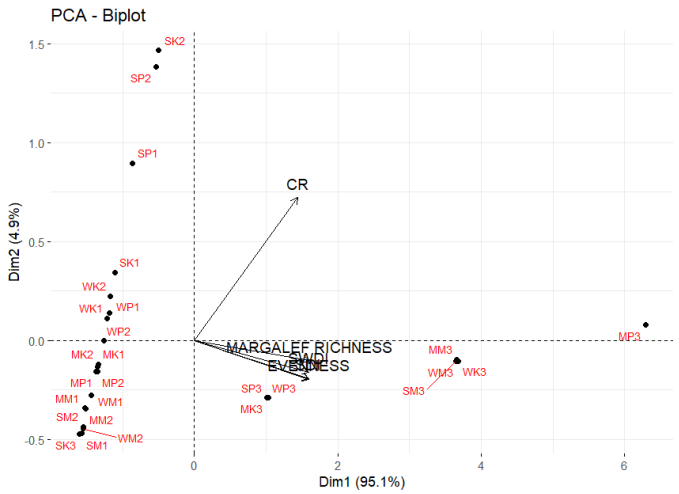


Fig.45 Biplot illustrates the PCA results performed on endophytic fungi

The plot shows the projection of the dependent variables (arrows) and observations (points) onto the principal component space. Variables are scaled to unit variance, and observations are centered. The arrows' lengths and directions indicate each variable's contribution to the principal components, while the points' positions reflect the observations' relative similarity.

Identification of Fungal Endophytes

Morphological Characteristics of Fungal Endophytes

Thirty-six fungal endophytes from *Lagenandra toxicaria* were selected for further studies based on the maximum frequency of occurrence, growth rate, and differences in appearance. The selected fungal endophytes were analyzed by studying their macro and micro morphological characteristics. The macromorphological observation of colony color from the front and reverse colony texture, shape, margin, elevation, exudates, reverse side morphology, and micro morphological characteristics like presence or absence of septa, type of conidiophores, shape, and color of conidia. Macromorphological features of fungal endophytes showed variation in color, texture, and pigment production. Fungal isolates FRWS8, FLWW5, FLWW17, FRWM12, FLWS32, FRWW18, FRWM25, FRWW30, FLWS1, FRhWW3, and FLWW12 were identified as species of *Aspergillus*. FRhWS1, FRWM3, FRWS24, FRWW10, and FRWW11 as species of *Fusarium*; FLWW15, FRhWS2, and FRhWW4 as species of *Penicillium* based on their phenotypic characters. All the isolated

endophytic fungi belong to the families Aspergillaceae (38.89%), Nectriaceae (16.67%), Glomerellaceae (8.34%), Trichocomaceae (5.56%), Didymellaceae (5.56%), Hypocreaaceae (5.56%), Botyosphaeriaceae (2.78%), Diaporthaceae (2.78%), Phanerochaetaceae (2.78%), Debaryomycetaceae (2.78%), Corynesporascaceae (2.78%), Mucoraceae (2.78%), Pleosporaceae (2.78%). A total of 97.22% of the isolates, representing 12 families belong to the phylum Ascomycota, while the remaining 2.78% (Mucoraceae) belong to the phylum Mucoromycota.

The diverse range of colony color, including white, yellow, green, grey, and black. These colors may be uniform or exhibit concentric zoning, adding to the complexity of identification. Texture also varies, with some isolates displaying a powdery, woolly, or cottony appearance. Colony shape and size are also important distinguishing features. Some isolates form circular colonies, while others are irregular or lobate. The elevation of the colonies ranges from flat to raised, with some exhibiting wavy or undulated margins. The presence of liquid droplets or an oily appearance is another characteristic used to identify certain isolates. This feature may be accompanied by a specific color or texture, further aiding in identification.

The mycelium, the vegetative part of the fungus, also exhibits distinct colors and textures. These characteristics can be used in conjunction with colony features to ensure accurate identification. Most colonies are circular or irregular, while FLWW11 and FRWM1 are seen with a radial pattern. The colonies are slightly raised/raised from the surface. FLWW11 is raised with furrows, FLWS21, FRWW11, and FRWS9 are umbonate, and FRhWW4, a bluish colony, is convex. The margins of the colonies vary from strain to strain. A slight wavy, smooth, or entire margin is generally occurring in the colonies while FLWW14 and FLWW6 have a lobate margin. The fungal exudates are seen as liquid droplets in the majority of the strains. FLWW6, FLWW17, and FRWS4 do not produce exudates. The reverse morphology of each strain is

slightly different from strain to strain; hence it can be used as a key character for the identification also (Table 30).

The fungal isolates exhibit diverse characteristics, including hyphae structure, shape of conidia, shape of vesicle, conidiophore, and color of conidia. Notably, septate and aseptate hyphae are present, with septate being more common. Conidia shapes range from ellipsoidal, globose, ovoid, sub-globose, fusiform, and crescent, while vesicle shapes include spherical, flask-shaped, pyriform, and clavate. Conidiophores are mostly unbranched, some showing sparse or branched structures. The color of conidia varies widely, featuring shades of brown, green, yellow, olive, blue-green, pinkish-white, and hyaline. Some isolates, like FLWW14, and FRWM27 have aseptate hyphae and brown conidia, whereas FRWS8 has septate hyphae and dark green conidia. Conidia, the asexual reproductive structure is spherical to ellipsoidal to fusiform in structure. They are pale yellow to green in color. The vesicles in the fungal hyphae in the strains are isolated in different shapes spherical, globose, and flask-shaped. FLWW5 stands out with its flask-shaped vesicle and brown conidia. The unique combination of these characteristics allows precise identification and classification of each fungal isolate, highlighting the vast diversity within this group of organisms. The conidiophores in which conidia are produced are either branched or branched. The detailed micromorphological characteristics of all isolates are mentioned in Table 31 & Fig.46.

Sl No.	Fungal isolates	Colony color		Texture	Shape	Margin	Elevation	Exudates	Reverse morphology
		Front	Reverse						
1	FLWW14	Greyish white	Black hue	Woolly	Irregular	Lobate	Raised	Yellowish	Equally distinctive, with a dark, almost black color
2	FRWS8	White to yellowish-green	Pale yellow	Powdery	Circular	Sightly wavy	Raised	Yellowish	Flat-slightly wrinkled, underlying pigmentation visible through the mycelium
3	FLWW5	Cinnamon-brown	Reddish-brown	Powdery	Circular	Slightly irregular	Flat to slightly raised	Liquid droplet	Flat-slightly wrinkled, whitish brown
4	FLWW15	Greenish-blue or blue-green hue	yellowish or cream	Powdery	Circular	Entire	Slightly raised	Liquid droplet	Yellowish colored center and a darker, concentric ring surrounding it
5	FLWW17	Dark green	Yellow to brown hue	Velvety	Circular	Smooth	Slightly raised	Nil	Yellow to brownish, slightly wrinkled
6	FLWW6	Yellowish black	Brown hue	Powdery	Circular	Lobate	Slightly raised	Nil	Darkly pigmented appearance

7	FRWS7	Blackish-green	Yellowish-brown	Cottony with a fluffy appearance	Circular	Entire	Slightly raised	Glossy appearance	Equally intriguing with pale brown
8	FRWM12	Yellow to brown hue	Brown	Powdery	Circular	Smooth	Slightly raised	Liquid droplet	Flat-slightly wrinkled, yellowish brown
9	FRWS4	Dark olive-green to brown	Golden-brown hue	Velvety with a woolly appearance	Irregular	Undulate	Flat	Nil	Equally intriguing, with dark brown coloration that may exhibit a concentric zonal pattern
10	FLWW11	Whitish-green	Yellowish-green	Cottony	Circular with a radial pattern	Wavy	Raised with furrows	Greenish	Yellow, green, and even white with concentric circle
11	FRhWS1	Whitish-pink	Pale yellow	Cottony with a wooly appearance	Circular	Slightly undulate	Slightly convex	Liquid droplet	Dark with a radial growth pattern
12	FLWW18	Greenish-brown	Creamy-yellow	Downy	Circular	Entire	Raised	Nil	Yellow with a radially wrinkled pattern
13	FLWS32	Olive-green	Pale yellow	Granular	Circular	Slightly undulate	Slightly raised	Yellow	Wrinkled pattern
14	FRWW18	Pale green	Yellow	Powdery	Circular	Slightly undulate	Slightly raised	Yellow	Wrinkled pattern

15	FRWM25	Greyish-black	Yellowish-brown	Granular	Circular	Undulate	Slightly raised	Nil	Wrinkled pattern
16	FRWW30	Yellowish-brown	Colourless	Powdery	Circular	Slightly irregular	Raised	Oily	Flat-slightly wrinkled, underlying pigmentation visible through the mycelium
17	FLWM1	Whitish-pink	Dark pink	Velvety	Circular	Slightly undulate	Slightly raised	Pinkish droplet	Dark with a radial growth pattern
18	FLWW9	Dark green	Pale yellow	Cottony	Circular	Slightly irregular	Slightly raised	Liquid droplets	Equally intriguing, exhibit a concentric zonal pattern
19	FRWM27	Beige	Cream	Glistening appearance	Circular	Entire	Slightly convex	Oily	Equally intriguing
20	FLWS1	Golden-yellow	Light brown	Powdery	Circular	Entire	Slightly raised	Liquid droplet	Equally intriguing with pale yellow to light brown, with a smooth and even texture
21	FRhWW3	Velvety green	Yellowish-brown	Velvety	Circular	Slightly undulate	Raised	Nil	Equally intriguing
22	FRhWM2	Greyish-green	Pale yellow	Velvety	Circular	Entire	Folded	Liquid droplets	Concentric zoning
23	FRhWS2	Deep green	Yellowish-orange	Powdery	Circular	Undulate	Moderately raised	Nil	Radial arrangement

24	FRWM3	Whitish-pink	Reddish-brown	Cottony with a wooly appearance	Irregular	Feathery	Slightly raised	Liquid droplet	Dark with a radial growth pattern
25	FLWS16	Cream with a yellow tinge	Tan	Floccose with downy appearance	Flat	Smooth	Slightly raised	Glistening appearance	Smooth appearance
26	FRWS24	Whitish pink	Reddish-brown	Cottony	Circular	Lobate	Slightly raised	Colourless	Smooth appearance
27	FRWW10	Pink	Yellow	Woolly	Circular	Filamentous	Umbonate	Nil	Equally intriguing
28	FLWW12	Cinnamon-green	Pale brown	Powdery	Circular	Slightly irregular	Flat to slightly raised	Liquid droplet	Flat-slightly wrinkled, whitish brown
29	FRWM1	Whitish-green	Yellowish-green	Cottony	Circular with a radial pattern	Wavy	Raised with furrows	Greenish	Yellow, green, and even white with concentric circle
30	FLWS21	Whitish-grey	Brown hue	Slightly rough	Irregular sectored	Lobate	Umbonate	Colourless	Concentric zones
31	FRWW11	Pink	Pink	Woolly	Circular	Filamentous	Umbonate	Nil	Equally intriguing
32	FRhWW4	Bluish-green	Deep Yellow	Powdery	Circular	Entire	Convex	Yellow	Wrinkled pattern
33	FRWS9	Dark grey	Black	Fluffy	Circular	Filamentous	Umbonate	Nil	Smooth
34	FLWM11	Whitish-grey with a hint of orange	Whitish-grey with a hint of orange	Cottony	Circular	Undulate	Flat	Orange	Distinct zone pattern

35	FLWW13	Black	Reddish-brown hue	Velvety	Circular	Entire	Slightly raised	Brown	Equally intriguing
36	FLWS5	Pale olivaceous-grey hue	Dark olivaceous-grey	Woolly	Irregular	Undulate	Flat	Black	Wrinkled pattern

Table 30: Macro-morphological characters of endophytic fungal isolates

Sl No.	Fungal isolates	Hyphae	Structure	Shape of conidia	Shape of vesicle	Conidiophore	Colour of conidia
1	FLWW14	Aseptate	Uniseriate	Ellipsoidal	Spherical	Unbranched	Brown
2	FRWS8	Septate	Uniseriate	Globose	Globose	Sparsely branched	Dark green
3	FLWW5	Septate	Uniseriate	Globose	Flask-shaped	Unbranched	Brown
4	FLWW15	Septate	Uniseriate	Ovoid	Globose	Branched	Blue-green
5	FLWW17	Septate	Uniseriate	Sub-globose	Flask-shaped	Unbranched	Pale yellow
6	FLWW6	Septate	Biseriate	Ellipsoidal	Globose	Branched	Dark brown
7	FRWS7	Septate	Biseriate	Oval	Pyriform	Branched	Dark olive green
8	FRWM12	Septate	Biseriate	Globose	Spherical	Branched	Pale yellow
9	FRWS4	Septate	Biseriate	Cresent	Clavate	Branched	Brown
10	FLWW11	Septate	Uniseriate	Ovoid	Globose	Branched	Olive green
11	FRhWS1	Septate	Uniseriate	Fusiform	Flask-shaped	Unbranched	Light green
12	FLWW18	Septate	Uniseriate	Globose	Flask-shaped	Branched	Dark brown
13	FLWS32	Septate	Uniseriate	Sub-globose	Flask-shaped	Unbranched	Yellow-green
14	FRWW18	Septate	Uniseriate	Sub-globose	Flask-shaped	Unbranched	Yellow
15	FRWM25	Septate	Uniseriate	Oval	Globose	Branched	Dark brown

16	FRWW30	Septate	Uniseriate	Ellipsoidal	Globose	Unbranched	Pale green
17	FLWM1	Septate	Uniseriate	Slightly curved	Clavate	Unbranched	Hyaline
18	FLWW9	Septate	Uniseriate	Ovoid	Sub-globose	Unbranched	Pale brown
19	FRWM27	Aseptate	Uniseriate	Oval	Flask-shaped	Unbranched	Creamy yellow
20	FLWS1	Septate	Uniseriate	Sub-globose	Flask-shaped	Unbranched	Pale green
21	FRhWW3	Septate	Uniseriate	Elliptical	Globose	Unbranched	Green
22	FRhWM2	Septate	Uniseriate	Elongated	Globose	Unbranched	Olive green
23	FRhWS2	Septate	Uniseriate	Globose	Globose	Slightly branched	Green
24	FRWM3	Septate	Uniseriate	Ellipsoidal	Nil	Unbranched	Pale yellow
25	FLWS16	Septate	Uniseriate	Ellipsoidal	Flask-shaped	Unbranched	Pale brown
26	FRWS24	Septate	Uniseriate	Fusiform	Nil	Unbranched	Pinkish white
27	FRWW10	Septate	Uniseriate	Ellipsoidal	Nil	Branched	Pale yellow
28	FLWW12	Septate	Uniseriate	Sub-globose	Flask-shaped	Unbranched	Dark green
29	FRWM1	Septate	Uniseriate	Sub-globose	Nil	Branched	Pale green
30	FLWS21	Septate	Uniseriate	Ellipsoidal	Nil	Branched	Dark brown
31	FRWW11	Septate	Uniseriate	Ellipsoidal	Nil	Unbranched	Yellow
32	FRhWW4	Septate	Biseriate	Spherical	Nil	Branched	Bluish-green
33	FRWS9	Septate	Uniseriate	Ellipsoidal	Nil	Unbranched	Dark brown
34	FLWM11	Septate	Uniseriate	Fusiform	Nil	Unbranched	Pale grey
35	FLWW13	Septate	Uniseriate	Ellipsoidal	Nil	Branched	Dark brown
36	FLWS5	Septate	Uniseriate	Fusiform	Nil	Unbranched	Hyaline

Table 31: Micro-morphological characters of endophytic fungal isolates

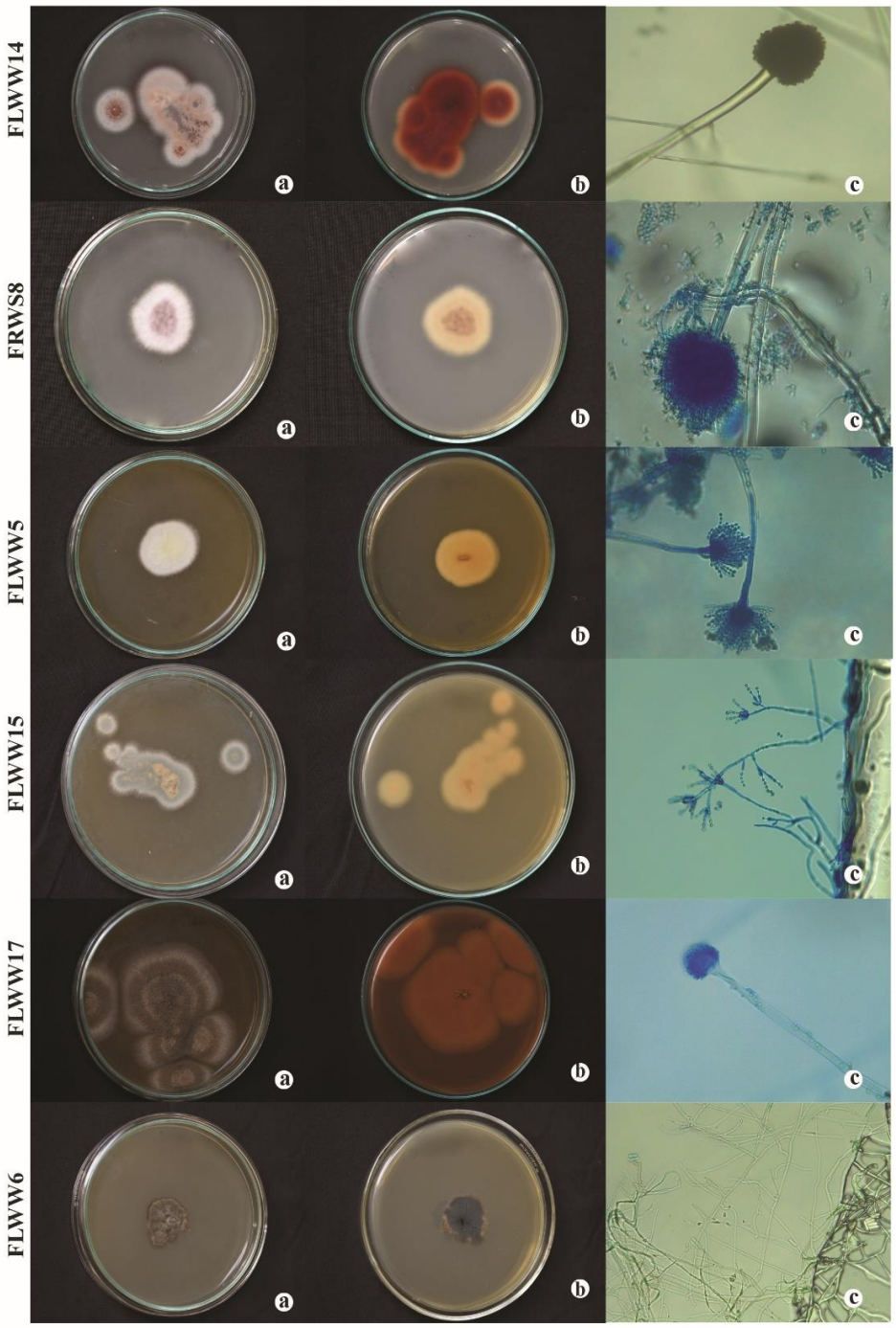


Fig.46 Endophytic fungal isolates. a - Colonies upper view; b - Colonies lower view; e - Cells

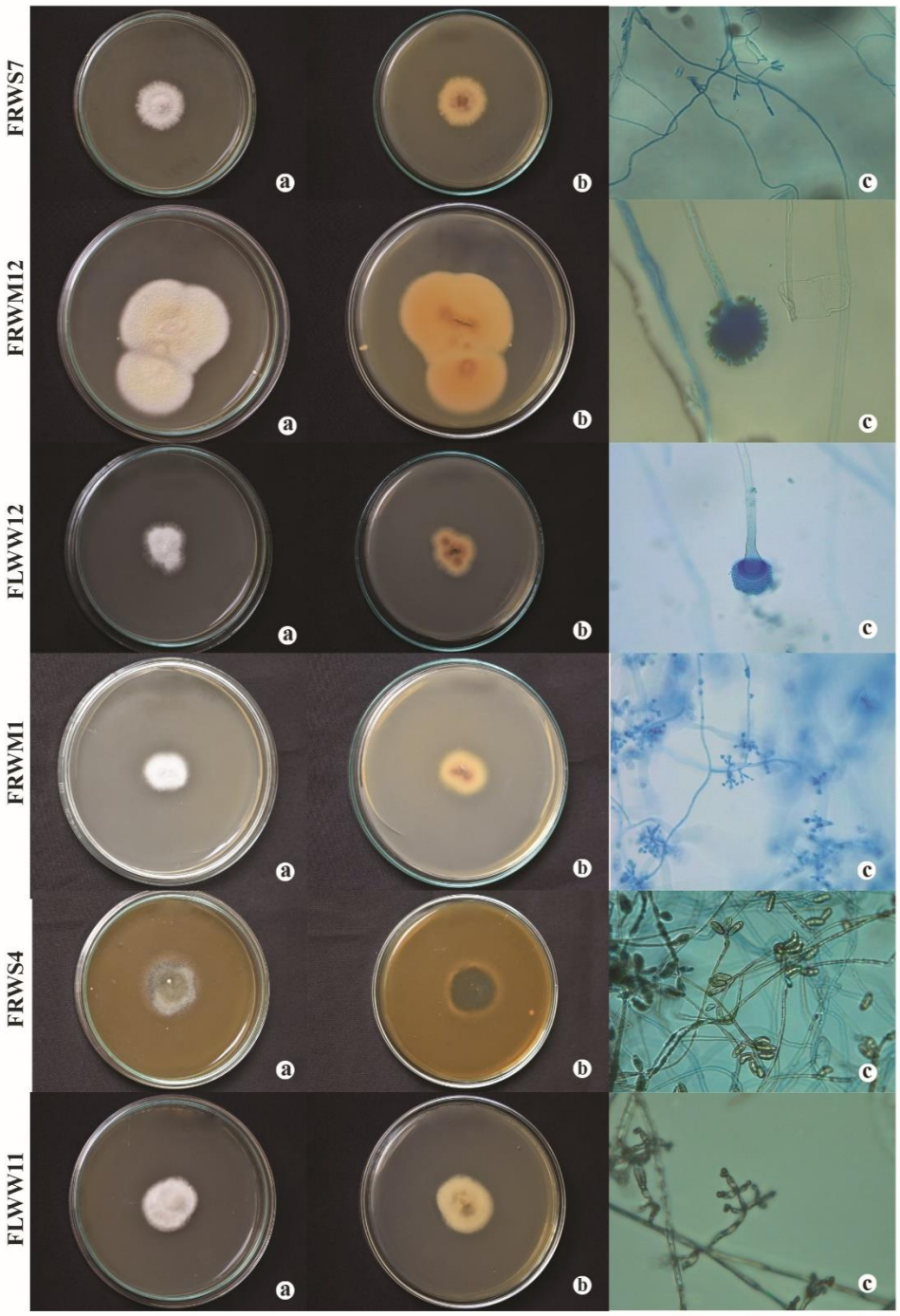


Fig.46a Endophytic fungal isolates. a - Colonies upper view; b - Colonies lower view; e - Cells

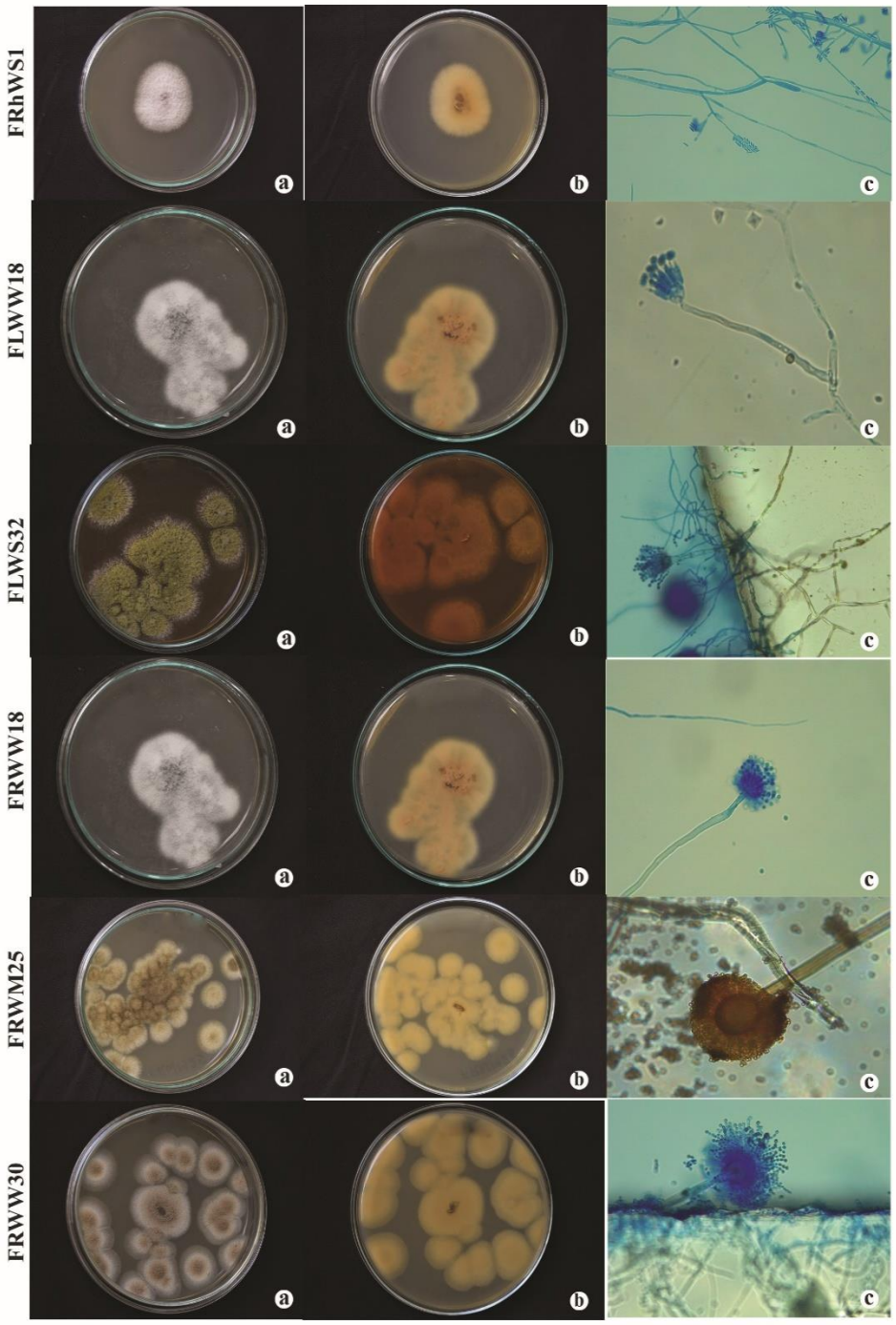


Fig.8b Endophytic fungal isolates. a - Colonies upper view; b - Colonies lower view; e - Cells

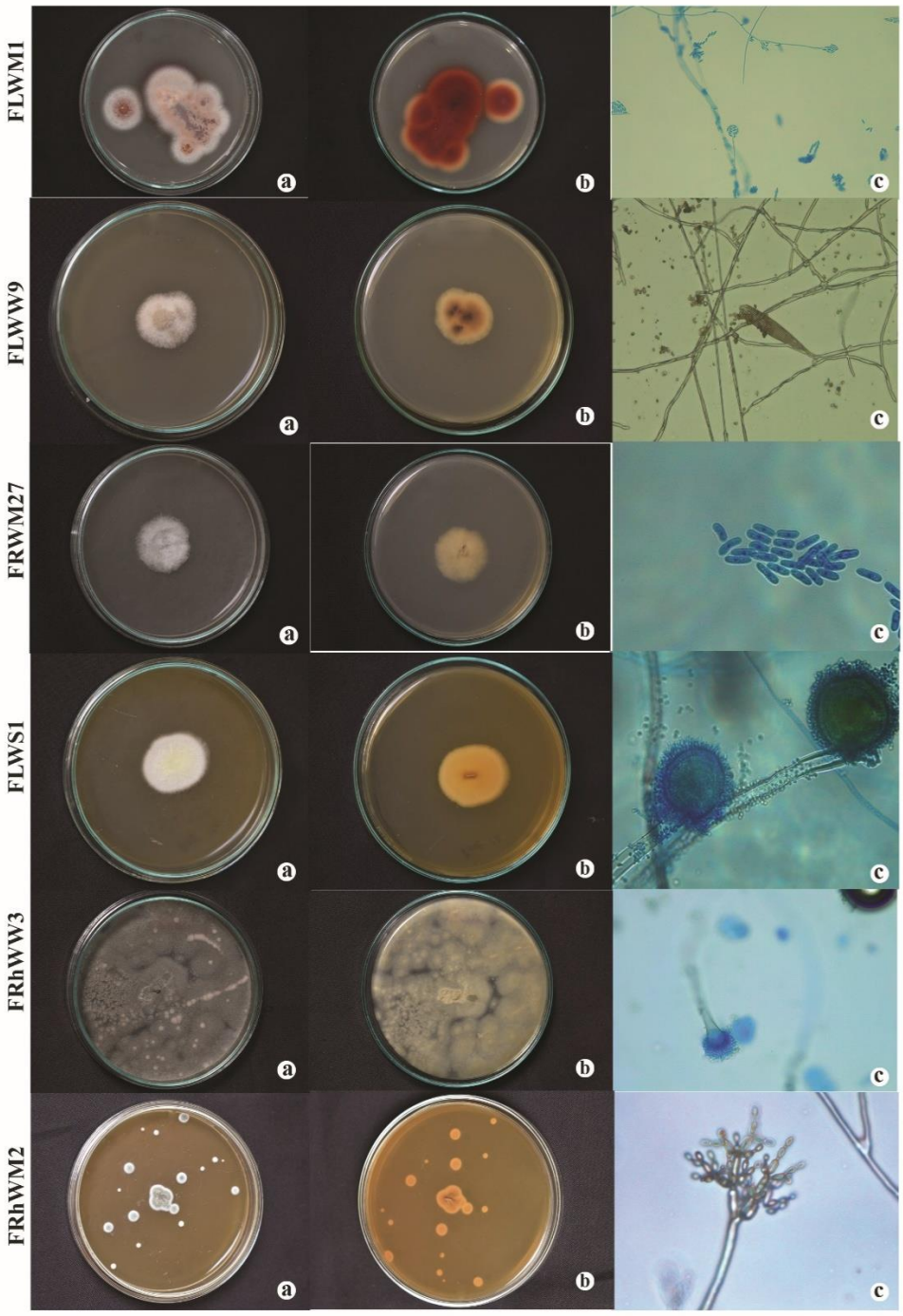


Fig.8c Endophytic fungal isolates. a - Colonies upper view; b - Colonies lower view; e - Cells

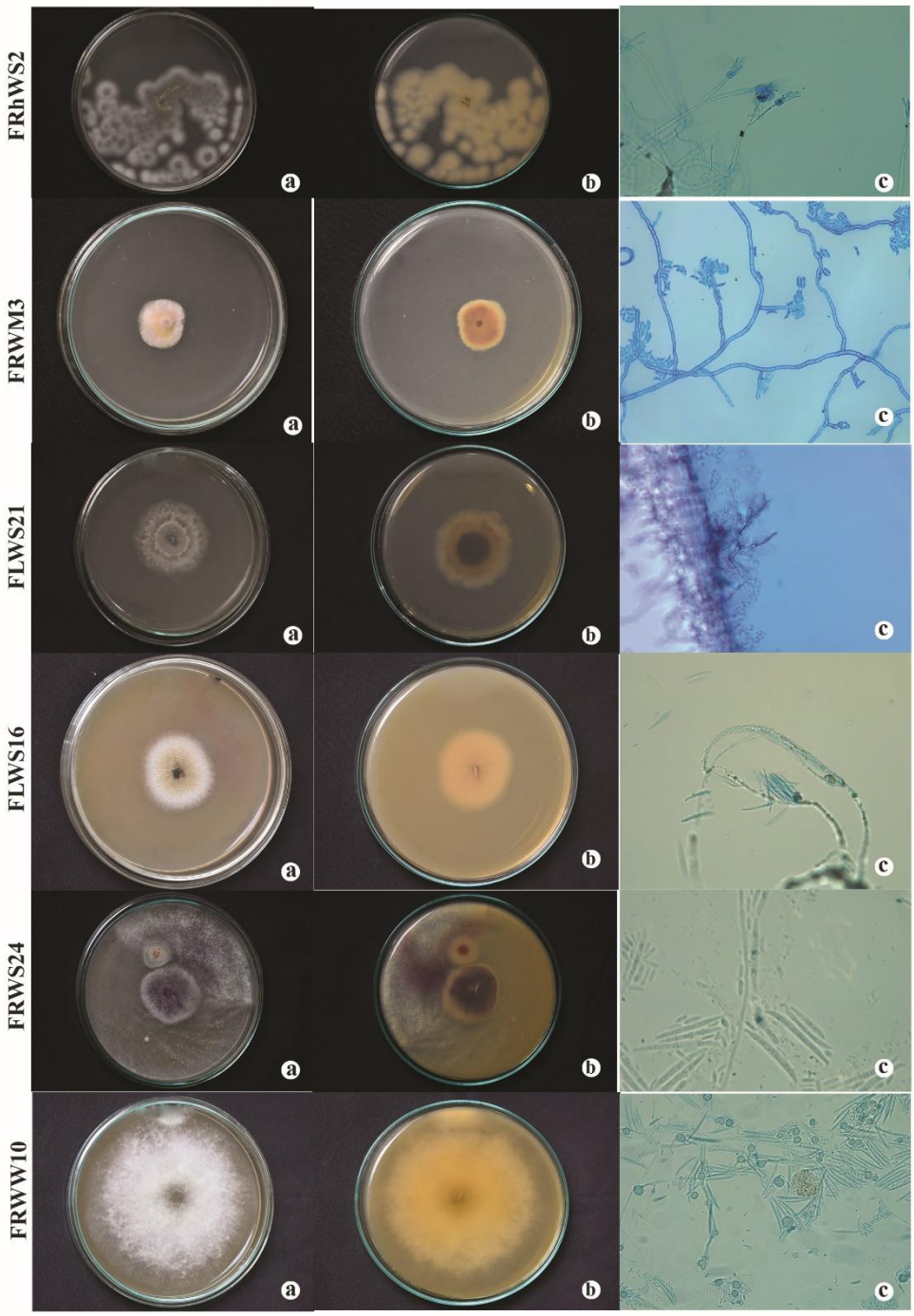


Fig.8d Endophytic fungal isolates. a - Colonies upper view; b - Colonies lower view; e - Cells

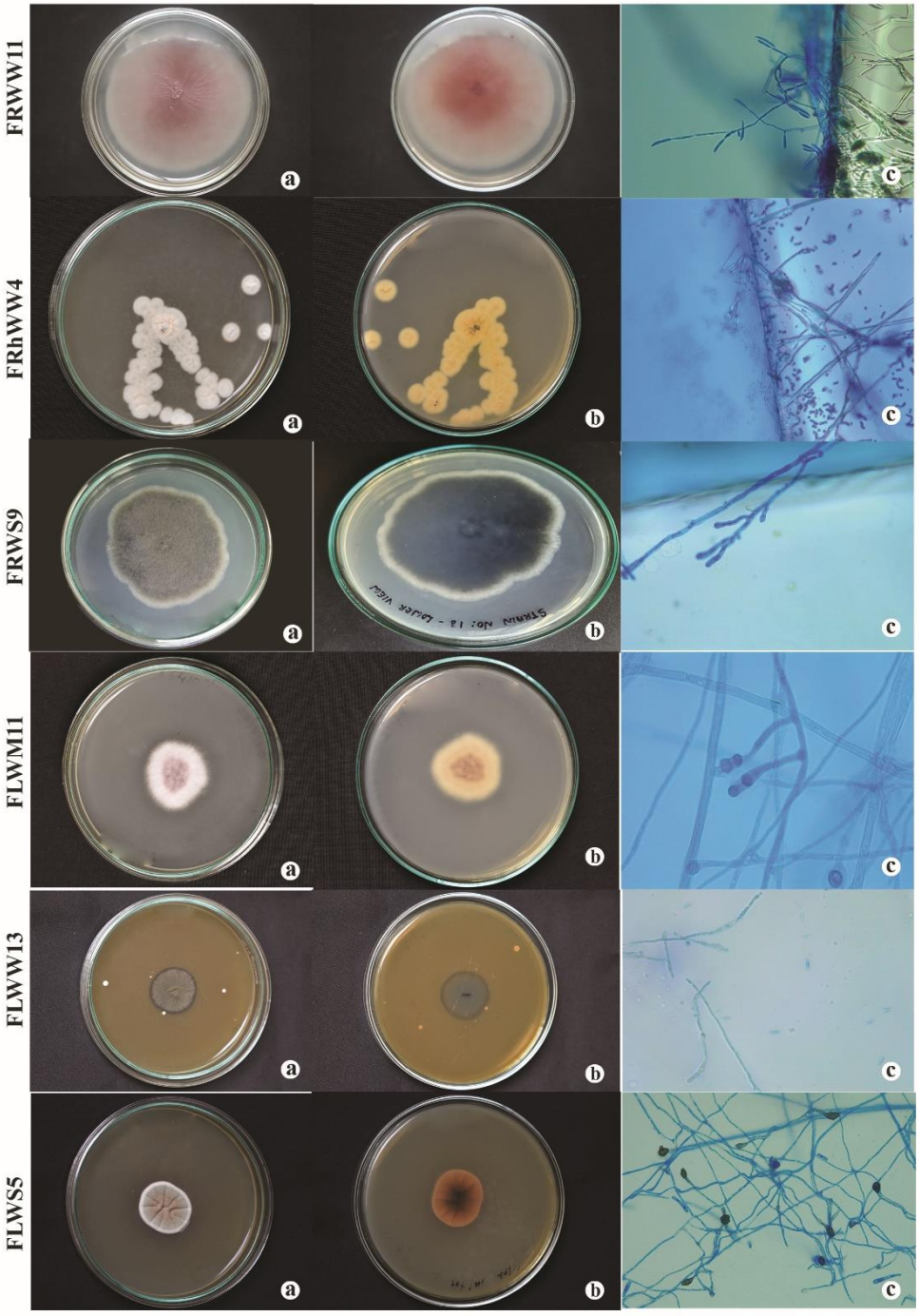


Fig.8e Endophytic fungal isolates. a - Colonies upper view; b - Colonies lower view; e - Cells

Molecular Taxonomical Identification

Molecular taxonomic characterization of the isolates involved genomic DNA extraction, PCR amplification of the ITS region, sequencing, and sequence analysis. The DNA quality was confirmed with A260/A280 ratios between 1.6 and 1.8. Genomic DNA was resolved on 1.0% agarose gels containing 10 µg/mL ethidium bromide. PCR amplification using universal primers ITS1 and ITS4 yielded products of approximately 540-600 bp, visualized on 1% agarose gels (Fig. 47). Sequence assembly was performed using BIOEDIT v7.2.5's contig assembly program, generating nearly full-length sequences. The sequences were analyzed in the BLAST alignment program of the Genbank database. To confirm the relationships between 30 other strains and the isolates, a maximum likelihood analysis was conducted with 1000 bootstrap replications, and phylogenetic trees were constructed using MEGA 11 (Fig.48 - Fig.83) and a tree constructed for showing the relationship of all isolated endophytic fungi (Fig.84). In the tree, each isolate is clustered in a strongly supported clade with a reference strain. Based on the similarity results, a comparison of the ITS gene sequences and morphological characteristics concluded the identity of isolates. Among the identified isolates, *Aspergillus aculeatus*, *Aspergillus terreus*, *Pencillium oxalicum*, *Aspergillus niger*, and *Fusarium odoratissimum* are the isolates that are most frequently colonized in the various segments in different seasons of 16.7%, 13.02%, 12.9%, 11.74%, and 11.07% respectively. The lowest percentage of colonization frequency was observed in the isolates *Didymella pedeiae* (0.003%), *Phanerochaete ordida* (0.006%), and *Fusarium sp.* 2 (0.007%) The colonization frequency of identified fungal isolates is represented in Fig.85. The details of the isolates with strain code, similarity index, identity, family, and accession number are shown in Table 32.

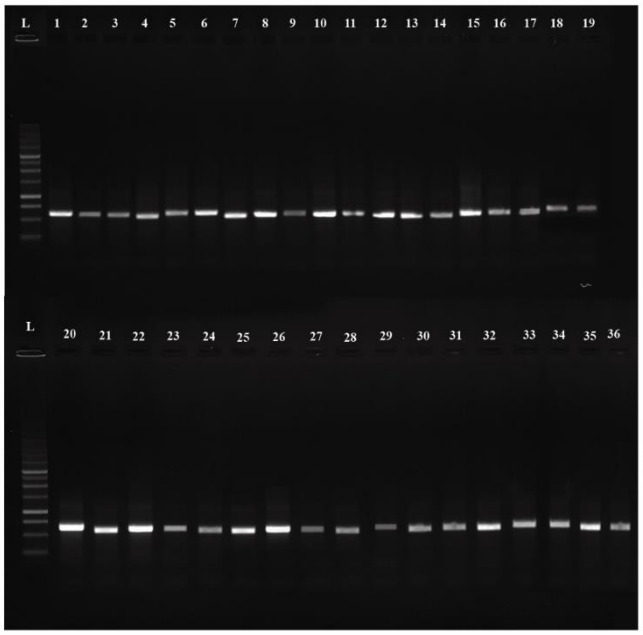


Fig. 47 ITS region amplification of endophytic fungal DNA

Sl No.	Strain code	% of similarity	Identity	Family	Accession No.
1	FLWW14	98.65	*Rhizopus sp.*	Mucoraceae	PQ165882
2	FRWS8	95.66	*Aspergillus aculeatus*	Aspergillaceae	PQ165881
3	FLWW5	99.49	*Aspergillus terreus*	Aspergillaceae	PQ197657
4	FLWW15	98.66	*Penicillium oxalicum*	Aspergillaceae	PQ197655
5	FLWW17	98.29	*Aspergillus trinidadensis*	Aspergillaceae	PQ197648
6	FLWW6	93.94	*Ectophoma multirostrata*	Didymellaceae	PQ219763
7	FRWS7	94.63	*Colletotrichum gloeosporioides*	Glomerellaceae	PQ219473
8	FRWM12	99.66	*Aspergillus sp.* 1	Aspergillaceae	PQ197647
9	FRWS4	98.99	*Curvularia geniculata*	Pleosporaceae	PQ166595
10	FLWW11	95.07	*Trichoderma harzianum*	Hypocreaceae	PQ166527
11	FRhWS1	94.82	*Fusarium proliferatum*	Nectriaceae	PQ197636
12	FLWW18	92.29	*Talaromyces verruculosus*	Trichocomaceae	PQ197638
13	FLWS32	99.66	*Aspergillus flavus*	Aspergillaceae	PQ168921
14	FRWW18	95.31	*Aspergillus sp.* 2	Aspergillaceae	PQ219309
15	FRWM25	98.71	*Aspergillus niger*	Aspergillaceae	PQ168922
16	FRWW30	94.85	*Aspergillus japonicus*	Aspergillaceae	PQ197427
17	FLWM1	97.67	*Fusarium odoratissimum*	Nectriaceae	PQ181486
18	FLWW9	98.59	*Corynespora cassicola*	Corynesporascaceae	PQ168926
19	FRWM27	83.76	*Meyerozyma guilliermondii*	Debaryomycetaceae	PQ181464
20	FLWS1	99.83	*Aspergillus sp.* 3	Aspergillaceae	PQ168931
21	FRhWW3	95.17	*Aspergillus fumigatus*	Aspergillaceae	PQ168932
22	FRhWM2	99.34	*Paecilomyces variotii*	Trichocomaceae	PQ197586
23	FRhWS2	99.16	*Penicillium chrysogenum*	Aspergillaceae	PQ181462
24	FRWM3	97.86	*Fusarium sp.* 1	Nectriaceae	PQ181461
25	FLWS16	97.19	*Phanerochaete sordida*	Phanerochaetaceae	PQ181460
26	FRWS24	96.90	*Fusarium verticilliodes*	Nectriaceae	PQ197587

27	FRWW10	99.11	*Fusarium chlamydosporum*	Nectriaceae	PQ181298
28	FLWW12	94.29	*Aspergillus sp.* 4	Aspergillaceae	PQ181297
29	FRWM1	92.03	*Trichoderma inhamatum*	Hypocreaceae	PQ197588
30	FLWS21	96.47	*Diaporthe penetriteum*	Diaporthaceae	PQ168940
31	FRWW11	93.32	*Fusarium sp.* 2	Nectriaceae	PQ181291
32	FRhWW4	98.97	*Penicillium shearii*	Aspergillaceae	PQ181289
33	FRWS9	93.04	*Lasiodiplodia theobromae*	Botryosphaeriaceae	PQ219310
34	FLWM11	98.65	*Colletotrichum aenigma*	Glomerellaceae	PQ168941
35	FLWW13	95.76	*Didymella pedeiae*	Didymellaceae	PQ361983
36	FLWS5	97.93	*Colletotrichum cigarro*	Glomerellaceae	PQ168942

Table 32: Identification of selected endophytic fungal isolates

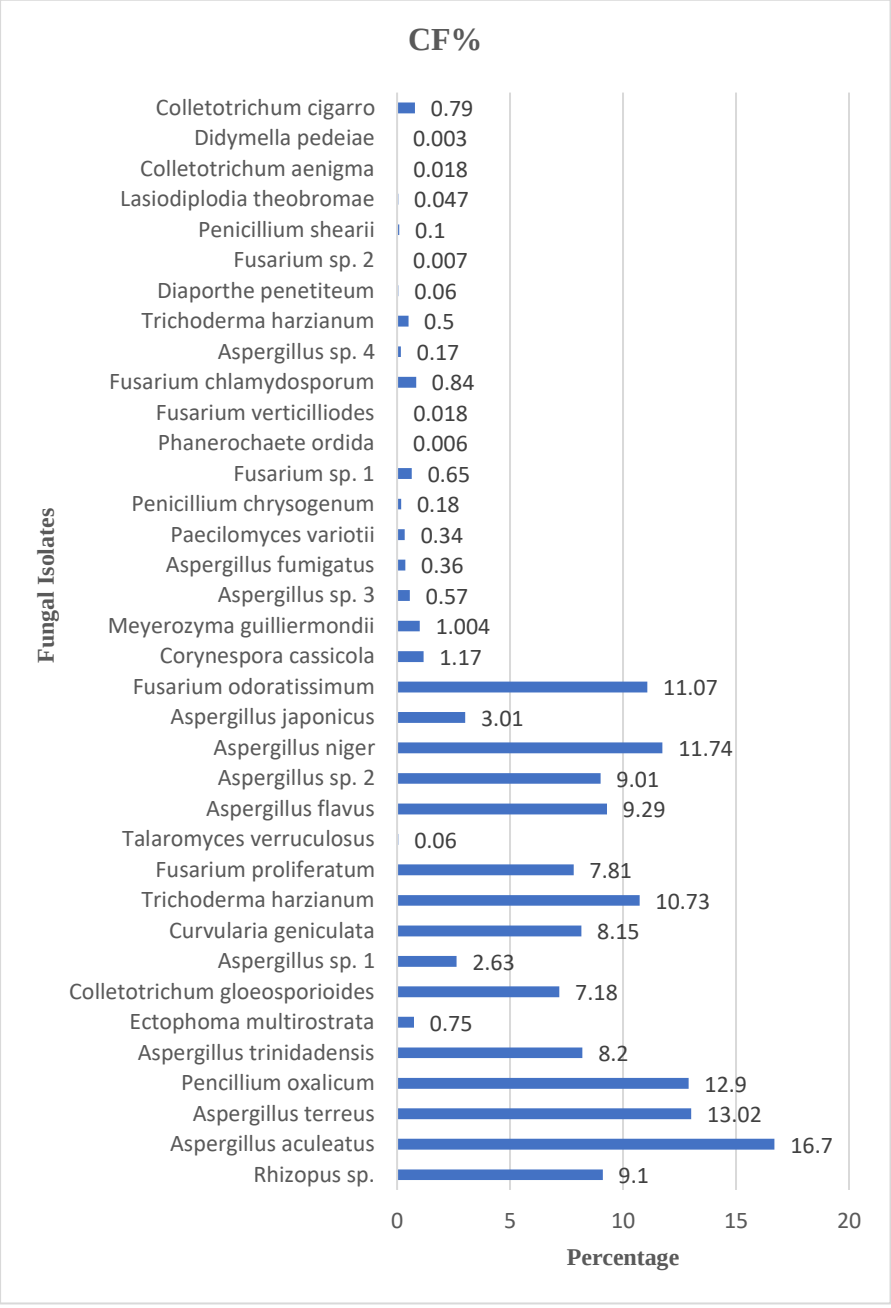


Fig.85 Colonization frequency of selected endophytic fungal isolates

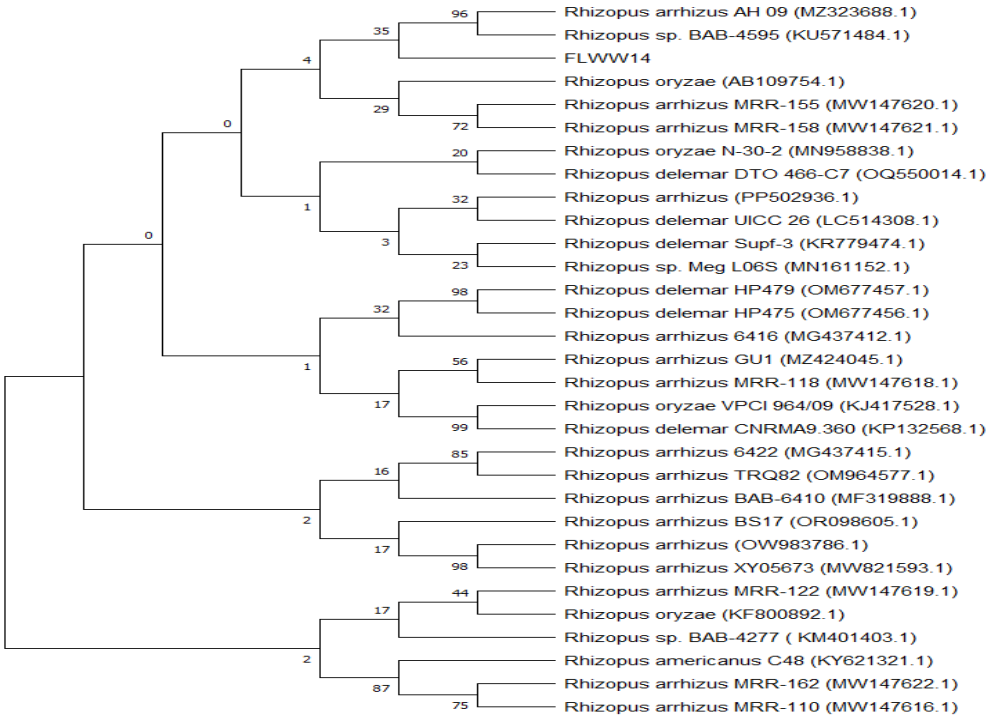


Fig.48 Maximum Likelihood phylogenetic tree of FLWW14

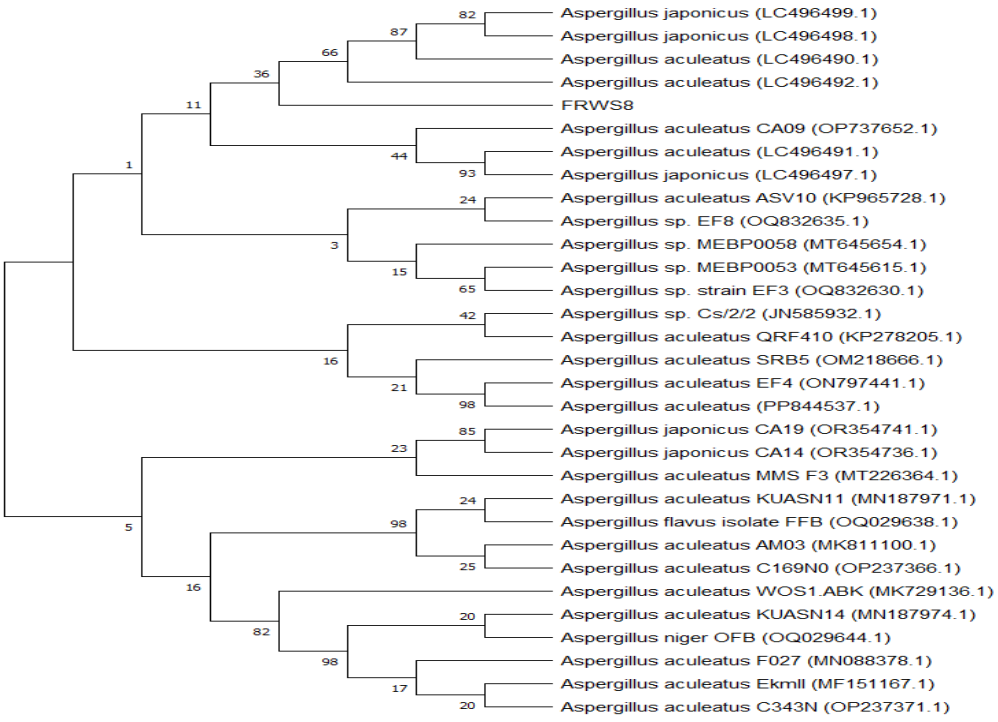


Fig.49 Maximum Likelihood phylogenetic tree of FRWS8

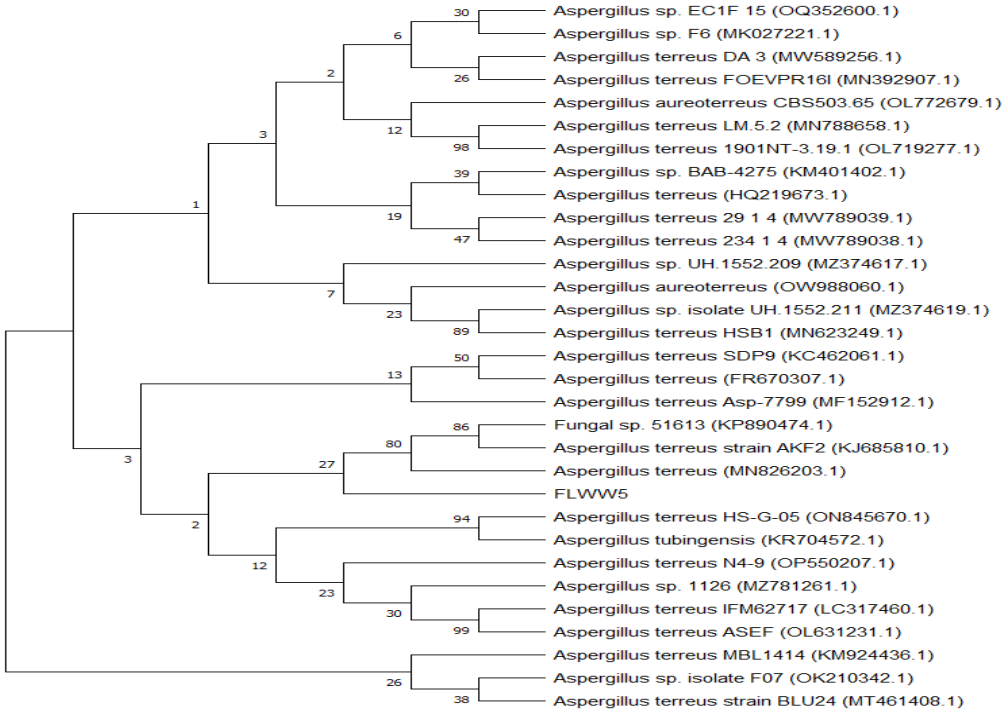


Fig.50 Maximum Likelihood phylogenetic tree of FLWW5

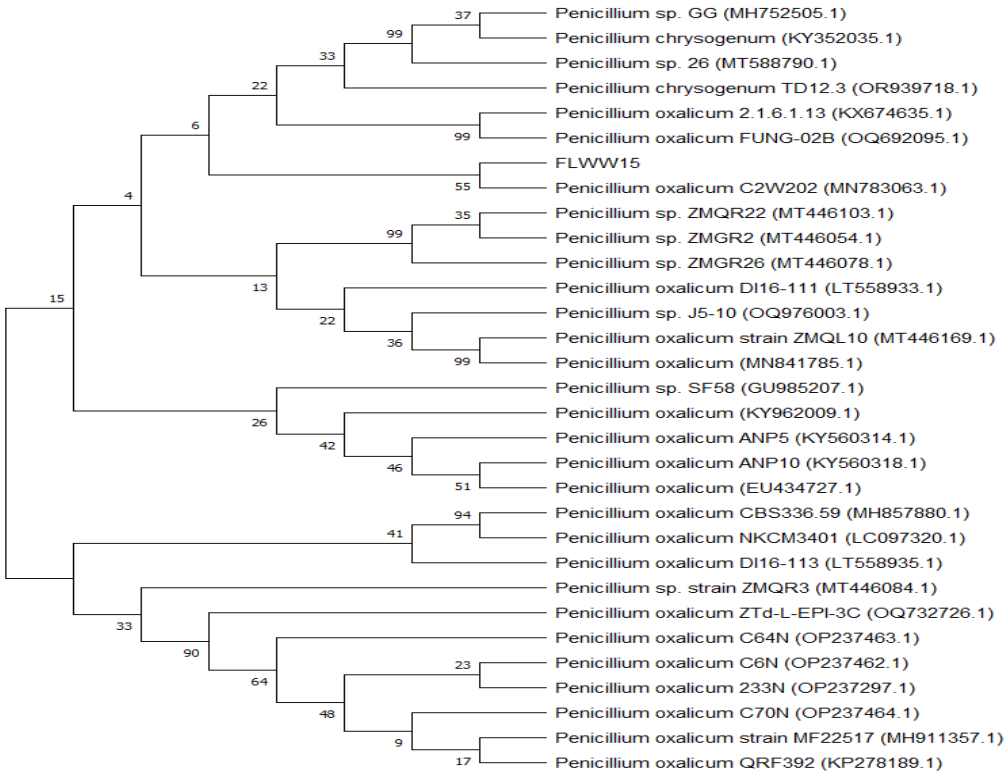


Fig.51 Maximum Likelihood phylogenetic tree of FLWW15

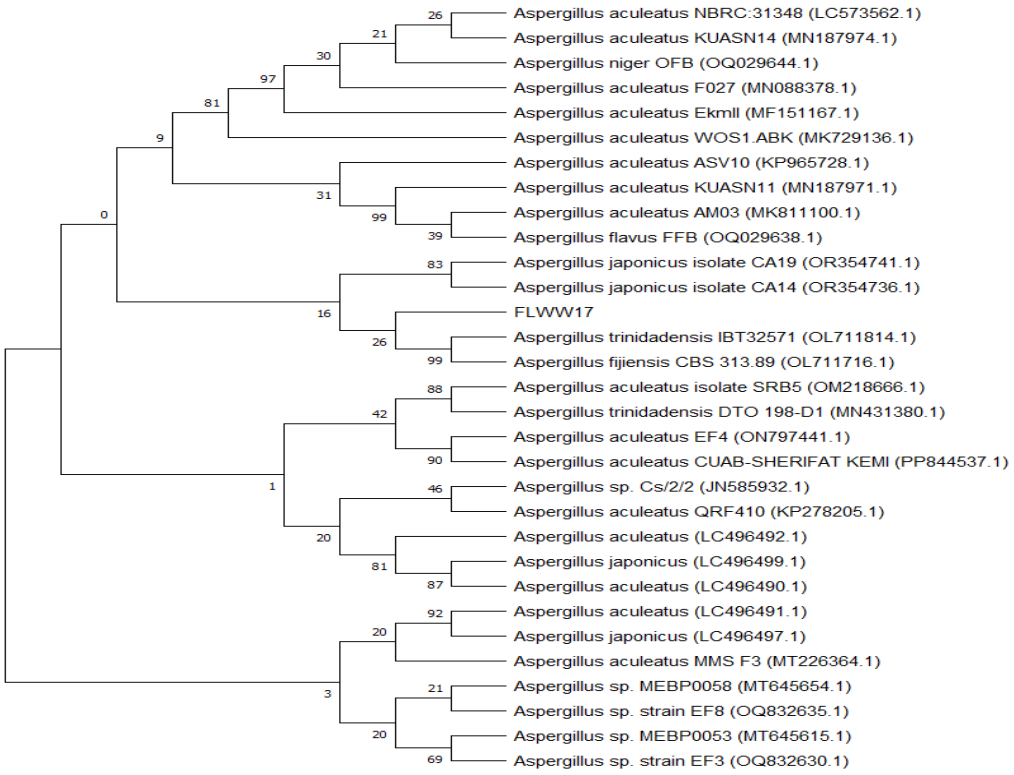


Fig.52 Maximum Likelihood phylogenetic tree of FLWW17

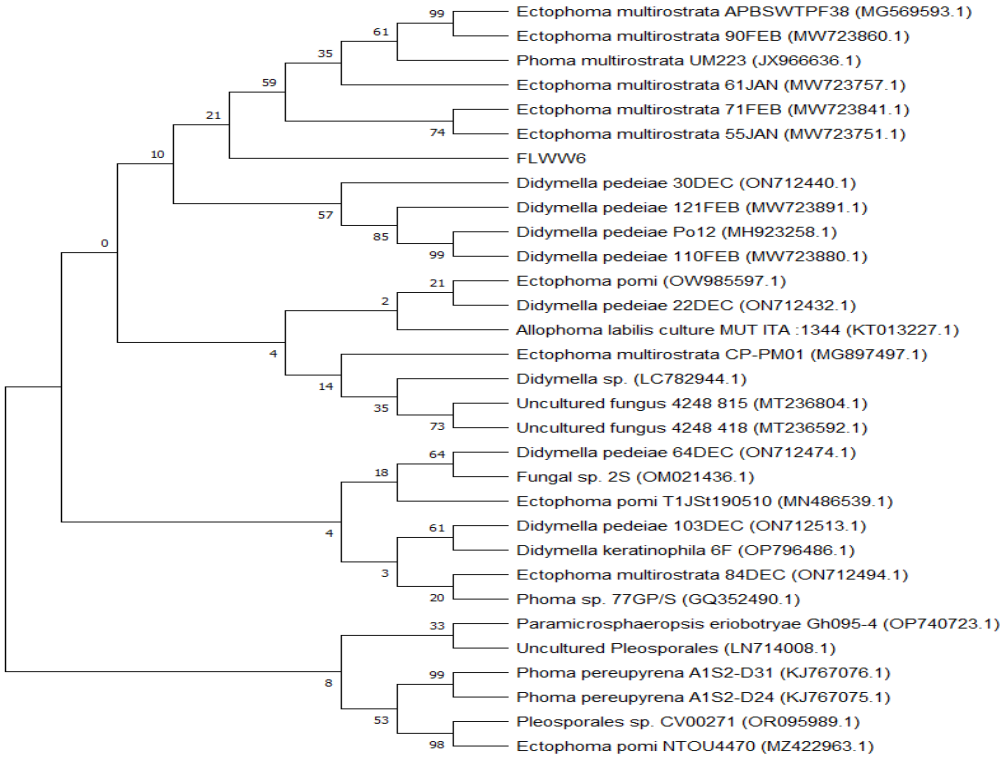


Fig.53 Maximum Likelihood phylogenetic tree of FLWW6

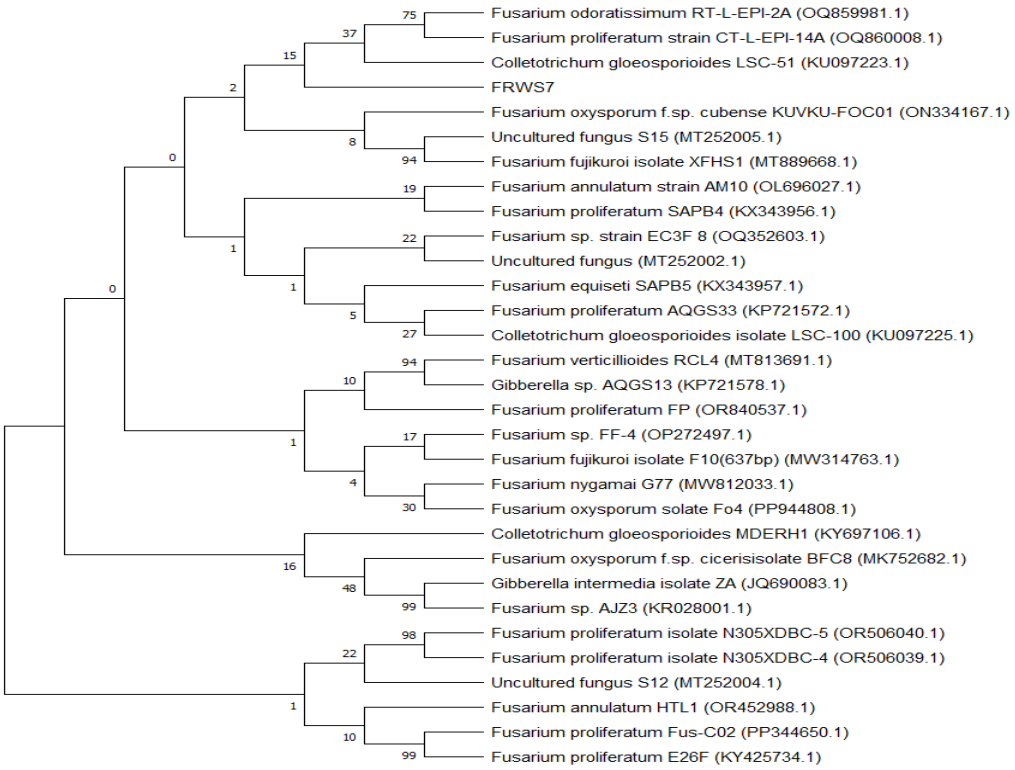


Fig.54 Maximum Likelihood phylogenetic tree of FRWS7

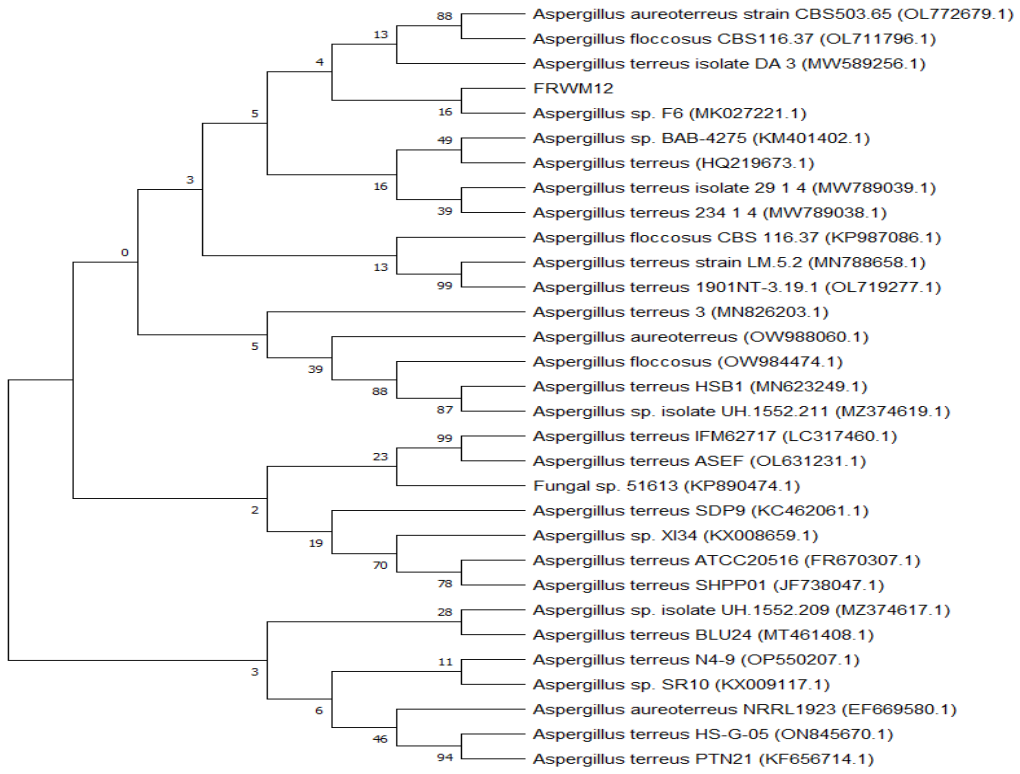


Fig.55 Maximum Likelihood phylogenetic tree of FRWM12

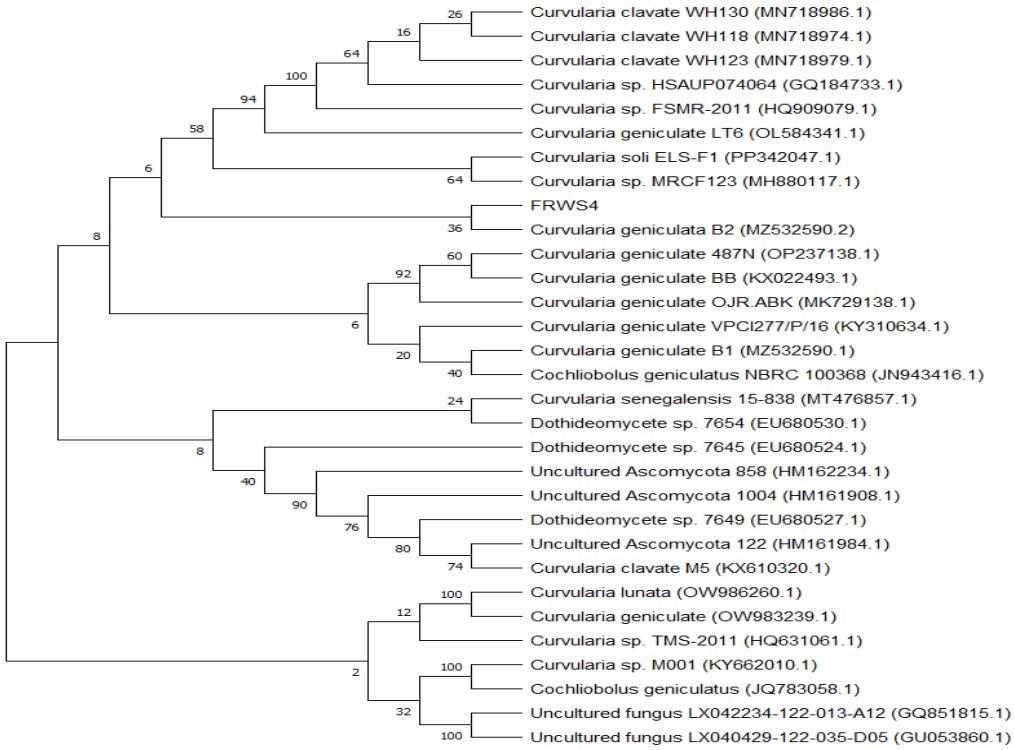


Fig.56 Maximum Likelihood phylogenetic tree of FRWS4

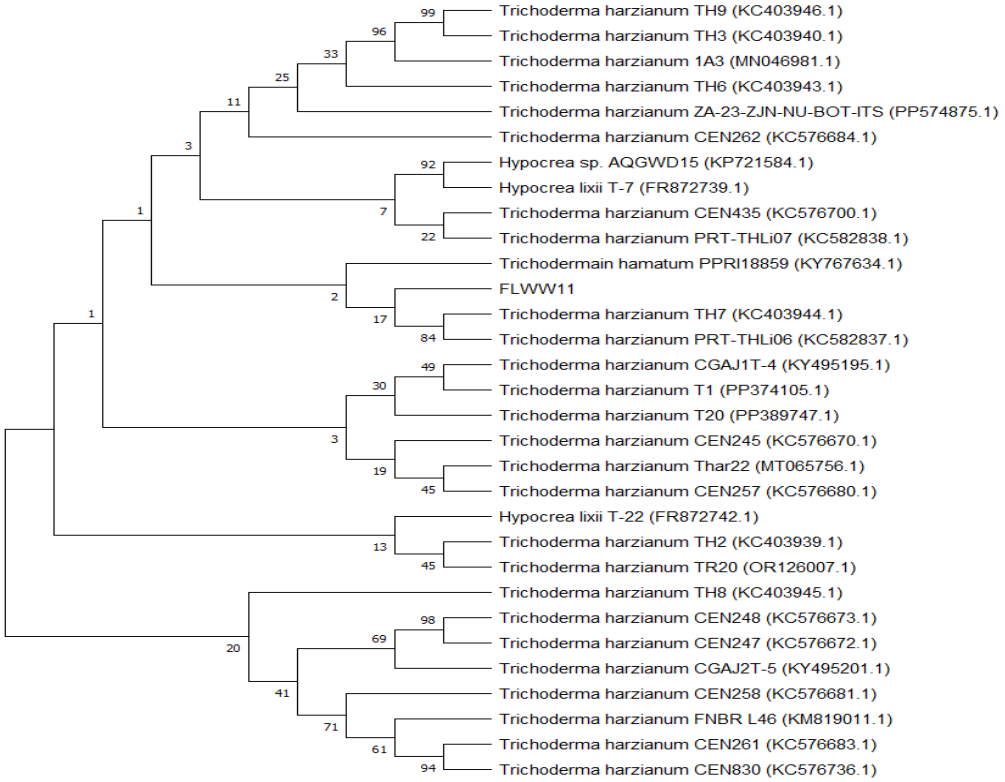


Fig.57 Maximum Likelihood phylogenetic tree of FLWW11

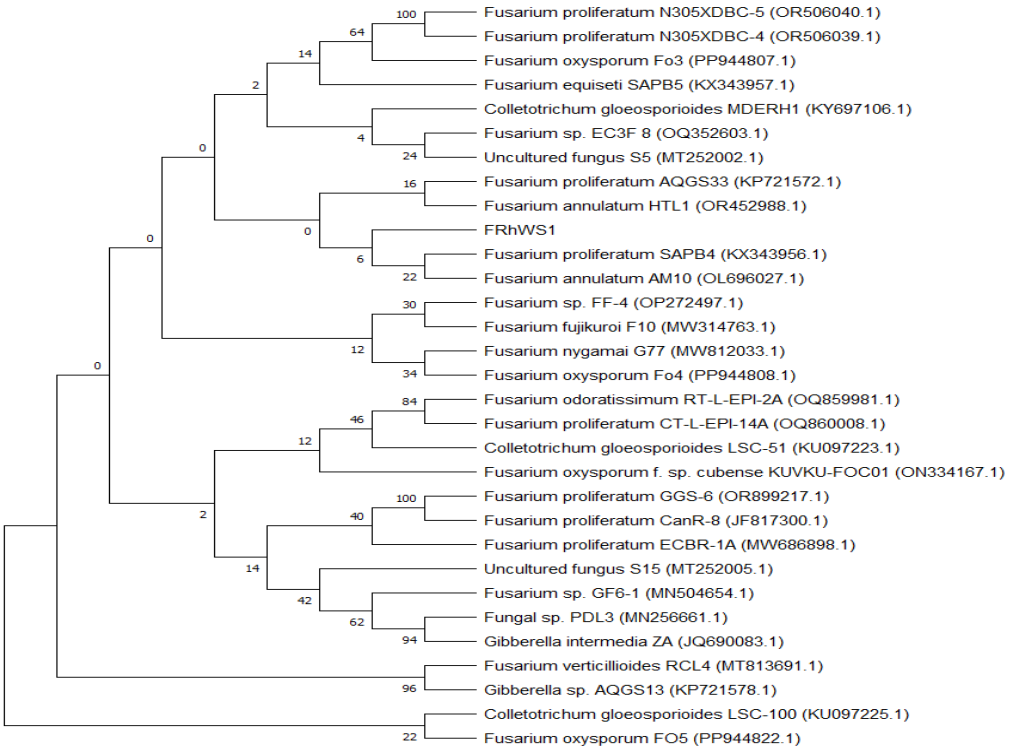


Fig.58 Maximum Likelihood phylogenetic tree of FRhWS1

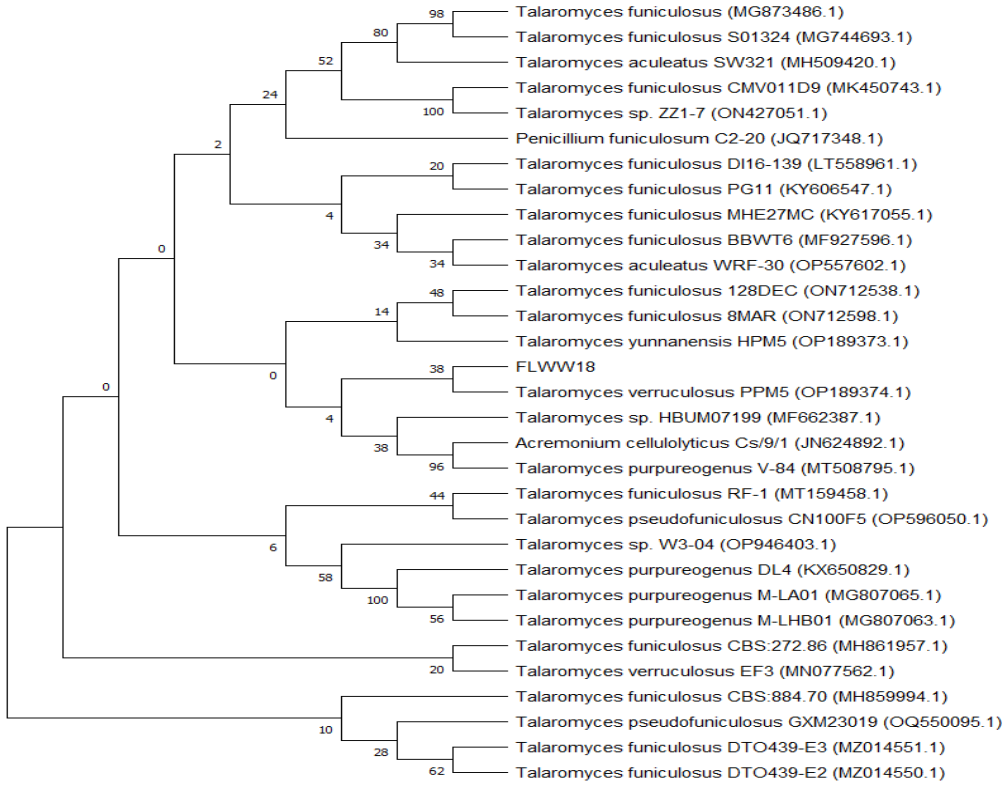


Fig.59 Maximum Likelihood phylogenetic tree of FLWW18

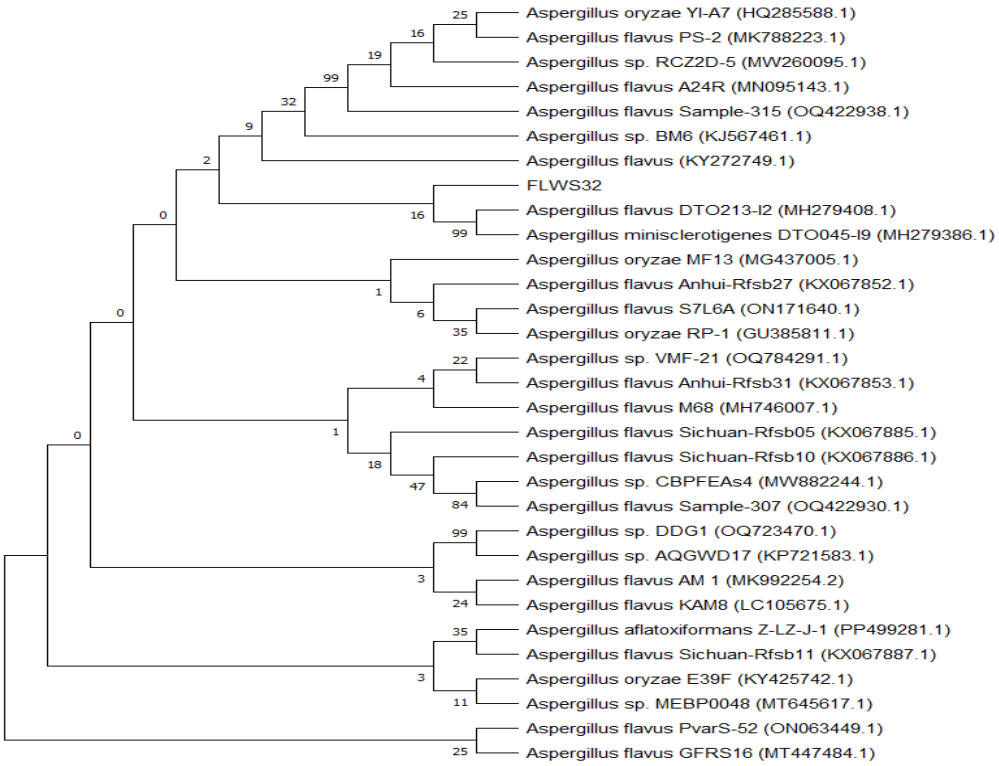


Fig.60 Maximum Likelihood phylogenetic tree of FLWS32

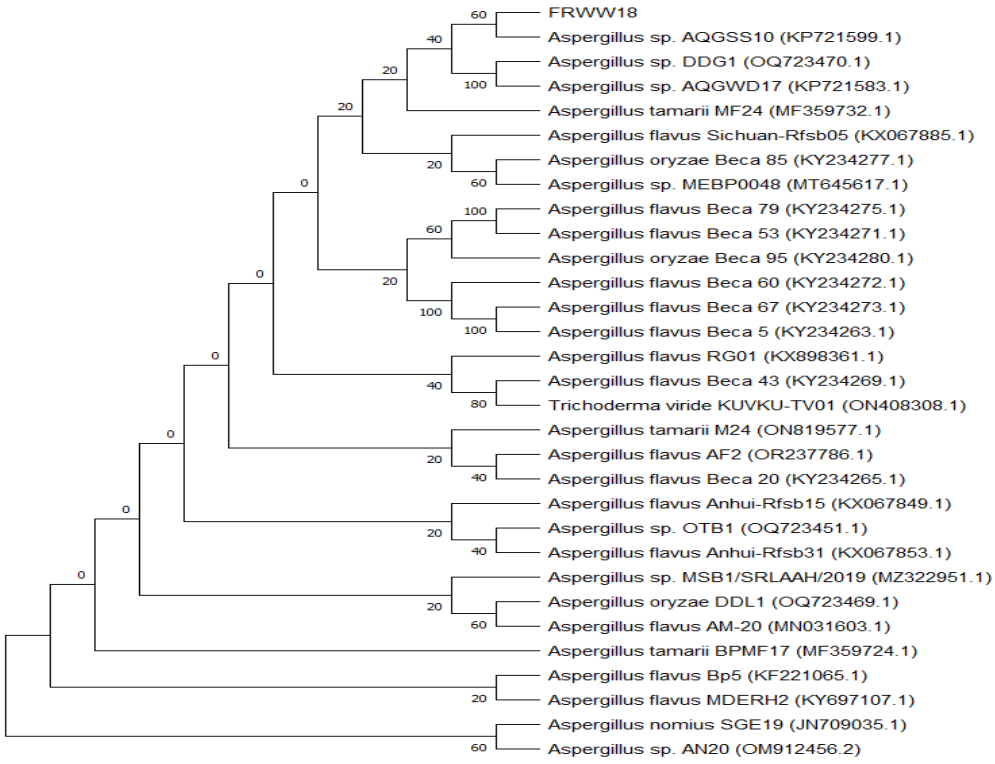


Fig.61 Maximum Likelihood phylogenetic tree of FRWW18

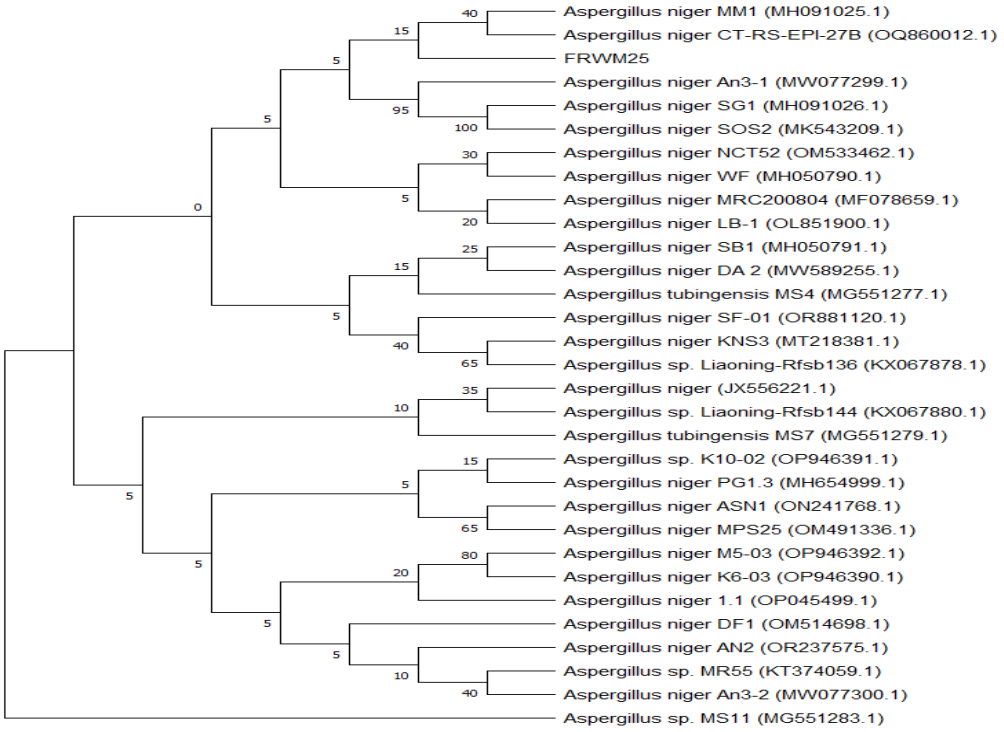


Fig.62 Maximum Likelihood phylogenetic tree of FRWM25

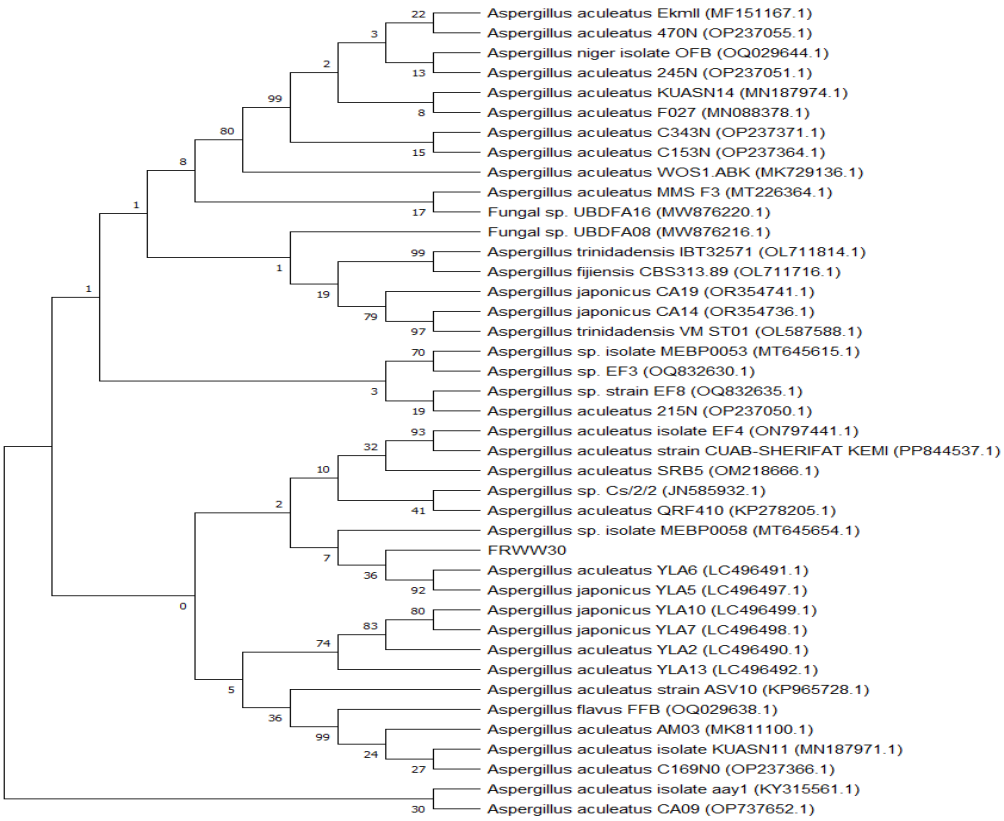


Fig.63 Maximum Likelihood phylogenetic tree of FRWW30

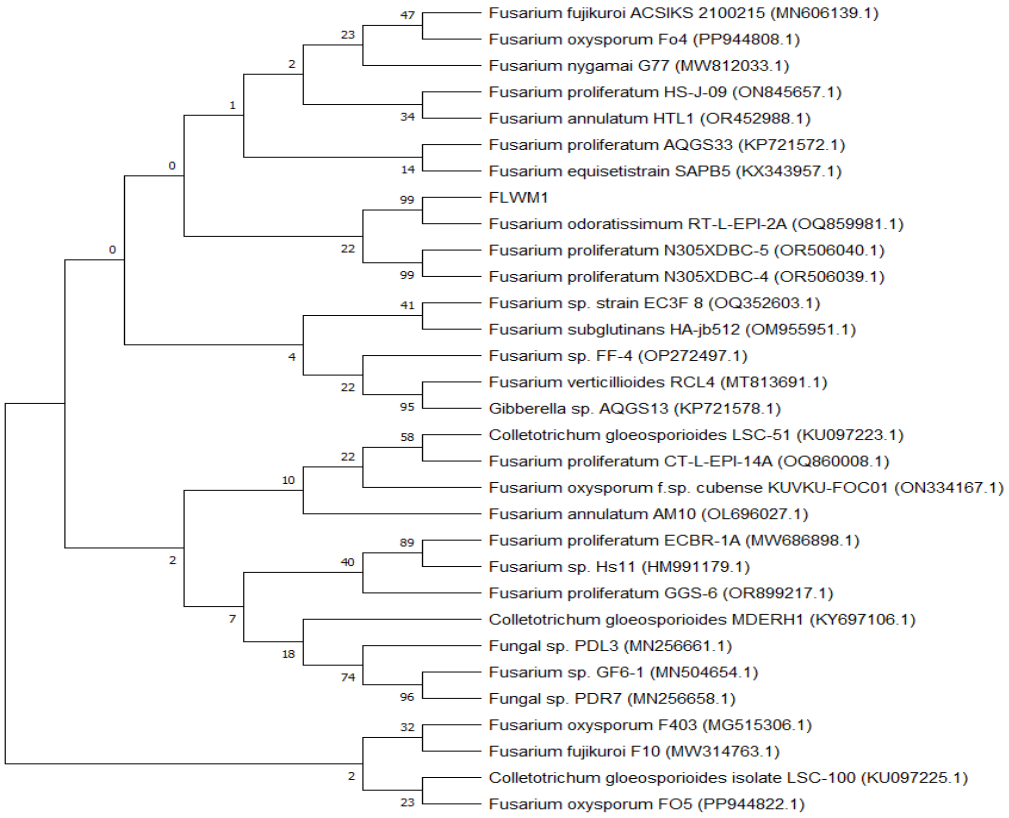


Fig.64 Maximum Likelihood phylogenetic tree of FLWM1

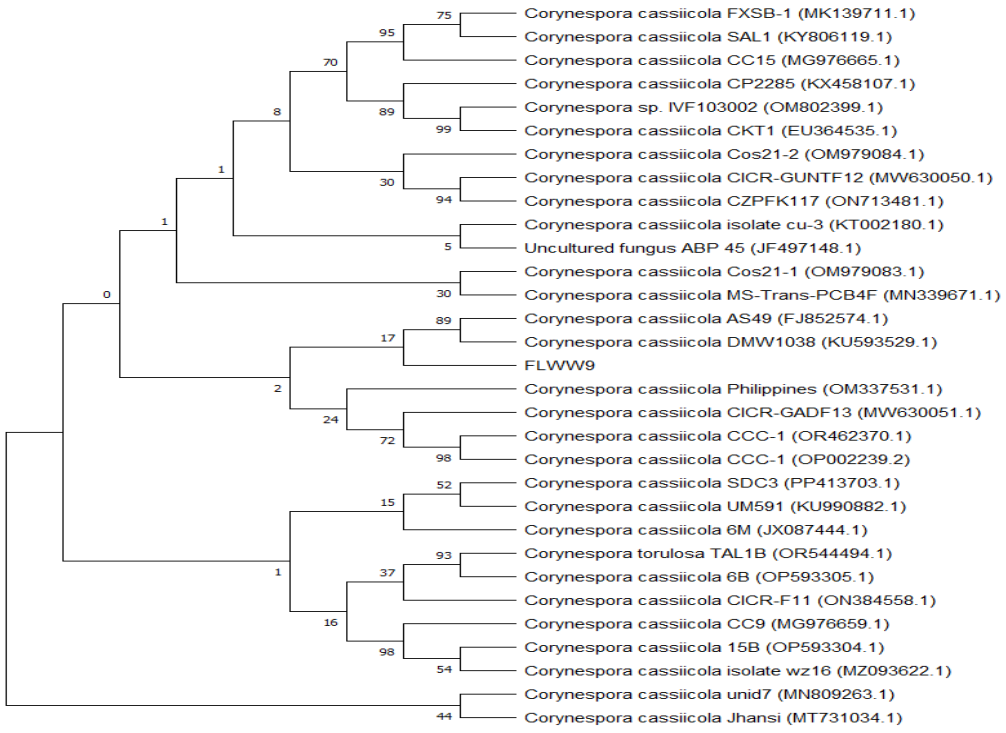


Fig.65 Maximum Likelihood phylogenetic tree of FLWW9

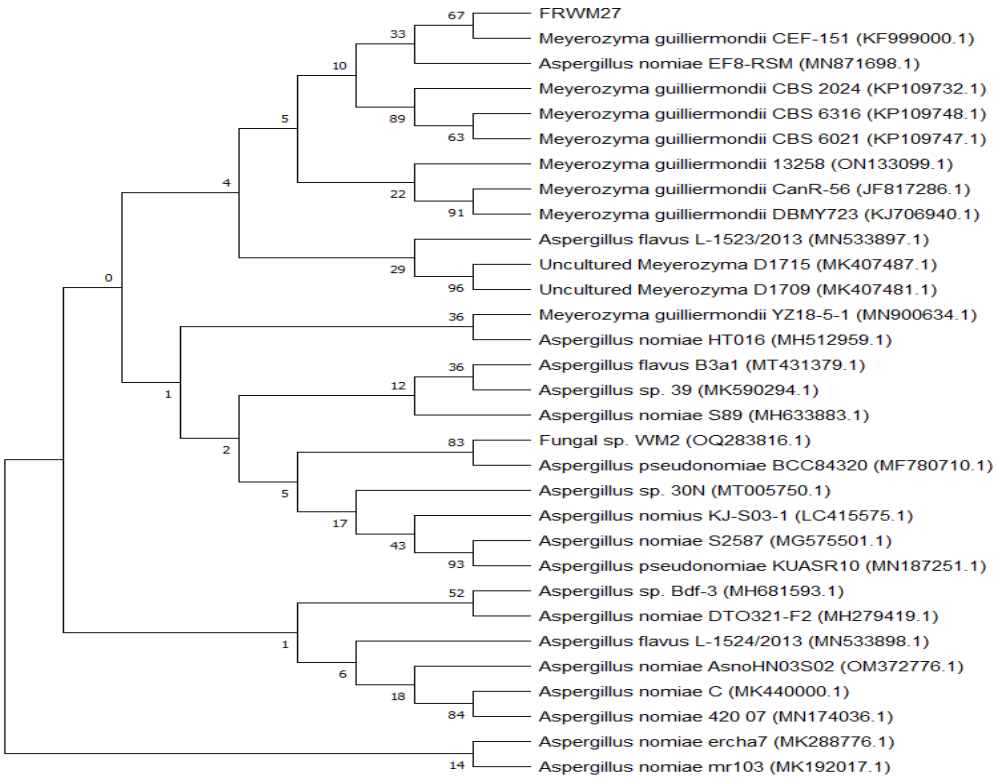


Fig.66 Maximum Likelihood phylogenetic tree of FRWM27

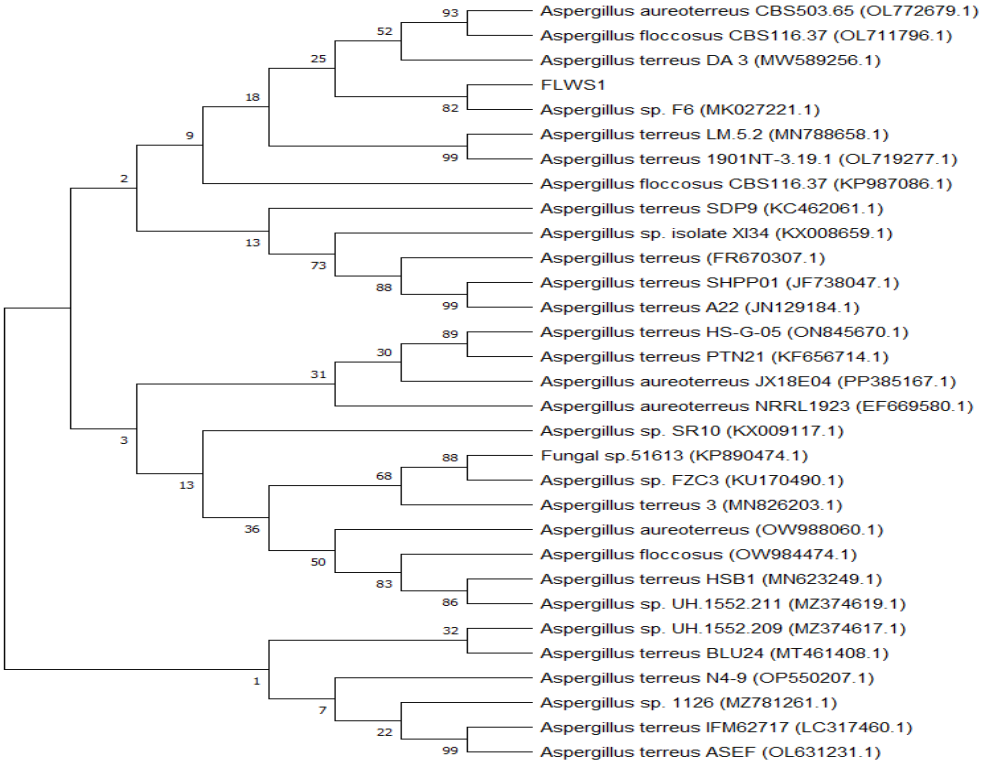


Fig.67 Maximum Likelihood phylogenetic tree of FLWS1

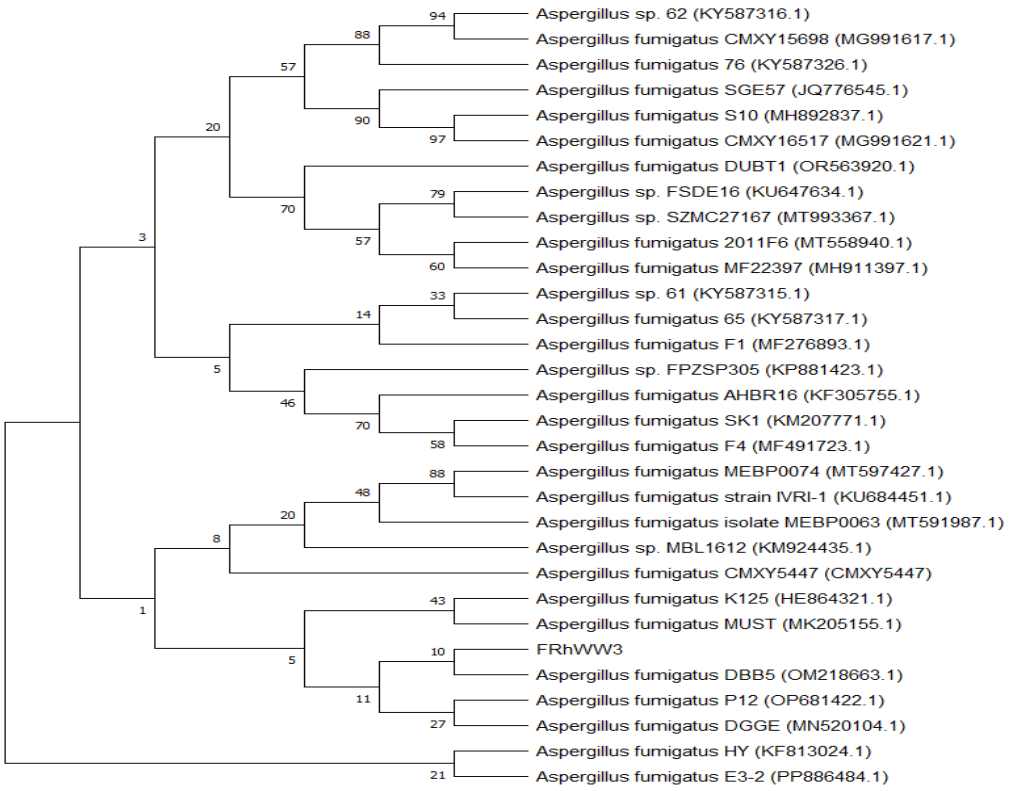


Fig.68 Maximum Likelihood phylogenetic tree of FRhWW3

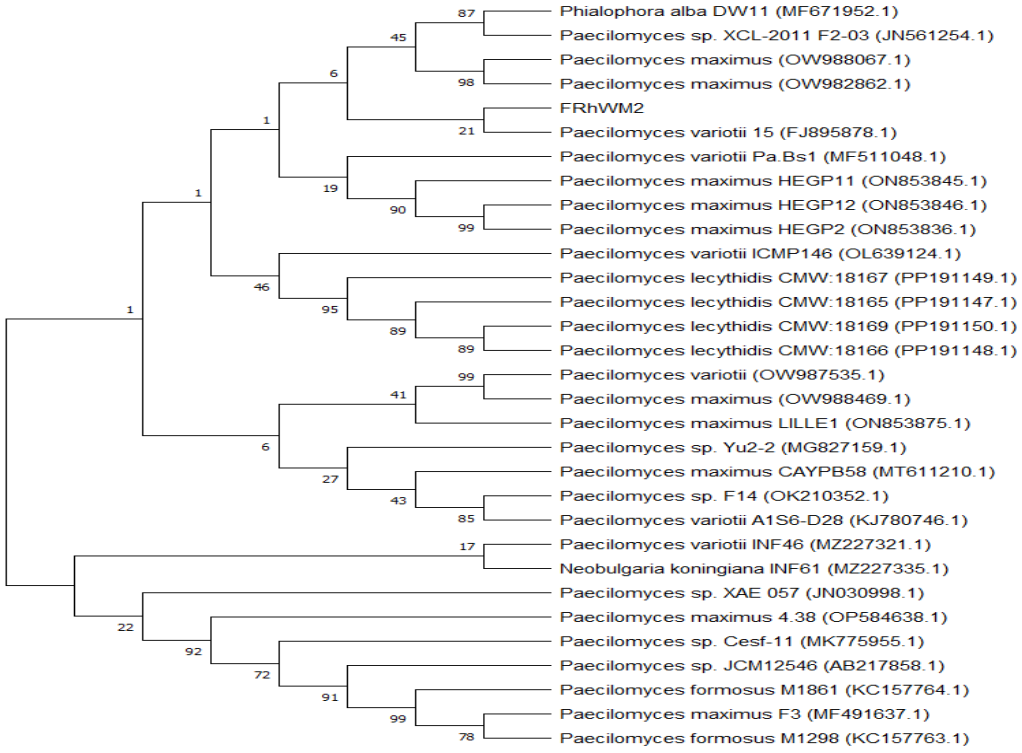


Fig.69 Maximum Likelihood phylogenetic tree of FRhWM2

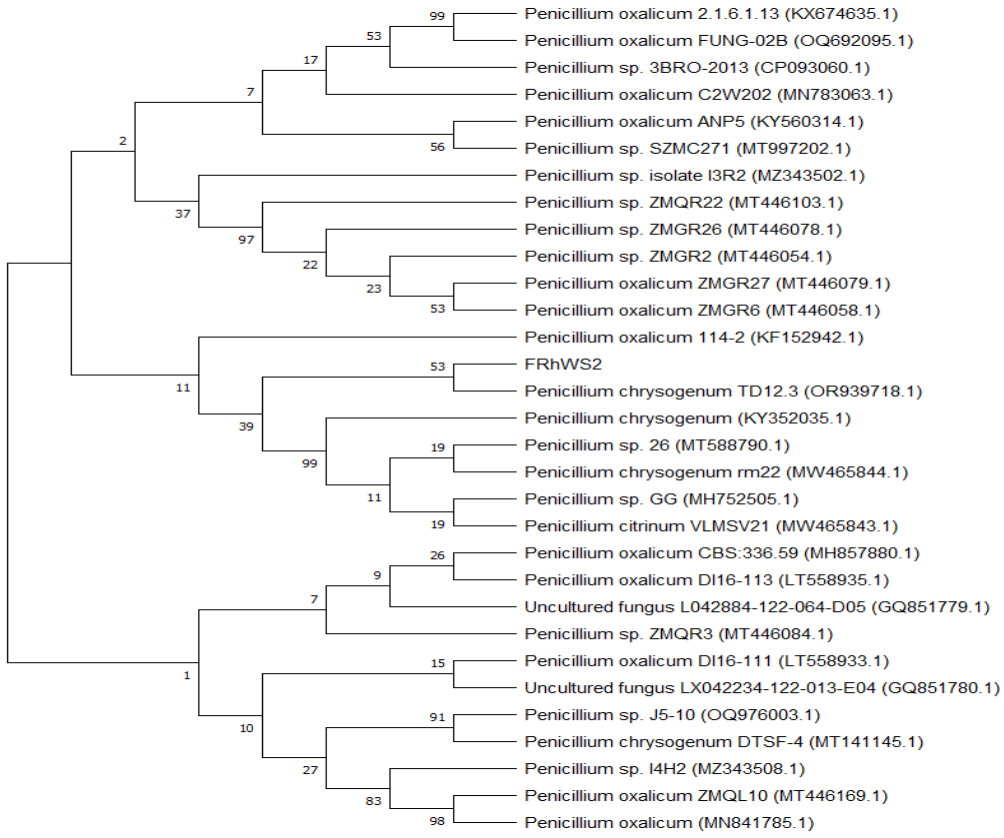


Fig.70 Maximum Likelihood phylogenetic tree of FRhWS2

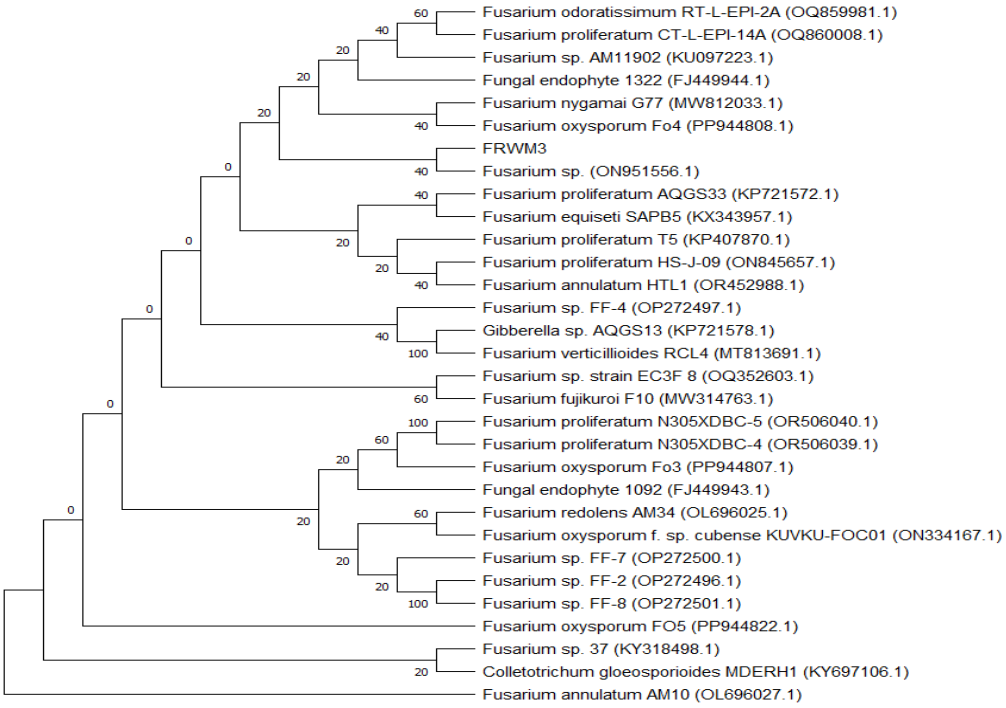


Fig.71 Maximum Likelihood phylogenetic tree of FRWM3

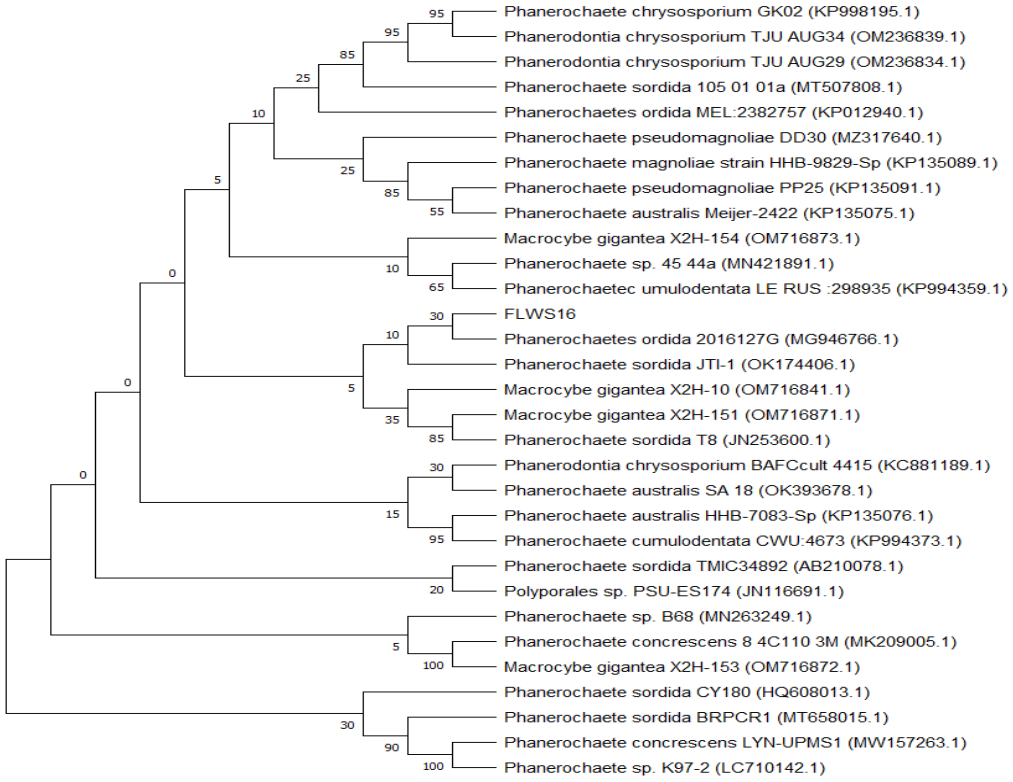


Fig.72 Maximum Likelihood phylogenetic tree of FLWS16

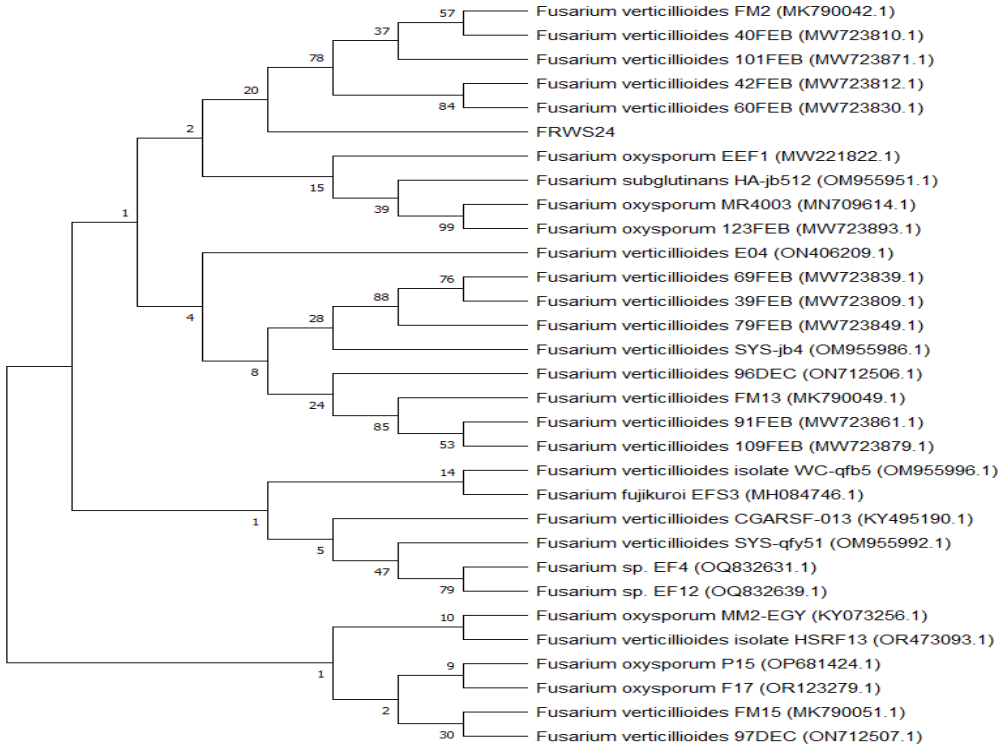


Fig.73 Maximum Likelihood phylogenetic tree of FRWS24

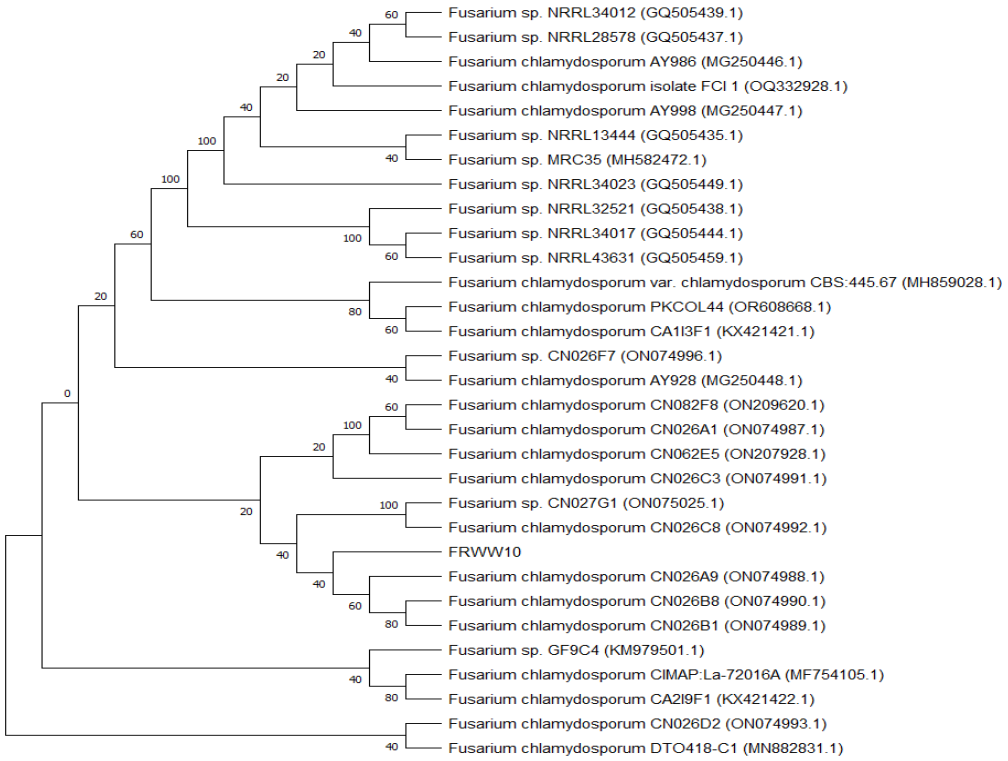


Fig.74 Maximum Likelihood phylogenetic tree of FRWW10

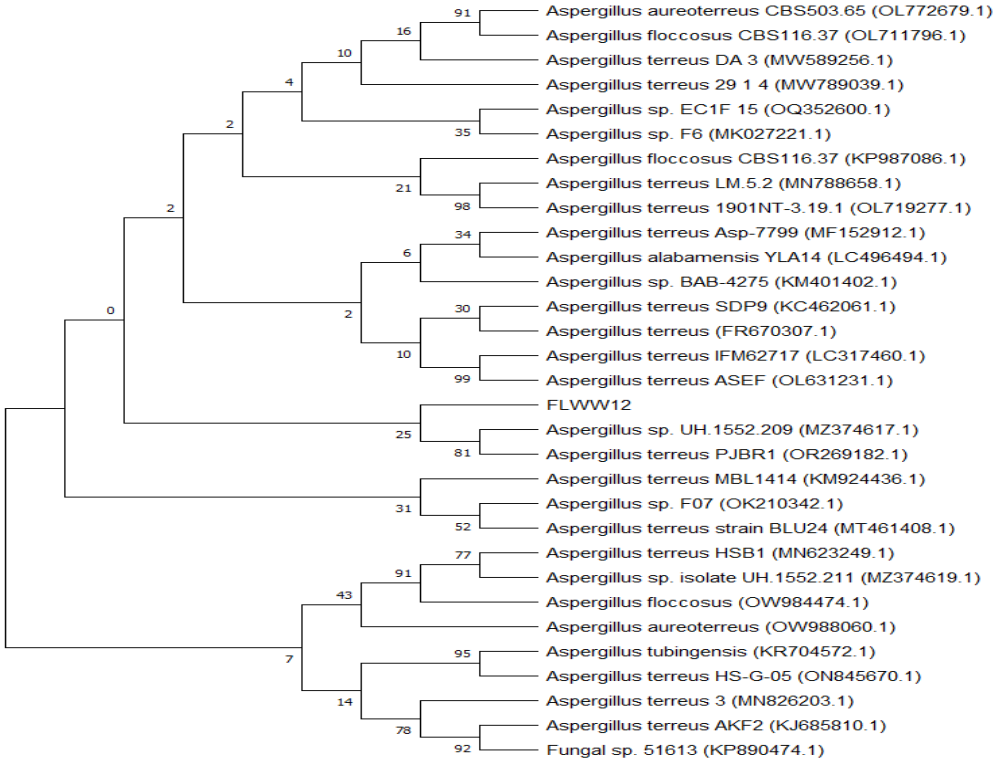


Fig.75 Maximum Likelihood phylogenetic tree of FLWW12

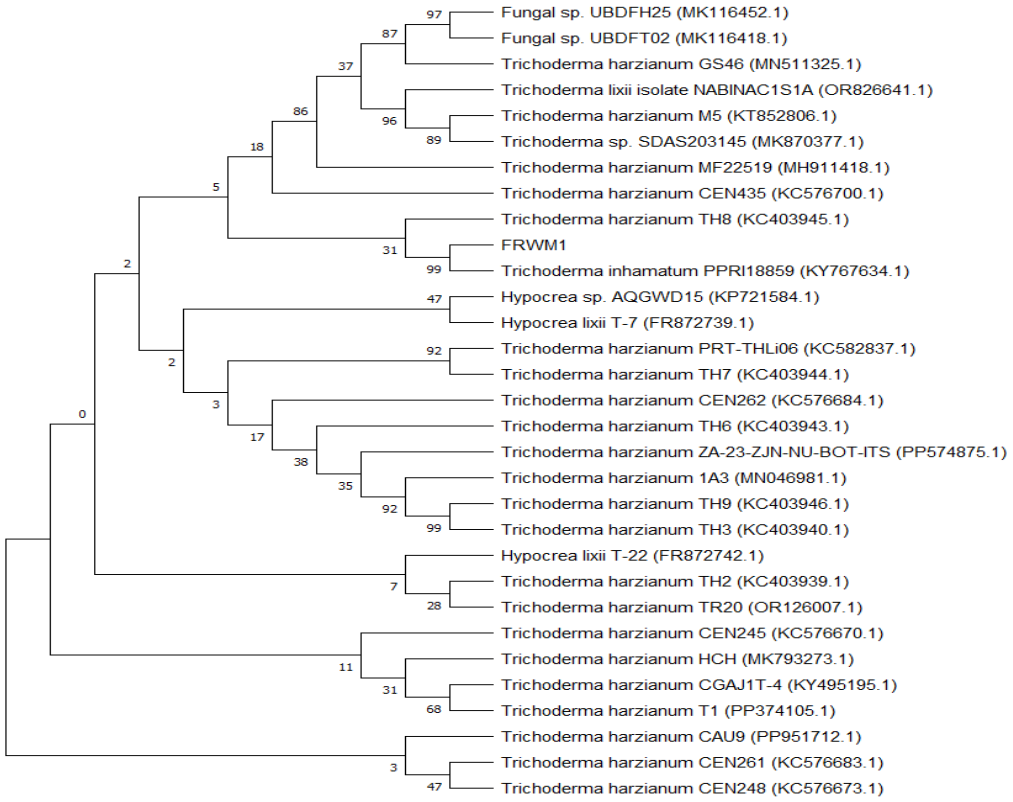


Fig.76 Maximum Likelihood phylogenetic tree of FRWM1

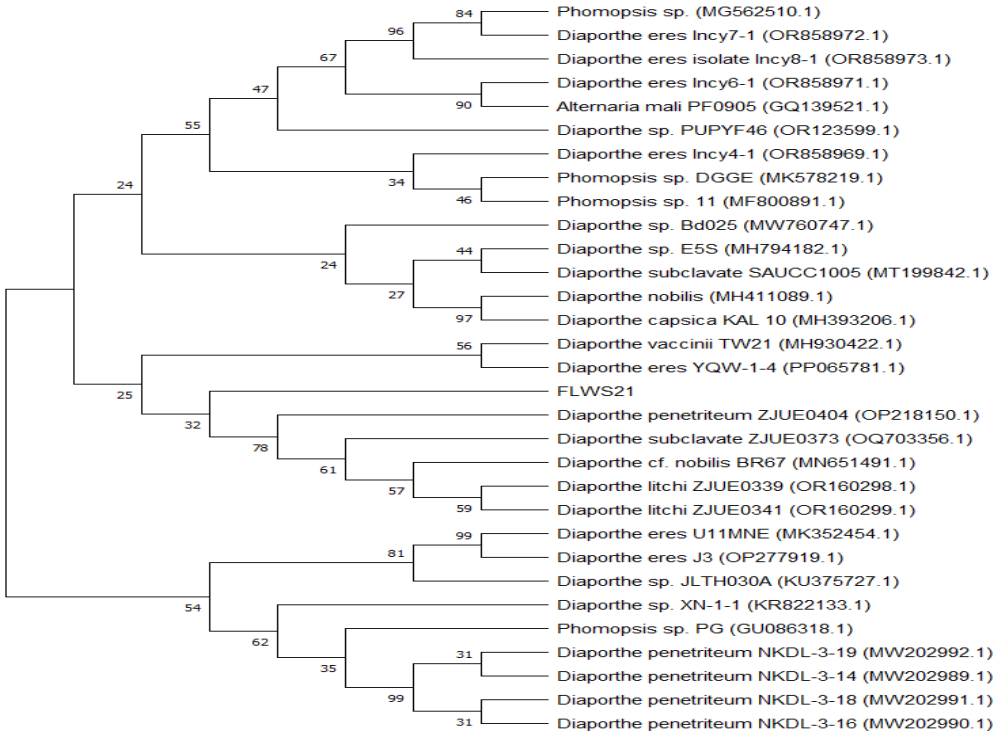


Fig.77 Maximum Likelihood phylogenetic tree of FLWS21

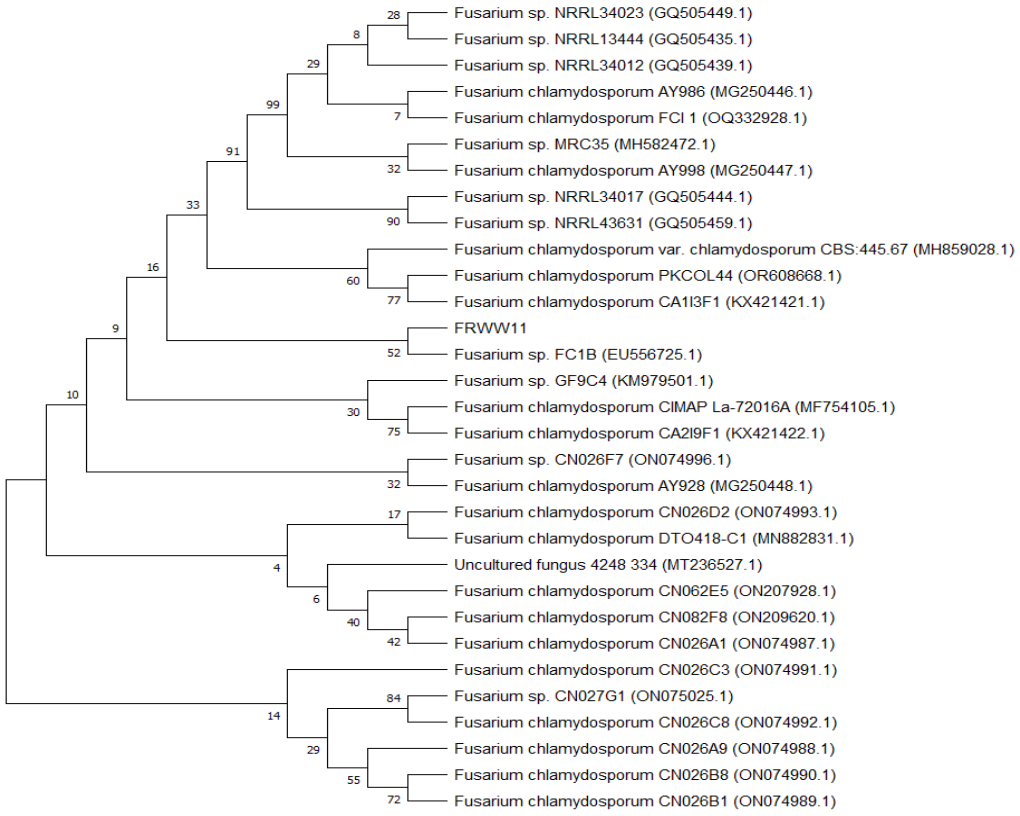


Fig.78 Maximum Likelihood phylogenetic tree of FRWW11

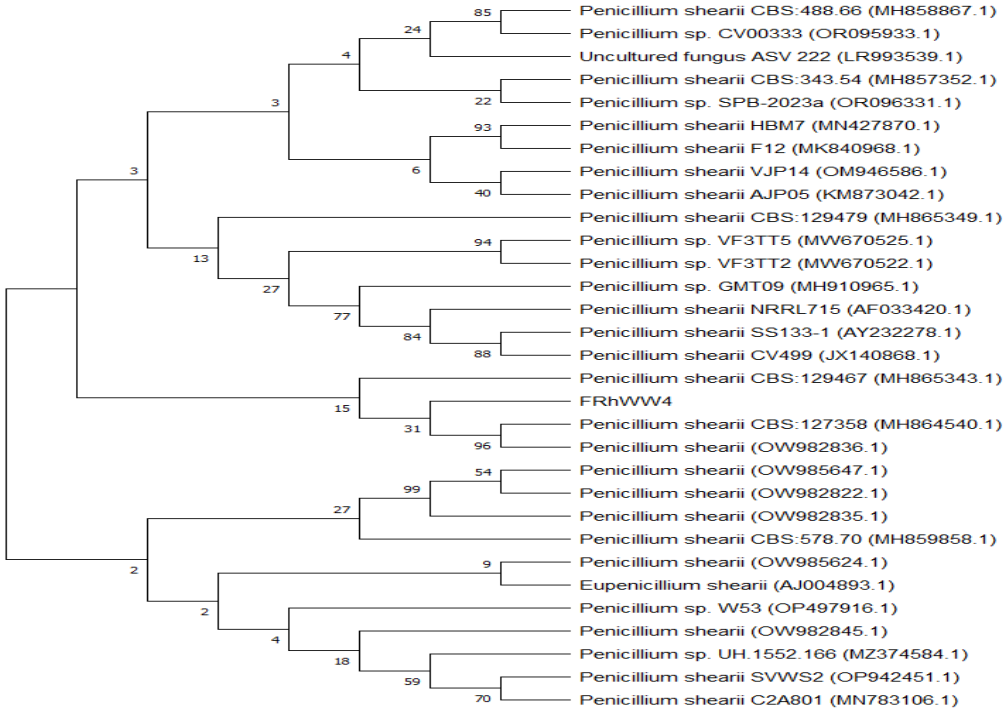


Fig.79 Maximum Likelihood phylogenetic tree of FRhWW4

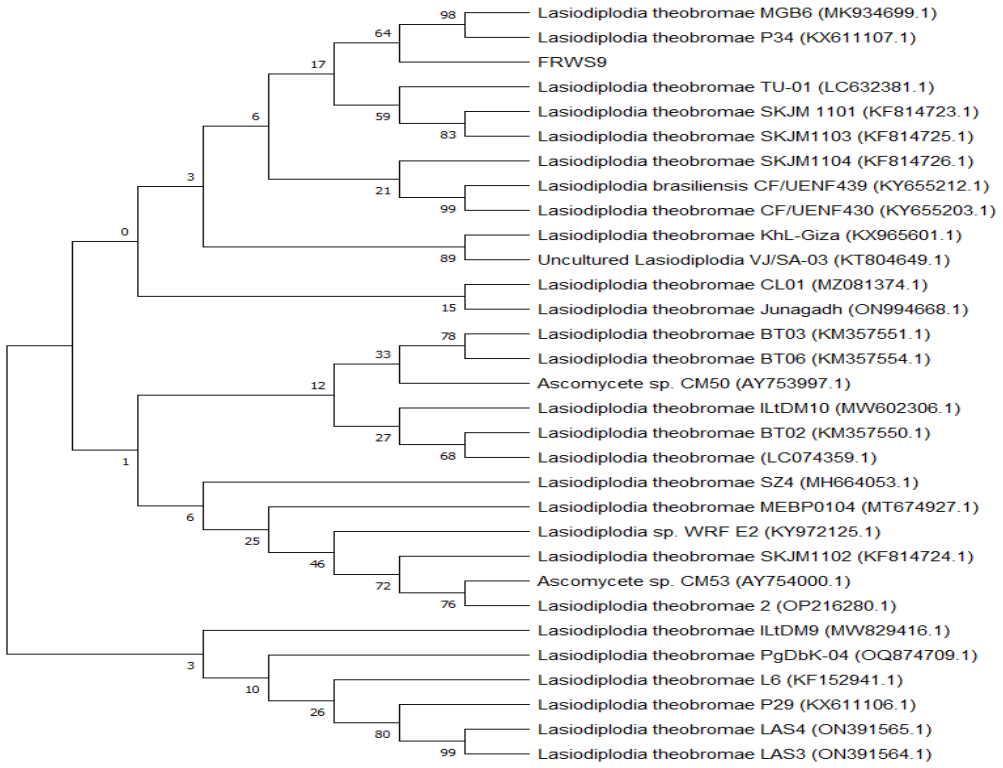


Fig.80 Maximum Likelihood phylogenetic tree of FRWS9

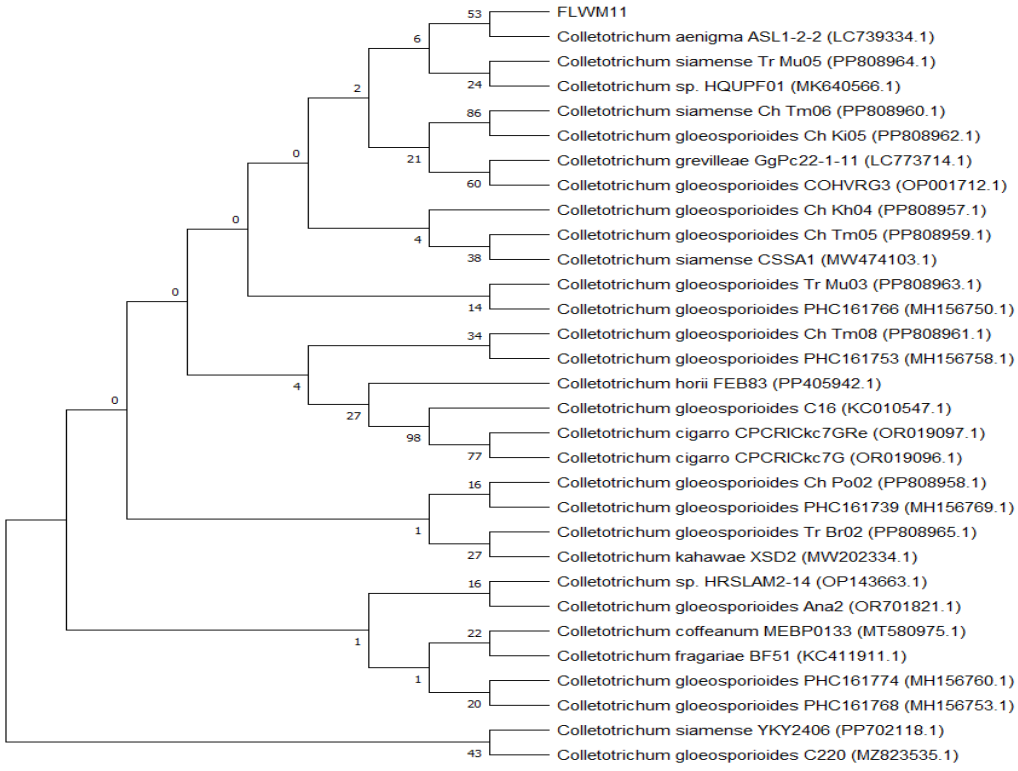


Fig.81 Maximum Likelihood phylogenetic tree of FLWM11

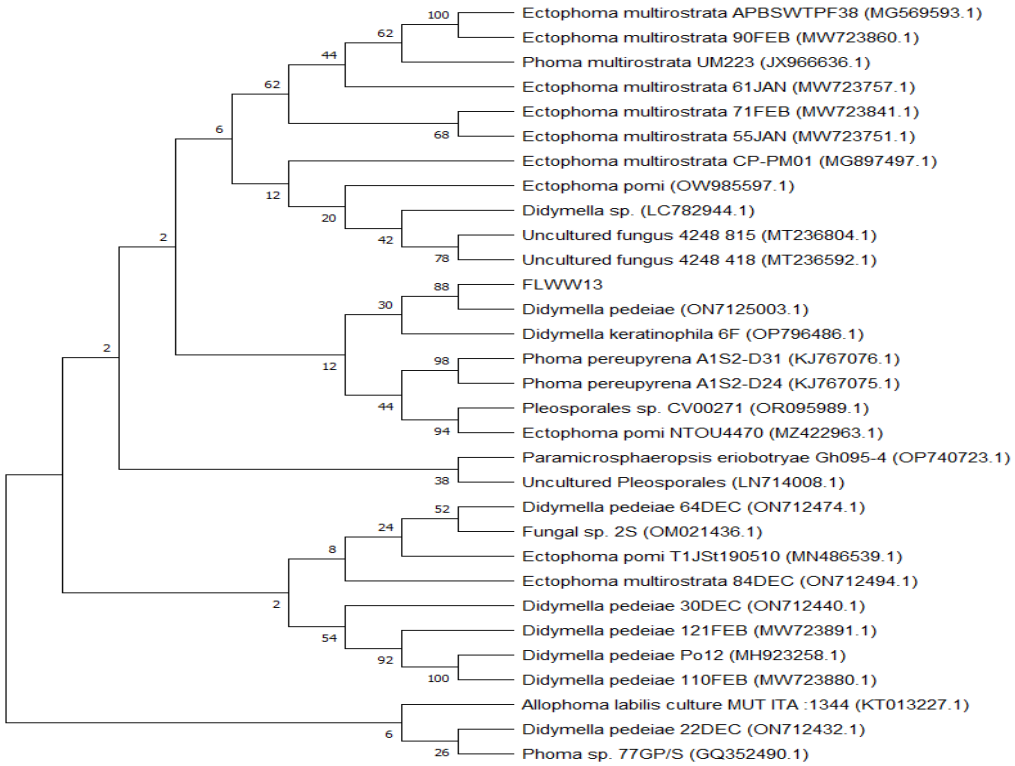


Fig.82 Maximum Likelihood phylogenetic tree of FLWW13

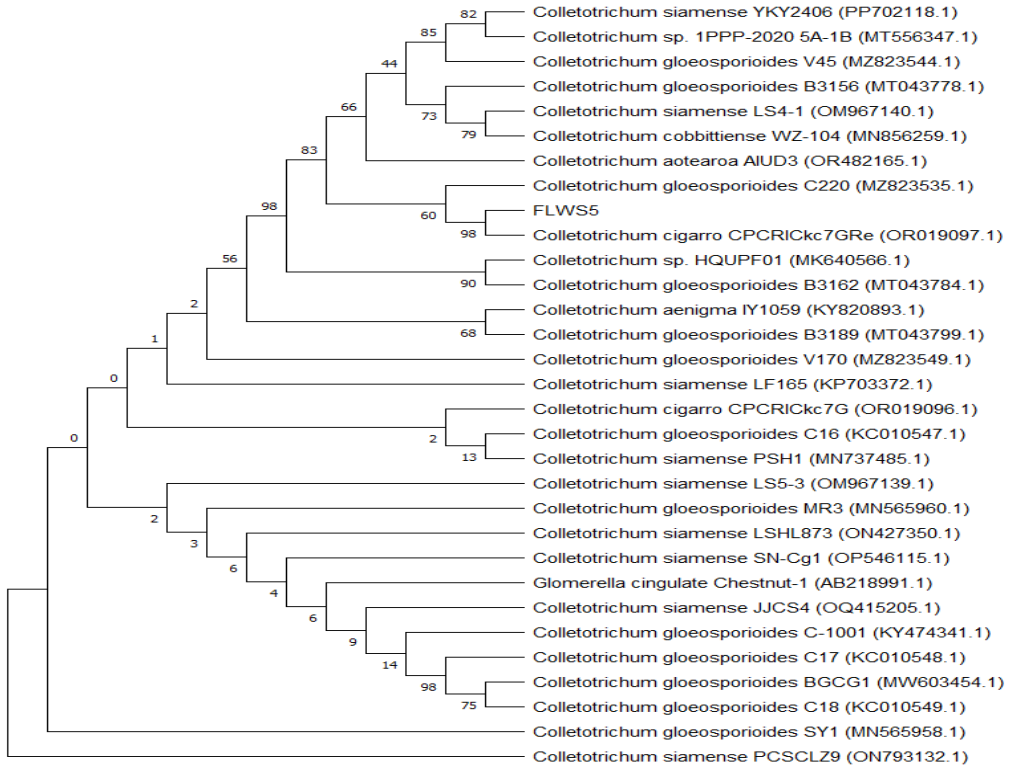


Fig.83 Maximum Likelihood phylogenetic tree of FLWS5

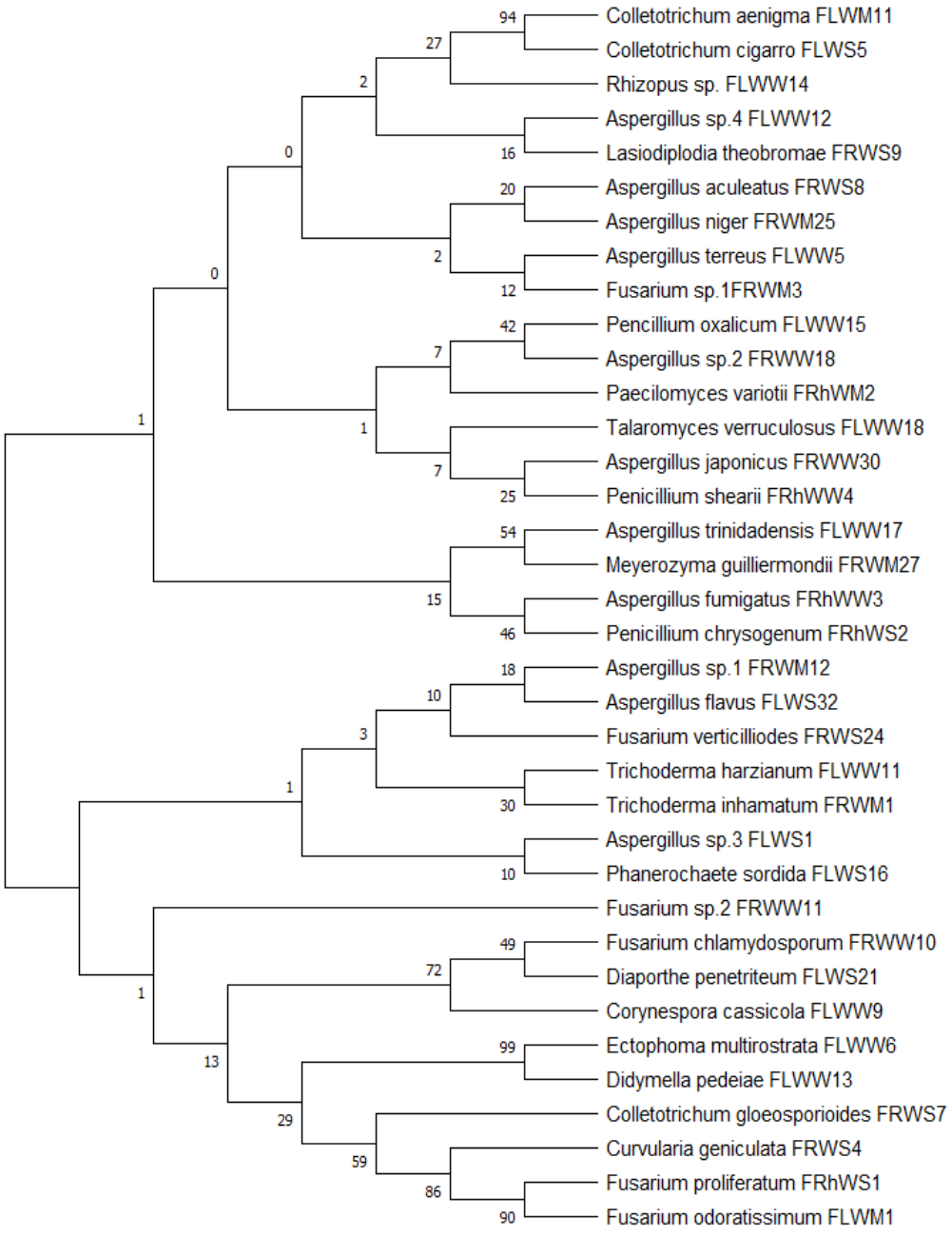


Fig:84 Maximum Likelihood phylogenetic tree showing the relationship of isolated endophytic fungi and their nearest relatives of all fungal isolates

Discussions

Endophytic bacteria and fungi associated with plant species have been investigated (Padhi and Tayung 2013; Rao and Satish, 2015). Medicinal plants from the tropical rainforests were known to be heavily manifested with microbial endophytes in their tissues. Medicinal plants worldwide harbor diverse endophytes, offering immense potential for discovery. Microbial endophytes, comprising fungi and bacteria, exhibit remarkable metabolic versatility, bridging the gap between microbes and their host plants. Recent efforts have focused on isolating endophytes from unexplored niches and medicinal plants to uncover potent natural products. Against this backdrop, this study aims to tap into the unexplored potential of endophytic bacteria and fungi associated with aquatic medicinal plants. Despite their rich microbial diversity, these plants have been understudied, presenting a unique opportunity for discovery.

Although numerous studies have focused on isolating endophytes from medicinal plants, underexplored aquatic plants remain an untapped resource. Various factors impact the presence and diversity of endophytes in their host plants, notably weather variability, stress conditions, and environmental characteristics of the host's ecosystem. These complex interactions between endophytes and their host plants highlight the need for further research into underexplored aquatic plant systems. Investigating these relationships can uncover novel endophytes with potential applications in medicine, agriculture, and biotechnology. In India, climatic conditions for the endophytic diversity within the host were reported to be more varied by the influence of seasons and habitat of the host (Mishra et al., 2012). From these observations, this study considered three different sites in Wayanad district in Kerala because of some particular characteristics i.e., 1. Wayanad is situated in the Western Ghats, a recognized biodiversity hotspot, making it an ideal location for discovering novel endophytes. 2. Wayanad experiences a distinct seasonal pattern, with the Monsoon season (June to September): High rainfall and humidity, ideal for plant

growth. Winter season (December to February): Cooler temperatures, influencing endophyte activity and diversity. Summer season (March to May): Warmer temperatures, potentially leading to changes in endophyte populations. 3. Compared to other regions in Kerala, Wayanad is relatively less explored, making it an attractive location for novel discoveries.

This study yielded 2362 endophytic bacterial isolates from *Lagenandra toxicaria*. The absence of microbial growth on control plates inoculated with the final rinse water from surface-sterilized tissues demonstrates the effectiveness of the sterilization protocol. Therefore, the isolated bacteria can be considered true endophytes, residing within the plant tissues. The isolates obtained from *L. toxicaria* are higher than the other aquatic species. In the previous studies of Zhang et al., 2014a; Zhang and Yao, 2015; Chen et al., 2012; El-Deeb et al., 2012; Shcherbakov et al., 2013; Ho et al., 2012 in different aquatic and wetland plant species such as *Najas marina, Nymphaea tetragona, Potamogeton crispus, Scirpus triqueter, Eichhornia crassipes* yield limited number of endophytic bacteria including *Bacillus, Pseudomonas, Aeromonas, Delftia, Staphylococcus*, etc. 120 endophytic bacterial isolates were obtained from the roots, stems, and leaves of aquatic plants, including *Potamogeton pectinatus*, *Myriophyllum spicatum,* and *Ceratophyllum demersum*. The most dominant genera were Pseudomonas (30%), Bacillus (20%), and Stenotrophomonas (15%) (Hallmann et al. (2006). The dominance of certain genera, such as Pseudomonas and Bacillus, may be due to their ability to form symbiotic relationships with the host plant. The present study also supports this statement that the members in genera Bacillus are the predominant species except for Pseudomonas. The comparison of the endophytic bacterial community differs between plant parts, locations, and also with seasons. Similarly, our study found that roots had the highest isolation frequency and colonization rate of endophytic bacteria. The results of this study are consistent with the study of Hallmann et al. (2006) found that the roots of plants harbored a higher diversity of endophytic bacteria compared to other plant parts. The

highest number of isolates was obtained from the roots of *Potamogeton pectinatus* (40%), followed by the stems of *Myriophyllum spicatum* (25%) and the leaves of *Ceratophyllum demersum* (20%). This suggests roots may be a preferred habitat for endophytic bacteria in aquatic plants (Sessitsch et al., 2012).

Akinsanya et al. (2015) isolated 29 culturable bacterial endophytes from surface-sterilized tissues of *Aloe vera*, including roots, stems, and leaves. Molecular characterization revealed a diverse community comprising 13 distinct genera. The dominant genera were Bacillus, Pseudomonas, and Enterobacter, accounting for 20.7%, 20.7%, and 13.8% of the isolates, respectively. Chen et al., (2012) unraveled the diversity of endophytic bacteria in four aquatic plants, uncovering distinct microbial consortia in different plant parts. *Pseudomonas spp.* dominated roots and stems across all plants, while *Enterobacter* and *Paenibacillus spp.* were widespread. *Aeromonas spp.* surprisingly thrived in *Phragmites communis* and *Nymphaea tetragona* stems. *Phragmites communis* harbored a diverse array of bacteria, including *Microbacterium* and *Flavobacterium.* The study exposed a remarkable diversity of endophytic bacteria, spanning 12 genera across five phyla, highlighting the complex plant-microbe interplay in aquatic plants. Lata et al., (2024), the study aimed to isolate, identify, and characterize endophytic bacteria from feather grass (*Chloris virgata*) collected from waterlogged rice fields. Forty-three bacterial isolates were obtained from roots, rhizomes, and leaves and subjected to identification by 16S rRNA sequencing. The identified isolates belong to Bacillus (25 isolates) and Glutamicibacter (5 isolates) genera, with Bacillus being dominant. Emitaro et al. (2024) investigated the diversity of bacterial endophytes in *Sesbania sesban*, *Leucaena diversifolia*, and *Calliandra calothyrsus*. Using morphological and molecular characterization, they recovered 27 pure isolates from the three plants, with 44.4% from leaves, 33.3% from stems, and 22.2% from roots. Analysis of 16S rRNA gene sequences revealed eight closely related bacterial genera, including Bacillus (33.3%),

Staphylococcus (22.2%), Alcaligens (11.1%), Pantoea (11.1%), Xanthomonas (7.4%), Sphingomonas (7.4%), Acinetobacter (3.7%), and Pseudomonas (3.7%). Notably, Bacillus was isolated from all three plants, highlighting its widespread presence. This study demonstrates the high diversity of endophytic bacteria associated with different parts of *L. diversifolia*, *S. sesban*, and *C. calothyrsus* growing in western Kenya. The findings provide valuable insights into the complex relationships between these plants and their endophytic microbial communities.

Seasonal variation also played a significant role in shaping the endophytic bacterial community. The observations from previous works of Liu et al., 2017 and Sessitsch et al. (2012) on the influence of climatic and seasonal conditions support the present study. Our study found that the summer season accommodated the highest percentage frequency of endophytic bacteria, which is consistent with the findings of Sessitsch et al. (2012). The Shannon-Wiener diversity index and evenness index values obtained in this study are also comparable to those reported in previous studies. A study by Miguel et al., 2016 found that the Shannon-Wiener diversity index values for plant endophytic bacterial communities ranged from 1.5 to 2.99. Our study found that this diversity index is ranging from 0.2 to 2.46. The findings of this study have implications for our understanding of the role of endophytic bacteria in plant health and ecosystem functioning. This suggests that the endophytic bacterial community in aquatic plants is complex and diverse (Ryan et al., 2008). Akinsanya et al., 2015a, study revealed the composition and diversity of microbial communities in several habitats. Phylogenetic analysis revealed four predominant phyla: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Notably, the roots exhibited a unique composition, with 23% of genera absent in other tissues. In contrast, stems were characterized by a higher abundance of Pseudomonas and unclassified Pseudomonadaceae. Alpha-diversity analysis yielded varying richness and inverse Simpson's diversity indices across tissues: 2.221 for leaves, 6.603 for roots, and 1.491 for stems.

These findings highlight tissue-specific differences in bacterial endophyte communities. A separate study on culturable endophytic bacteria in *Aloe vera* plants identified Pseudomonas and Bacillus as dominant genera. Interestingly, four species were evenly distributed across root, stem, and leaf tissues, suggesting a consistent endophytic profile.

The Simpson's Diversity Index (SDI) revealed a high level of diversity among the endophytic bacterial communities in the root, leaf, and rhizome samples from different locations and seasons. The highest diversity index was observed in root samples from Pakkom and leaf samples from Mattilayam during the summer season, indicating a more even distribution of bacterial species. In contrast, the lowest diversity index was observed in rhizome samples from Mattilayam, suggesting a less diverse community. The Margalef Richness Index also showed a similar pattern, with the highest richness observed in root samples from Kottavayal during the summer season. The evenness index revealed, the highest evenness observed in rhizome samples from Kottavayal root samples from Pakkom during the summer season and leaf samples from Mattilayam in winter indicating a more even distribution of bacterial species. However, the lowest evenness was observed in rhizome samples from Mattilayam, suggesting a less even distribution. The significant impact of plant parts on endophytic bacterial diversity is also consistent with previous studies. For example, a study on the endophytic bacteria of maize found that the diversity of bacteria varied across different plant parts (Farooq, 2014). This suggests that different plant parts may provide different habitats for endophytic bacteria, leading to variations in diversity. The interaction effects between location, season, and plant parts were less pronounced but still significant. That means the combined effects of these factors on endophytic bacterial diversity are complex and require further investigation. A similar study on the endophytic bacteria of wheat found that the interaction between location and season significantly impacted bacterial diversity (Pang et al., 2022).

The endophytic fungal diversity data from our study revealed a high level of diversity, with a total of 1408 isolates belonging to 12 families. This is consistent with other studies that have found a high level of endophytic fungal diversity in various plant species (Farooq, 2014). The present study observed a higher percentage frequency in leaf samples than root and rhizome. This is consistent with previous studies that have found a higher diversity of endophytic fungi in leaf samples (Hamzah et al., 2018). The data shows that the isolation frequency of endophytic fungi was higher in summer (60%) compared to winter (40%) and monsoon (35%). This suggests summer is the best season for isolating endophytic fungi from aquatic plants. This data is supported by a previous study that found a higher diversity of endophytic fungi in summer (Mishra et al., 2012). The colonization rate, Shannon-Wiener diversity index, and Simpson's diversity index were higher in root samples than leaf and rhizome samples, suggesting that roots may be a preferred habitat for endophytic fungi in aquatic plants (Qiao et al., 2019). Interestingly, no fungal isolates were detected in rhizome samples from Pakkom during the monsoon season, and overall, the fungal diversity in the rhizome was found to be extremely low, approaching negligible levels. This may be due to the rhizomes producing toxic chemical compounds to fungi, such as alkaloids, glycosides, or terpenoids, which can inhibit fungal growth and colonization. These chemical compounds can be produced in response to environmental cues, such as the monsoon season. They can help the rhizome maintain a balanced microbiome that favors beneficial bacterial endophytes over fungal endophytes.

Diversity indices in endophytic fungi found in aquatic plants are used to assess the variety and distribution of fungal species within a given environment. A higher Shannon-Wiener Index indicates a more diverse community with a balanced distribution of species i.e., it indicates that the endophytic fungal community is rich and even. In aquatic plants, a higher index suggests a complex fungal community that might contribute to the health and stability of the plant ecosystem (Tedersoo et al., 2014). The Shannon-Wiener diversity

index (SWDI) and Simpson's diversity index (SDI) values from our study are higher than those reported in other studies (Sessitsch et al., 2012). The Margalef richness index values from our study are also higher than those reported in other studies (Radu et al., 2022). The evenness index values from our study are consistent with other studies that have found a high level of evenness in endophytic fungal communities (Vannier et al., 2020). A comprehensive study of 30 aquatic plant species in southwest China yielded 1,689 fungal isolates, representing three phyla and 154 genera, from 15,373 plant tissue segments. The aquatic environment and similar climatic conditions likely influence this remarkable diversity of endophytic fungi. The average isolation frequency across all plants was 11%, with no significant difference among root, stem, and leaf tissues. Despite the relatively low isolation frequency, the endophytic fungal community exhibited high diversity (H' = 5.23) and evenness (0.72). Notably, Qiao et al. (2021) found that increasing altitude correlated with decreased isolation frequency, fungal species richness, and diversity index of endophytic fungi. This study highlights the complex relationships between aquatic plants, their endophytic fungi, and environmental factors.

The present study challenges the findings of Sandberg et al. (2014), who reported a high diversity of endophytes in aquatic plants despite low isolation frequencies. In contrast, our research reveals that isolation frequency and diversity of fungal endophytes are positively correlated. Notably, summer samples exhibited higher isolation frequencies and diversity of fungal endophytes. Furthermore, our study demonstrates that fungal diversity varies with environmental conditions and the site of collection. Analysis revealed significant variations in endophytic community diversity across different sites. This site-specific diversity is consistent with previous literature (Petrini et al., 1993; Sieber, 2007), which attributes variations in species diversity to factors such as local environmental conditions, plant host specificity, and geographic location.

The present investigation revealed that the diversity is higher in roots than leaves which is higher than that of the rhizome of *Lagenandra toxicaria*. This finding coincides with the findings of Kohout et al. studies carried out in the aquatic plants in Norway and Kandalepas who studied the leaves from two freshwater plant species collected from Louisiana wetlands. It could be observed that plant parts of the same site had a high similarity of species. Also, isolates from the plant parts of leaf and root in the same plant and the different plant samples from the same location showed similarity in species distribution, and the isolates from the locations Kottavayal and Pakkom showed similar species.

The diversity of endophytic fungi associated with aquatic/riparian plants is a fascinating area of research with potential applications in medicine and agriculture. The major fungal genera isolated from the roots of aquatic plants in the study of You et al., 2015 were Aspergillus, Fusarium, Penicillium, and Talaromyces. They sequenced the fungal isolates' internal transcribed spacer (ITS) regions and conducted a phylogenetic analysis. This study concluded that fungal diversity was influenced by environmental conditions and the host plant species in the two wetlands. In the present study, the isolated endophytic fungal genera are almost similar to that of You et al., 2015 and the ITS region was sequenced for the identification studies. The most dominant genera obtained in this study were *Aspergillus, Fusarium*, and *Penicillium*. This is also consistent with previous studies that have found these genera dominant in aquatic plants (Vannier et al., 2020). The presence of Nectriaceae (16.67%) and Glomerellaceae (8.34%) in the study is also consistent with other studies (Kaliane et al., 2018) that have found these families to be common among endophytic fungi. The presence of Mucoromycota (2.78%) in our study is less common, which is supported by the study of Govindan and Kumar, 2020 in *Oryza sativa*. The comprehensive study on the incidence of endophytic fungi from the Western Ghats of India corresponded to those studies investigated by Raviraja et al. (2006), Naik et al. (2008), and Nalini and Prakash, (2017). Verma

et al. (2014) have isolated 1897 fungal endophytes from 2700 different tissues of *Madhuca indica*. Several medicinal plants are intensively screened for fungal endophytes by Sowparthani and Rajagopal, (2011), Orlandelli et al. (2012), Gautam et al. (2013), Zhang and Yao, (2015), Yokoya et al. (2017).

In the current study, MANOVA is a statistical technique used for analyzing the effect of multiple variables to identify significant differences in endophytic microbial communities across different locations, seasons, and plant parts. The microbial diversity was analyzed by comparing the individual factors and pairwise factors, which provide a comprehensive understanding of the pattern and correlations between them. In the bacteria, the analysis revealed a significant variation in endophytic bacterial diversity based on location, season, and plant species, with a p-value of <0.01. Nevertheless, the paired comparison revealed no significant difference, indicating that while location, season, and plant species impact endophytic bacterial diversity, paired conditions do not have a significant effect. From this, principal component analysis was also analyzed and a biplot was generated to show all dependent variables contributed equally to the principal component.

In the fungi, the MANOVA analysis revealed highly significant effects ($p < 0.01$) of individual factors, including location, season, and plant species and their interaction effects. These results indicate that endophytic fungal diversity is significantly influenced by these factors and their interactions, with a significance level of 1%. This suggests a complex interplay between location, season, and plant species in shaping endophytic fungal communities. The principal component analysis (PCA) revealed that CR disproportionately influences the first principal component (PC1), as evidenced by its longer vector. In contrast, the remaining factors exhibit a high degree of positive correlation, as indicated by their proximity to each other in the vector space. Notably, the similar lengths of their vectors suggest that these factors have a relatively uniform impact on the PCA, with no single factor dominating the others.

Similarly, Arnold and Lutzoni (2007) and Wilson and Carroll (1994) substantiated that endophyte colonization is shaped by a combination of factors, including geographic location, climate, seasonality, host plant identity, and specific host tissues. These factors profoundly impact the colonization patterns and diversity of endophytic microorganisms. Singh et al. (2017) conducted a similar study on endophytic fungi diversity using MANOVA, revealing significant differences ($p \leq 0.001$) in diversity measures across locations, seasons, and tissue types. Their analysis of the Shannon-Wiener index (H′) and species richness data showed significant variations in endophytic fungal diversity across these factors. Additionally, the interactive effect of location, season, and tissue type was found to impact dominant endophytes ($\%D \geq 0.5$), with tissue type having the strongest effect, as evident from the tissue-type-based grouping of isolates in the biplot analysis. This study underscores the importance of considering multiple factors when analyzing endophytic fungal diversity.

This pioneering research provides the first comprehensive insights into the endophytic bacteria inhabiting *L. toxicaria*. The plant's microbial community offers a valuable source of potential endophytes with applications in drug development. The discovery of novel endophytic microbes from unexplored regions holds immense promise for uncovering naturally occurring solutions. This study underscores the significance of exploring diverse ecosystems to identify biologically active compounds, driving innovation in pharmaceutical research.

Conclusion

In short, this chapter aimed to isolate, identify, and analyze the diversity of endophytic bacteria and fungi from *Lagenandra toxicaria*, an aquatic medicinal plant. Studying the percentage frequency, isolation frequency, colonization rate, and colonization frequency of endophytic isolates is crucial for understanding the ecology and dynamics of these microorganisms within plants. These metrics provide insights into the abundance, diversity, and potential interactions

between endophytes and host plants. The Shannon-Wiener diversity index (SWDI) and Simpson's diversity index (SDI) indices help to understand the structure of endophytic communities, including the number of species, their relative abundance, and distribution and quantitatively measure the biodiversity, to compare diversity levels between different plant parts, seasons, and locations. Evenness indices can indicate community stability, with higher evenness values suggesting a more stable community. Margalef richness and SWDI enable comparisons of community richness and diversity between different studies.

MANOVA helped to compare multiple groups (e.g., plant parts, seasons, locations) based on multiple dependent variables (e.g., endophytic diversity indices) to identify significant differences between groups, controlling for multiple comparisons. Biplot analysis provides the graphical representation of complex data, facilitating identify patterns and correlations between variables and groups. Biplot analysis aids in interpreting MANOVA results, illustrating significant differences and relationships. PCA reduces the dimensionality of large datasets, simplifies analysis and interpretation, identifies underlying factors or patterns in the data, and reveals structure and relationships. PCA facilitates the visualization of high-dimensional data that can inform future research directions, experimental design, and sampling strategies. The morphological, biochemical, and molecular analysis helps to characterize the bacterial and fungal colonies and individual cells for identification at the species level. The next chapter deals with the antagonistic properties of these selected endophytic bacterial and fungal isolates.

Chapter 4

Antimicrobial, Antioxidant, and Phytochemical Analysis of Selected Endophytic Bacterial and Fungal Isolates

Introduction

In recent decades, investigating natural products from endophytes has experienced a significant resurgence, particularly following the discovery of taxol-producing fungal endophytes. This finding has underscored the importance of endophytes as a promising source of innovative antimicrobial compounds with substantial pharmaceutical potential. Some of the significant antimicrobial agents, since increasing multidrug-resistant microorganisms against the existing antibiotics, have become one of the major areas of concern across the globe. The ability of endophytes to produce antimicrobial compounds is closely tied to their unique evolutionary history, which has enabled them to acquire genetic information from their plant hosts (Strobel, 2003). This adaptation allows endophytes to thrive within plants. Notably, endophytes function as in-planta chemical synthesizers, acting as a selective force driving microbes to produce antagonistic substances with low toxicity. These substances effectively combat pathogens while bolstering plant defense mechanisms. (Owen and Hundley, 2004). The production of phytochemicals by endophytic microorganisms is crucial for plant protection. These bioactive compounds confer resistance to pests, diseases, and environmental stresses, ensuring plant survival and fitness. Endophyte-derived phytochemicals have significant medicinal applications. They exhibit antibacterial, antifungal, and anticancer properties, making them valuable for drug discovery and development. The unique plant-microbe interactions facilitated by phytochemical production promote biodiversity. This diversity is essential for ecosystem resilience and adaptability.

Endophytes form an important component of microbial biodiversity, secreting various bioactive metabolites that bear antimicrobial and antioxidant agents (Strobel and Daisy, 2003). Antimicrobial agents are substances that target and eliminate or inhibit the growth of microorganisms. These agents disrupt vital

cellular components essential for metabolism, thereby suppressing the synthesis of functional biomolecules or hindering normal cellular activities. The discovery of antibiotics has revolutionized the field, with an extensive array of antimicrobial agents being continually explored from diverse sources. Among these, plants and microorganisms stand out as superior and economically viable options, offering cost-effective solutions and ensuring the sustainability of these sources (Bbosa et al., 2014). Extraction of new antimicrobial metabolites is a multidisciplinary endeavor that includes metabolites bearing antimicrobial activity can be defined as low molecular weight compounds that are active at low concentrations against pathogenic microorganisms (Strobel and Daisy, 2003).

Antioxidant activity involves the scavenging or inhibiting free radicals, thereby protecting cells from oxidative stress and potential harm caused by these highly reactive molecules. Endophytic microbes, such as fungi and bacteria, have been found to possess antioxidant activity, producing compounds that can scavenge free radicals and protect their host plants from oxidative damage. The antioxidant activity of endophytic microbes is significant because it can contribute to the overall health and resilience of the host plant. Endophytic microbes can help promote plant growth, improve disease resistance, and enhance tolerance to environmental stresses by reducing oxidative stress. Additionally, the antioxidant compounds produced by endophytic microbes have potential applications in medicine and industry, such as in developing natural antioxidants for food preservation and pharmaceuticals. This chapter deals with the production of secondary metabolites, phytochemical analysis, and its antagonistic properties of antimicrobial and antioxidant activities.

Materials and Methods

Production of Secondary Metabolites from Selected Endophytic Bacterial and Fungal Isolates

A loopful of bacteria was inoculated in the nutrient agar plate for 48 hr at 36±2°C. After the incubation, cultured endophytic bacteria were sub-cultured in

the autoclaved Nutrient Broth. The process of liquid shake fermentation production of the secondary metabolites was carried out for the selected endophytic isolates. The broth media were incubated at 36°C, under shaking at 120 rpm for 10 days (Nisa et al., 2019). After incubation, broth media were centrifuged (10,000 rpm, 10 min, 4°C) to collect cell-free supernatant. The supernatant was then filtered through sterilized 0.22 µm pore-size filters (Millex-GS, Millipore, USA). Fungal isolates were cultured on Potato Dextrose Agar (PDA) plates at 28°C ± 2°C for 5-10 days. Actively growing mycelial plugs (5mm diameter) were aseptically transferred to 500ml Erlenmeyer flasks containing 250ml Potato Dextrose Broth. The flasks were incubated at 30°C with orbital shaking (120 rpm) for 10 days, with regular monitoring for contamination.

Extraction of Secondary Metabolites from Endophytic Bacterial and Fungal Isolates

Once the fermentation process was over, the culture medium of both bacteria and fungi was used to separate cells and fermented broth by using centrifugation and filtration through muslin cloth. Ethyl acetate (1:1, v/v) was added to the cell-free supernatant, shaken vigorously, and transferred to separating funnels. After phase separation, the organic phase was collected, and the process was repeated three times. The combined organic phases were then evaporated to dryness using a rotary evaporator at 40°C, resulting in ethyl acetate extracts (Rajaofera et al., 2019). Upon harvest of fermented fungal biomass on the 10^{th} day, methanol was added into the flasks equal to 10% of the volume of whole broth, and the entire culture was high shear homogenized to disrupt fungal cells. Here, the fungal mass and broth were taken together and fractioned in a separating funnel with an equal volume of ethyl acetate. The organic layer (upper layer of ethyl acetate) containing the dissolved metabolites was collected separately. Then, all these fractions were pooled and rotary evaporated. The dried crude extracts were weighed and labeled in separate vials and used for various biological attributes. The dried bacterial and fungal crude extracts were dissolved in DMSO to known concentrations and assessed for their bioactive

potential viz. antibacterial activity, antioxidant activity, and phytochemical analysis.

Extraction of Secondary Metabolites from *L. toxicaria*

The plant parts, such as the leaf, root, and rhizome of *L. toxicaria* from three different seasons, were washed thoroughly, removed excess water content, and air-dried. Then the plant materials were weighed, ground, and transferred to the Soxhlet apparatus with ethyl acetate (Nikhal et al., 2010). The dried samples were packed in a thimble and kept in a Soxhlet extractor. The Soxhlet assembly was run with ethyl acetate for 6-8 hours at a temperature of 77.1°C in a heating mantle. After 8 hours, the ethyl acetate fraction was concentrated. The dried crude extracts were dissolved in DMSO to known concentrations and assessed for their bioactive potential, viz. antibacterial activity, antioxidant activity, and phytochemical analysis.

Qualitative Phytochemical Screening of Crude Extracts of Endophytes and Plant Samples

A qualitative and preliminary phytochemical screening was done according to established protocols to identify the chemical nature of the active components present in the endophytic bacterial and fungal crude extracts. All the crude extracts were checked for the presence of various secondary metabolites.

Test for Alkaloid

To detect alkaloids, 1 ml of the sample was mixed with 1 ml of concentrated HCl. A few drops of Mayer's reagent were then added. The appearance of a brown or yellow precipitate indicated the presence of alkaloids (Santhi and Sengottuvel, 2016).

Test for Flavonoid

To test the presence of flavonoids, 1 ml of the sample was mixed with 0.5 ml of a 10% ammonium hydroxide solution; the observation was a yellow fluorescence color (Prabhavathi et al., 2016).

Test for phenol

A ferric chloride test was used to test the presence of phenol by the 1 ml of extract treated with a few drops of 5% ferric chloride, a dark green color indicating the presence (Thakur and Sahani, 2019).

Test for saponin

For saponin, a 1 ml sample was quivered with 1 ml distilled water and was warmed until it boiled. The blend was shuddered to lather emergence (Yadav and Agarwala, 2011).

Test for Sterol

To perform the Liebermann-Burchard test, 1 ml of sample was mixed with 4 ml of chloroform, followed by adding a few drops of concentrated sulfuric acid. The mixture was then shaken well and allowed to stand. The formation of distinct color rings at the interface indicated the presence of specific compounds. A reddish-brown ring signified the presence of sterols, whereas a bluish-brown ring denoted the presence of phytosterols (Thakur and Sahani, 2019).

Test for Tannin

The ferric chloride test was employed to detect the presence of tannins in the sample. To perform the test, 1 ml of sample was mixed with a few drops of 10% alcoholic ferric chloride. The addition of ferric chloride resulted in a bluish-black color, which faded upon adding a few drops of sulfuric acid. This was followed by forming a yellowish-brown precipitate, confirming the presence of tannins in the sample (Thakur and Sahani, 2019).

Test for Terpenoid

The Liebermann-Burchard test was employed to detect the presence of terpenoids in the sample. The test was conducted by mixing 1 ml of sample with a few drops of acetic acid, followed by the addition of 1-2 ml of concentrated sulfuric acid. A distinct red-brown color formed at the junction, indicating the presence of terpenoids in the sample (Thakur and Sahani, 2019).

Test for Cardiac Glycoside

To detect flavonoids, 2.5 ml of glacial acetic acid was mixed with 1 ml of sample, followed by adding a few drops of 5% ferric chloride. Subsequently, 1 ml of sulfuric acid was added to the mixture. The formation of a distinct brown ring indicated the presence of flavonoids (Raja et al., 2021).

Test for Carbohydrate

The Molisch test was employed to detect the presence of carbohydrates in the extract. The test procedure involved adding two drops of alcoholic α-naphthol solution to the extract, shaking well, and then slowly adding a few drops of concentrated sulfuric acid along the sides of the test tube. The formation of a distinct violet ring at the junction indicated the presence of carbohydrates (Thakur and Sahani, 2019).

Test for Coumarin

Mix the extract with 10% sodium hydroxide solution. If coumarin is present, a yellow or orange color will develop (Dewick, 2002).

Test for Quinone

1 ml of sample was added with 1 ml of concentrated sulphuric acid, the red color indicates the presence of quinone (Krvavych et al., 2014).

Test for Glycoside

Add Borntrager's reagent (ferric chloride and hydrochloric acid) to the extract. A brown or greenish-black color indicates the presence of glycosides (Silverstein and Bassler, 1962).

Test for Protein

A biuret test was used, 2 ml of the sample was treated with 10% sodium hydroxide and slowly added a few drops of copper sulfate. The blue-pink color indicates the presence of protein (Thakur and Sahani, 2019).

Quantitative Phytochemical Screening of Crude Extracts of Endophytes and Plant Samples

Total Alkaloid Content

The test extract was treated with 2 N HCl, filtered, and 1 ml of the solution was transferred to a separating funnel. Chloroform (10 ml) was added, and the

mixture was shaken. The pH was adjusted to 7.0 using 0.1 N NaOH. Phosphate buffer (5 ml, pH 7.4) and bromocresol green solution were added, and the mixture was shaken. The complex was extracted with chloroform by vigorous shaking. Atropine standard solutions (20, 40, 60, 80, and 100 µg/ml) were prepared. The absorbance of the complex was measured at 470 nm against a blank (prepared without extract) (Ajanal et al., 2012). All tests were performed in triplicates and results were expressed as atropine equivalent (µg of AE/mg extracts).

Total Flavonoid Content

Flavonoid detection was performed using the aluminum chloride (AlCl3) method. 0.5 ml of sample was mixed with 2% ethanolic AlCl3 and incubated for 1 hour at room temperature. The formation of a yellow color indicated the presence of flavonoids. The absorbance of the mixture was measured spectrophotometrically at 420 nm against a blank. All tests were performed in triplicates. Results were expressed as quercetin equivalent (µg of quercetin per mg of extract).

Total Phenolic Content

To estimate total phenolic content, 1 mg/ml of extract was mixed with 3.6 ml of tenfold diluted Folin-Ciocalteu reagent and allowed to rest for 5 minutes at room temperature. The reaction was terminated by adding 7.5% sodium carbonate, and the mixture was incubated for 1.5 hours. The absorbance was measured using a UV spectrophotometer at 765 nm (Kim et al., 2003). Gallic acid was used as the standard. All the tests were carried out in triplicates and results were expressed as gallic acid equivalent (µg of GAE/mg extracts).

Total Saponin Content

To estimate saponin content, test extract was dissolved in 1 ml of 50% methanol. Then, 1 ml of 8% vanillin reagent was added, mixed well, and followed by the addition of 2 ml of 72% sulphuric acid. The mixture was heated in a water bath at 60°C for 10 minutes and subsequently cooled in an ice-cold bath for 5 minutes. The absorbance was measured at 544 nm against a reagent blank (Hiai

et al., 1976). Diosgenin was used as the standard material and compare the assay with diosgenin equivalents. All the tests were carried out in triplicates and the results were expressed as µg Diosgenin equivalents per mg crude extract.

Total Sterol Content

The Liebermann-Burchard reagent was employed for sterol estimation. The reagent, composed of 0.5 ml concentrated sulfuric acid in 10 ml acetic anhydride, was mixed with 1 ml of extract, 4 ml chloroform, and 2 ml LB reagent. After 15 minutes of incubation in the dark, the mixture turned green and was measured spectrophotometrically at 640 nm against a blank (Attarde et al., 2010). The results were expressed as Beta-Sitosterol equivalent (µg of Beta-Sitosterol per mg of extract), with all tests performed in triplicates.

Total Tannin Content

Total phenolic content was estimated using the Folin-Ciocalteu assay. 1 ml of sample extract (1 mg/ml) was diluted to 6.5 ml with distilled water, then mixed with 1 ml of Folin-Ciocalteu reagent and 1.5 ml of 20% sodium carbonate. After 1 hour of incubation, the absorbance was measured at 725 nm against a blank (Afify et al., 2012). Tests were performed in triplicates, and results were expressed as µg of Tannic Acid Equivalent (TAE) per mg of extract.

Total Terpenoid Content

The extract was mixed with 3 ml chloroform, allowed to rest for 5 minutes, and then 500 µl sulphuric acid was added. After a 2-hour incubation at room temperature in the dark, reddish-brown precipitation formed. The supernatant was carefully decanted, and 1.5 ml 95% methanol was added, vortexing until the precipitation dissolved. Absorbance was measured at 538 nm (Ghorai et al., 2012). Tests were performed in triplicates, and results were expressed as linalool equivalent (µg of linalool/mg extract).

Total Cardiac Glycoside Content

1 ml of extract was mixed with 5 ml of Baljet's reagent (47.5 ml of 1% picric acid with 2.5 ml of 10% NaOH) and incubated for an hour. Then the mixture was mixed with 10 ml of distilled water and the absorbance was measured at

495nm (Solich et al., 1992). All the tests were carried out in triplicates and results were expressed as securidaside equivalent (µg of securidaside/mg extracts).

Total Carbohydrate Content

Polysaccharide content was estimated using the anthrone method. Sample solution was mixed with 4 ml of anthrone reagent (0.2% anthrone in concentrated sulphuric acid), then heated for 8 minutes at boiling point. The reaction was rapidly cooled in an ice bath. Absorbance was measured at 630 nm against a blank. This assay was based on the protocol by Hedge and Hofreiter (1962). All tests were conducted in triplicates to ensure accuracy. Results were expressed as micrograms of glucose equivalents per milligram of extract, providing a quantitative measure of polysaccharide content.

Total Coumarins Content

The plant extract was treated with lead acetate in a test tube. After shaking, 7 ml of distilled water and 8 ml of 1M hydrochloric acid solution were added and mixed thoroughly. The mixture was allowed to stand for 30 minutes at room temperature. The absorbance was then measured at 327 nm using a UV-visible spectrophotometer, following the method described by Hedge and Hofreiter (1962). This experiment was conducted in triplicates to ensure reliability. Results were expressed as micrograms of esculin equivalents per milligram of extract, providing a quantitative measure of flavonoid content.

Total Quinone Content

The extract was reacted with 8-hydroquinone reagent and ammonium hydroxide, followed by dilution with distilled water. After heating for 20 minutes in a water bath, the mixture was further diluted with 10 ml of distilled water. The resulting solution's absorbance was measured at 685 nm, adopting the method outlined by Singh et al. (2015). Triplicate tests ensured reliability. Results were expressed as micrograms of 2-hydroxymethylanthraquinone equivalents per milligram of extract, quantifying anthraquinone content.

Total Glycoside Content

Lead acetate solution was mixed with 1g of sample in test tubes and filtered. Chloroform was added to the filtrate, mixed thoroughly, and allowed to settle. The lower partition was collected and evaporated to dryness. Glacial acetic acid, 0.1ml of iron (lll) chloride, and sulphuric acid were added, mixed vigorously, and left for a few minutes. The absorbance was measured using a spectrophotometer at 568nm (Dewick, 2002). The experiments were conducted in triplicate to ensure reliability. Results were expressed as micrograms of quercetin equivalents per milligram of extract.

Total Protein Content

A 1 ml sample was mixed with 4 ml of freshly prepared alkaline solution, comprising 50 ml 2% Na2CO3 in 0.1 N NaOH, 1 ml 0.5% CuSO4·5H2O, and 1% sodium potassium tartrate. After 10-minute incubation at room temperature, 0.5 ml Folin-Ciocalteau reagent was added, followed by 1.5-hour incubation. The absorbance was measured at 660 nm against a blank, adopting the protocol outlined by Lowry et al. (1951). Triplicate tests ensured reliability. Results were expressed as micrograms of Bovine Serum Albumin (BSA) equivalents per milligram of extract (µg BSA/mg), quantifying protein content.

Antimicrobial Potential of Crude Extracts of Endophytic Bacterial and Fungal Isolates, and Plant Extracts

For screening of antimicrobial activity, the agar well diffusion method was employed against nine different test strains (Yadav et al., 2014). The pathogenic cultures used in this study were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh. These cultures were utilized to evaluate the antimicrobial activity of the extract. The test panel consisted of eight bacterial strains, including three Gram-positive bacteria, *Bacillus subtilis*, *Enterococcus faecalis*, and *Staphylococcus aureus*, and five Gram-negative bacteria, *Pseudomonas aureginosa*, *Salmonella typhi*, *Escherichia coli*, *Proteus vulgaris*, and *Vibrio flavialis*, and one pathogenic fungus *Candida albicans* were selected for this study. A loopful of cultures was inoculated into test tubes containing Muller Hilton broth and incubated at 37°C for 24 hrs. After incubation, the

standard inoculum was prepared by inoculating 100 µl (107 CFU/ml) of fresh bacterial culture into Muller Hilton broth and mixed properly. Wells of about 6 mm diameter were prepared and filled with 50 µl of endophytic and plant extracts. Standard antibiotic Azithromycin and an equal quantity of DMSO were used as a positive and negative control respectively. The endophytic and plant extracts demonstrated inhibitory effects against test organisms, evident from the clear zones formed around the wells containing these extracts. As the extracts diffused through the agar, their antimicrobial properties became apparent, inhibiting the growth of surrounding microorganisms and creating distinct inhibition zones. All the experiments were carried out in triplicates. Results were recorded as the zone of inhibition in mm (of diameter). The mean values of inhibition zones were calculated (Perez et al., 1990; Rojas et al., 2006) after 24 hrs of incubation.

Process Optimization of Fermentation Conditions for the Production of Active Metabolites from Selected Endophytic Isolates

Based on the analysis of the antibacterial activity of different extracts, some isolates were shortlisted for further study. Microbial biomass and crude metabolite production were further studied for the chosen species by optimizing parameters like temperature, pH, and incubation period in shake culture conditions. The isolate was cultivated in the specific medium in a 250 ml Erlenmeyer flask containing 100 ml of the medium at three different pH (3.0, 5.0, 7.0). The flasks were then incubated in a rotary shaker for 15th and 21st days at 25ºC, 27ºC, and 30ºC. The experiment was carried out in triplicate. The culture filtrates were extracted using ethyl acetate after the 15th and 21st days of incubation. Effects of temperature, pH, and incubation period on the production of crude metabolite were studied, and proper optimization parameters were selected for further extraction of crude metabolites.

Large-scale production of Secondary Metabolites from Selected Isolates for the Isolation of Pure Molecules

To isolate pure molecules from the microbial source, the selected endophytic isolate should be cultured in a large liquid medium for the production of

secondary metabolites by the process of liquid shake fermentation. Isolate was freshly sub-cultured and then inoculated into 3 litres (3000 ml) of autoclaved broth in 6 conical flasks of 1-liter capacity each. Each flask was inoculated with 20-25 agar plugs (8mm diameter) of actively growing cultures. The flasks were then incubated under continuous shaking conditions for optimized conditions. The filtrate was extracted three times with an equal volume of ethyl acetate. Solid residues obtained by evaporating organic extracts under reduced pressure were used for the evaluation of antibacterial activity.

Determination of Antimicrobial Activity in Optimized Condition

The antimicrobial activity was calculated using the extract from the selected potential endophytic isolate. The test was performed at four different concentrations, 250, 500, 750, and 1000 µg/ml, employing the well diffusion method (Dilika et al., 2000).

Determination of Diethyl Phthalate in Endophytic and Plant Samples Ethyl Acetate Extracts

A qualitative and quantitative evaluation of Diethyl phthalate in endophytic isolate and plant ethyl acetate extracts was conducted using the High-Performance Liquid Chromatography (HPLC) technique. Shimadzu LC2010AHT HPLC system was used for the analysis. Purospher STAR RP-18e (5 µm) column was used as the stationary phase. Methanol: deionized water, in the ratio 98: 2 v/v with a flow rate of 1 ml/min was used as the mobile phase. The column oven temperature was maintained at 30℃ and the samples were analyzed using a UV detector at a wavelength of 225 nm. Chemical screening of selected endophytes and plant samples was used to identify the compound Diethyl phthalate, which was assumed to be present in the plant extract as stated in the previous literature.

Antioxidant Activity

Crude extracts of microbial and plant materials were evaluated for antioxidant activity using different methods as discussed below.

Free Radical Scavenging Assay

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity

The antioxidant potential of extracts/fractions was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, as described by Blois (1958). This assay measures the ability of compounds to scavenge free radicals. The DPPH free radical possesses an unpaired electron, resulting in a strong absorption at 517 nm and a purple color. Addition of an antioxidant quenches this free electron, decolorizing the solution to yellow. A 0.1 mM DPPH solution in methanol was prepared. 100 µl of this solution was mixed with 30 µl of extract/fractions at concentrations of 20-100 µg/ml. After 30-minute incubation, absorbance was measured at 517 nm. Ascorbic acid served as the standard antioxidant. All tests were performed in triplicate. Percent inhibition was calculated using:

% inhibition = [Absorbance of control – Absorbance of test sample / Absorbance of control] × 100

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) Radical Scavenging Activity

The antioxidant activity of test samples was evaluated using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method. Stock solutions of 7.4 mM ABTS and 2.6 mM potassium persulfate were prepared. Equal quantities of these solutions were mixed and allowed to react for 16 hours at room temperature in darkness. The resulting ABTS+ solution was diluted with ethanol to achieve an absorbance of 0.70 ± 0.02 units at 734 nm. 30 µl of each extract (20-100 µg/ml) was mixed with 200 µl diluted ABTS+ solution. Absorbance was measured at 734 nm after 5-minute incubation (Thaipong et al., 2006). Trolox served as the reference compound. All determinations were performed in triplicate. Percent inhibition was calculated using:

% inhibition = [Absorbance of control – absorbance of the test sample/Absorbance of control] × 100

Reducing Power Assay of the Extract

Ferric Reducing Power Assay (FRAP)

The antioxidant potential of extracts was evaluated using the Ferric Reducing Power Assay, based on Oyaizu's method (1986) with modifications. This assay measures the ability of antioxidant compounds to donate electrons and reduce ferric ions. The assay procedure involved mixing 100 µl of extract (20-100 µg/ml in methanol) with 625 µl phosphate buffer (0.2M), 625 µl 1% potassium ferricyanide [K3Fe(CN)6], and 625 µl 10% trichloroacetic acid (TCA). The mixture was incubated at 50°C for 20 minutes, followed by centrifugation at 3000 rpm for 10 minutes. Then, 100 µl supernatant was mixed with 100 µl distilled water and 70 µl 0.1% ferric chloride (FeCl3), and absorbance was measured at 700 nm. All readings were performed in triplicate. The results indicated that increased absorbance with increasing extract concentration correlated with enhanced reducing power, reflecting the electron-donating capacity of antioxidant compounds and their potential antioxidant activity.

Results

Phytochemical Analysis

Qualitative Analysis of Endophytic Bacteria

The phytochemical qualitative analysis revealed diverse compounds across the 35 isolates (Table 33). Alkaloids were present in a few isolates, including BLWW1, BLWW4, BLWS6, BLWM1, and BLWS9, while flavonoids were detected in isolates such as BRhWW11 and BRhWS22. There are no bacterial isolates that showed the presence of phenol, glycoside, and carbohydrate. Saponin was found in several isolates, including BRWW6, BLWW1, BLWS11, BLWS6, BLWW10, BLWW9, BLWS4, BLWS1, BRhWS1, BLWS10, etc. Sterols were present in several isolates, including BRWS13, BLWS11, BLWM1, BLWS1, and all the rhizome isolates. At the same time, tannins were found in several isolates, including BRWW12, BRWS15, BRWW1, BLWS7, BLWW5, BRWW7, BRhWM3, etc. Terpenoids were present in a few isolates, including BRWS15, BRWS3, BLWW1, BLWW4, BLWS10, BLWM1, BRhWW11, and BRhWS27, suggesting a potential role in antioxidant activity,

while cardiac glycosides were detected in isolates like BRWM3, BRWM2, BLWW4, BLWW2, BLWS10, BLWM1, BLWW10, BLWW9, BLWS2, BLWS1, and BLWS3. Glycosides were found in several isolates, including BRhWS1 and BRhWM3, indicating a wide distribution of this compound. Coumarins were present in a few isolates, including BRWW6, BRWM3, BLWW4, BLWS2, BRhWS1, BRhWW11, BRhWW7, and BRhWM3. Quinones were found in several isolates, including BRWW6, BLWW4, BLWW1, BLWS7, BLWS10, BLWS2, and BRhWS1. Protein was present in a few isolates, including BRWS5, BLWS7, BLWS10, BLWS9, BLWS4, BRhWW10, BRhWS27, and BRhWS22. However, BLWW7 and BLWM4 did not show the presence of any of the chemicals. The analysis revealed a diverse range of phytochemicals across the isolates, with potential applications in various fields. Quantitative analysis of these phytochemicals was conducted using the isolates showing positive results.

Sl No.	Isolates	Al	Fl	Ph	Sa	St	Ta	Te	Cg	Gl	Ch	Co	Qu	Pr
1	BRWW12	-	-	-	-	-	++	-	-	-	-	-	-	-
2	BRWW6	-	-	-	+++	-	-	-	-	-	-	+	+++	-
3	BRWS13	-	-	-	-	+++	-	-	-	-	-	-	-	-
4	BRWS15	-	-	-	-	-	+++	++	-	-	-	-	-	+
5	BRWS1	-	-	-	-	-	-	-	-	-	-	-	-	-
6	BRWS3	-	-	-	-	-	-	+++	-	-	-	-	-	-
7	BRWM2	-	-	-	-	-	-	-	++	-	-	-	-	-
8	BRWM3	-	-	-	-	-	-	-	++	-	-	+	-	-
9	BLWW1	++	-	-	+++	-	+	+++	-	-	-	-	++	-
10	BLWW4	++	-	-	-	-	-	++	++	-	-	+	++	-
11	BLWW2	-	-	-	-	-	-	-	++	-	-	-	-	-
12	BLWS11	-	-	-	+++	++	-	-	-	-	-	-	-	-
13	BLWS6	++	-	-	+++	-	+	-	-	-	-	-	-	-
14	BLWS7	-	-	-	-	-	++	-	-	-	-	-	++	+
15	BLWW5	-	-	-	-	-	+++	-	-	-	-	-	-	-
16	BLWW7	-	-	-	-	-	+	-	-	-	-	-	-	-
17	BLWW8	-	-	-	-	-	-	-	-	-	-	-	-	-
18	BLWM4	-	-	-	-	-	-	-	-	-	-	-	-	-
19	BLWM1	+++	-	-	-	++	++	+	++	-	-	-	-	-
20	BLWW10	-	-	-	++	-	-	-	++	-	-	-	-	-
21	BLWW9	-	-	-	+++	-	-	-	++	-	-	-	-	-
22	BLWS10	-	-	-	++	-	-	++	++	-	-	-	+	++
23	BLWS9	+++	-	-	-	-	-	-	-	-	-	-	-	++
24	BLWS4	-	-	-	+++	-	+	-	-	-	-	-	-	+
25	BLWS2	-	-	-	-	-	+	-	++	-	-	+	++	-
26	BLWS1	-	-	-	++	+++	-	-	++	-	-	-	-	-
27	BLWS3	-	-	-	-	-	+	-	+++	-	-	-	-	-

28	BRhWS1	-	-	-	+	++	++	-	-	-	-	+	+++	-
29	BRhWS8	-	-	-	-	+++	+	-	-	-	-	-	-	-
30	BRhWW11	-	++	-	-	+++	-	-	-	-	-	+++	-	-
31	BRhWW7	-	-	-	+	++	+	-	-	-	-	+	-	-
32	BRhWM3	-	-	-	++	+++	+++	-	-	-	-	+	-	-
33	BRhWW10	-	-	-	++	+++	+++	-	-	-	-	-	-	+
34	BRhWS27	-	-	-	++	++	++	-	-	-	-	-	-	++
35	BRhWS22	-	+	-	++	+++	++	-	-	-	-	-	-	+

Table 33: Qualitative analysis by crude extracts of bacterial endophytes

Qualitative Analysis of Endophytic Fungi

The phytochemical analysis revealed a diverse range of compounds across the 36 isolates, with varying degrees of presence (Table 34). Isolates FRhWW3 and FRhWM2 showed a strong presence of multiple compounds, while FLWW9, FLWM11, FRWM3, FRhWS2, FLWM1, and FRWS24 showed a limited presence. Alkaloids were present in several isolates, including FRWS8, FLWW6, FRWS7, FRWM12, FRWW12, FLWW11, FLWS32, FRWM27, FLWS21, FRWW10, FRWW11, FRWS9, and FLWS5 indicating a potential role in biological activity. Flavonoids were detected in isolates like FLWW14, FLWW12, FLWS16, and FLWW18, suggesting a limited presence. Phenols were found in various isolates, including FRWS8, FLWW14, FRWS4, FRWM12, FRWS7, FLWW18, FRWW30, FLWS16, FRWS9, FLWS5 and FLWW12, indicating a wide distribution. Saponins were present in several isolates, including FLWW14, FRWS8, FLWW6, FRWS4, FRhWS1, FRWW18, FRhWW3, FRhWM2, and FLWS32 while sterols were detected in few isolates like FRWS7, FLWS32, FRhWW3, FRhWM2, and FRhWW4. Tannins were found in various isolates, including FRWS8, FRWS4, FLWW18, FLWM1, FRhWW3, FRhWM2, FLWS21, FRWW10, and FRhWW4, suggesting a potential role in antioxidant activity. Terpenoids were present in a few isolates, including FLWW14, FRWS8, FLWW15, FLWW6, FLWW12, FLWW11, FRhWS1, FLWW18, FLWS32, FRWW18, FLWS1, FRhWM2, FRhWW3, FRhWS2, FRhWM3, FLWS21, FLWS16, FRWW10, FRhWW4, FLWM11 while cardiac glycosides were detected in isolates like FLWW15, FLWW6, FRWS7, FRWM1, FRhWS1, FRWW18, FLWM1, FRhWW3, FRhWM2, FRhWS2, FRWM3, FLWS21 and FRWM25. Glycosides were found in several

isolates, including FLWW5, FRWM12, FRWM1, FLWW11, FLWS32, FRWW18, FRWW30, FRhWW3, FRWM3, FLWS21, FLWS16, and FRWW10, indicating a wide distribution. Carbohydrate content was present in few isolates FLWW5, FLWW12, FLWS32, FLWS1, FRhWW3, FRhWM2, FRWW10, and FRhWW4. Coumarin was observed in FRWS8, FLWW12, FLWW11, FRhWS1, FLWS1, FRhWW3, FRhWM2, FLWS16, FRhWW4, and FLWW13. Quinone was detected in FLWW15, FLWW17, FLWW6, FLWW12, FRWM1, FLWW11, FLWS32, etc. Proteins were present in most of the isolates, except FRWS8, FLWW6, FRWS4, FLWW11, FLWS32, FLWM1, FRhWW3, FRhWS2, FRWM3, FRWS24, FRhWW4, and FLWS5. The presence of these compounds suggests that the isolates may have potential biological activities, including antioxidant, anti-inflammatory, and antimicrobial properties. Quantitative analysis of these phytochemicals was conducted using the isolates showing positive results.

Sl No.	Isolates	Al	Fl	Ph	Sa	St	Ta	Te	Cg	Gl	Ch	Co	Qu	Pr
1	FLWW14	-	+++	+	+	-	-	+++	-	-	-	-	-	++
2	FRWS 8	+++	-	+++	++	-	++	+++	-	-	-	++	-	-
3	FLWW5	-	-	-	-	-	-	-	-	++	+++	-	-	++
4	FLWW15	+	-	-	-	-	-	+++	+	-	-	-	+++	+++
5	FLWW17	-	-	-	-	-	-	-	-	-	-	-	+++	++
6	FLWW6	++	-	-	+++	-	-	+++	+++	-	-	-	+++	-
7	FRWS7	+++	-	++	-	++	-	-	+	-	-	-	-	++
8	FRWM12	+++	-	++	-	-	-	-	-	+	-	-	-	+
9	FLWW12	++	+++	++	-	-	-	+	-	-	+	++	++	++
10	FRWM1	-	-	-	-	-	-	-	+	+	-	-	++	++
11	FRWS4	-	-	+++	++	-	+	-	-	-	-	-	-	-
12	FLWW11	++	-	-	-	-	-	+	-	++	-	++	++	-
13	FRhWS1	-	-	-	+++	-	-	+	+++	-	-	++	-	+
14	FLWW18	+	+++	+	-	-	+++	++	-	-	-	-	-	+
15	FLWS32	+++	-	-	+++	+++	-	++	-	++	++	-	+++	-
16	FRWW18	-	-	-	+++	-	-	+	+	+	-	-	++	++
17	FRWM25	-	-	-	-	-	-	-	-	-	-	-	++	++
18	FRWW30	-	-	+++	-	-	-	-	-	+	-	-	-	++
19	FLWM1	-	-	-	-	-	++	-	+++	-	-	-	-	-
20	FLWW9	-	-	-	-	-	-	-	-	-	-	-	-	+
21	FRWM27	+++	-	-	-	-	-	-	-	-	-	-	+++	++
22	FLWS1	-	-	-	-	-	-	+	+	-	++	++	+	+
23	FRhWW3	-	-	-	+++	++	++	+	+	+	+	++	++	-
24	FRhWM2	-	-	-	++	++	++	+++	++	++	+	++	+++	+
25	FRhWS2	-	-	-	-	-	-	-	+	-	-	-	-	-
26	FRWM3	-	-	-	-	-	-	-	++	+++	-	-	-	-
27	FLWS21	++	-	-	-	-	++	+	+++	++	-	-	-	+

28	FLWS16	-	++	++	-	-	-	++	-	+	-	+	+	+
29	FRWS24	-	-	-	-	-	-	-	+	-	-	-	-	-
30	FRWW10	++	-	-	-	-	+	+++	-	++	+++	-	++	+
31	FRWW11	++	-	-	-	-	-	-	+++	-	-	-	-	+
32	FRhWW4	-	-	-	+	++	++	+	-	-	+++	++	+	-
33	FRWS9	++	-	++	++	-	-	+++	-	-	-	-	-	+
34	FLWM11	-	-	-	-	-	-	+++	-	-	-	-	-	+++
35	FLWW13	-	-	++	-	-	-	-	-	+	-	++	-	+
36	FLWS5	++	-	+	++	-	-	-	+	-	-	-	+++	-

Table 34: Qualitative analysis by crude extracts of fungal endophytes

Qualitative Analysis of Plant Extracts

The phytochemical qualitative analysis of leaf, root, and rhizome isolates revealed varying levels of bioactive compounds (Table 35). The summer leaf extract showed the presence of all tested phytochemicals studied while the root showed the presence except coumarin and quinones in the rhizome extract, alkaloids, flavonoids, and phenols are absent. Extracts from the summer season showed more phytochemical contents than the winter and monsoon.

Sl No.	Isolates	Al	Fl	Ph	Sa	St	Ta	Te	Cg	Gl	Ch	Co	Qu	Pr
1	LS	+++	+++	+++	+++	+++	++	+++	++	+	++	+	+	++
2	RS	+++	+++	+++	+++	++	++	+++	-	+	++	-	-	++
3	RhS	-	-	-	+++	++	+++	+++	++	++	++	+	+	++
4	LW	++	++	++	++	+	+	+	+	+	+	+	+	+
5	RW	++	+	-	++	+	++	+	-	-	+	++	-	+
6	RhW	-	+	-	+	-	++	++	-	+	-	+	-	-
7	LM	++	++	++	+	++	+	+	++	+	+	+	+	-
8	RM	+	++	+	+	-	+	+	-	+	++	+	++	+
9	RhM	+	-	+	-	-	+	++	+	-	+	-	+	-

Table 35: Qualitative analysis by crude extracts of the plant parts

*LS: Leaf in summer; RS: Root in summer; RhS: Rhizome in summer; LW: Leaf in winter; RW: Root in winter; RhW: Rhizome in winter; LM: Leaf in monsoon; RM: Root in monsoon; RhM: Rhizome in monsoon

Quantitative Analysis

Alkaloid

Table 36 presents the quantitative analysis of alkaloid content in 19 fungal isolates, expressed as mean ± standard deviation. The alkaloid content varied widely among the isolates, ranging from 54.845 ± 3.36 (FLWW15) to 844.65 ± 134.99 (FRWM12) µg AE/mg. Isolates FRWS8, FLWS32, and FRWS7 showed high alkaloid content, with values above 700 µg AE/mg. Isolates BLWS9, FRWS9, FRWW10, FRWM12, FLWS21, and FRWM27 showed moderate to high alkaloid content, ranging from 500-700 µg AE/mg. Isolates FLWW6,

FLWW11, FLWW12, BLWM1, BLWW1, and BLWW4 showed moderate alkaloid content, with values ranging from 300-500 µg AE/mg. Isolates FLWW15, FLWW18, FLWS5, and FRWW11 showed low alkaloid content, with values below 300 µg AE/mg. When comparing the alkaloid content of the isolates with the alkaloid content in the leaf and root extracts, there is a greater difference in the summer. That means high alkaloid content is obtained in the leaf and root extract.

Sl No.	Isolates	Concentration (µg AE/mg)
1	FRWS7	744.01 ± 10.46^{af}
2	FRWS8	805.56 ± 14.98^{be}
3	FRWS9	625.74 ± 14.97^{c}
4	FLWW15	54.845 ± 3.36^{d}
5	FLWW6	324.16 ± 13.26^{h}
6	FLWW11	338.28 ± 26.73^{h}
7	FLWW12	376.58 ± 17.84^{hi}
8	FLWS32	768.21 ± 21.44^{a}
9	FRWM12	844.65 ± 134.99^{e}
10	FRWM27	700.01 ± 9.42^{f}
11	FRWW10	551.32 ± 32.48^{k}
12	FLWW18	58.048 ± 3.33^{d}
13	FLWS21	529.59 ± 17.82^{jk}
14	FLWS5	261.44 ± 10.37^{g}
15	FRWW11	215.26 ± 7.15^{g}
16	BLWS9	506.35 ± 14.91^{jk}
17	BLWM1	486.59 ± 12.24^{j}
18	BLWW1	319.64 ± 11.49^{h}
19	BLWW4	427.47 ± 7.16^{i}
20	LS	1181.01 ± 7.15^{l}
21	RS	1273.2 ± 2.15^{m}
22	LW	734.85 ± 8.76^{ef}
23	RW	893.11 ± 12.69^{e}
24	LM	472.90 ± 9.16^{i}
25	RM	378.90 ± 15.34^{hi}
26	RhM	456.99 ± 8.69^{jk}

Table 36: Alkaloid content of crude extracts

Flavonoid

The flavonoid content varied significantly among the isolates, ranging from 5.146 ± 0.345 (BRhWS22) to 189.04 ± 5.265 (FLWW12) µg QE/mg (Table 37). Isolates FLWW12 and FLWW14 showed high flavonoid content, with values above 180 µg QE/mg. Isolate FLWW18 showed moderate flavonoid content, with a 143.14 ± 6.341 µg QE/mg value. Isolate FLWS16 showed relatively low

flavonoid content, with a value of 115.14 ± 4.669 µg QE/mg. Isolates BRhWS22 and BRhWW11 showed very low flavonoid content, with values below 100 µg QE/mg. Here, the plant extracts also show higher flavonoid content in summer than in winter and monsoon. But as compared with the alkaloid content, the flavonoid quantity is less than that of the alkaloid content.

Sl No.	Isolates	Concentration (µg QE/mg)
1	FLWW14	182.06±6.560[a]
2	FLWW12	189.04±5.265[a]
3	FLWW18	143.14±6.341[b]
4	FLWS16	115.14±4.669[c]
5	BRhWS22	5.146±0.345[d]
6	BRhWW11	82.19±1.651[e]
7	LS	369.02±5.341[f]
8	RS	254.45±6.581[g]
9	LW	112.70±3.759[c]
10	RW	185.83±5.72[a]
11	RhW	159.88±7.438[ab]
12	LM	95.29±1.698[e]
13	RM	47.52±1.997[h]

Table 37: Flavonoid content of crude extracts

Phenol

The phenol content varied significantly among the isolates, ranging from 42.163 ± 1.905 (FLWW14) to 215.19 ± 4.066 (FRWS8) µg GAE/mg (Table 38). Isolate FRWS8 showed the highest phenol content, with a 215.19 ± 4.066 µg GAE/mg value. Isolates FRWS4 and FRWW30 showed high phenol content, with values above 180 µg GAE/mg. Isolates FRWS7, FLWS16, and FRWM12 showed moderate phenol content, with values ranging from 130-160 µg GAE/mg. Isolates FRWS9, FLWW12, and FLWW13 showed relatively low phenol content, with values ranging from 100-130 µg GAE/mg. Isolates FLWS5, FLWW18, and FLWW14 showed the lowest phenol content, with values below 100 µg GAE/mg. In the case of plant extracts, the phenol content is three to four times more than that of the isolates in all seasons. They show a huge difference in the phenol content.

Sl No.	Isolates	Concentration (µg GAE/mg)
1	FRWS4	186.73±6.395[a]
2	FRWS7	154.64±4.480[b]
3	FRWS8	215.19±4.066[c]

4	FRWS9	130.06±5.791[d]
5	FLWW12	126.73±4.305[d]
6	FLWW13	104.76±4.085[g]
7	FLWW14	42.163±1.905[h]
8	FRWW30	202.65±12.065[f]
9	FRWM12	135.81±4.324[de]
10	FLWW18	88.626±4.556[i]
11	FLWS16	141.5±5.962[e]
12	FLWS5	45.8±2.968[h]
13	LS	808.626±4.56[j]
14	RS	754.64±4.480[k]
15	LW	473.64±5.986[l]
16	LM	591.55±3.174[m]
17	RM	304.17±2.651[n]
18	RhM	296.77±7.92[n]

Table 38: Phenol content of crude extracts

Saponin

The saponin content varied widely among the isolates, ranging from 130.99 ± 6.148 (BRhWW11) to 927.64 ± 20.86 (FRhWW3) µg DE/mg (Table 39). Isolate FRhWW3 showed the highest saponin content, with a value of 927.64 ± 20.86 µg DE/mg. Isolates FRWW18, BLWW1, and FLWW6 showed high saponin content, with values above 850 µg DE/mg. Isolates FRWS8, BLWS4, and BLWS11 showed moderate to high saponin content, with values ranging from 750-850 µg DE/mg. Isolates FRWS4, FLWM11, FLWS32, and BRWW6 showed moderate saponin content, with values ranging from 600-750 µg DE/mg. Isolates FLWS5, BLWS1, and BLWS10 showed relatively low saponin content, with values ranging from 400-600 µg DE/mg. Isolates FLWW14, FRhWW4, BRhWW7, BRhWM3, BRhWW10, and BRhWM27 showed low saponin content, with values below 400 µg DE/mg. The leaf extract shows a saponin concentration of 892.52±7.571 µg DE/mg, the fungal isolate FLWW6 and the bacterial isolate BLWW1 shows the comparative amount of saponin with a concentration of 870-875 µg DE/mg. In the case of root extract, there is no comparison with the isolates obtained from the root. The saponin concentration in the root extract is 618.52±2.50 in summer. In the rhizome extract, the saponin concentration is 621.68±0.798, and the bacterial isolate BRhWW10 showed a similar concentration.

Sl No.	Isolates	Concentration (µg DE/mg)
1	FRWS4	643.74±12.008[a]
2	FRWS8	807.95±11.699[b]
3	FLWM11	687.61±7.607[c]
4	FLWW6	868.88±5.299[d]
5	FLWW14	289.31±5.726[f]
6	FLWS32	746.75±11.052[g]
7	FRWW18	875.75±11.762[d]
8	FLWS5	540.88±4.864[e]
9	FRhWS1	624.52±3.899[f]
10	FRhWW4	256.86±4.725[m]
11	FRhWW3	927.64±20.86[n]
12	FRhWM2	400.65±14.016[op]
13	BLWS1	458.38±6.355[h]
14	BLWS4	842.72±6.495[i]
15	BLWS10	521.4±5.470[j]
16	BLWS11	765.18±5.035[k]
17	BLWW1	872.41±2.019[d]
18	BLWS6	724.09±3.553[l]
19	BLWW9	745.34±8.376[g]
20	BLWW10	620.52±5.350[f]
21	BRWW6	693.95±9.272[c]
22	BRhWS1	310.38±5.645[q]
23	BRhWW7	413.87±9.638[o]
24	BRhWM3	392.52±7.571[p]
25	BRhWW10	278.9±5.883[f]
26	BRhWM27	353.44±11.287[s]
27	BRhWW11	130.99±6.148[r]
28	LS	892.52±7.571[d]
29	RS	618.52±2.503[f]
30	RhS	621.68±0.798[f]
31	LW	874.02±6.489[d]
32	RW	628.7±1.386[f]
33	RhW	690.34±2.893[f]
34	LM	401.66±4.768[o]
35	RM	392.64±8.267[p]

Table 39: Saponin content of crude extracts

Sterol

The sterol content varied widely among the isolates, ranging from 160.69±3.569 (FRhWW3) to 1112±29.885 (BRhWW11) µg/mg (Table 40). Isolates BLWS1, BRWS13, BRhWM3, and BRhWS22 showed high steroid content, with values above 210 µg/mg. Isolates FLWS32, BLWS11, and BRhWW10 showed moderate to high steroid content, ranging from 180 to 210 µg/mg. Isolates FRWS7, FRhWW3, FRhWM2, FRhWW4, BLWM1, BRhWS1, BRhWS8, and BRhWM27 showed moderate steroid content, with values ranging from 160-

180 µg/mg. In the quantitative analysis of sterol, found that the root and leaf extracts showed the presence of high concentration. While there is no comparative concentration can be seen in the isolates.

Sl No.	Isolates	Concentration (µg/mg)
1	FRWS7	160.7±7.115[a]
2	FLWS32	190.69±10.019[c]
3	FRhWW3	160.69±3.569[a]
4	FRhWM2	173.62±2.864[abc]
5	FRhWW4	174.21±3.777[abc]
6	BLWS1	216.45±4.607[d]
7	BLWS11	185.81±7.427[bc]
8	BLWM1	171.97±7.345[abc]
9	BRWS13	256.95±11.825[e]
10	BRhWS1	167.87±3.878[ab]
11	BRhWS8	181.64±6.319[bc]
12	BRhWW7	170.19±7.970[ab]
13	BRhWM3	219.52±7.343[d]
14	BRhWW10	187.73±5.807[bc]
15	BRhWM27	178.37±14.888[abc]
16	BRhWS22	211.72±7.456[d]
17	BRhWW11	1112±29.885[f]
18	LS	387.73±5.807[g]
19	RS	197.69±0.019[c]
20	RhS	263.09±11.895[e]
21	LW	379.44±4.913[g]
22	RW	316.04±0.084[h]
23	LM	185.56±3.815[bc]

Table 40: Sterol content of crude extracts

Tannin

Table 41 presents the quantitative analysis of tannin content in 33 endophytic isolates, expressed as mean ± standard deviation. The tannin content varied widely among the isolates, ranging from 2.1957 ± 0.092 (BLWS4) to 79.1858 ± 3.047 (FLWW18) µg TAE/mg (Table 41). Isolates FRWM1, BRhWM3, and FLWW11 showed high tannin content, with values above 25 µg TAE/mg. Isolates BLWW5, BRWW12, BRhWW10, and BRWS15 showed moderate to high tannin content, with values ranging from 15-25 µg TAE/mg. Isolates FRWS4, FRhWW4, FRWM27, FLWM1, FLWS32, FRhWW3, FRhWM2, FRhWW4, BLWS7, and BRhWS22 showed moderate tannin content, with values ranging from 10-15 µg TAE/mg. Isolates BRhWS8, BLWW8, BLWW1, BLWS6, BLWS4, BLWS3, FRWW10, and BRhWS1 showed relatively low

tannin content, with values ranging from 5-10 µg TAE/mg. Isolates BLWS2, BLWS4, BLWS6, and BLWW1 showed the lowest tannin content, with values below 5 µg TAE/mg. As 33 isolates showed the presence of tannin content, the concentration is highest in the leaf, root, and rhizome. No proportional concentration can be seen in isolates.

Sl No.	Isolates	Concentration (µg TAE/mg)
1	FRWS4	11.2436 ± 0.982^{a}
2	FRWS8	12.4742 ± 0.552^{ab}
3	FLWM1	12.6098 ± 0.950^{ab}
4	FLWW15	16.5582 ± 0.838^{fgh}
5	FLWW11	25.592 ± 1.573^{k}
6	FLWW13	17.6316 ± 0.662^{hi}
7	FLWS32	14.5553 ± 0.597^{cde}
8	FRWM27	11.0113 ± 0.288^{a}
9	FRWM1	28.6089 ± 1.798^{m}
10	FRWW10	9.0949 ± 0.928^{s}
11	FLWW18	79.1858 ± 3.047^{l}
12	FLWS21	14.7449 ± 0.426^{de}
13	FRhWW3	14.7323 ± 0.583^{de}
14	FRhWM2	15.071 ± 0.156^{def}
15	FRhWW4	11.9736 ± 0.212^{ab}
16	BLWS2	2.203 ± 0.329^{n}
17	BLWS3	8.6133 ± 0.435^{rs}
18	BLWS4	2.1957 ± 0.092^{n}
19	BLWS6	4.4502 ± 0.395^{o}
20	BLWS7	11.2296 ± 0.563^{a}
21	BLWM1	17.2544 ± 1.386^{gh}
22	BLWW1	5.2179 ± 0.358^{op}
23	BLWW5	19.8995 ± 0.766^{j}
24	BLWW8	6.5242 ± 0.503^{pq}
25	BRWS15	19.0855 ± 0.859^{ij}
26	BRWW12	15.7852 ± 0.262^{efg}
27	BRhWS8	7.2369 ± 0.213^{qr}
28	BRhWW7	13.6122 ± 0.653^{bcd}
29	BRhWM3	27.9963 ± 0.701^{m}
30	BRhWW10	21.8083 ± 0.057^{m}
31	BRhWM27	12.0736 ± 0.719^{ab}
32	BRhWS22	13.0923 ± 0.104^{bc}
33	BRhWS1	12.637 ± 0.463^{ab}
34	LS	90.949 ± 0.928^{t}
35	RS	167.52 ± 4.386^{u}
36	RhS	314.7449 ± 0.426^{v}
37	LW	68.45 ± 0.825^{w}
38	RW	95.63 ± 1.279^{t}
39	RhW	40.55 ± 2.145^{n}
40	LM	71.06 ± 0.596^{l}
41	RM	79.44 ± 1.704^{l}
42	RhM	30.15 ± 0.084^{m}

Table 41: Tannin content of crude extracts

Terpenoid

Table 42 presents the quantitative analysis of terpenoid content in 25 isolates, expressed as mean ± standard deviation. The terpenoid content varied widely among the isolates, ranging from 0.566 ± 0.189 (FRhWW3) to 544.77 ± 12.829 (FLWW6) µg linalool/mg (Table 42). Isolates FLWM11 and FRWS9 showed high terpenoid content, with values above 350 µg linalool/mg. Isolates FRWS8, FLWW14, FRhWM2, and BLWW1 showed moderate to high terpenoid content, with values ranging from 200-350 µg linalool/mg. Isolates FLWW12, FLWS32, and FLWW18 showed moderate terpenoid content, with values ranging from 150-200 µg linalool/mg. Isolates FLWS16, FLWS21, and BLWS10 showed relatively low terpenoid content, with values ranging from 100-150 µg linalool/mg. Isolates FLWW11, FRWW18, FLWS1, FRhWS1, FRhWW3, FRhWW4, BLWW4, BRWS15, and BLWM1 showed low terpenoid content, with values below 100 µg linalool/mg. As a larger number of isolates show the presence of terpenoids, the concentration is highest in the plant extracts of the summer and winter seasons.

Sl No.	Isolates	Concentration (µg linalool/mg)
1	FRWS8	335.64 ± 6.154^{a}
2	FRWS9	354.31 ± 13.913^{b}
3	FLWM11	508.8 ± 25.260^{c}
4	FLWW15	251.66 ± 23.618^{e}
5	FLWW6	544.77 ± 12.829^{d}
6	FLWW11	83.6367 ± 6.308^{l}
7	FLWW12	179.92 ± 3.909^{f}
8	FLWW14	342.42 ± 14.374^{ab}
9	FLWS32	184.56 ± 5.723^{fg}
10	FRWW10	238.14 ± 9.910^{e}
11	FRWW18	35.21 ± 1.114^{jk}
12	FLWW18	185.05 ± 4.885^{fg}
13	FLWS1	23.8033 ± 1.868^{ijk}
14	FLWS16	177.42 ± 2.692^{f}
15	FLWS21	128.34 ± 4.462^{m}
16	FRhWS1	21.57 ± 0.981^{ij}
17	FRhWW3	0.5667 ± 0.189^{h}
18	FRhWM2	216.04 ± 6.232^{p}
19	FRhWW4	32.6833 ± 1.966^{jk}
20	BLWS10	103.22 ± 3.223^{n}
21	BLWW1	200.63 ± 4.805^{g}
22	BLWW4	40.7367 ± 3.872^{k}

23	BRWS3	155.29±7.187^{o}
24	BRWS15	34.91±2.439^{ik}
25	BLWM1	14.6612±1.252^{hi}
26	LS	816.04±6.232^{q}
27	RS	744.77±12.829^{r}
28	RhS	440.7367±2.208^{s}
29	LW	643.7±5.72^{t}
30	RW	459.27±3.825^{bc}
31	RhW	553.6±3.572^{d}
32	LM	290.13±6.89^{a}
33	RM	219.55±2.285^{p}
34	RhM	157.25±0.984^{f}

Table 42: Terpenoid content of crude extracts

Cardiac Glycoside

The cardiac glycoside content varied widely among the 27 isolates, ranging from 4.6733 ± 0.357 (FRhWS2) to 24.9863 ± 0.803 (FRhWS1) µg/mg (Table 43). Isolates FRhWS1, FLWS21, and FLWM1, FRWW11, showed exceptionally high cardiac glycoside content, with values above 20 µg/mg. A few isolates, including FLWM1, and FLWW6 exhibited moderate to high cardiac glycoside content, with values ranging from 15-20 µg/mg. Many isolates, such as FLWW15, FRWM1, BLWS1, BLWS10, BRWM2, and BLWW10 showed moderate cardiac glycoside content, with values between 10-15 µg/mg. Some isolates, like BLWW9, and BLWS2 had relatively low cardiac glycoside content, with values ranging from 5-10 µg/mg. Finally, a few isolates, including FRhWS2 and FRWS7 showed low cardiac glycoside content, with values below 5 µg/mg. In the case of cardiac glycoside, the highest concentration can be seen in leaf and rhizome extracts, 16.52±1.652 µg/mg and 17.238±0.52 µg/mg respectively in summer. The fungal isolate FLWS21 shows a comparative amount of cardiac glycoside with the leaf extract with a concentration of 16.352±0.635 µg/mg.

Sl No.	Isolates	Concentration (µg/mg)
1	FRWS24	8.3994±0.538^{ijk}
2	FRWS7	4.9103±0.122^{ab}
3	FLWW15	5.63±0.492^{abcdef}
4	FLWW6	14.8883±0.689^{n}
5	FRWM1	5.2117±0.289^{abcd}
6	FRWW18	8.8333±0.966^{jk}
7	FLWS1	7.5007±0.548^{hi}

8	FLWS21	16.352 ± 0.635^{p}
9	FLWM1	15.1443 ± 0.825^{no}
10	FLWS5	6.1693 ± 0.170^{cdefg}
11	FRWW11	16.616 ± 0.582^{p}
12	FRWM3	9.414 ± 0.258^{k}
13	FRhWS1	24.9863 ± 0.803^{r}
14	FRhWS2	4.6733 ± 0.357^{a}
15	FRhWW3	6.1613 ± 0.939^{cdefg}
16	FRhWM2	11.435 ± 0.755^{l}
17	BLWS1	5.4963 ± 0.435^{abcde}
18	BLWS10	5.399 ± 0.210^{abcd}
19	BLWM1	5.996 ± 0.071^{bcdefg}
20	BLWS2	7.323 ± 0.618^{hi}
21	BLWS3	13.811 ± 0.264^{m}
22	BLWW2	6.2173 ± 0.069^{defg}
23	BLWW4	5.5787 ± 0.272^{abcdef}
24	BLWW9	8.0937 ± 0.624^{ij}
25	BLWW10	5.9267 ± 0.295^{bcdefg}
26	BRWM2	5.76 ± 0.510^{acdef}
27	BRWM3	6.9768 ± 0.057^{gh}
28	LS	16.52 ± 1.652^{p}
29	RhS	17.238 ± 0.52^{p}
30	LW	8.40 ± 0.629^{ij}
31	LM	3.65 ± 0.097^{q}
32	RhM	5.55 ± 0.426^{abcde}

Table 43: Cardiac glycoside content of crude extracts

Carbohydrate

Table 44 presents the quantitative analysis of carbohydrate content in 8 fungal isolates, expressed as mean ± standard deviation. The carbohydrate content varied widely among the isolates, ranging from 1.0846 ± 0.061 (FRhWW3) to 279.06 ± 8.019 (FRhWW4) µg glucose/mg. Whereas the leaf, root, and rhizome extracts show the highest concentration. Isolate FLWW5 showed high carbohydrate content, with a value of 207.35 ± 3.213 µg glucose/mg. Isolate FRWW10 showed moderate carbohydrate content, with 164.97 ± 3.920 µg glucose/mg. Isolates FLWS32 and FLWS1 showed relatively low carbohydrate content, ranging from 40-50 µg glucose/mg. Isolates FLWW12, FRhWM2, and FRhWW3 showed the lowest carbohydrate content, below 10 µg glucose/mg. A moderate amount of carbohydrates was observed in plant samples.

Sl No.	Isolates	Concentration (µg glucose/mg)
1	FLWW5	207.35 ± 3.213^{a}
2	FLWW12	9.558 ± 0.556^{b}
3	FLWS32	51.777 ± 3.079^{d}

4	FRWW10	164.97±3.920^{e}
5	FLWS1	46.7502±2.350^{d}
6	FRhWW3	1.0846±0.061^{c}
7	FRhWM2	3.2861±0.296^{bc}
8	FRhWW4	279.06±8.019^{f}
9	LS	81.06±0.415^{g}
10	RS	62.61±0.256^{h}
11	RhS	49.702±2.056^{d}
12	LW	75.66±2.567^{g}
13	RW	52.91±1.89^{d}
14	LM	12.57±0.024^{i}
15	RM	24.22±0.541^{j}
16	RhM	56.18±0.615^{d}

Table 44: Carbohydrate content of crude extracts

Coumarin

Table 45 presents the quantitative analysis of coumarin content in 18 isolates, expressed as mean ± standard deviation. The coumarin content varied widely among the isolates, ranging from 0.0705 ± 0.003 (BRhWS1) to 4.126 ± 0.112 (BRhWW11) µg esculin/mg. Isolates FRWS8 and FLWW13 showed high coumarin content, with values above 0.75 µg esculin/mg. Isolates FLWW12, FLWS1, FRhWS1, and FRhWM2 showed moderate coumarin content, ranging from 0.5-0.75 µg esculin/mg. Isolates FLWW11, FLWS16, FRhWW3, and FRhWW4 showed relatively low coumarin content, ranging from 0.3 to 0.5 µg esculin/mg. Isolates BLWS2, BLWW4, BRWM3, BRWW6, BRhWS1, BRhWW7, and BRhWM3 showed low coumarin content, with values below 0.3 µg esculin/mg. The coumarin content of the leaf extract is 0.813±0.035 µg esculin/mg and the isolate FLWW13 has a comparable amount of coumarin with the concentration of leaf extract. While the concentration in rhizome extract in summer is 1.913±1.15 µg esculin/mg and it is not comparable with any of the isolates.

Sl No.	Isolates	Concentration (µg esculin/mg)
1	FRWS8	0.7603±0.038^{g}
2	FLWW11	0.4022±0.030^{d}
3	FLWW12	0.6539±0.009^{f}
4	FLWW13	0.7712±0.052^{g}
5	FLWS1	0.5286±0.094^{e}
6	FLWS16	0.3512±0.045^{cd}
7	FRhWS1	0.5623±0.005^{e}
8	FRhWW3	0.4042±0.012^{d}

9	FRhWM2	0.5379±0.054^{e}
10	FRhWW4	0.3154±0.013^{c}
11	BLWS2	0.1536±0.005^{b}
12	BLWW4	0.0847±0.004^{ab}
13	BRWM3	0.087±0.010^{ab}
14	BRWW6	0.1198±0.008^{ab}
15	BRhWS1	0.0705±0.003^{a}
16	BRhWW7	0.1182±0.017^{ab}
17	BRhWM3	0.1351±0.009^{ab}
18	BRhWW11	4.126±0.112^{h}
19	LS	0.813±0.035^{g}
20	RhS	1.913±1.15^{f}
21	LW	1.134±0.771^{i}
22	RW	0.812±0,538^{g}
23	RhW	0.521±0.049^{e}
24	LM	0.95±0.007^{j}
25	RM	0.778±0.056^{g}

Table 45: Coumarin content of crude extracts

Quinone

Table 46 presents the quantitative analysis of quinone content in 25 isolates, expressed as mean ± standard deviation. The quinone content varied widely among the isolates, ranging from 0.0384 ± 0.0006 (BLWS10) to 0.276 ± 0.012 (BRWW6) µg 2-HQ /mg. Isolates FLWW17, FLWS32, FLWW15, FLWW6, and BRhWS1 showed high quinone content, with values above 0.24 µg 2-HQ /mg. Isolates FRWM25, FRWM1, FRWW10, FLWS1, and FRhWM2 showed moderate quinone content, ranging from 0.18-0.24 µg 2-HQ /mg. Isolates FLWW11, FRWM26, FRWW18, FRhWW3, BLWS2, and BLWW4 showed relatively low quinone content, ranging from 0.1-0.18 µg 2-HQ /mg. Isolates FLWS16, FRhWW4, and BLWS10 showed low quinone content, with values below 0.1 µg 2-HQ /mg. The quinone in the leaf extract is 0.719±0.083 µg 2-HQ /mg, which is noncomparable with any of the isolates.

Sl No.	Isolates	Concentration (µg 2-HQ /mg)
1	FLWW11	0.1595±0.016^{ab}
2	FLWW12	0.1182±0.014^{e}
3	FLWW17	0.2642±0.035^{hi}
4	FLWS32	0.2476±0.010^{ij}
5	FRWM25	0.1827±0.006^{bcd}
7	FRWM27	0.2614±0.005^{hi}
8	FRWM1	0.1833±0.005^{bcd}
9	FRWW10	0.1857±0.007^{cd}
10	FRWW18	0.1544±0.006^{a}

11	FLWS1	0.1943±0.007^{d}
12	FLWS16	0.0709±0.018^{f}
13	FLWS5	0.2439±0.012^{ij}
14	FLWW15	0.268±0.011^{hi}
15	FLWW6	0.2668±0.013^{hi}
16	FRhWW3	0.1634±0.008^{abc}
17	FRhWM2	0.2207±0.018^{k}
18	FRhWW4	0.0484±0.002^{g}
19	BLWS2	0.1676±0.009^{abc}
20	BLWS7	0.2308±0.019^{jk}
21	BLWS10	0.0384±0.0006^{g}
22	BLWW1	0.1065±0.005^{e}
23	BLWW4	0.1653±0.009^{abc}
24	BRWW6	0.276±0.012^{h}
25	BRhWS1	0.2565±0.011^{hi}
26	LS	0.719±0.083^{l}
27	RhS	0.248±0.171^{hi}
28	LW	0.396±0.093^{m}
29	LM	0.129±0.042^{e}
30	RM	0.056±0.0007^{g}
31	RhM	0.036±0.002^{g}

Table 46: Quinone content of crude extracts

Glycoside

The glycoside content varied widely among the 15 isolates, ranging from 0.4 ± 0.05 (FRWW18) to 80.9333 ± 0.731 (FRWM3) units (Table 47). Isolate FRWM3 showed exceptionally high glycoside content, followed by isolates FLWW5, FLWS21, and FLWW11, which exhibited high glycoside content with values above 39 µg QE/mg. Isolates FRWW10 and FLWS32 showed moderate to high glycoside content, ranging from 30-36 µg QE/mg. Several isolates, including FLWS16, FLWS5, FRWM12, and FRhWW3, showed moderate glycoside content, with values between 11-20 µg QE/mg. Isolates FLWW13, FRWM1, and FRWW30 had relatively low glycoside content, with values ranging from 6-7 µg QE/mg. Finally, isolate FRWW18 showed the lowest glycoside content, with a value of 0.4 µg QE/mg. Here the isolate FRWM3 showed higher glycoside concentrations than the leaf and root extracts.

Sl No.	Isolates	Concentration (µg QE/mg)
1	FLWW5	43.9367±1.546^{a}
2	FLWW11	39.1267±1.463^{b}
3	FLWW13	7.1013±0.066^{c}
4	FLWS32	30.3063±1.464^{d}
5	FRWM12	11.3386±0.426^{e}

6	FRWM1	6.9543±0.725[c]
7	FRWW10	36.5347±0.480[f]
8	FRWW18	0.4±0.05[g]
9	FRWW30	7.1667±0.351[c]
10	FLWS16	13.7747±0.594[h]
11	FLWS21	41.669±0.728[i]
12	FLWS5	12.7467±0.116[h]
13	FRWM3	80.9333±0.731[j]
14	FRhWW3	19.33±0.378[k]
15	FRhWM2	26.465±0.588[l]
16	LS	26.73±1.248[l]
17	RS	53.67±1.546[m]
18	RhS	34.55±1.09[b]
19	LW	33.71±0.825[b]
20	RhW	12.30±0.613[h]
21	LM	7.27±0.167[c]
22	RM	7.31±0.425[c]

Table 47: Glycoside content of crude extracts

Protein

The protein content varied widely among the 33 isolates, ranging from 0.722 ± 0.05281 (FLWS1) to 102.98 ± 1.490 (FLWM11) µg BSA/mg (Table 48). Isolate FLWM11 showed exceptionally high protein content, followed by isolates FLWW15 and FLWW14, which exhibited high protein content with values above 57 µg BSA/mg. Several isolates, including FLWW17, FLWW12, FRWM27, and FRWW30 showed moderate to high protein content, with values ranging from 40-57 µg BSA/mg. Many isolates, such as FLWW5, FRWS7, FRWM25, FRWM1, FLWW18, FLWS21, FRWW11, FRhWM2, BLWS10, BLWM4, BLWS9, and BRhWM27 showed moderate protein content, with values between 20-40 µg BSA/mg. Some isolates, like FLWW9, FLWW13, BLWS7, and BLWS4, had relatively low protein content, with values ranging from 1-10 µg BSA/mg. Finally, isolates FLWS1 showed the lowest protein content, with values below 1 µg BSA/mg. The protein content of the leaf extract is 101.98±2.938 µg BSA/mg, and the fungal isolate FLWM11 shows a comparatively equal concentration. In the case of the root, the protein concentration is 62.71±1.969 µg BSA/mg and the rhizome have a concentration of 33.51±1.725 µg BSA/mg. Leaf extracts of summer have a greater concentration of protein presence.

Sl No.	Isolates	Concentration (µg BSA/mg)
1	FRWS9	13.2919±0.511[a]
2	FLWM11	102.98±1.490[b]
3	FLWW15	93.6015±2.506[c]
4	FLWW14	57.257±2.110[d]
5	FLWW17	53.8744±2.186[e]
6	FLWW5	31.4781±0.733[ij]
7	FRWS7	30.5784±1.364[ik]
8	FLWW9	8.0153±0.210[n]
9	FLWW12	46.2611±0.868[p]
10	FLWW13	2.004±0.040[o]
11	FRWM25	33.5031±1.076[j]
12	FRWM12	5.6937±0.779[n]
13	FRWM27	41.9405±1.272[q]
14	FRWM1	39.4465±1.401[r]
15	FRWW10	13.0211±0.676[a]
16	FRWW18	62.6713±3.306[f]
17	FRWW30	50.647±1.645[g]
18	FLWW18	20.354±1.098[m]
19	FLWS1	0.722±0.052[o]
20	FLWS16	14.3585±1.949[ah]
21	FLWS21	29.6288±2.858[ik]
22	FRWW11	20.9457±0.656[m]
23	FRhWS1	16.2748±1.895[h]
24	FRhWM2	21.4713±0.944[m]
25	BLWS4	1.0749±0.085[o]
26	BLWS7	5.7871±0.591[n]
27	BLWS9	28.8163±1.122[k]
28	BLWS10	21.8003±0.695[m]
29	BLWM4	26.0735±0.756[l]
30	BRWS15	19.4427±0.884[m]
31	BRhWW10	12.388±0.446[a]
32	BRhWM27	26.015±1.670[l]
33	BRhWS22	14.1628±0.406[ah]
34	LS	101.98±2.938[b]
35	RS	62.71±1.969[f]
36	RhS	33.51±1.725[j]
37	LW	49.73±1.939[s]
38	RW	41.88±0.859[q]
39	RM	20.11±0.059[m]

Table 48: Protein content of crude extracts

Antimicrobial Activity of Endophytic Bacteria

The antimicrobial activity of the bacterial isolates was evaluated against a range of microorganisms, including Gram-positive and Gram-negative bacteria and fungi. The results showed that several isolates exhibited significant antimicrobial activity, while others showed moderate or low activity (Table 49). The isolates with high antimicrobial activity were BLWS10, BLWW10, and

BLWS11. These isolates demonstrated broad-spectrum activity, inhibiting the growth of multiple microorganisms. The inhibition zones ranged from 15 mm to 18 mm, indicating strong antimicrobial activity (Fig.86).

The zone of inhibition values ranged from 0 to 18.83 mm, indicating varying levels of antimicrobial activity among the isolates. Among 35 isolates, 14 (40%) showed antimicrobial activity on *Bacillus subtilis* with zones of inhibition ranging from 7.5 to 14.6 mm. 8 isolates (22.85%) showed antimicrobial activity against *Pseudomonas aureginosa*, of inhibition ranging from 7.1 to 13.47 mm. The zone of inhibition ranging from *Salmonella typhi* showed the highest range of inhibition from 7.6 to 18.83 mm by 13 isolates (37.14%). 6 isolates (17.14%) had inhibition against *Enterococcus faecalis,* and *Candida albicans* ranging from 7.47 to 13.77 mm, and 7.97 to 11.07 mm respectively. The inhibition ranged from 7.46 to 11.4 mm was shown by 5 isolates (14.28%) against *E. coli*. Among the total isolates, only 3 isolates (8.57%) showed a zone of inhibition against *Staphylococcus aureus* ranging from 7.47 to 14.33 mm, and 2 isolates (5.71%) against *Proteus vulgaris* of 12.67 and 11.53 mm. 7 isolates (20%) showed antimicrobial activity against *Vibrio fluvialis* ranging from 7.4 to 14.63 mm. There are 2 isolates (5.71%) that showed no antimicrobial activity, with zones of inhibition of 0 mm.

BLWS10 showed strong antimicrobial activity against *Salmonella typhii* at 18.83±1.68 mm followed by BLWW10 (17.73±1.80 mm), BLWS11 (16.3±3.37 mm), BLWW5 (16.13±1.90 mm), and BLWS6 (15.76±3.95). All are shown their inhibition on *S. typhi*. BRWS15, BLWS6, and BLWM1 showed moderate antimicrobial activity against *P. vulgris,* and *V. fluvialis* of 12.67±0.98 mm, 13.17±3.57 mm, and 12.0±3.65 respectively. BRWS1 and BLWW4 showed moderate antimicrobial activity against *S. typhii* of 11.37±4.48 mm, and 13.03±2.54 mm. BLWW1 and BLWS10 showed moderate antimicrobial activity against *Pseudomonas aureginosa* of diameter 12.6±5.13 mm and 11.36±0.40 mm. BRhWS1 showed a low activity of 9.63±0.351, 7.1±0.10, 7.6±0.26, and 7.97±0.25 against *B. subtilis*, *P. aureginosa, S. typhi,* and *C.*

albicans respectively. BLWS10, in particular, showed maximum antimicrobial activity, inhibiting the growth of 5 tested microorganisms, with inhibition zones ranging from 11.07±0.21mm to 18.83±1.68 mm. This isolate may produce potent antimicrobial compounds that can effectively target a wide range of microorganisms, making it a promising candidate for further study. Most isolates showed low antimicrobial activity, with less than 10 mm inhibition zones.

The inhibition zone shown by leaf, root, and rhizome extracts in the summer against *B. subtilis* is 22.6±2.22mm, 19.53±0.87mm, and 25.2±2.72mm, respectively. The inhibition zone shown by leaf, root, and rhizome extracts against *P. aureginosa* is 8.9±0.75mm, 8.97±1.747, and 14.87±0.7mm, respectively. The inhibition zone of antimicrobial activity against *S. typhi* by leaf, root and rhizome extracts are 22.07±1.92mm, 20.47±1.21mm, and 19.73±1.47mm. Antimicrobial activity against *Enterococcus faecalis* showed an inhibition zone of 18.33±0.80mm in leaf extract, 16.01±0.1mm in root extract, and 15.63±0.98mm in rhizome extract. Antimicrobial activity against *E. coli* showed an inhibition zone of 17.5±1.31 in leaf extract, 20.7±3.65mm in root extract, and 26.67±1.24mm in rhizome extract. The inhibition zone of antimicrobial activity against *Staphylococcus aureus* by leaf, root and rhizome extracts are 29.5±1.23mm, 27.4±3.62mm, and 28.17±2.03mm, respectively. The inhibition zone of antimicrobial activity against *P. vulgaris* by leaf, root and rhizome extracts are 27.4±2.42mm, 18.2±1.83mm, and 27.87±0.81mm, respectively. Antimicrobial activity against *V. fluvialis* showed an inhibition zone of 25.0±4.03mm in leaf extract, 25.57±1.11mm in root extract, and 24.9±0.78mm in rhizome extract. The leaf showed activity against *C. albicans* of 12.4±2.64 mm. The root extract has an inhibition zone of 12.43±2.67mm and the rhizome extract of 11.8±1.21mm. Samples show relatively low antimicrobial activity compared to summer, winter, and monsoon. No isolates have considerable antimicrobial activity except the bacterial isolate BRhWS8, which showed an antifungal activity with an inhibition zone of 10.07±0.21mm.

These isolates may not possess significant antimicrobial compounds or require further optimization to exhibit significant activity. The antimicrobial activity of the isolates may be due to the production of secondary metabolites, such as antibiotics, bacteriocins, or other bioactive compounds. These compounds can interact with microbial cells, disrupting their membranes, inhibiting protein synthesis, or interfering with DNA replication. The varying levels of antimicrobial activity among the bacterial isolates may be due to differences in their metabolic profiles, growth conditions, or genetic makeup. Further studies are needed to identify the specific compounds responsible for the antimicrobial activity and to explore their potential applications.

The results indicate that a significant proportion of the isolates (66%) exhibited antimicrobial activity against one or more of the test microorganisms, with varying levels of inhibition. The most active isolates were from the *Bacillus* and *Pseudomonas* genera. These findings suggest that the isolates are potential sources of novel antimicrobial compounds.

Antimicrobial Activity of Endophytic Fungi

The fungal antimicrobial activity of the isolates was evaluated against various bacterial test organisms and fungi, *Candida albicans*. The results showed that most isolates unveiled significant antimicrobial activity at different levels (Table 50). Fungal isolates FLWS21 and FRWW11 showed more antimicrobial activity against all test bacterial organisms and no inhibition on *C. albicans*. The other isolates with high antimicrobial activity were FLWW5, FLWW13, FLWS5, and FRWS7. These isolates showed potent antimicrobial properties, inhibiting the growth of a diverse array of microorganisms. The inhibition zones ranged from 15.73 mm to 22.07 mm, indicating strong antimicrobial activity.

The zone of inhibition values ranged from 0 to 22.07 mm, indicating varying levels of antimicrobial activity among the isolates (Fig.87). Among 36 isolates, 15 (41.66%) showed antimicrobial activity on *Bacillus subtilis* with zones of inhibition ranging from 6.0 to 22.07 mm. 13 isolates (36.11%) showed antimicrobial activity against *Pseudomonas aureginosa*, of inhibition ranging

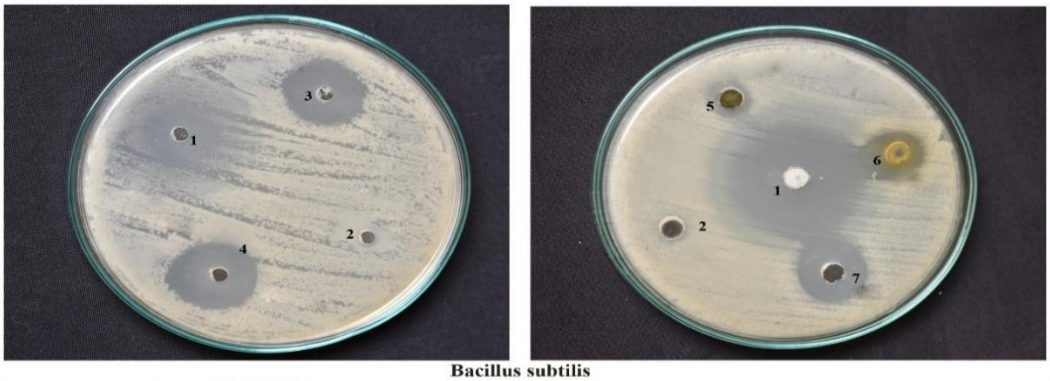
from 8.9 to 16.4 mm. The zone of inhibition ranging from 6.8 to 16.33 mm was shown by 21 isolates (58.33%) against *Salmonella typhi*. 19 isolates (52.77%) had inhibition against *Enterococcus faecalis* ranging from 9.87 to 19.07 mm. The inhibition ranged from 8.13 to 15.06 mm was shown by 10 isolates (27.77%) against *E. coli*. Among the total isolates, only 1 isolate (2.77%) showed a zone of inhibition against *C. albicans* of 10.2 mm and 9 isolates (25 %) against *S. aureus* ranging from 11.36 to 18.5 mm. The zone of inhibition ranging from 9.77 to 17.4 mm was shown by 14 isolates (38.88%) against *P. vulgaris*. 16 isolates (44.44%) against *V. fluvialis* ranging from 8.4 to 18.3 mm. FLWW5 showed strong antimicrobial activity against *B. subtilis* (22.07±0.31 mm), and *S. aureus* (17.43±0.89 mm). FLWW13, FRWS7, FLWS21, and FRWM1 showed strong activity on *E. faecalis* of 19.07±6.63 mm, 17.7±3.89 mm, 17.7±1.02, and 16.7±2.65 mm. FRWS8, FLWW6, FRWS4, FRhWW4, FLWM11 showed no antimicrobial activity against any of the test organisms. The highest inhibition against *P. aureginosa* and *S. typhi* was showed by FLWS21 of 16.4±2.22 mm and 16.33±2.41 mm. Among the isolates, FLWS21 inhibits *E. coli* of diameter 15.06±4.26 mm. FRWW10, FLWS5, FLWW5, and FLWS32 exhibit the highest inhibition on *S. aureus* ranging from 16.6±2.41 and 17.4±2.55 mm. FLWS5 and FRWW10 showed the highest zone of inhibition against *P. vulgaris* of 17.4±2.55 and 16.6±2.41 mm. The diameter of 18.3±0.91 mm zone of inhibition exhibited by FLWS5 against *V. fluvialis*. The isolates FRWS24, FRWM3, FRhWM2, FRhWW3, FRWM12, and FLWW11 showed moderate antimicrobial activity, with inhibition zones ranging from 10 mm to 15 mm. While their activity is not as high as the high-activity isolates, they may still possess compounds with some antifungal potential.

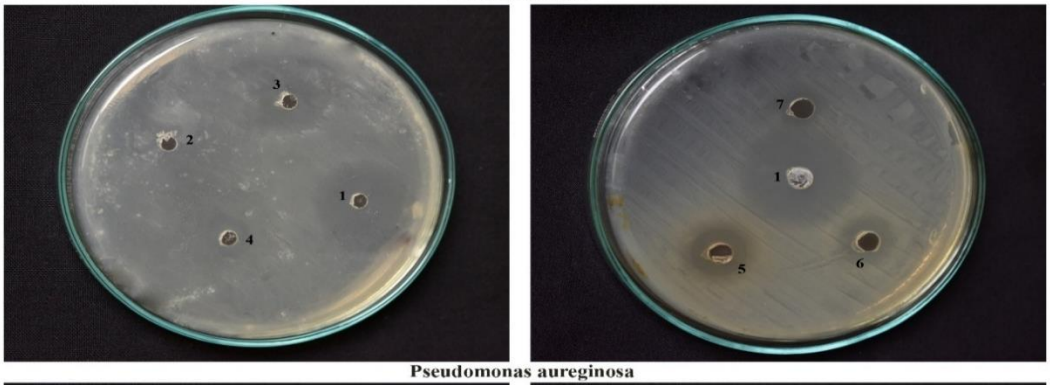
The inhibition zone of antimicrobial activity of leaf extract against *B. subtilis* is 22.6±2.22mm, the isolate FLWW5 also showed a similar inhibition zone on the same of 22.07±0.31mm. The root and rhizome extracts showed an inhibition zone of 19.53±0.87mm and 25.2±2.72mm. In the case of *P. aureginosa*, leaf extract has an inhibition zone of 8.9±0.75mm and root with 8.97±1.747mm. The

FRWW18 isolate has a similar activity zone of inhibition of 9.23±0.73mm is noted. Then the rhizome extract has an inhibition zone of 14.87±0.71mm. The activity against *S. typhi* by leaf, root, and rhizome extracts are 22.07±1.92mm, 20.47±1.21mm, and 19.73±1.47mm respectively. The leaf extract showed an inhibition zone of 18.33±0.80 against *E. faecalis* and the isolate FLWW13 showed a similar value on the activity of 19.07±6.63. The root extract showed 01±0.1mm and the isolate FRSW7 has an inhibition zone of 17.7±3.89mm against *E. faecalis*. The rhizome extract has an activity zone of 15.63±0.98mm. The activity against *E. coli* by the leaf, root, and rhizome extracts is 17.5±1.31mm, 20.7±3.65mm, and 26.67±1.24mm. The inhibition zone of activity shown by the leaf, root, and rhizome extracts against *S. aureus* are 29.5±1.23, 27.4±3.62, and 28.17±2.03. The inhibition zone of activity shown by the leaf, root, and rhizome extracts against *P. vulgaris* is 27.4±2.42mm, 18.2±1.83, and 27.87±0.81mm. The inhibition zone of antimicrobial activity of leaf extract against *V. fluvialis* is 25.0±4.03mm, the root is 25.57±1.11 and the rhizome is 24.9±0.78. Here the leaf extract has 12.4±2.64 mm activity against *C. albicans*. While root and rhizome have activity ranges of 12.43±2.67 and 11.8±1.21mm (Fig.88).

The majority of the isolates showed no antifungal activity, except FRhWM2. These isolates may not possess significant antifungal compounds or may require further optimization to exhibit significant activity. The antifungal activity of the isolates may be due to the production of secondary metabolites, such as polyketides, terpenes, or other bioactive compounds. These compounds can interact with fungal cells, disrupting their membranes, inhibiting cell wall synthesis, or interfering with metabolic processes. The results indicate that a significant proportion of the fungal isolates (86.11%) exhibited antimicrobial activity against one or more of the test organisms, with varying levels of inhibition. The most active isolates were FLWW5, FLWS21, and FRWW11 which showed broad-spectrum antimicrobial activity. These findings suggest

that the fungal isolates have potential as sources of novel antimicrobial compounds.





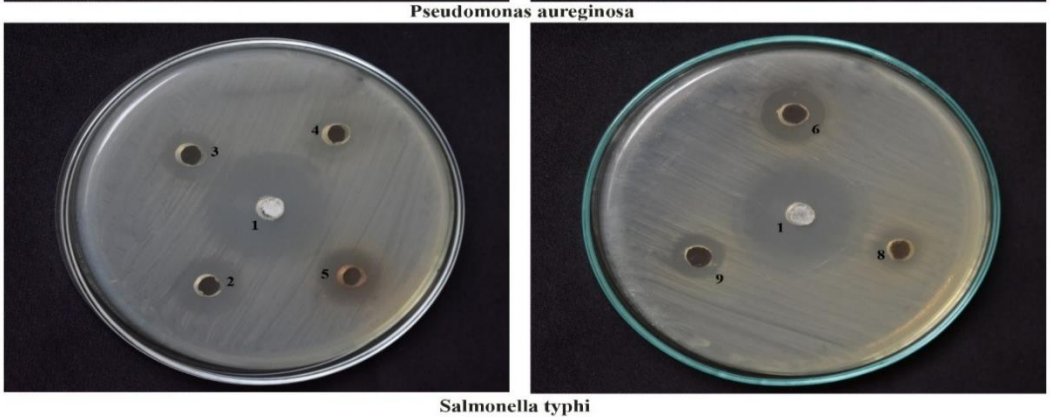
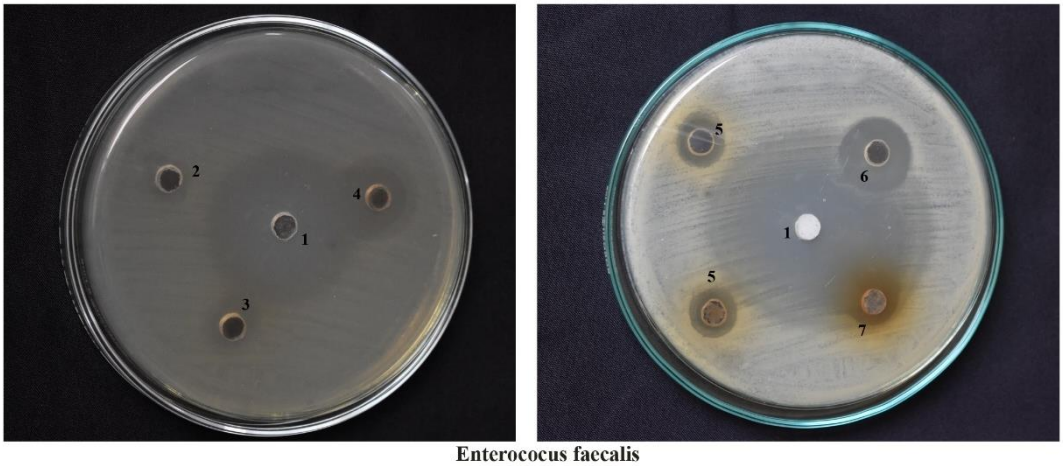
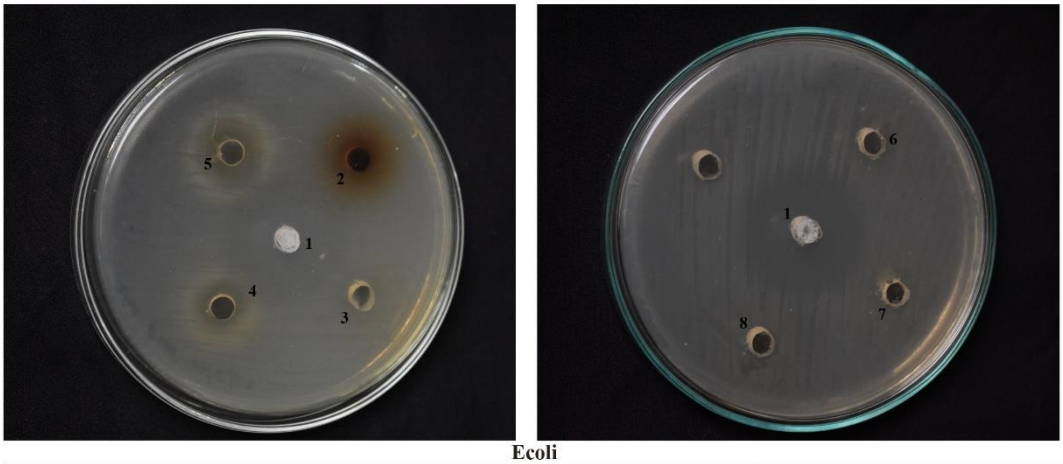


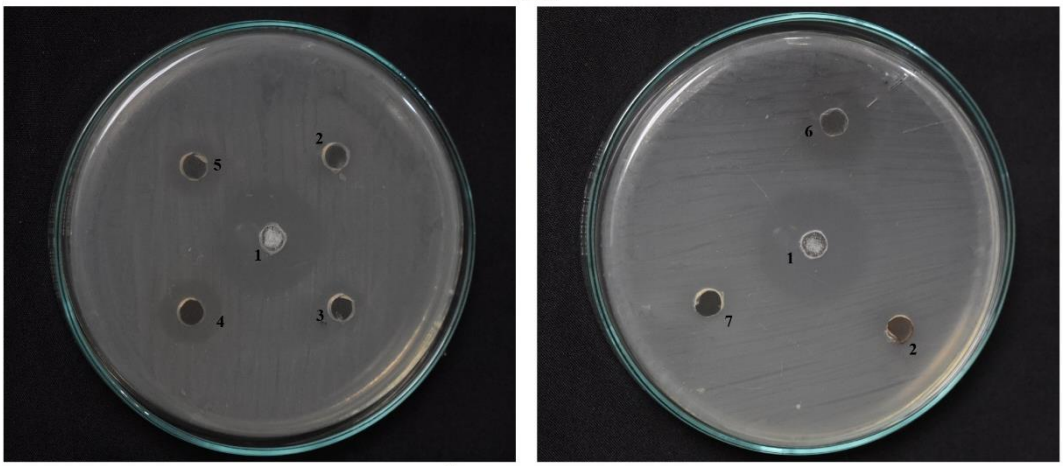
Fig.86a Antimicrobial activity of endophytic bacterial isolates
B. subtilis: 1- +ve control; 2- -ve control; 3- BLWW2; 4- BRWM2; 5- BLWM1; 6- BLWS10; 7- BLWS2
P. aureginosa: 1- +ve control; 2- -ve control; 3- BLWW1; 4- BRhWW7; 5- BLWW9; 6- BLWW10; 7- BLWS4
S. typhi: 1- +ve control; 2- -ve control; 3- BLWM1; 4- BRWS1; 5- BLWW4; 6- BLWS10; 7- BLWW10; 8- BRhWS1



Enterococus faecalis

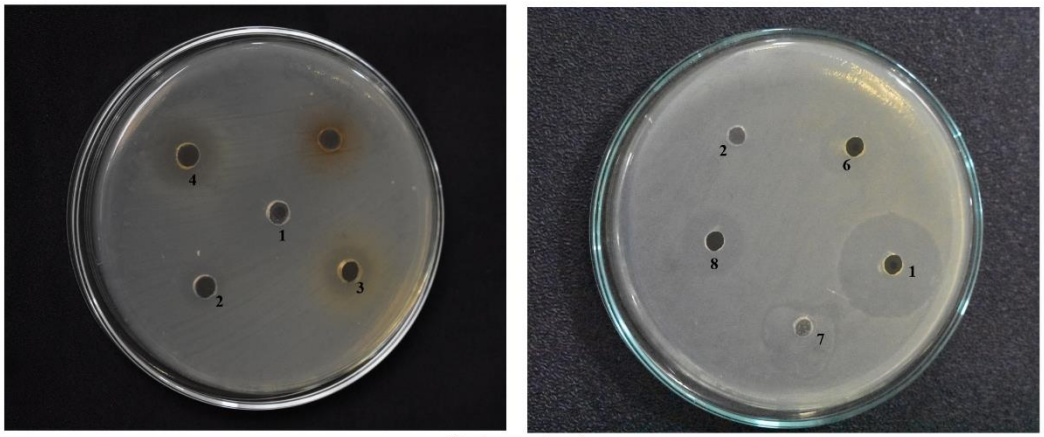


Ecoli

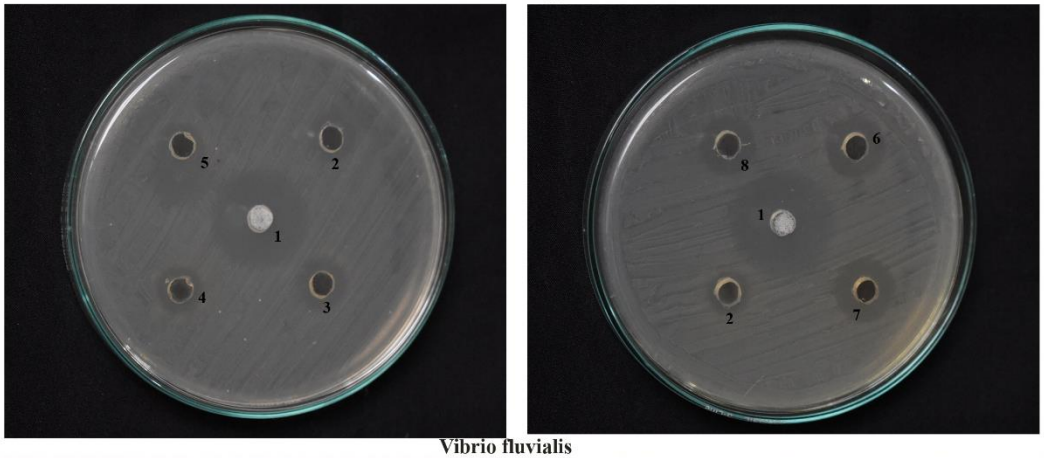


Staphylococus aureus

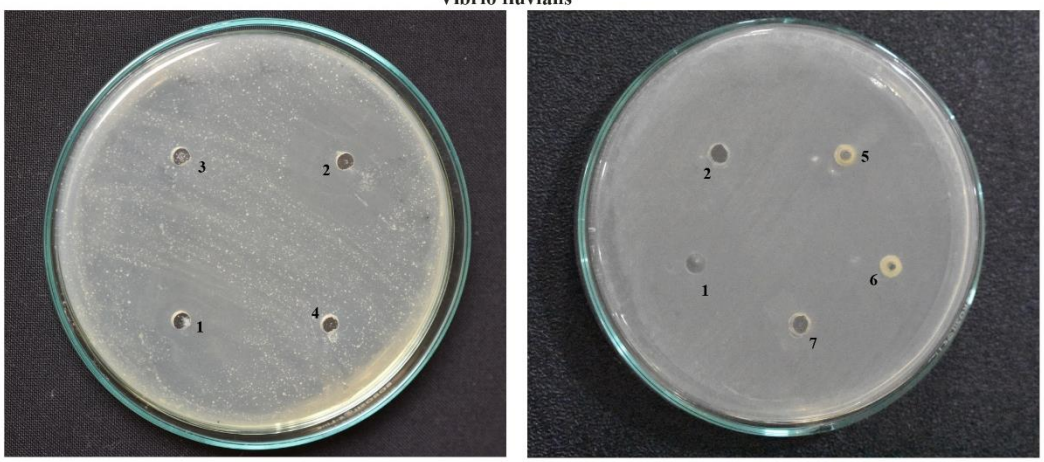
Fig.86b Antimicrobial activity of endophytic bacterial isolates
***E. faecalis*: 1- +ve control; 2- -ve control; 3- BRhWM3; 4- BLWS10; 5- BLWW9; 6- BRWW12; 7- BLWS7; 8- BRhWW7**
***E. coli*: 1- +ve control; 2- -ve control; 3- BRhWS27; 4- BRhWS22; 5- BLWS2; 6- BLWS3; 7- BLWM1; 8- BLWW10**
***S. aureus*: 1- +ve control; 2- -ve control; 3- BRWS3; 4- BRWW12; 5- BRhWW11; 6- BLWW10; 7- BLWS11**



Proteus vulgaris



Vibrio fluvialis



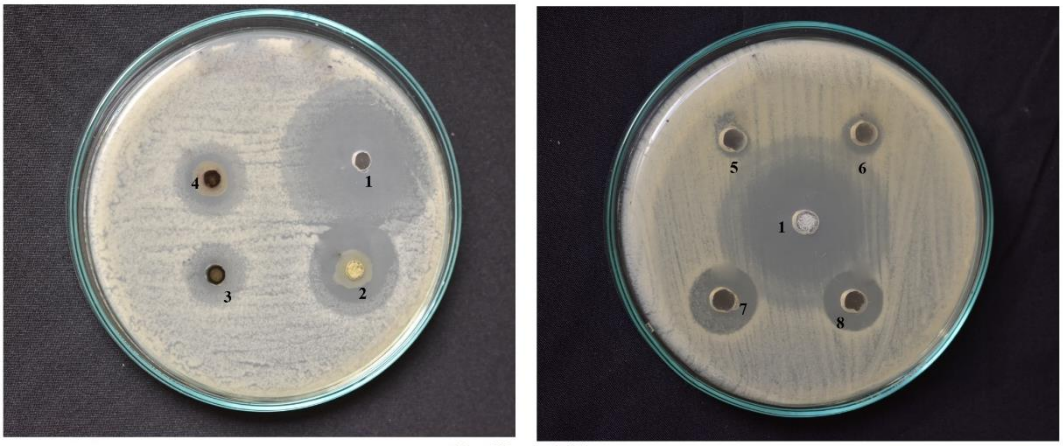
Candida albicans

Fig.86c Antimicrobial activity of endophytic bacterial isolates

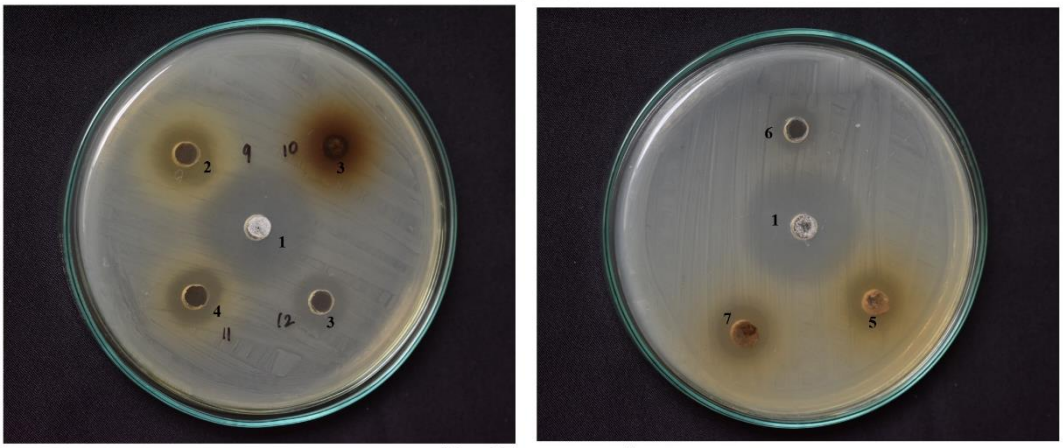
P. vulgaris: **1- +ve control; 2- -ve control; 3- BLWM4; 4- BRWS15; 5- BLWS6; 6- BLWW5; 7- BRWW12; 8- BRWS 13**

V. fluvialis: **1- +ve control; 2- -ve control; 3- BLWW7; 4- BLWW9; 5- BLWS1; 6- BLWW2; 7- BLWS11; 8- BLWS6**

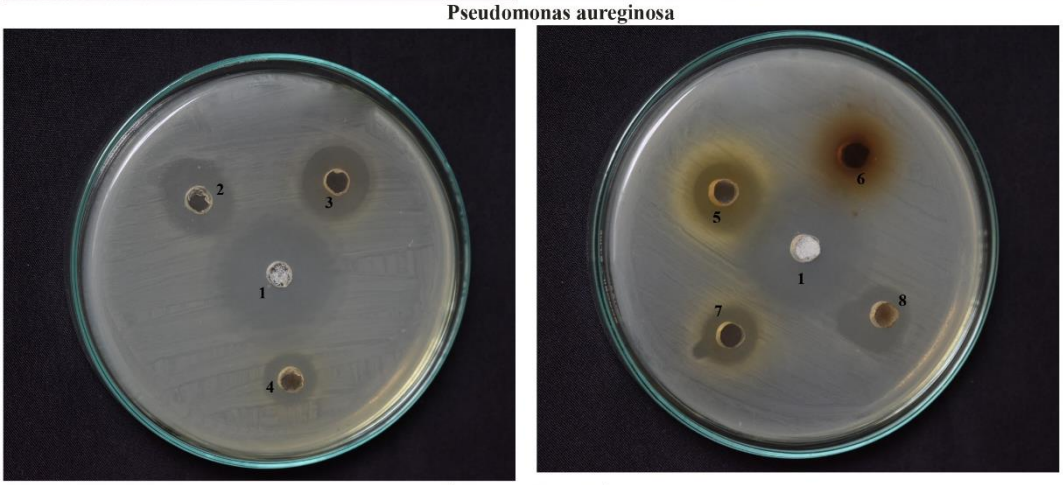
C. albicans: **1- +ve control; 2- -ve control; 3- BLWS10; 4- BRhWS8; 5- BRWW6; 6- BRWS13; 7- BLWS7**



Bacillus subtilis



Pseudomonas aureginosa



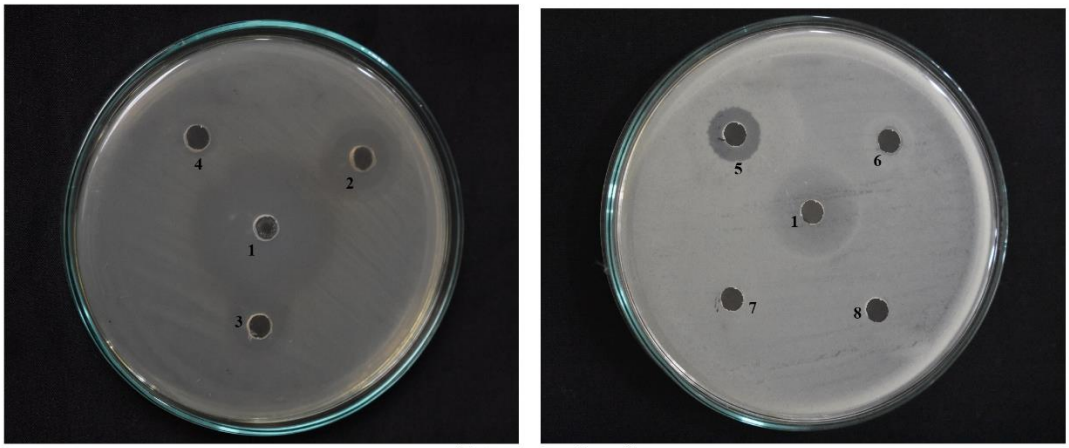
Salamonella typhi

Fig.87a Antimicrobial activity of endophytic fungal isolates

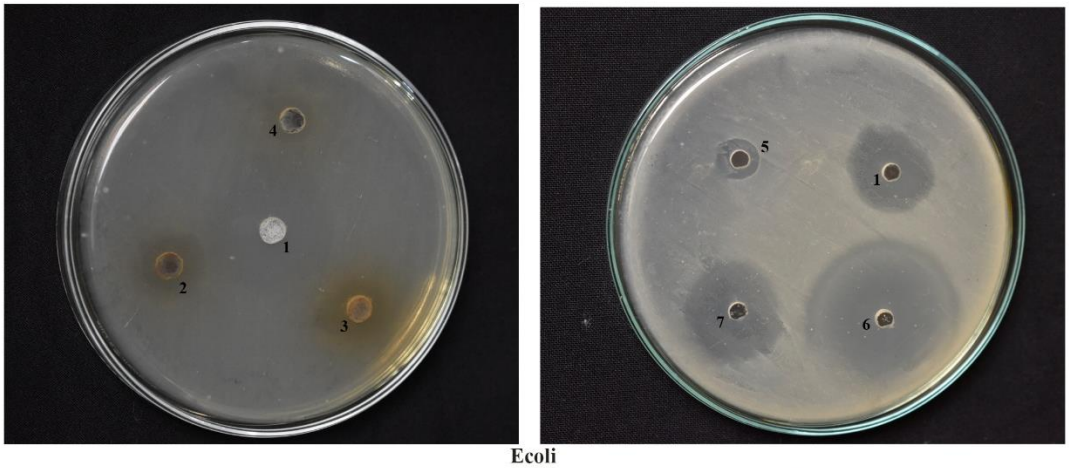
B. subtilis: **1- +ve control; 2- FLWW5; 3- FLWM1; 4- FLWS21; 5- FRhWW3; 6- FLWS32; 7- FLWW9; 8- FRWM12**

P. aureginosa: **1- +ve control; 2- FRWW11; 3- FLWS1; 4- FLWS16; 5- FRWW10; 6- FLWW13; 7- FLWM1; 8- FLWS21**

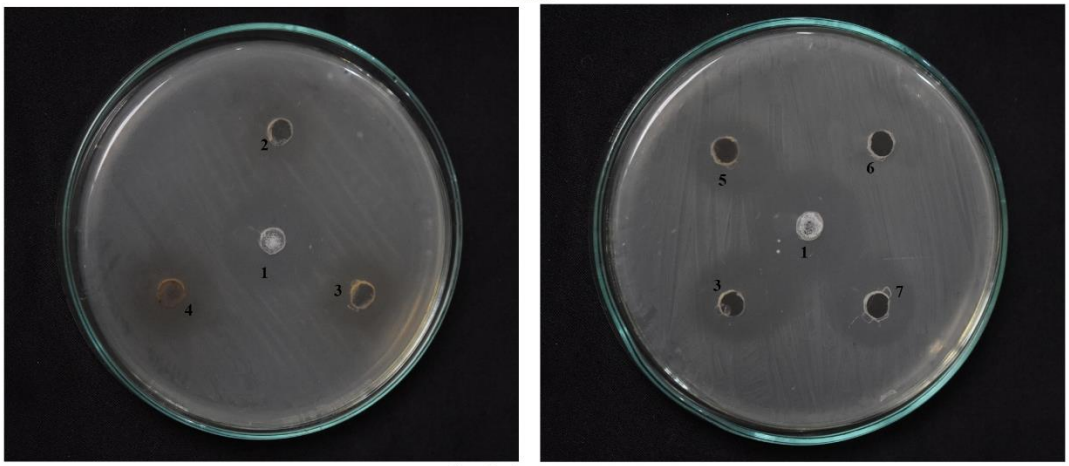
S. typhi: **1- +ve control; 2- FRWW11; 3- FLWW13; 4- FRWM3; 5- FLWS5; 6- FRhWS2; 7- FLWS32; 8- FLWW6**



Enterococcus faecalis

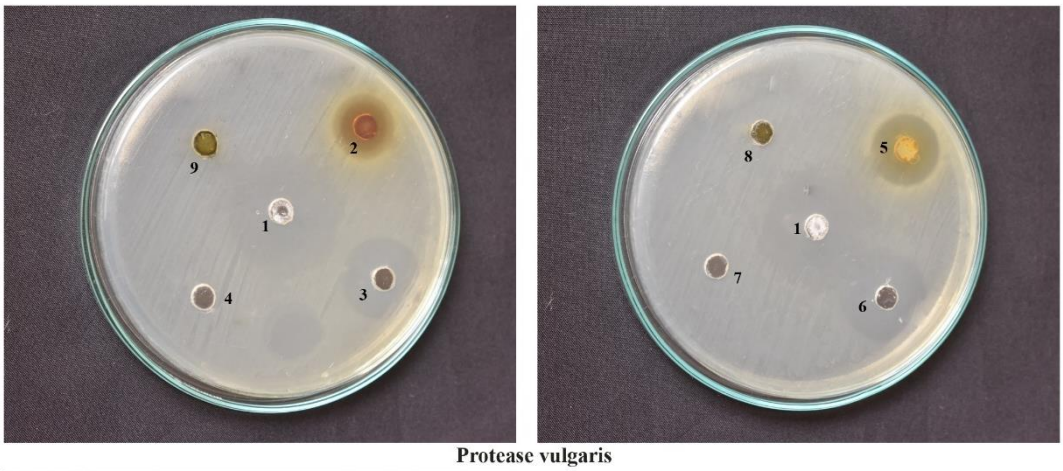


Ecoli

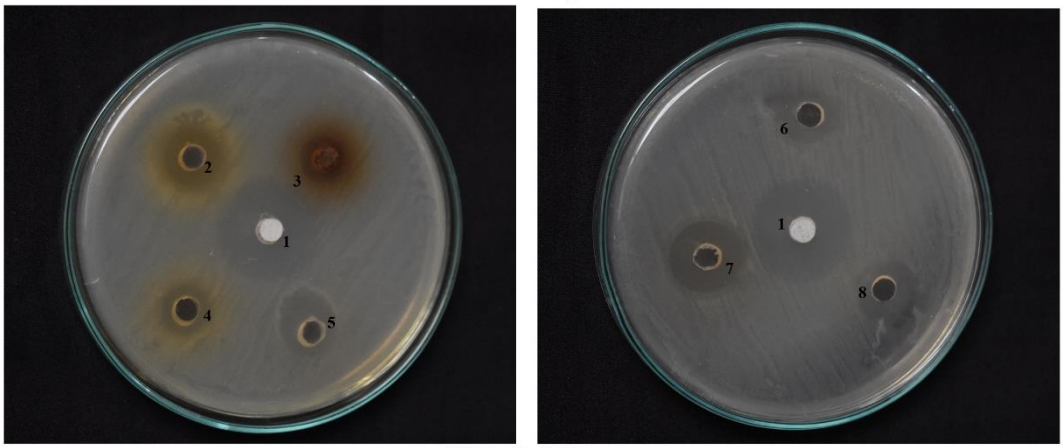


Staphylococcus aureus

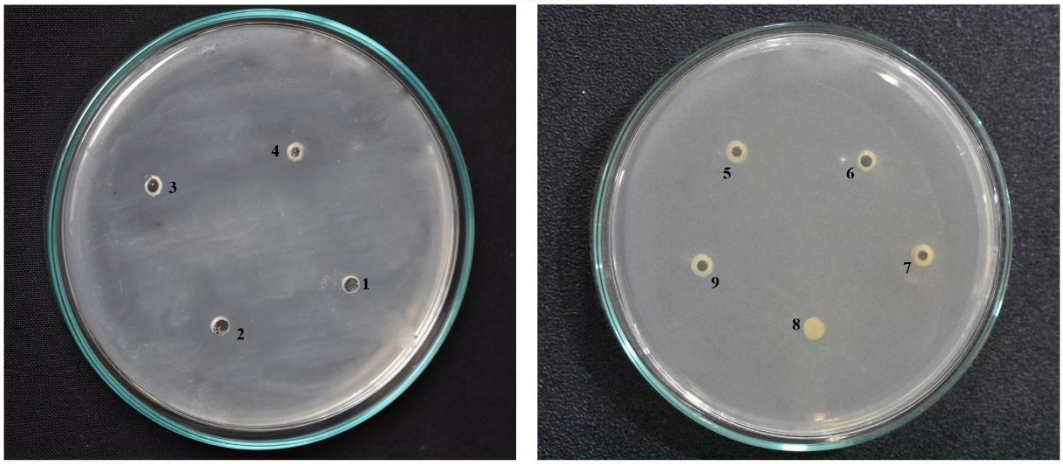
Fig.87b Antimicrobial activity of endophytic fungal isolates
E. faecalis: **1- +ve control; 2- FLWW13; 3- FLWS5; 4- FLWM11; 5- FRWS7; 6- FRWM1; 7- FLWS21; 8- FRWW10**
E. coli: **1- +ve control; 2- FLWW6; 3- FLWW11; 4- FLWW14; 5- FLWS16; 6- FLWS21; 7- FLWS1**
S. aureus: **1- +ve control; 2- FLWW13; 3- FLWS1; 4- FRWM12; 5- FLWS5; 6- FRWW10; 7-FRWW11**



Protease vulgaris



Vibrio fluvialis



Candida albicans

Fig.87c Antimicrobial activity of endophytic fungal isolates
P. vulgaris: **1- +ve control; 2- FRWS7; 3- FRWM1; 4- FLWW15; 5- FRWW10; 6- FLWS5; 7- FRhWW3**
V. fluvialis: **1- +ve control; 2-FLWS21; 3- FLWW14; 4- FLWW5; 5- FLWS1; 6- FRWW11; 7- FLWS5; 8- FRWM3**
C. albicans: **1- +ve control; 2- FLWW14; 3- FRhWM2; 4- FLWS1; 5- FRWW30; 6- FLWW11; 7- FRWM1; 8- FLWW5; 9- FRWS7**

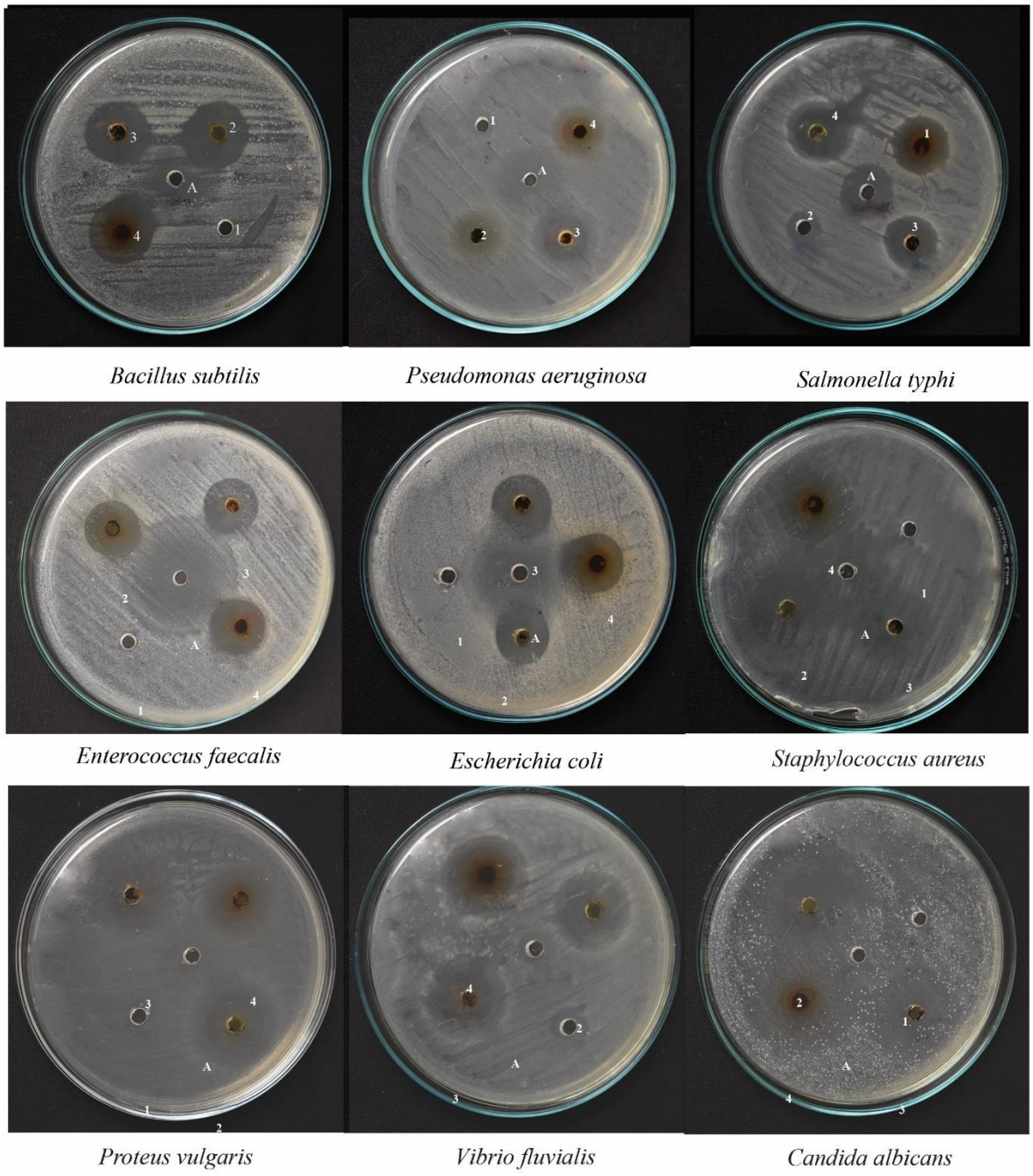


Fig. 88 Antimicrobial activity of ethylacetate extract of plant parts. A - control; 1 - -ve control; 2 - Leaf; 3 - Rhizome; 4 - Root.

No.	Bacterial isolates	Bacillus	Pseudomonas	Salmonella	Enterococcus	E. coli	Staphylococcus	Proteus	Vibrio	Candida
1	BRWW12	0	0	0	9.0±0.435^{a}	0	9.40±0.90^{a}	0	0	0
2	BRWW6	0	0	0	0	0	0	0	0	0
3	BRWS13	0	0	15.46±3.95^{de}	0	0	0	0	0	0
4	BRWS15	0	0	0	0	0	0	12.67±0.98^{a}	0	0
5	BRWS1	0	0	11.37±4.48^{bc}	0	0	0	0	0	0
6	BRWM2	14.27±5.82^{de}	0	0	0	0	0	0	0	0
7	BRWM3	0	0	15.2±4.43^{d}	0	0	0	0	0	0
8	BRWS3	0	0	0	0	0	0	0	0	0
9	BLWW1	0	12.6±5.13^{cd}	0	0	0	0	0	0	0
10	BLWW4	0	0	13.03±2.54^{cd}	0	0	0	0	0	0
11	BLWW2	14.6±4.16^{e}	0	14.23±1.73^{cd}	0	0	0	0	14.63±1.78^{de}	0
12	BLWS11	0	0	16.3±3.37^{de}	0	0	0	0	14.63±0.78^{e}	0
13	BLWS6	0	0	15.76±3.95^{de}	0	0	0	0	13.17±3.57^{cd}	0
14	BLWS7	11.57±4.60^{bcde}	0	0	7.47±0.51^{c}	0	0	0	0	0
15	BLWW5	0	0	16.13±1.90^{de}	0	0	0	0	0	0
16	BLWW7	0	0	0	0	0	0	0	9.6±1.65^{b}	0
17	BLWW8	0	10.6±1.61^{bc}	0	0	0	0	0	0	0
18	BLWM4	0	0	0	0	0	0	11.53±3.17^{b}	0	0
19	BLWM1	9.20±1.25^{ab}	0	9.50±2.77^{ab}	0	0	0	0	12.0±3.65^{c}	0
20	BLWW10	0	13.0±3.16^{d}	17.73±1.80^{ef}	0	0	14.33±1.81^{b}	0	0	0
21	BLWW9	0	13.47±3.97^{d}	0	13.77±4.14^{b}	0	0	0	8.9±0.26^{ab}	0
22	BLWS10	12.17±0.20^{bcde}	11.36±0.40^{bcd}	18.83±1.68^{ef}	12.2±0.50^{c}	0	0	0	0	11.07±0.21^{b}
23	BLWS9	10.83±3.13^{abcd}	0	14.5±2.26^{cd}	0	0	0	0	0	0

24	BLWS4	0	7.67±0.55^{a}	0	0	0	0	0	0	0
25	BLWS2	14.07±5.26^{de}	0	0	0	7.5±0.61^{a}	0	0	0	0
26	BLWS1	11.7±1.87^{bcde}	0	0	0	0	0	0	7.4±0.40^{a}	0
27	BLWS3	7.5±0.458^{a}	0	0	0	0	0	0	0	0
28	BRhWS1	9.63±0.351^{abc}	7.1±0.10^{a}	7.6±0.26^{a}	0	0	0	0	0	7.97±0.25^{a}
29	BRhWS8	0	0	0	0	7.46±0.37^{a}	0	0	0	10.07±0.21^{b}
30	BRhWW11	0	0	0	0	0	7.47±0.51^{c}	0	0	9.17±0.21^{c}
31	BRhWW7	9.03±0.45^{ab}	10.07±0.21^{b}	0	11.97±0.25^{c}	0	0	0	0	9.83±0.35^{e}
32	BRhWM3	10.9±0.361^{bcd}	0	0	7.5±0.46^{d}	0	0	0	0	7.6±0.60^{f}
33	BRhWW10	0	0	0	0	7.6±0.36^{a}	0	0	0	0
34	BRhWS27	12.87±0.32^{cde}	0	0	0	9.4±0.78^{b}	0	0	0	0
35	BRhWS22	10.2±0.265^{abc}	0	0	0	11.4±0.36^{c}	0	0	0	0
36	LS	22.6±2.22^{g}	8.9±0.75^{e}	22.07±1.92^{h}	18.33±0.80^{f}	17.5±1.31^{d}	29.5±1.23^{e}	27.4±2.42^{d}	25.0±4.03^{f}	12.4±2.64^{b}
37	RS	19.53±0.87^{f}	8.97±1.747^{e}	20.47±1.21^{g}	16.01±0.1^{e}	20.7±3.65^{e}	27.4±3.62^{d}	18.2±1.83^{c}	25.57±1.11^{f}	12.43±2.67^{b}
38	RhS	25.2±2.72^{h}	14.87±0.71^{d}	19.73±1.47^{f}	15.63±0.98^{e}	26.67±1.24^{f}	28.17±2.03^{de}	27.87±0.81^{d}	24.9±0.78^{f}	11.8±1.21^{b}
39	LW	19.3±1.24^{f}	7.8±0.83a	14.91±1.28^{bc}	11.87±2.13^{c}	18.25±0.54^{d}	13.51±2.31^{b}	12.95±2.15^{a}	7.51±0.54^{a}	11.24±3.45^{b}
40	RW	13.0±0.71^{cd}	0	18.97±2.68^{ef}	7.91±0.54^{d}	7.65±1.89^{a}	8.46±1.97^{ac}	17.56±2.54^{c}	9.59±0.14^{b}	8.54±2.18b^{c}
41	RhW	12.01±3.25^{bcde}	10.54±1.72^{bc}	11.21±0.48^{bc}	12.01±1.01^{c}	10.98±1.102^{c}	14.65±0.021^{f}	21.54±0.31^{e}	12.04±2.13^{c}	0
42	LM	9.14±0.83^{ab}	0	0	9.25±1.15^{a}	15.63±0.24^{g}	21.54±2.68^{g}	11.74±2.98^{b}	18.57±3.65^{g}	0
43	RM	9.18±0.52^{ab}	0	7.71±1.62^{a}	8.45±0.05^{d}	16.21±1.01d^{g}	9.46±2.541^{a}	15.42±1.031^{f}	7.96±1.11^{a}	7.89±1.18^{a}
44	RhM	7.32±0.85^{a}	0	0	10.76±1.26g	8.11±0.36^{ab}	13.54±0.025^{b}	12.01±3.14^{a}	0	0

Table 49: Antimicrobial activity of endophytic bacteria against test organisms

No.	Fungal isolates	Bacillus	Pseudomonas	Salmonella	Enterococcus	E. coli	Staphylococcus	Proteus	Vibrio	Candida
1	FLWW14	0	0	0	0	11.66 ± 2.1^{cd}	0	0	11.67 ± 2.08^{cde}	12.67 ± 4.61^{a}
2	FRWS8	0	0	12.33 ± 4.04^{a}	0	12.67 ± 4.61^{a}	0	0	0	0
3	FLWW5	22.07 ± 0.31^{j}	0	11.2 ± 2.26^{cdef}	11.6 ± 2.75^{ab}	0	17.43 ± 0.89^{d}	0	11.23 ± 0.83^{bcd}	0
4	FLWW15	0	0	0	0	0	0	0	8.4 ± 0.435^{b}	0
5	FLWW17	0	0	12.96 ± 0.57^{defg}	0	0	0	0	14.57 ± 4.28^{defgh}	0
6	FLWW6	0	0	13.03 ± 5.25^{ab}	0	12.70 ± 4.67^{a}	0	0	13.1 ± 5.37^{a}	0
7	FRWS7	0	11.43 ± 3.82^{abc}	10.6 ± 2.79^{cde}	17.7 ± 3.89^{de}	0	0	13.9 ± 3.15^{bcde}	0	0
8	FRWM12	11.7 ± 2.91^{bcde}	0	6.8 ± 5.93^{bc}	12.36 ± 2.71^{abc}	0	13.33 ± 5.77^{a}	0	0	0
9	FLWW12	0	0	10.07 ± 2.67^{cd}	14 ± 5.53^{abcd}	12.77 ± 4.35^{cde}	0	10.97 ± 1.01^{ab}	0	0
10	FRWM1	15.43 ± 4.46^{ghi}	14.47 ± 2.15^{cdef}	0	16.7 ± 2.65^{cde}	0	0	15.36 ± 1.30^{def}	12.23 ± 1.70^{cdef}	0
11	FRWS4	0	0	0	0	0	0	0	0	0
12	FLWW11	0	0	11.43 ± 4.47^{cdefg}	12.1 ± 4.91^{abc}	11.17 ± 1.56^{cd}	0	0	0	0
13	FRhWS1	0	11.9 ± 3.47^{abcde}	14.2 ± 3.39^{defg}	0	0	0	0	0	0
14	FLWW18	0	12.56 ± 1.07^{bcde}	13.63 ± 1.02^{defg}	0	8.13 ± 1.05^{b}	0	9.77 ± 1.85^{a}	14.9 ± 3.50^{efgh}	0
15	FLWS32	8.2 ± 1.15^{a}	0	15.7 ± 2.95^{fg}	0	0	17.56 ± 1.36^{d}	0	15.07 ± 3.26^{fgh}	0
16	FRWW18	15.56 ± 1.46^{hi}	9.23 ± 0.73^{a}	0	13.63 ± 1.15^{abcd}	0	15.63 ± 2.35^{cd}	9.8 ± 2.3^{a}	0	0
17	FRWM25	0	0	14.0 ± 1.68^{defg}	0	0	0	12.07 ± 3.33^{abc}	0	0
18	FRWW30	0	0	0	12.9 ± 4.16^{abc}	0	0	0	0	0
19	FLWM1	11.06 ± 2.19^{bcd}	13.3 ± 2.48^{bcdef}	0	14.07 ± 2.59^{abcd}	0	0	0	9.63 ± 1.21^{bc}	0
20	FLWW9	13.43 ± 1.41^{defgh}	0	15.53 ± 2.38^{efg}	14.5 ± 3.15^{abcde}	0	0	12.57 ± 0.81^{abcd}	0	0
21	FRWM27	14.367 ± 2.27^{efghi}	11.6 ± 1.11^{abcd}	13.96 ± 4.82^{defg}	12.3 ± 2.17^{abc}	0	0	0	12.37 ± 1.94^{cdef}	0
22	FLWS1	9.4 ± 1.05^{ab}	15.03 ± 4.27^{ef}	10.57 ± 0.85^{cde}	0	9.77 ± 2.11^{bc}	12.83 ± 5.43^{bc}	12.57 ± 4.69^{abcd}	15.53 ± 2.93^{fghi}	0
23	FRhWW3	6.0 ± 0.45^{i}	0	0	0	0	0	0	0	0

24	FRhWM2	0	0	0	0	9.3±1.69^{efg}	0	0	0	10.2±1.05^{bc}
25	FRhWS2	0	0	15.23±1.43^{efg}	13.47±4.32^{abcd}	0	0	0	0	0
26	FRWM3	0	0	12.2±1.92^{defg}	14.57±0.71^{abcde}	0	0	0	13.1±1.83^{defg}	0
27	FLWS21	16.67±1.85^{i}	10.9±0.41^{ab}	13.13±2.01^{defg}	17.7±1.02^{de}	15.06±4.26^{e}	15.93±3.36^{cd}	14.7±0.64^{cdef}	17.4±2.8^{hi}	0
28	FLWS16	12±1.15^{bcd}	14.76±3.69^{def}	11.33±1.68^{cdefg}	0	13.4±1.53^{de}	0	0	16.3±2.11^{ghi}	0
29	FRWS24	12.7±4.61^{cd}	0	0	0	0	0	0	0	0
30	FRWW10	0	13.3±4.23^{bcdef}	14.73±1.23^{defg}	15.97±1.89^{bcde}	0	18.5±1.03^{d}	16.6±2.41^{ef}	14.5±3.19^{defgh}	0
31	FRWW11	10.6±1.08^{abc}	16.4±2.22^{f}	16.33±2.41^{g}	14.2±3.31^{abcd}	10.2±1.05^{bc}	16.4±2.23^{cd}	13.86±2.32^{bcde}	14.4±0.96^{defgh}	0
32	FRhWW4	0	0	0	0	0	0	0	0	0
33	FRWS9	0	11.4±3.85^{abc}	0	12.16±3.75^{abc}	0	0	13.43±5^{bcde}	0	0
34	FLWM11	0	0	0	0	0	0	0	0	0
35	FLWW13	0	0	16.23±6.52^{fg}	19.07±6.63^{e}	12.17±3.09^{cde}	11.36±9.92^{b}	10.9±2.36^{ab}	0	0
36	FLWS5	14.7±4.158^{fghi}	8.9±1.5^{a}	15.73±3.62^{fg}	9.87±1.42^{a}	0	17.73±2.37^{d}	17.4±2.55^{f}	18.3±0.91^{i}	0
37	LS	22.6±2.22^{j}	8.9±0.75^{a}	22.07±1.92^{h}	18.33±0.80^{e}	17.5±1.31^{i}	29.5±1.23^{f}	27.4±2.42^{g}	25.0±4.03^{k}	12.4±2.64^{c}
38	RS	19.53±0.87^{bcd}	8.97±1.747^{a}	20.47±1.21^{h}	16.01±0.1^{de}	20.7±3.65^{h}	27.4±3.62^{e}	18.2±1.83^{f}	25.57±1.11^{k}	12.43±2.67^{c}
39	RhS	25.2±2.72^{k}	14.87±0.71^{def}	19.73±1.47^{h}	15.63±0.98^{bcde}	26.67±1.24^{j}	28.17±2.03^{ef}	27.87±0.81^{g}	24.9±0.78^{jk}	11.8±1.21^{bc}
40	LW	19.3±1.24^{bc}	7.8±0.83^{g}	14.91±1.28^{defg}	11.87±2.13^{ab}	18.25±0.54^{d}	13.51±2.31^{a}	12.95±2.15^{a}	7.51±0.54^{a}	11.24±3.45^{b}
41	RW	13.0±0.71^{defgh}	0	18.97±2.68^{ef}	7.91±0.54^{f}	7.65±1.89^{k}	8.46±1.97^{ac}	17.56±2.54^{f}	9.59±0.14^{bc}	8.54±2.18^{d}
42	RhW	12.01±3.25^{bcd}	10.54±1.72^{a}	11.21±0.48^{bcde}	12.01±1.012^{abc}	10.98±1.102^{cb}	14.65±0.021^{ac}	21.54±0.31^{e}	12.04±2.13^{cdef}	0
43	LM	9.14±0.83^{ab}	0	0	9.25±1.154^{a}	15.63±0.24^{e}	21.54±2.68^{g}	11.74±2.98^{b}	18.57±3.65^{g}	0
44	RM	9.18±0.52^{ab}	0	7.71±1.62^{c}	8.45±0.054^{d}	16.21±1.01^{k}	9.46±2.541^{ac}	15.42±1.031^{fef}	7.96±1.11^{a}	7.89±1.18^{bc}
45	RhM	7.32±0.85^{a}	0	0	10.76±1.26^{g}	8.11±0.36^{ef}	13.54±0.025^{a}	12.01±3.14^{a}	0	0

Table 50: Antimicrobial activity of endophytic fungi against test organisms

Process optimization for the production of active metabolites from *Diaporthe penetriteum* FLWS21 isolated from *L. toxicaria*

The process optimization study was carried out to determine the supporting conditions for the enhanced growth in the production of crude/secondary metabolites by selected endophytic *Diaporthe penetiteum* FLWS21 and its potential activities against test-chosen bacteria. Different parameters like temperature, pH, and incubation period were optimized concerning fungal biomass and crude metabolite production in shake culture conditions for maximum production of active metabolites by the antagonistic strains against bacterial pathogen *E. faecalis*, since in the preliminary screening FLWS21 showed maximum inhibitory activity against this test pathogen. The mycelial mats were filtered and dried at 50°C until constant weight was obtained. The final fungal biomass was recorded in mg/100mL. Effects of temperature (25°C, 27°C, 30°C), pH (3.0, 5.0, 7.0), and incubation period (15th and 21st day) were studied by inoculating the fungus in Potato Dextrose Broth (PDB) and the effect on crude metabolite production was observed.

The production of fungal biomass and metabolites exhibited a significant increase between the 15th and 21st days of incubation, across all three temperature conditions (25°C, 27°C, and 30°C) and pH levels (3.0, 5.0, and 7.0). This pattern was consistent across all experimental conditions, indicating a robust growth pattern. The production of crude extract followed a similar pattern, with a marked increase observed between the 15th and 21st days of incubation, under all temperature and pH conditions (Fig.89, 90, 91). This suggests a strong correlation between fungal growth and metabolite production. Notably, the optimal conditions for maximum fungal growth were observed at pH 7.0, with the highest values recorded after 21 days of incubation at 30°C. This indicates that the fungal isolate thrives in neutral pH environments and elevated temperatures. The maximum fungal biomass production and crude extract production values achieved at each temperature were: 310 mg and 0.71 mg at 25°C, 350 mg and 0.88 mg at 27°C, and 391 mg and 1.89 mg at 30°C, respectively.

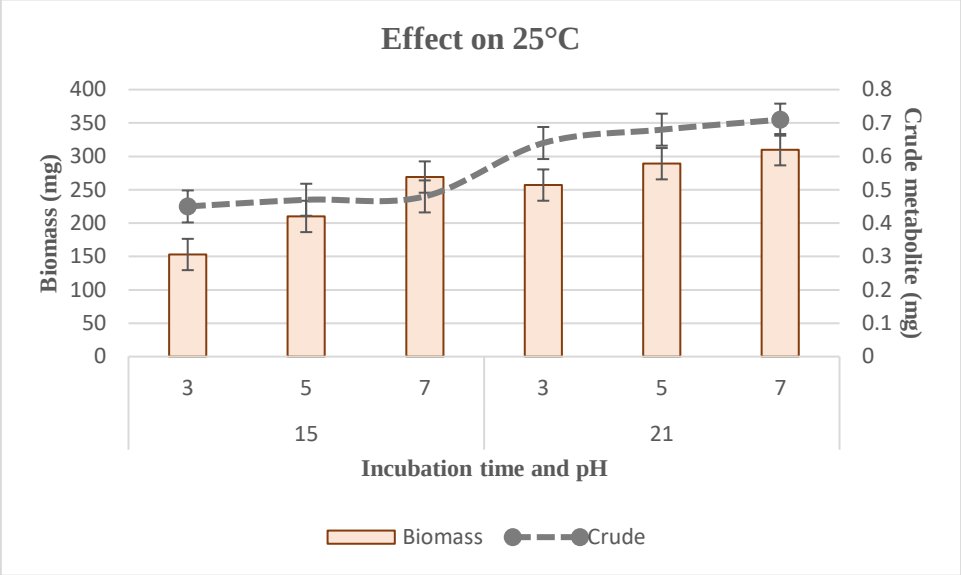


Fig.89 Effect of pH and incubation time on the growth and production of crude metabolite at 25°C in PDB

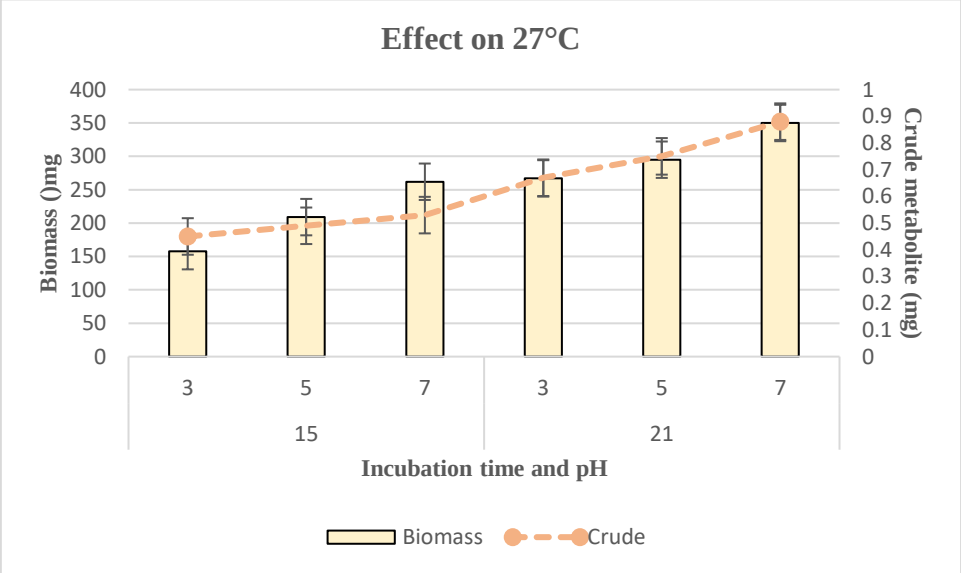


Fig.90 Effect of pH and incubation time on the growth and production of crude metabolite at 27°C in PDB

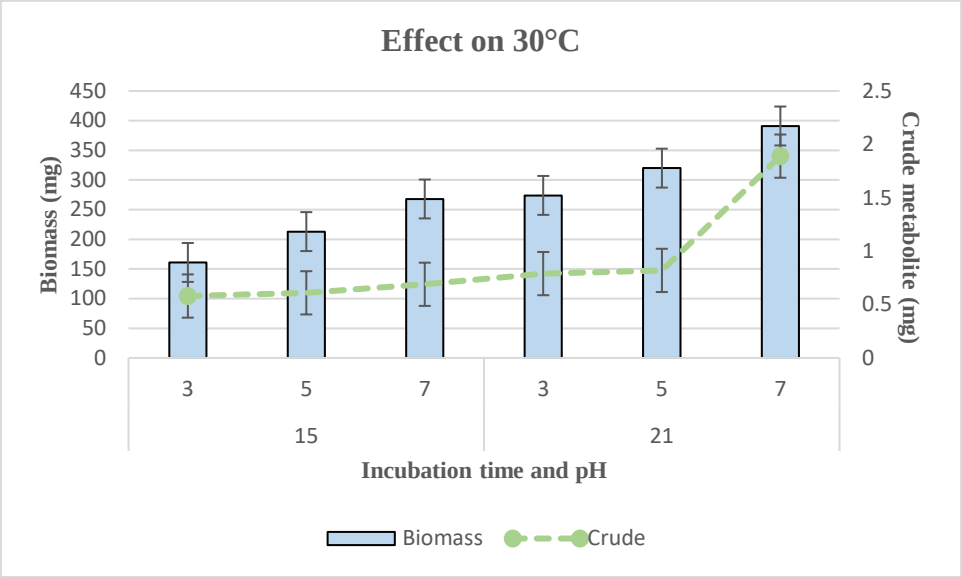


Fig.91 Effect of pH and incubation time on the growth and production of crude metabolite at 30°C in PDB

Antimicrobial Activity under Optimized Conditions

Under the optimized condition the crude extract was prepared and the antimicrobial activity was determined in four various concentrations (250, 500, 750, and 1000 µg/ml) of *Diaporthe penetriteum* FLWS21 by well diffusion method. The antimicrobial activity is measured in terms of zone of inhibition (in mm). At lower concentrations (250 and 500), the FLWS21 isolate showed moderate antimicrobial activity against most test organisms, with the zone of inhibition ranging from 8.4 to 19.6 mm. However, at higher concentrations (750 and 1000), the antimicrobial activity increased significantly, with zone of inhibition ranging from 14.1 to 26.5 mm. The FLWS21 isolate showed the highest antimicrobial activity against *B. subtilis*, with a zone of inhibition of 26.5 mm at 1000µg/ml. It also showed significant activity against Pseudomonas, Enterococcus, and Staphylococcus, with zones of inhibition ranging from 20.5 to 23.7 mm at 1000 µg/ml (Fig.92).

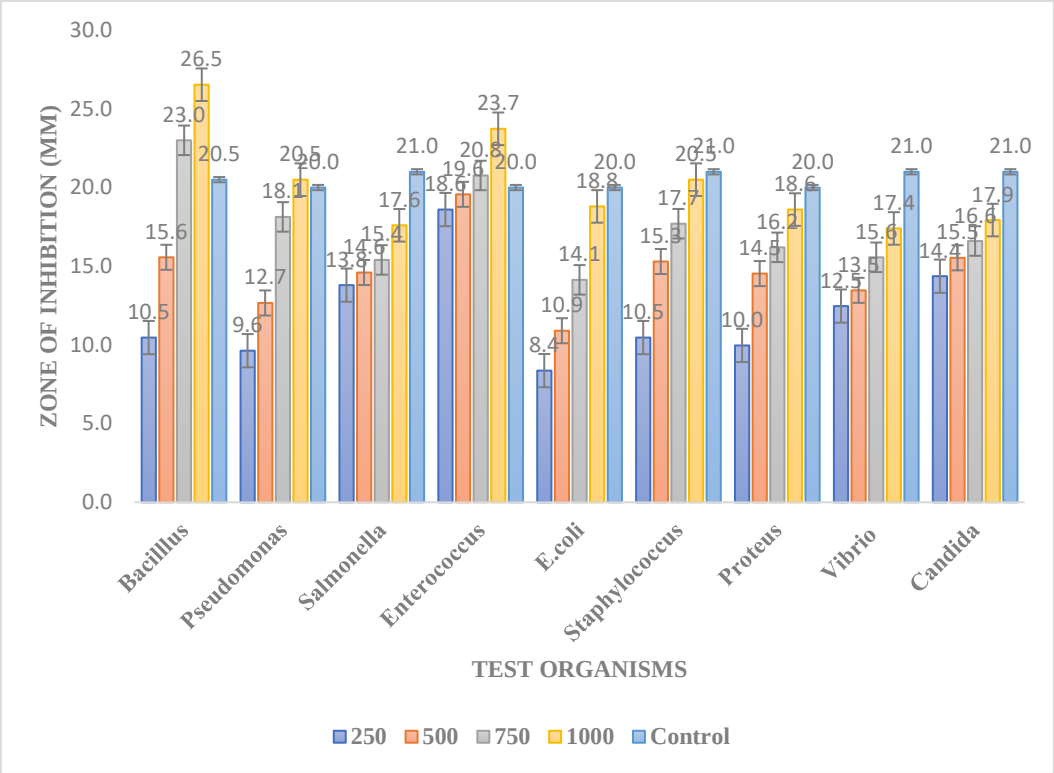


Fig.92 Antimicrobial activity of *Diaporthe penetriteum* FLWS21 in different concentrations

Diethyl Phthalate profiling from *Diaporthe penetriteum* FLWS21 by HPLC

High-Performance Liquid Chromatography (HPLC) was employed to isolate and purify the target compound, diethyl phthalate, from crude extracts of the fungal isolate Diaporthe penetriteum and leaf extract. To verify the recovery of diethyl phthalate, a known amount of standard was added to the medium, and the peak area was compared before and after the addition. HPLC analysis confirmed the presence of diethyl phthalate, exhibiting a retention time identical to the authentic standard. This confirms the successful identification of diethyl phthalate, a phthalate compound, from the specified fungal isolate and leaf extract. The plate shows a typical chromatogram of the fungal extract before the secondary metabolite optimization and after the optimization. The compound present in the pre-optimization extract was 2.155 ppm with a retention time of 2.093 of area 91235 with peak height 19458 (Fig.93). After the optimization, the compound was increased by its concentration of 11.540 ppm with a retention time of 2.102 of area 489673 with a peak height of 95300 and one of another

phthalate dioctyl phthalate also showed its presence in the concentration of 0.127 ppm (Fig.94). As predicted the presence of diethyl phthalate in the plant extract, the compound was identified in the leaf sample of concentration 1.475 ppm with a retention time of 2.099 of area 62366 with peak height 15889 (Fig.95).

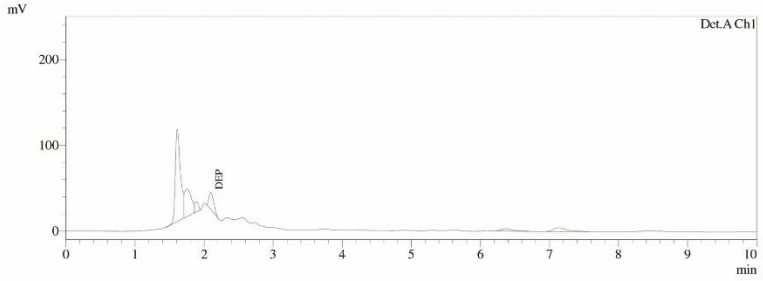


Fig. 93 HPLC analysis ofethylacetate extracted pre-optimized culture filtrate for DEP

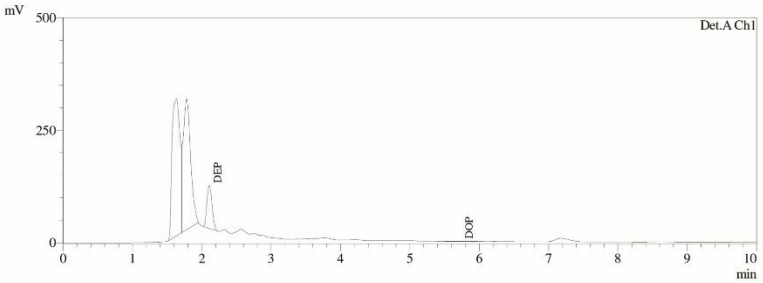


Fig. 94 HPLC analysis of ethyl acetate extracted optimized culture filtrate for DEP

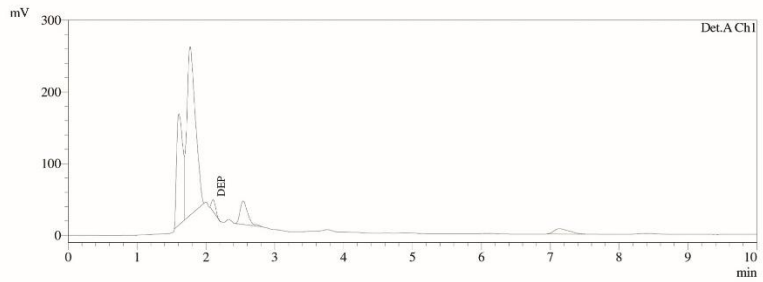


Fig. 95 HPLC analysis of ethyl acetate extracted leaf for DEP

Antioxidant Activity of Endophytic Bacteria

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity of Endophytic Bacteria

Endophytic bacterial extract's free radical scavenging activity can be analyzed using DPPH (1, 1-diphenyl-2-picrylhydrazyl), a stable free radical that can absorb light at 517 nm. The light absorption of the free radical reduces when it is scavenged by antioxidants present in the endophytic bacterial isolates. The intensity of light from the samples was measured using a spectrophotometer. The presence of antioxidants changes the purple color of the DPPH solution which can be measured at 517 nm by checking the output intensity of light from the sample solution. Different concentrations of endophytic bacterial extracts can be used to determine the free radical scavenging activity of the extract. Each experiment was replicated thrice to reduce the errors. After that, we determined the IC50 value which is the concentration of isolate needed to scavenge 50% of the free radical of DPPH. One with the lowest value of IC50 is most efficient in free radical scavenging since it has a higher antioxidant potential even in small concentrations. We used ascorbic acid as the standard for this experiment. 35 endophytic bacterial isolates were used to test the DPPH scavenging activity at different concentrations (20, 40, 60, 80, and 100 µg/mL). Results showed a difference in free radical scavenging activity between different extracts. The IC50 values of bacterial extracts are represented in Fig.96.

The extracts with higher DPPH activity or higher antioxidant potential are BLWM4, BLWS1, BLWS3, and BRWS15 with the following IC50 values 58.69µg/mL, 59.87µg/mL, 60.33µg/mL, and 60.4µg/mL respectively against 51.93 µg/mL of ascorbic acid (Table 51). This means only 58.69µg/mL of BLWM4 is needed to scavenge 50% DPPH. Hence it has the highest antioxidant potential among all the endophytic bacterial samples. These isolates possess antioxidants that can neutralize free radicals' effect with less extract. The lowest free radical scavenging activity was shown by BRhWS8 endophytic bacterial extract with an IC50 value of 108.01 µg/mL. BRhWS8 bears the lowest

antioxidant potential because 108.01 µg/mL of isolate is needed to scavenge 50% of DDPH. BLWM1, BRhWM27, BRWW12, and BLWW9 showed slightly more antioxidant potential than BRhWS8 with nearly the same IC50 values around 140 µg/mL. The endophytic bacterial isolates BLWS2, BLWW1, BRWS3, BRWS15, and BRWW6 showed higher DPPH scavenging activity comparable to the positive control (Ascorbic acid). The compounds in these extracts possess antioxidants that can effectively scavenge free radicals. The majority of bacterial extracts showed medium to low antioxidant potential with varying levels of free radical scavenging activity. The highest scavenging activity of DPPH radicals by the crude extracts of leaf extract in summer was 75.80 µg/ml and the isolate BLWS6 showed a similar value of 83.373 µg/ml. The highest scavenging activity of DPPH radicals by the crude extracts of leaf extract in summer and winter was 60.62 µg/ml and 60.84 µg/ml. The root and rhizome in summer showed an activity of 67.65 µg/ml and 85.60 µg/ml respectively.

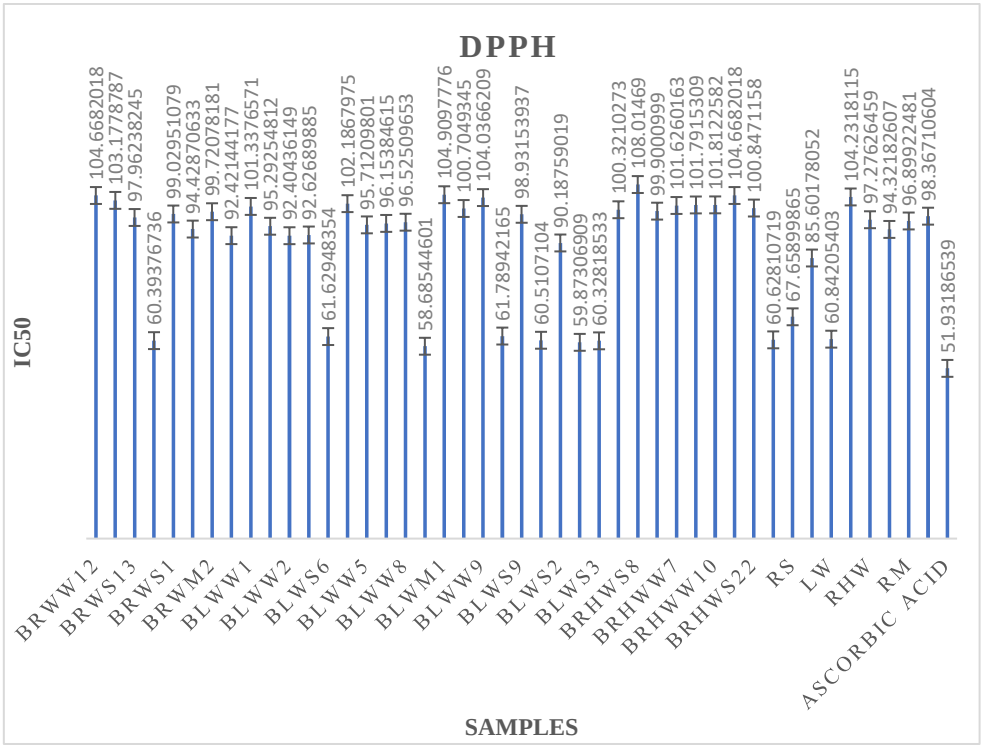


Fig.96 IC50 value of bacterial isolates for DPPH activity

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity of Endophytic Fungi

DPPH (1, 1-diphenyl-2-picrylhydrazyl) activity of 36 endophytic fungal extracts was determined. Light intensity was measured at 517 nm in the spectrophotometer after the incubation period, where the sample and DPPH were allowed to react. The colour change was evident in the highest concentration (100 µg/ml) and varied between samples. IC50 values of all the isolates were plotted in a graph to better understand the data. IC50 represents the concentration of a sample that can scavenge 50% free radicals by the antioxidants present in different fungal isolates. To obtain a standardized result, five different concentrations (20, 40, 60, 80, and 100 µg/ml) of the fungal extract were used to determine the % inhibition followed by IC50 values. Three replicates of each concentration were done. Fig.97 was represented plotted based on IC50 values of different fungal extracts.

The experiment showed the highest antioxidant potential in FRWW11 (59.59 µg/ml), and FRWW30 (60.15 µg/ml) are needed to scavenge 50% of DPPH. FLWW12 shows moderate antioxidant potential than the above-mentioned isolates with a 64.08 µg/ml IC50 value. The lowest IC50 value among all the fungal extracts was FLWS16 (99.82µg/ml). The extracts of FLWM11, FRWS24, FRWM25, and FRWM27 showed IC50 values of 98.54µg/ml, 94.91 µg/ml, 94.2 µg/ml, and 92.52 µg/mL respectively (Table 52).

FRWW11 showed the lowest IC50 value which is slightly above Ascorbic acid and can have antioxidant potential. This extract can be used to scavenge free radicals. FRWW30 showed the lowest IC50 value among all and this causes it to be the highest antioxidant potential sample. These samples with high IC50 values may not have significant free radical scavenging activity which may need further optimization to exhibit antioxidant activity. Those samples showed low IC50 values can be analyzed further to identify the bioactive compound responsible for free radical scavenging activity. Chromatography, spectroscopy, and various bioassays can be used to find out the phytochemicals behind this

antioxidant potential. The potent samples should be analyzed against different free radicals to discover the range of free radicals they can scavenge. Furthermore, the isolation of specific compounds can lead to practical application of these compounds in the future.

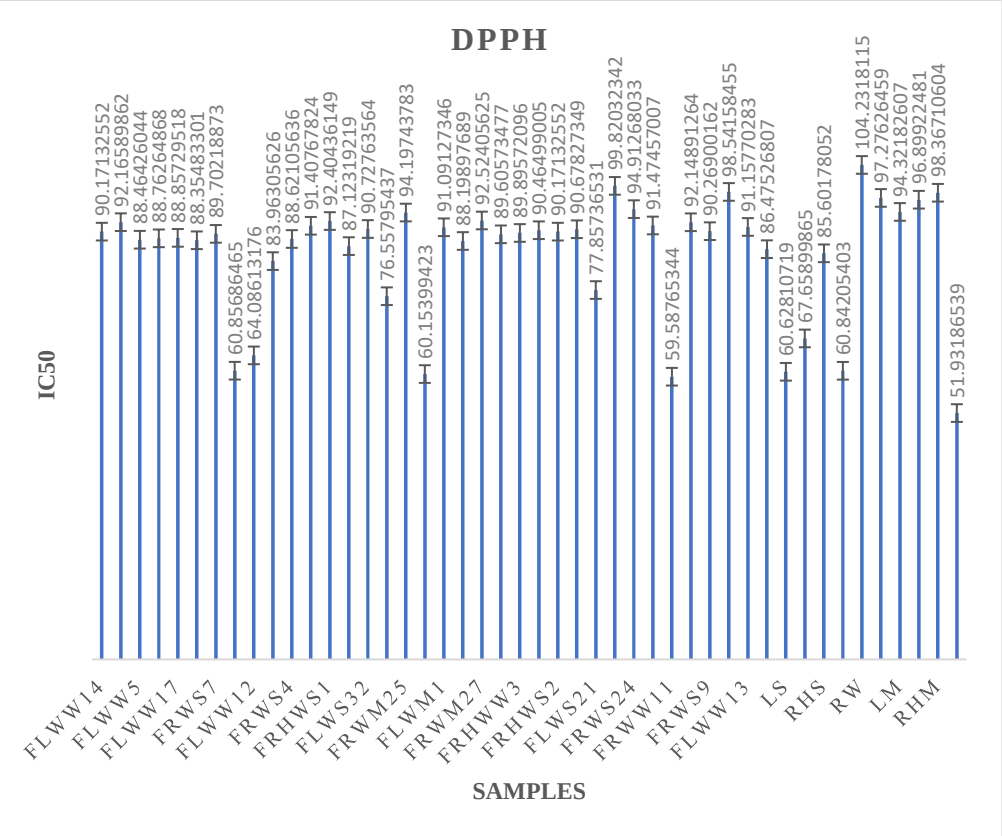


Fig.97 IC50 value of fungal isolates for DPPH activity

No.	Bacterial isolates	20	40	60	80	100
1	BRWW12	8.6067±0.42^{c}	16.9933±0.76^{abcd}	26.9300^{abcdefgh}	38.0833±0.52^{abcd}	49.9400±1.47^{abc}
2	BRWW6	7.5133±0.31^{c}	15.3167±0.69^{a}	28.0933±1.05^{defghij}	38.1633±1.40^{abcd}	51.6033±1.75^{abcde}
3	BRWS13	8.3800±0.91^{c}	16.2367±0.72^{abc}	27.0400±0.37^{abcdefghi}	42.9800±0.83^{ghi}	53.5167±0.77^{abcdefgh}
4	BRWS15	21.8167±1.18^{b}	38.4033±0.91^{e}	48.5867±0.95^{l}	58.7467±0.24^{k}	86.2633±0.95^{ijk}
5	BRWS1	8.2267±0.99^{c}	15.6267±0.67^{ab}	26.3467±1.38^{abcde}	42.3133±1.72^{efghi}	53.5300±0.79^{abcdefgh}
6	BRWS3	7.9867±0.38^{c}	18.3267±0.84^{d}	29.7767±2.05^{j}	41.5900±2.06^{defgh}	56.413±3.77^{efgh}
7	BRWM2	8.0167±0.73^{c}	16.2400±1.14^{abc}	26.1633±0.48^{abc}	43.2633±1.13^{ghi}	51.9000±0.28^{abcde}
8	BRWM3	7.2167±0.37^{c}	15.6467±1.12^{ab}	28.5400±0.54^{ghij}	45.6667±2.54^{ij}	57.6500±0.39^{fgh}
9	BLWW1	7.1600±0.425^{c}	16.3467±0.51^{abc}	27.1267±1.99^{abcdefghi}	39.4100±0.91^{cdefg}	52.7800±2.98^{abcdefg}
10	BLWW4	7.6467±0.51^{c}	16.3867±1.41^{abcd}	28.1000±0.28^{defghij}	44.8667±1.91^{hij}	54.6000±2.41^{bcdefgh}
11	BLWW2	8.1667±0.99^{c}	16.4400±0.91^{abcd}	28.1867±0.24^{efghij}	45.4300±3.77^{hij}	57.5767±2.85^{fgh}
12	BLWS11	8.1900±0.31^{c}	15.9333±2.01^{ab}	27.6500±0.37^{abcdefghi}	45.2867±1.07^{hij}	57.9300±0.83^{gh}
13	BLWS6	21.1600±0.54^{b}	37.8900±0.77^{e}	47.9367±0.25^{kl}	58.7167±0.67k	83.3733±0.69^{ij}
14	BLWS7	7.52000.51^{c}	16.7167±0.96^{abcd}	26.7200±1.11^{abcdefg}	39.7600±0.77^{cdefg}	51.6233±0.62^{abcde}
15	BLWW5	7.8433±0.70^{c}	17.3567±1.39^{bcd}	26.06±0.104^{ab}	45.2467±1.55^{hij}	54.9367±2.39^{cdefgh}
16	BLWW7	8.7667±0.91^{c}	16.5567±0.33^{abcd}	27.3967±1.03^{abcdefghi}	42.6167±3.51^{fghi}	55.5033±4.74^{defgh}
17	BLWW8	8.5400±0.19^{c}	15.7567±0.21^{ab}	27.4600±0.46^{abcdefghi}	42.6167±3.57^{fghi}	55.3833±2.91^{defgh}
18	BLWM4	21.0933±2.16^{b}	38.6267±1.06^{e}	48.4033±1.11^{l}	62.6933±1.95^{l}	88.5633±1.41^{k}
19	BLWM1	7.7867±0.35^{c}	16.6067±0.38^{abcd}	26.7800±0.29^{abcdefg}	34.9800±6.89^{a}	52.5967±0.49^{abcdef}
20	BLWW10	8.0400±0.91^{c}	16.5033±0.89^{abcd}	27.5633±0.61^{abcdefghi}	36.3133±0.74^{abc}	55.4233±0.69^{defgh}

21	BLWW9	7.7800±0.19^{c}	17.2233±1.05^{abcd}	28.0433±1.51^{cdefghij}	38.7300±0.33^{abcde}	49.4700±1.55^{ab}
22	BLWS10	21.6167±1.51^{b}	37.4067±2.26^{e}	48.4500±1.01^{l}	60.0567±0.45^{kl}	81.6233±3.43^{i}
23	BLWS9	8.4267±1.21^{c}	16.5367±0.45^{abcd}	27.7500±1.92^{abcdefghi}	39.9267±2.31^{cdefg}	54.2967±4.52^{bcdefgh}
24	BLWS4	20.3000±1.30^{b}	37.9333±1.54^{e}	46.5533±1.07^{k}	57.7900±1.62^{k}	88.3900±1.27^{k}
25	BLWS2	8.5800±0.47^{c}	16.5967±0.82^{abcd}	28.4000±0.68^{fghij}	47.8767±1.28^{j}	58.2800±2.76^{h}
26	BLWS1	21.3967±1.51^{b}	38.3133±1.13^{e}	48.4600±0.68^{l}	59.5933±0.91^{kl}	87.3700±1.06^{jk}
27	BLWS3	21.8133±0.81^{b}	38.5700±0.52^{e}	48.6300±0.88^{l}	58.8967±0.81^{k}	86.2500±1.02^{ijk}
28	BRhWS1	12.4167±4.57^{a}	15.8967±0.78^{ab}	25.9700±0.11^{a}	38.6800±1.24^{abcde}	54.2700±4.22^{bcdefgh}
29	BRHWS8	7.7933±0.23^{c}	17.1900±0.51^{abcd}	27.1600±0.91^{abcdefghi}	35.3467±1.51^{ab}	48.8267±0.695^{a}
30	BRhWW11	7.6567±41^{c}	17.1567±0.12^{abcd}	28.7700±1.42^{hij}	38.6500±1.29^{abcde}	53.5233±3.21^{abcdefgh}
31	BRhWW7	7.7733±0.302^{c}	17.4467±0.67^{bcd}	28.9033±0.71^{ij}	39.4267±0.99^{cdefg}	50.8267±2.49^{abcd}
32	BRhWM3	7.2233±0.81^{c}	17.9200±0.41^{cd}	27.9200±0.36^{bcdefghi}	38.2700±0.92^{abcd}	52.0867±5.07^{abcde}
33	BRhWW10	7.4900±0.51^{c}	17.3433±0.74^{bcd}	28.1067±0.83^{defghij}	38.9200±0.97^{bcdef}	51.6100±6.55^{abcde}
34	BRhWM27	7.4867±0.82^{c}	16.4633±0.91^{abcd}	26.2300±0.37^{abcd}	38.7400±0.59^{abcde}	50.2900±2.02^{abcd}
35	BRhWS22	7.2833±0.94^{c}	17.2967±0.74^{bcd}	26.5167±0.53^{abcdef}	37.8667±1.53^{abcd}	54.5067±1.338^{bcdefgh}
36	LS	28.48±1.64^{e}	34.45±0.75^{e}	56.47±1.48^{m}	65.35±4.81^{lm}	75.80±1.22^{ij}
37	RS	25.34±2.85^{b}	38.41±2.15^{e}	47.93±1.63^{kl}	55.78±1.61^{k}	68.76±2.77^{gh}
38	RhS	12.48±1.01^{d}	21.83±2.04^{f}	37.13±1.19^{jk}	45.86±3.52^{hij}	58.31±1.36^{h}
39	LW	28.4±1.62^{e}	32.9±0.37^{e}	56.47±1.48^{m}	65.35±4.81^{lm}	75.80±1.22^{ij}
40	RW	7.83±1.57^{c}	16.89±2.68^{abcd}	27.23±0.487^{bcdefgh}	38.7600±0.77^{cdefg}	49.871±1.47^{abc}
41	RhW	12.392±2.58^{a}	19.58±1.45^{f}	26.14±2.86^{ab}	44.2467±1.55^{hij}	51.68±1.75^{abcde}
42	LM	16.28±3.58^{d}	17.28±1.07^{bcd}	29.83±1.64^{ij}	43.6167±3.51^{fgh}	53.67±0.77^{abcdefgh}
43	RM	13.25±1.114^{e}	20.31±0.93^{g}	28.25±0.28^{defghij}	42.6167±3.57^{fghi}	51.7100±6.55^{abcde}

44	RhM	8.83±0.541^{c}	18.56±2.87^{h}	34.52±4.65^{m}	39.67±0.77^{cdefg}	50.1900±2.02^{abcd}
45	Ascorbic acid	7.91±2.64^{c}	17.91±1.23^{cd}	24.96±0.58^{ab}	45.467±1.55^{hij}	53.967±1.338^{bcdefgh}

Table 51: DPPH activity by crude extracts of bacterial endophytes

No.	Fungal isolates	20	40	60	80	100
1	FLWW14	10.1267±0.551^{abc}	18.9667±0.786^{abcde}	30.2533±1.421^{bcdefg}	46.5867±2.549^{ghijklm}	56.9533±3.537^{defg}
2	FRWS8	8.5667±1.489^{abc}	16.3167±2.725^{a}	27.6400±2.890^{ab}	41.5067±0.725^{abc}	61.3133±1.02^{ij}
3	FLWW5	8.3467±0.866^{abc}	17.1033±0.991^{ab}	29.3967±2.151^{abcde}	49.12670.716^{lmn}	58.8833±1.175^{ghij}
4	FLWW15	8.8367±0.615^{abc}	21.3600±1.517^{ef}	34.4500±2.544^{hijk}	48.5033±0.962^{jklmn}	56.4667±0.834^{defg}
5	FLWW17	8.7067±1.983^{abc}	20.9433±1.944^{def}	28.5267±0.801^{abcd}	47.4367±2.51^{hijklm}	58.6000±1.012^{fghij}
6	FLWW6	8.6067±0.447^{abc}	16.8100±1.035^{ab}	26.4533±1.803^{a}	49.5167±2.59^{mno}	60.5733±0.455^{hij}
7	FRSW7	10.8567±0.645^{c}	20.3067±3.258^{cdef}	31.5167±3.156^{cdefghij}	45.7300±1.41^{defghijk}	56.8367±2.803^{defg}
8	FRWM12	21.7233±2.684^{ef}	36.2533±1.161^{i}	50.5233±1.241^{n}	61.2567±2.175^{q}	82.5900±2.02^{m}
9	FLWW12	17.3533±2.956^{d}	30.2700±1.415^{h}	45.8033±2.709^{m}	63.0133±1.167^{qr}	78.1700±1.304^{l}
10	FRWM1	7.9333±0.776^{a}	19.4800±0.743^{bcde}	34.3767±2.321^{hijk}	52.1900±1.831^{op}	59.2533±1.204^{ghij}
11	FRWS4	9.0333±0.574^{abc}	22.5100±2.514^{f}	35.2600±1.741^{jk}	43.5700±0.649^{bcdefg}	57.3033±2.817^{efg}
12	FLWW11	10.5267±0.814^{abc}	25.4067±0.983^{g}	33.3033±2.054^{fghijk}	44.3700±1.849^{cdefgh}	52.5867±1.941^{abc}
13	FRhWS1	9.4200±0.690^{abc}	19.0833±0.182^{abcde}	31.0933±1.449^{bcdefghi}	40.8833±1.175^{ab}	58.1667±1.742^{efghi}
14	FLWW18	21.5233±1.245^{e}	31.9167±1.589^{h}	40.9933±1.606^{l}	50.6567±1.018^{nop}	61.4967±0.668^{j}
15	FLWS32	9.5567±0.540^{abc}	17.5067±0.705^{abc}	33.7733±1.263^{ghijk}	46.0000±2.865^{efghijkl}	55.2667±3.865^{cde}
16	FRWW18	8.7367±0.301^{abc}	16.4667±1.063^{a}	34.6667±0.941^{ijk}	44.7067±3.292^{ccdefghi}	59.0300±0.614^{ghij}
17	FRWM25	9.3100±0.671^{abc}	18.6000±1.016^{abcde}	31.6533±1.21^{defghijk}	41.5567±1.258^{abc}	55.2333±2.15^{cde}
18	FRWW30	22.1067±2.01^{efg}	37.3867±0.945^{i}	47.5833±1.407^{mn}	61.2267±1.925^{q}	85.9667±1.651^{n}
19	FLWM1	10.6367±0.963^{bc}	18.3033±1.042^{abcd}	32.6233±0.531^{efghijk}	43.2867±1.139^{bcdef}	57.1067±0.737^{defg}
20	FLWW9	10.4167±1.79^{abc}	18.5600±0.745^{abcd}	31.7167±1.709^{defghijk}	46.3733±1.086^{fghijklm}	59.0767±0.489^{ghij}
21	FRWM27	8.9067±0.789^{abc}	16.6533±1.622^{ab}	33.4767±1.226^{fghijk}	41.4467±1.257^{abc}	57.1967±1.056^{efg}
22	FLWS1	8.1267±0.175^{ab}	18.6067±1.184^{abcde}	33.3833±2.994^{fghijk}	43.1667±0.799^{bcdef}	59.1300±0.622^{ghij}
23	FRhWW3	10.8867±1.63^{c}	18.2767±1.017^{abcd}	32.7933±1.312^{efghijk}	44.0267±1.786^{bcdefg}	57.9767±0.670^{efgh}

24	FRhWM2	9.440±0.589^{abc}	19.1900±0.441^{abcde}	33.4100±2.005^{fghijk}	44.4767±1.406^{cdefghi}	56.3933±0.828^{defg}
25	FRhWS2	9.4467±1.08^{abc}	17.2267±0.952^{ab}	32.2833±0.943^{defghijk}	44.1600±1.107^{bcdefg}	58.5033±0.579^{efghij}
26	FRWM3	9.0600±0.545^{abc}	18.6133±1.135^{abcde}	31.4067±0.876^{cdefghij}	45.3667±0.996^{defghij}	56.9167±2.555^{defg}
27	FLWS21	15.8667±3.508^{d}	27.4533±1.596^{g}	39.4933±2.631^{l}	48.8200±1.773^{klmn}	64.3733±2.837^{k}
28	FLWS16	8.0933±0.851^{ab}	17.3100±0.872^{ab}	30.1467±1.035^{bcdefg}	41.3767±1.138^{abc}	50.4700±1.22^{a}
29	FRWS24	8.3733±0.231^{abc}	17.0100±0.377^{ab}	30.0600±1.314^{abcdefg}	42.5400±2.065^{bcd}	55.3500±2.325^{cdef}
30	FRWW10	9.0667±0.366^{abc}	18.3867±0.431^{abcd}	31.9200±0.339^{defghijk}	43.1533±1.066^{bcdef}	57.4067±1.329^{efgh}
31	FRWW11	24.2567±1.665^{g}	37.5967±1.796^{i}	50.4733±1.388^{n}	64.9500±1.795^{r}	82.4700±1.26^{m}
32	FRhWW4	10.7900±1.96^{c}	18.6100±0.888^{abcde}	32.4467±1.255^{efghijk}	42.7600±1.812^{bcde}	56.0900±1.001^{defg}
33	FRWS9	9.6133±0.527^{abc}	18.0300±1.754^{abc}	28.9500±0.714^{abcde}	47.6900±0.505^{ijklmn}	57.2000±1.337^{efg}
34	FLWM11	8.9500±0.425^{abc}	18.2667±0.814^{abcd}	29.6100±1.572^{abcdef}	41.9900±1.634^{bc}	51.170±0.630^{ab}
35	FLWW13	9.7367±0.816^{abc}	18.7767±0.211^{abcde}	30.6033±0.811^{bcdefgh}	44.5333±1.575^{cdefghi}	57.2133±0.910^{efg}
36	FLWS5	9.4567±1.01^{abc}	19.1700±1.023^{abcde}	31.6933±1.093^{defghijk}	52.7100±3.091^{p}	56.4500±1.401^{defg}
37	LS	28.48±1.64^{f}	34.45±0.75^{hi}	56.47±1.48^{n}	65.35±4.81^{qr}	75.80±1.22^{kl}
38	RS	25.34±2.85^{ef}	38.41±2.15^{i}	47.93±1.63^{mn}	55.78±1.61^{op}	68.76±2.77^{ij}
39	RhS	12.48±1.01^{abc}	21.83±2.04^{ef}	37.13±1.19^{l}	45.86±3.52^{ijklm}	58.31±1.36^{efg}
40	LW	7.83±1.57^{abc}	16.89±2.68^{abcd}	27.23±0.487^{bcdefgh}	38.7600±0.77^{cdefg}	49.871±1.47^{l}
41	RW	12.392±2.58^{ab}	19.58±1.45^{f}	26.14±2.86^{ab}	44.2467±1.55^{cdefghi}	51.68±1.75^{ab}
42	RhW	16.28±3.58^{d}	17.28±1.07^{ab}	29.83±1.64^{abcdef}	43.6167±3.51^{bcdef}	53.67±0.77^{abc}
43	LM	13.25±1.114^{g}	20.31±0.93^{cdef}	28.25±0.28^{abcde}	42.6167±3.57^{bcde}	51.7100±6.55^{ab}
44	RM	8.83±0.54^{bc}	18.56±2.87^{abcde}	34.52±4.65^{ijk}	39.67±0.77^{cdefg}	50.1900±2.02^{ab}
45	RhM	7.91±2.64^{abc}	17.91±1.23^{bcd}	24.96±0.58^{ab}	45.467±1.55^{hijkl}	53.967±1.338^{bc}
46	Ascorbic acid	23.880±0.63^{fg}	38.6933±0.628^{i}	65.1700±5.963^{o}	78.700±0.569^{s}	89.5067±0.963^{o}

Table 52: DPPH activity by crude extracts of fungal endophytes

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) Radical Scavenging Activity of Endophytic Bacteria

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay is widely used to analyze the antioxidant potential of various extracts, including bacterial isolates. ABTS cation radical is blue-green. It is generated through a reaction with potassium persulfate. During the free radical scavenging activity, antioxidants can donate electrons to ABTS free radicals, reducing it to a less coloured form. The absorbance is measured at 734 nm. The results are represented in terms of Trolox equivalent. Trolox is a water-soluble vitamin E analog. The isolates were allowed an incubation period to react with the free radical ABTS and the light intensity was measured using a spectrophotometer at 734 nm. The bacterial isolates showed various activities, with some showing higher antioxidant potential and others with moderate or lower levels of free radical scavenging activity. The activity is concentration-dependent, and higher concentrations showed higher activity. 35 bacterial endophytic samples were analyzed for ABTS free radical scavenging activity. Three replicates of five different concentrations (20, 40, 60, 80, and 100 µg/ml) of each bacterial isolate were allowed to react with the ABTS free radical. IC50 values were determined which is the half maximal inhibitory concentration.

Trolox, the standard showed an IC50 value of 75.39 µg/ml (Fig.98). BLWS6 showed nearly the same IC50 value of the standard with 78.78 µg/ml half maximal inhibitory concentration. BRWS3, BLWS10, BLWS2, BRWS15, BRWW6, and BLWW1 showed slightly higher IC50 values but had higher antioxidant potential like BLWS6 with IC50 values 80.44 µg/ml, 80.89 µg/ml, 81.02 µg/ml, 81.34 µg/ml, 81.45 µg/ml, and 83.77µg/ml. The rest of the 28 samples showed a very high IC50 value above 400 µg/ml. BLWS9 showed the highest IC50 value, which explains its low antioxidant potential indicated by an IC50 value of 506.59 µg/ml. This is a significantly high concentration to achieve antioxidant potential. Bacterial isolates such as BRhWS8, BRhWW11, BLWS1, and BLWS3 showed less free radical scavenging activity reflected by IC50

values of 496.03 µg/ml, 486.38 µg/ml, 477.55 µg/ml and 474.38 µg/ml. Inhibition of ABTS radicals by rhizome extract in monsoon is 77.06 µg/ml and the isolate BLWS6 showed a similar value of 78.77 µg/ml. The root extract showed an inhibition of 80.97 µg/ml and the isolate BRWS3 with a similar value (Table 53). The antioxidant potential comparable to the standard Trolox is BLWS6 which can be analysed further to ascertain the potential antioxidant. This may have practical applications to scavenge free radicals.

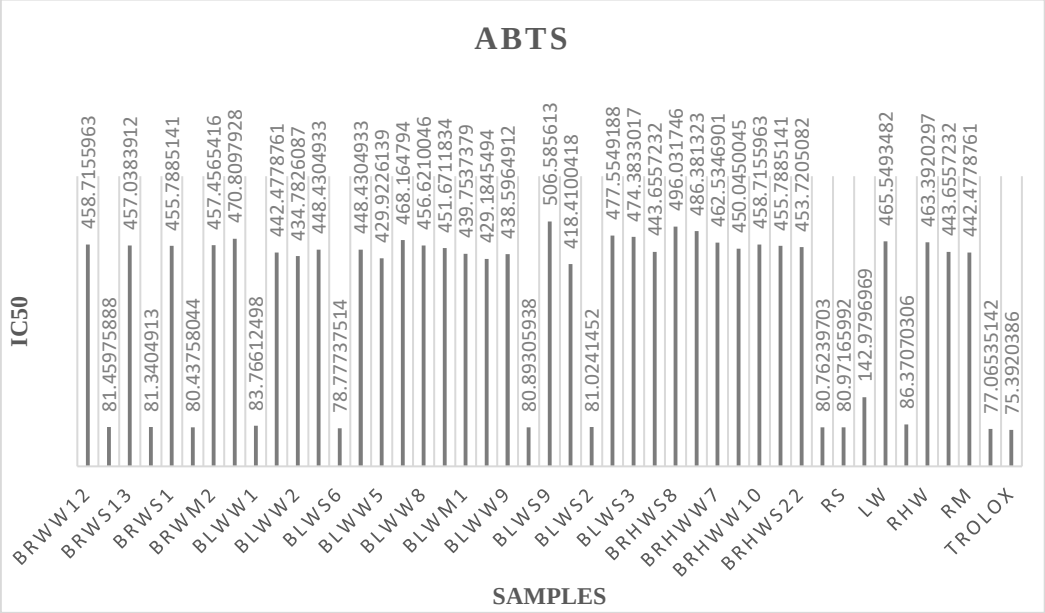


Fig.98 IC50 value of bacterial isolates for ABTS activity

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) Radical Scavenging Activity of Endophytic Fungi

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical activity of 36 endophytic fungal isolates was determined. Samples were allowed to react with ABTS free radicals. Each sample was incubated to react with free radicals, and the percentage of inhibitions was obtained by spectrophotometer analysis at 734 nm. IC50 values of all the fungal isolates were determined, and a graph was plotted based on the concentration. IC50 values gave an insight into the concentration of the isolate needed to scavenge half the number of free radicals present. This will help us predict the potential of antioxidants in the fungal isolates, thereby exploring the application of the isolate as a free radical scavenger. Trolox was used as a standard for the experiment.

FLWS16 showed the lowest IC50 value, holding the highest antioxidant potential for free radical scavenging with 82.76 µg/ml needed to scavenge half of the free radicals (Fig.99). FLWS16, FLWW12, FLWW18, and FLWW14 showed IC50 values below 90 µg/ml. 29 fungal isolates showed IC50 values above 400 µg/ml. Scavenging inhibition of ABTS radicals by the leaf extract in summer is 80.76 µg/ml, and the isolate FLWS16 showed approximately similar value. The root and rhizome extracts showed a value of 8.33±1.19 µg/ml and 37.75±1.55 and the positive control ascorbic acid of 70.15±0.33 µg/ml (Table 54). No isolates showed similar values of root, rhizome, and ascorbic acid values. The highest among them was FRWM12, with an IC50 value of 574.71µg/ml. It may not have a significant antioxidant activity or radical scavenging ability. However, FLWS16 is a potential candidate for high antioxidant activity.

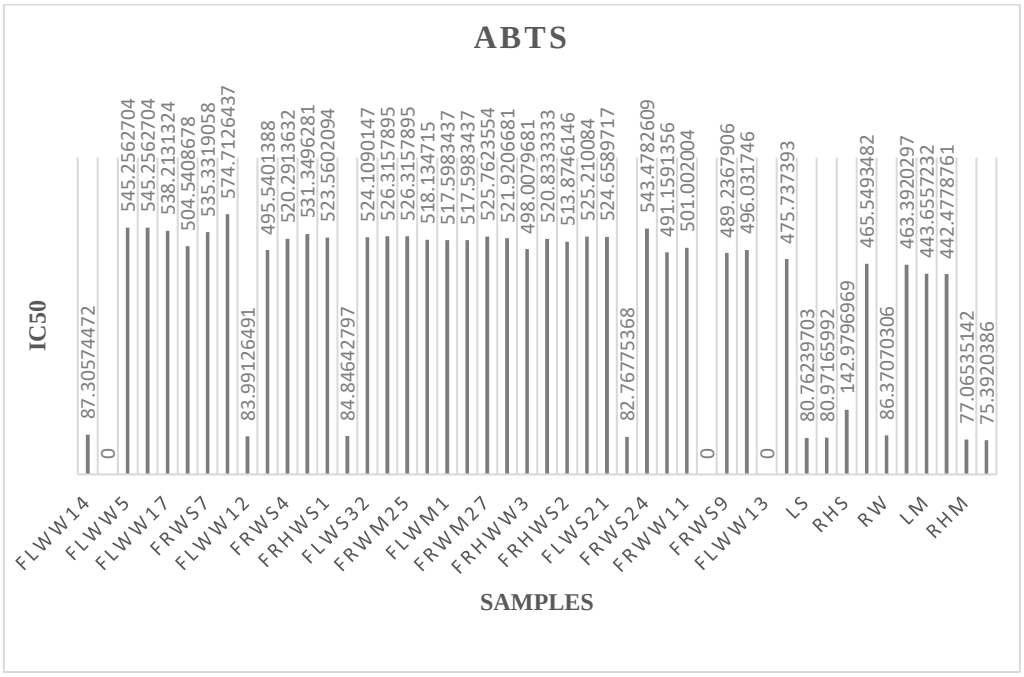


Fig.99 IC50 value of fungal isolates for DPPH activity

No.	Bacterial isolates	20	40	60	80	100
1	BRWW12	1.8667±0.39^{abc}	3.8067±0.67^{ab}	5.7633±0.41^{a}	7.4833±0.58^{a}	12.6467±0.56^{a}
2	BRWW6	15.3500±1.23^{f}	28.440±1.21^{de}	38.7233±0.29^{f}	49.2533±0.74^{d}	57.9600±0.28^{def}
3	BRWS13	2.0867±0.35^{abcd}	2.9167±0.43^{ab}	5.9100±0.12^{a}	8.3067±0.84^{ab}	12.2933±1.11^{ab}
4	BRWS15	18.4867±0.71^{h}	28.9233±0.56^{e}	38.8200±0.26^{f}	48.7433±0.76^{d}	57.6900±2.02^{de}
5	BRWS1	2.5167±0.438^{cd}	3.5733±0.31^{ab}	6.1767±0.62^{ab}	7.8433±0.19^{ab}	12.2133±0.367^{ab}
6	BRWS3	17.3433±0.835^{g}	29.3333±0.59^{e}	38.7667±0.72^{f}	49.1633±0.54^{d}	58.9633±0.49^{fg}
7	BRWM2	1.7733±0.29^{abc}	3.9433±0.64^{ab}	5.8967±0.22^{a}	7.6800±0.68^{ab}	12.4300±0.63^{a}
8	BRWM3	1.7500±0.11^{abc}	3.7200±0.26^{ab}	5.9833±0.12^{ab}	8.5933±0.34^{ab}	11.0600±0.39^{bc}
9	BLWW1	17.7867±0.285^{gh}	27.6400±0.48^{d}	36.8700±0.26^{e}	46.9900±0.63^{ab}	56.9967±0.89^{d}
10	BLWW4	1.3367±0.27^{a}	3.0267±0.66^{ab}	5.6300±0.37^{a}	8.5500±0.52^{ab}	12.7833±0.72^{a}
11	BLWW2	1.8300±0.062^{abc}	3.1100±0.39^{ab}	6.3967±0.42^{ab}	8.6333±0.34^{ab}	12.9367±0.65^{a}
12	BLWS11	2.3933±0.65^{cd}	3.0400±0.32^{ab}	6.3433±0.54^{ab}	7.8800±0.27^{ab}	12.7200±0.45^{a}
13	BLWS6	17.7733±0.33^{gh}	28.7900±2.02^{de}	39.8867±1.47^{f}	50.8400±1.86^{e}	59.9667±1.14^{g}
14	BLWS7	1.9267±0.090^{abc}	3.5667±0.32^{ab}	6.0300±0.101^{ab}	8.4300±0.51^{ab}	12.3567±0.98^{ab}
15	BLWW5	1.9800±0.39^{abc}	3.2800±0.35^{ab}	6.1800±0.26^{ab}	8.6267±0.33^{ab}	13.2767±0.41^{a}
16	BLWW7	1.9467±0.070^{abc}	3.3100±0.66^{ab}	5.8733±0.25^{a}	7.7700±0.26^{ab}	12.0500±0.29^{abc}
17	BLWW8	2.2567±0.238^{bcd}	2.8700±0.06^{ab}	5.9233±0.11^{a}	7.8700±0.49^{ab}	12.6400±0.48^{a}
18	BLWM4	2.3433±0.309^{cd}	3.2700±0.66^{ab}	6.1467±0.31^{ab}	7.9600±0.43^{ab}	12.5300±0.79^{a}
19	BLWM1	1.7933±0.050^{ac}	3.2733±0.66^{ab}	6.1500±0.229^{ab}	8.4333±0.32^{ab}	12.9167±0.24^{a}
20	BLWW10	2.0467±0.097^{abcd}	3.0433±0.085^{ab}	7.4833±0.65^{c}	7.9733±0.36^{ab}	13.1267±0.67^{a}
21	BLWW9	2.8000±0.17^{d}	3.6000±0.19^{ab}	6.0500±0.29^{ab}	7.9633±0.87^{ab}	13.0700±0.81^{a}
22	BLWS10	18.0900±0.33^{h}	28.2233±0.48^{de}	38.8067±0.09^{f}	48.7233±0.81^{d}	58.8067±0.22^{efg}
23	BLWS9	1.4900±0.33^{ab}	2.8267±0.16^{a}	5.8700±0.14^{a}	7.4833±0.48^{a}	10.7733±0.852^{c}
24	BLWS4	2.4033±0.42^{cd}	4.1433±0.71^{ab}	7.2800±0.82^{c}	9.0033±0.56^{b}	12.5767±0.898^{a}

25	BLWS2	18.2267±0.377^{h}	28.7167±1.16^{de}	38.9967±0.44^{f}	49.0167±0.54^{d}	58.0233±0.145^{def}
26	BLWS1	1.8700±0.61^{abc}	4.0700±0.61^{ab}	6.8667±0.65^{bc}	7.2800±0.58^{a}	11.0800±0.367^{bc}
27	BLWS3	2.4100±0.37^{cd}	4.0667±0.52^{ab}	6.3533±0.581^{ab}	8.1100±0.16^{ab}	10.7900±0.769^{c}
28	BRhWS1	2.2900±0.13^{bcd}	4.2300±0.94^{b}	5.9167±0.032^{a}	8.6000±0.12^{ab}	12.2200±0.465^{ab}
29	BRHWS8	1.9300±0.95^{abc}	3.1600±0.91^{ab}	6.0433±0.075^{ab}	7.5667±0.52^{a}	10.8467±0.352^{c}
30	BRhWW11	1.8767±0.23^{abc}	3.6367±0.15^{ab}	6.0800±1.38^{ab}	7.6233±0.61^{ab}	11.0367±0.736^{bc}
31	BRhWW7	1.9633±0.97^{abc}	3.5433±0.48^{ab}	6.3167±0.49^{ab}	7.8400±0.65^{ab}	11.9133±0.831^{abc}
32	BRhWM3	2.3167±0.46^{cd}	3.6733±0.47^{ab}	5.8967±0.14^{a}	8.1867±0.48^{ab}	12.4300±0.425^{a}
33	BRhWM10	2.0733±0.59^{abcd}	3.5900±0.27^{ab}	5.9500±0.19^{a}	7.8733±0.68^{a}	12.2600±0.426^{ab}
34	BRhWM27	1.8833±0.23^{abc}	3.4033±0.24^{ab}	6.0933±0.42^{ab}	7.6333±0.42^{ab}	12.6400±0.105^{a}
35	BRhWS22	1.8333±0.20^{abc}	3.9200±0.25^{ab}	5.9300±0.09^{a}	7.7933±0.15^{ab}	12.5200±0.35^{a}
36	LS	21.27±1.4^{i}	29.68±0.53^{de}	38.26±0.35^{f}	47.56±1.78^{d}	59.08±1.67^{g}
37	RS	19.43±2.29^{h}	29.75±0.98^{de}	38.25±1.09^{f}	48.47±1.17^{d}	58.33±1.19^{fg}
38	RhS	4.99±0.18^{d}	11.06±1.39^{f}	17.65±0.64^{g}	28.96±0.91^{g}	37.75±1.55^{h}
39	LW	1.6900±0.11abc	3.690±0.26ab	5.873±0.12ab	8.6933±0.34ab	11.3567±4.98ab
40	RW	17.67±0.285gh	27.630±0.48d	36.700±0.26e	46.9900±0.83ab	53.1767±0.61a
41	RhW	1.467±0.27a	3.017±0.66ab	5.5400±0.37a	8.4500±0.52ab	12.1500±2.29abc
42	LM	1.7900±0.062abc	3.1200±0.39ab	6.2967±0.42ab	8.593±1.48ab	12.5400±0.84a
43	RM	2.3283±0.65cd	3.0500±0.32ab	6.453±0.54ab	7.9800±1.97ab	12.9300±0.79a
44	RhM	17.33±0.33gh	28.7900±2.02de	39.97±1.47f	50.8400±1.86e	63.1167±0.24a
45	Ascorbic acid	13.6667±0.55e	24.0033±0.33^{c}	36.1167±0.23^{d}	52.1800±1.97^{f}	70.1500±0.33^{h}

Table 53: ABTS activity by crude extracts of bacterial endophytes

No.	Fungal isolates	20	40	60	80	100
1	FLWW14	16.6067±0.85^{f}	26.80±0.67^{a}	36.20±1.135^{f}	43.41±1.02^{f}	55.51±0.78^{f}
2	FRWS8	.0000	.0000	.0000	.0000	.0000
3	FLWW5	2.6333±0.69^{d}	3.6567±0.43^{ef}	5.0600±0.307^{a}	7.5633±0.445^{abcd}	9.0900±0.202^{b}
4	FLWW15	1.5967±0.27^{abc}	3.4800±0.56^{def}	5.7700±0.105^{abc}	7.4500±0.125^{abc}	9.0300±0.72^{b}
5	FLWW17	1.5867±0.54^{abc}	3.5933±0.31^{def}	5.4300±0.443^{abc}	7.7267±0.37^{abcde}	9.2433±0.359^{b}
6	FLWW6	1.7533±0.26^{abc}	3.5300±0.59^{def}	6.2467±0.411^{cde}	8.4333±0.26^{abcde}	9.5500±0.285^{bc}
7	FRSW7	1.9867±0.09^{abcd}	3.8400±0.643^{ef}	5.5300±0.367^{abc}	7.6767±0.43^{abcd}	9.1500±0.304^{b}
8	FRWM12	1.2800±0.04^{a}	3.7433±0.255^{ef}	5.9500±0.175^{bcde}	7.3867±0.67^{a}	7.9000±0.413^{a}
9	FLWW12	16.9733±0.56^{f}	28.3333±0.48^{b}	37.6567±0.401^{g}	46.3400±0.92^{g}	56.5700±0.849^{g}
10	FRWM1	1.8567±0.454^{abc}	3.7033±0.66^{fe}	6.6633±0.435^{de}	8.3400±0.353^{abcde}	9.6833±0.436^{bc}
11	FRWS4	1.6933±0.27^{abc}	3.6800±0.43^{ef}	5.7900±0.766^{abc}	7.8100±0.651^{abcde}	9.6100±0.525^{bc}
12	FLWW11	1.4033±0.122^{ab}	3.7267±0.19^{ef}	5.5133±0.411^{abc}	7.9667±0.136^{abcde}	9.2600±0.512^{bc}
13	FRhWS1	1.7467±0.122^{abc}	3.5533±0.40^{def}	5.7900±0.151^{abc}	8.1700±0.37^{abcde}	9.2233±0.147^{b}
14	FLWW18	18.1700±0.602^{g}	28.8000±1.11^{b}	37.5467±0.401^{g}	42.9500±0.68^{f}	57.5933±0.218^{h}
15	FLWS32	1.8533±0.092^{abc}	3.5067±0.35^{def}	5.9267±0.085^{bcd}	7.400±0.456^{ab}	9.7400±0.401^{bc}
16	FRWW18	1.6067±0.16^{abc}	3.7433±0.13^{ef}	5.7533±0.133^{abc}	7.9967±0.236^{abcde}	9.2267±0.37^{b}
17	FRWM25	1.6067±0.03^{abc}	3.8067±0.32^{ef}	6.0633±0.080^{bcde}	7.6567±0.037^{abcd}	9.2867±0.405^{bc}
18	FRWW30	1.7333±0.31^{abc}	4.0900±0.13^{f}	5.9833±0.141^{bcde}	7.6067±0.475^{abcd}	9.5700±0.370^{bc}
19	FLWM1	1.5133±0.29^{abc}	3.6733±0.14^{ef}	5.5000±0.193^{abc}	8.6033±0.584^{cde}	9.3067±0.217^{bc}
20	FLWW9	1.3467±0.585^{ab}	3.3967±0.29^{def}	6.1133±0.289^{cde}	8.0467±0.138^{abcde}	9.5233±0.332^{bc}
21	FRWM27	2.0533±0.102^{bcd}	3.6733±0.51^{ef}	5.2333±0.123^{ab}	7.7733±0.243^{abcde}	9.6900±0.141^{bc}
22	FLWS1	1.6600±0.01^{abc}	3.6300±0.44^{def}	5.8267±0.318^{abcd}	7.6967±0.61^{abcd}	9.6467±0.115^{bc}
23	FRhWW3	1.3167±0.29^{ab}	3.5633±0.33^{def}	6.0000±0.817^{bcde}	8.5333±0.42^{abcde}	9.9767±0.176^{bcde}
24	FRhWM2	1.5567±0.27^{abc}	3.1533±0.70^{def}	6.1233±0.722^{cde}	7.3833±0.903^{a}	9.9600±0.085^{bcde}
25	FRhWS2	2.2100±0.37^{cd}	3.4533±0.27^{def}	5.5733±0.402^{abc}	8.1667±0.112^{abcde}	9.7067±0.124^{bc}
26	FRWM3	1.3967±1.39^{a}	3.2467±0.36^{def}	5.4367±0.26^{abc}	8.1000±0.15^{abcde}	9.6200±0.35^{bc}

27	FLWS21	1.7233±0.26^{abc}	3.0200±0.11^{de}	6.1000±0.16^{cde}	7.7733±0.453^{abcde}	9.5433±0.63^{bc}
28	FLWS16	18.9933±0.812^{h}	29.1667±0.44^{b}	38.2267±1.01^{g}	46.3267±1.21^{g}	57.4467±1.30^{h}
29	FRWS24	1.4300±0.19^{ab}	2.9500±0.76^{de}	5.7633±0.241^{abc}	7.4867±0.311^{abc}	9.3333±0.64^{bc}
30	FRWW10	1.7600±0.066^{abc}	3.1500±0.51^{def}	6.1333±0.112^{cde}	8.0767±0.22^{abcde}	10.6533±0.61^{de}
31	FRWW11	2.173±0.25^{cd}	3.2600±0.93^{def}	6.036±0.80^{bcde}	8.386±0.64^{abcde}	9.8967±0.402^{bcd}
32	FRhWW4	.0000	.0000	.0000	.0000	.00
33	FRWS9	1.7533±0.24^{abc}	3.2333±0.56^{def}	6.0700±0.204^{bcde}	8.7167±0.28^{de}	10.2167±1.03.^{cde}
34	FLWM11	1.7533±0.71^{abc}	3.063±0.83^{de}	6.1067±0.15^{cde}	8.90±0.144^{e}	9.81±0.29^{bcd}
35	FLWW13	.0000	.0000	.0000	.0000	.0000
36	FLWS5	1.5100±0.31^{abc}	2.6800±0.67^{d}	6.7700±0.121^{e}	8.5967±0.442^{bcde}	10.8100±0.86^{e}
37	Leaf	21.27±1.4^{h}	29.68±0.53^{b}	38.26±0.35^{g}	47.56±1.78^{g}	59.08±1.67^{g}
38	Root	19.43±2.29^{g}	29.75±0.98^{b}	38.25±1.09^{g}	48.47±1.17^{g}	58.33±1.19^{g}
39	Rhizome	4.99±0.18^{cd}	11.06±1.39^{g}	17.65±0.64^{h}	28.96±0.91^{i}	37.75±1.55^{j}
40	LW	1.6900±0.11^{abc}	3.690±0.26^{def}	5.873±0.12^{abc}	8.6933±0.34^{bcde}	11.3567±4.98^{de}
41	RW	17.67±0.285^{g}	27.630±0.48^{ab}	36.700±0.26^{ef}	46.9900±0.83^{ab}	53.1767±0.61^{f}
42	RhW	1.467±0.27^{a}	3.017±0.66^{de}	5.5400±0.37^{abc}	8.4500±0.52^{bcd}	12.1500±2.29^{ef}
43	LM	1.7900±0.062^{abc}	3.1200±0.39^{def}	6.2967±0.42^{cde}	8.593±1.48^{bcd}	12.5400±0.84^{fg}
45	RM	2.3283±0.65^{cd}	3.0500±0.32^{de}	6.453±0.54^{bcd}	7.9800±1.97^{abcde}	12.9300±0.79^{g}
46	RhM	17.33±0.33^{g}	28.7900±2.02^{b}	29.97±1.47^{h}	50.8400±1.86^{h}	63.1167±0.24^{g}
47	Ascorbic acid	13.6667±0.55^{e}	24.0033±0.33^{c}	36.12±0.234^{f}	52.1±1.97^{h}	70.15±0.33^{i}

Table 54: ABTS activity by crude extracts of fungal endophytes

Ferric Reducing Power Assay (FRAP) of Endophytic Bacteria

Ferric Reducing Power Assay (FRAP) is another method to assess the antioxidant power of substances. This assay measures the ability of a sample to convert or reduce ferric ions (Fe 3+) to ferrous ions (Fe 2+). The FRAP reagent preparation involves mixing acetate buffer (pH 3.6), TPTZ (2,4,6-tripyridyl-s-triazine) solution in hydrochloric acid and ferric chloride. The fungal extract was allowed to react for 30 minutes at room temperature. If the solution contains antioxidants, then it can convert ferric ions to ferrous ions which in turn forms a complex with TPTZ producing a blue color. The intensity of the blue color is measured with the help of a spectrophotometer at a wavelength of 593 nm. The absorbance is directly proportional to the antioxidant power or the free radical scavenging ability of the sample. Ascorbic acid is used as a standard solution to create the calibration curve. IC50 values of 35 samples were determined after calculating the percentage inhibition of five different concentrations (20, 40, 60, 80, and 100 µg/ml) with 3 replicates.

Ascorbic acid showed an IC50 value of 51.93 µg/ml (Fig.100). BLWS4 bacterial sample portrayed 93.04 µg/ml with the highest antioxidant potential. This is followed by BLWS10, BLWS6, BLWW1, BRWS3, and BRWS15 with IC50 values 93.23µg/ml, 93.25 µg/ml, 95.63 µg/ml, 96.21 µg/ml, and 96.51 µg/ml. The remaining samples showed IC50 values above 100 µg/ml. The highest IC50 was shown by BLWW5 with a half maximum inhibitory concentration of 137.77 µg/ml. The other high IC50 values following the highest one are of BRhWM3, BLWS1, BLWW7, and BLWS11 with IC50 values of 135.50 µg/ml, 135.43 µg/ml, 135.39 µg/ml, and 135.17 µg/ml respectively. The leaf extract showed an inhibitory concentration of 66.06±1.62 µg/ml, the root showed 62.17±5.55 µg/ml, and the rhizome of 58.31±1.36 µg/ml. While the positive concentration of ascorbic acid was 89.5067±0.96381 µg/ml (Table 55). There are no comparative concentrations can be seen in the isolates. The highest antioxidant potential sample's IC50 value is around 40 µg/ml more than that of the standard. This may contain antioxidants that can scavenge free radicals. It

needs further analysis and experimentation to find out the antioxidant molecule to develop a practical application of the samples.

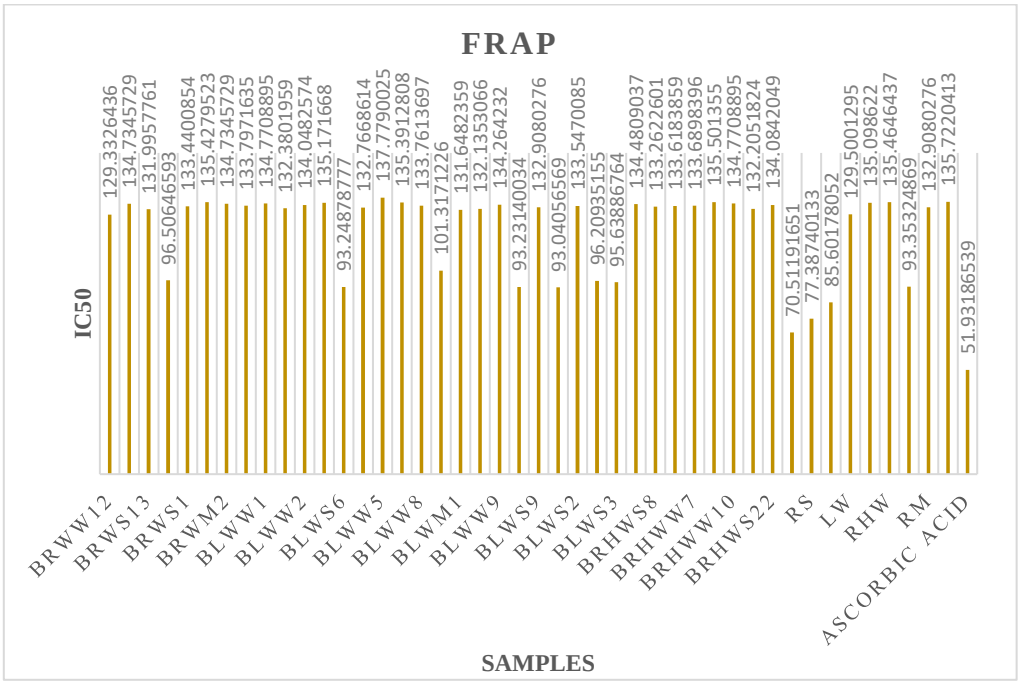


Fig.100 IC50 value of bacterial isolates for FRAP activity

Ferric Reducing Power Assay (FRAP) of Endophytic Fungi

Ferric Reducing Power Assay (FRAP) of endophytic fungi was also performed to analyze the free radical scavenging ability of the fungal extract. Ascorbic acid was used as a standard to draw the calibration curve. The samples were allowed to react with the FRAP reagent for 30 minutes. After the incubation, the color intensity was measured with the help of a spectrophotometer. Intensity was measured at a wavelength of 593 nm. IC50 values were determined for 36 fungal isolates. The reaction was performed for 5 different concentrations. Three replicates of each concentration were done to reduce the error.

73.42 µg/ml was the lowest IC50 value by FLWW5 obtained from endophytic fungal samples which is comparable to the standard solution's IC50 value, 51.93 µg/ml (Fig.101). The samples that showed slightly higher IC50 values are FLWM12, FLWW12, and FRWW18 with IC50 values of 73.92 µg/ml, 77.65 µg/ml, and 83.53 µg/ml respectively. The lowest antioxidant potential was shown by FLWW13 with 191.28 as the IC50 value. FRhWW4 and FRWS8 are

the other two samples of high IC50 values with values of 190.25 µg/ml and 183.41 µg/ml. The leaf extract showed an inhibitory concentration of 66.06 µg/ml, root of 62.17 µg/ml, and 58.31 µg/ml. The positive control ascorbic acid showed a concentration of 89.5067 µg/ml. There are no comparative concentrations can be seen in the isolates (Table 56).

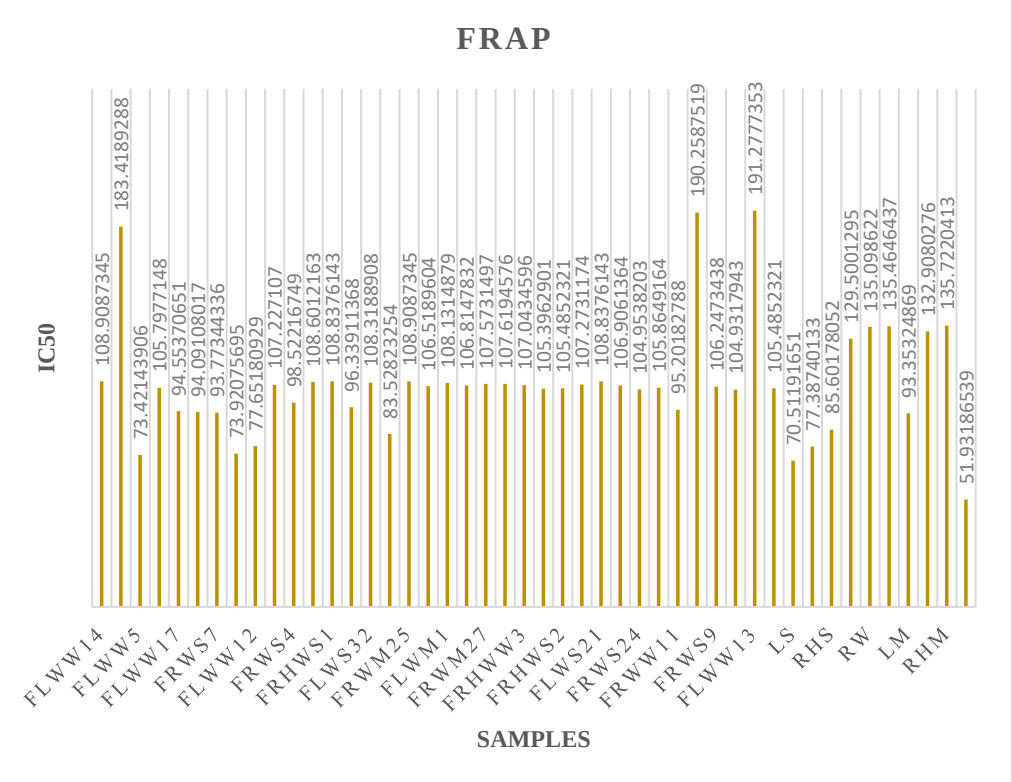


Fig.101 IC50 value of bacterial isolates for FRAP activity

No.	Bacterial isolates	20	40	60	80	100
1	BRWW12	9.4433±0.92522^{a}	18.5433±0.96231^{a}	25.6367±0.83692^{a}	31.0633±0.57134^{a}	35.52±0.936^{ab}
2	BRWW6	11.5433±1.33028^{cdefg}	16.15±0.53075^{bcd}	24.6567±1.11733^{a}	28.87±0.38743^{bcd}	34.99±0.65871^{ab}
3	BRWS13	9.84±0.38432^{ab}	18.2567±0.53799^{a}	24.9733±0.68625^{a}	30.0467±0.21548^{ab}	35.0467±0.8529^{ab}
4	BRWS15	15.8067±0.49652^{i}	25.0133±0.70493^{f}	34.1967±0.46822^{bcd}	38.7967±0.50639^{e}	49.2667±0.44015^{d}
5	BRWS1	10.3467±0.6426^{abcd}	17.27±0.64023^{ab}	24.8667±0.15177^{a}	29.3267±1.07188^{bcd}	35.0733±1.50364^{ab}
6	BRWS3	11.5067±1.19559^{cdefg}	15.9967±0.51695^{bcd}	25.01±0.78313^{a}	28.6433±1.14727^{cd}	34.6067±0.73214^{ab}
7	BRWM2	10.1233±0.62405^{abc}	15.85±0.44034^{bcd}	25.6733±0.9806^{a}	28.5867±0.49359^{cd}	34.9933±0.66108^{ab}
8	BRWM3	10.6067±1.15175^{abcd}	16.37±0.95168^{cd}	24.66±0.67668^{a}	29.1433±0.84358^{bcd}	35.4267±0.8471^{ab}
9	BLWW1	11.8533±0.39577^{defg}	15.4433±1.05747^{cd}	25.4367±0.966^{a}	29.14±0.26058^{bcd}	34.5033±0.8955^{ab}
10	BLWW4	10.32±1.2005^{abcd}	16.44±0.82164^{bcd}	25.6367±1.09564^{a}	28.8767±0.5519^{bcd}	35.9633±0.3421^{b}
11	BLWW2	10.6367±0.6698^{abcd}	16.3533±1.429^{bcd}	25.2833±0.9693^{a}	28.8±0.51507^{bcd}	35.18±1.06532^{ab}
12	BLWS11	10.8733±0.39526^{acdef}	16.1433±0.48232^{bcd}	23.99±0.44844^{a}	28.2433±0.32254^{f}	35.7633±1.18162^{ab}
13	BLWS6	18.0533±0.72037^{j}	29.1167±0.5208^{h}	36.3267±0.50738^{d}	40.61±0.64156^{bcd}	48.43±1.34637^{d}
14	BLWS7	11.6967±0.50143^{cdefg}	16.3833±1.19132^{bcd}	24.6133±1.1191^{a}	28.68±0.45177^{bcd}	36.2533±0.91817^{b}
15	BLWW5	10.98±0.52307^{abcdef}	14.8767±0.5186^{d}	24.89±0.54525^{a}	28.62±1.28946^{cd}	33.8667±0.5960^{ab}
16	BLWW7	11.6833±0.89579^{cdefg}	15.36±0.91411^{cd}	24.5533±0.5014^{a}	28.7533±0.77488^{bcd}	35.02±0.62698^{ab}
17	BLWW8	11.7033±0.67686^{cdefg}	15.5333±0.57047^{cd}	25.32±0.43313^{a}	28.8133±0.74842^{bcd}	35.4333±0.7892^{ab}
18	BLWM4	13.63±0.97555^{h}	23.7167±0.44546^{e}	31.6133±1.1238^{b}	38.8067±0.3139^{e}	46.35±0.91537^{c}
19	BLWM1	10.7767±0.47248^{abcde}	15.7667±1.12882^{bcd}	24.9967±0.7585^{a}	29.86±0.77891^{bc}	36.2133±0.46307^{b}
20	BLWW10	11.3333±1.04639^{bcdefg}	16.0033±0.7157^{bcd}	24.61±0.55435^{a}	29.3533±0.17243^{bcd}	36.34±0.12124^{b}
21	BLWW9	11.53±1.20254^{cdefg}	15.56±0.60357^{cd}	25.1033±0.73921^{a}	28.61±0.59093^{cd}	35.4567±1.1400^{ab}
22	BLWS10	18.3133±1.08786^{j}	28.8833±0.50461^{h}	35.73±0.43555^{cd}	40.8067±0.89389^{f}	48.68±1.22135^{ab}
23	BLWS9	12.43±0.7919^{fgh}	16.88±0.86643^{bc}	25.5267±0.4598^{a}	29.1367±0.69241^{bcd}	34.8967±2.0152^{ab}
24	BLWS4	18.5333±0.32747^{j}	28.8533±1.01687^{h}	36.03±0.53675^{cd}	40.7967±0.95616^{f}	48.7333±0.65896^{ab}
25	BLWS2	12.7833±0.29023^{gh}	16.2233±1.20006^{bcd}	25.03±0.29309^{a}	28.83±0.27221^{bcd}	35.25±1.49523^{ab}

26	BLWS1	16.4567±1.04644^{i}	26.4133±0.9538^{g}	33.3867±1.38868^{bc}	39.5833±1.22075^{ef}	48.7733±1.0062^{d}
27	BLWS3	16.48±0.68637^{i}	27.4367±1.37791^{g}	34.3167±0.96303^{bcd}	39.6433±0.56854^{ef}	48.45±1.04014^{d}
28	BRhWS1	11.1733±1.0416^{bcdefg}	15.7333±0.52918^{bcd}	24.59±0.47149^{a}	29.1867±0.40919^{bcd}	35.16±0.61147^{ab}
29	BRHWS8	11.5333±0.79002^{ab}	15.75±0.50715^{bcd}	25.6867±0.6961^{a}	28.8033±0.71654^{bcd}	35.4833±0.77655^{ab}
30	BRhWW11	12.33±0.49689^{efgh}	16.1133±0.57622^{bcd}	25.08±0.48384^{a}	29.1567±0.45742^{bcd}	35.0467±1.83233^{ab}
31	BRhWW7	11.88±0.62217^{defg}	15.6433±0.57744^{cd}	24.4833±0.60435^{a}	28.6233±0.56039^{cd}	36.05±0.68826^{b}
32	BRhWM3	11.3333±1.14997^{bcdefg}	15.5333±1.16423^{cd}	24.4±0.82018^{a}	28.92±0.57974^{bcd}	34.9167±0.54537^{ab}
33	BRhWW10	11.9033±0.54994^{defg}	15.3233±0.05132^{cd}	25.27±0.38197^{a}	28.7667±0.56854^{bcd}	34.9367±0.40004^{ab}
34	BRhWS27	11.6567±0.63375^{cdefg}	16.08±0.47445^{bcd}	24.8233±0.45081^{a}	29.3167±0.63956^{bcd}	36.1±0.54617^{b}
35	BRhWS22	11.2867±0.87048^{bcdefg}	15.7567±0.57457^{bcd}	24.5233±0.82682^{a}	29.17±0.87126^{bcd}	35.43±0.70661^{ab}
36	LS	19.48±1.24j	30.73±1.22^{i}	46.35±1.23^{f}	57.44±1.82^{j}	66.06±1.62^{g}
37	RS	17.83±0.87^{ij}	27.82±1.24^{gh}	38.01±1.69^{d}	53.09±3.51^{i}	62.17±5.55^{g}
38	RhS	12.48±1.01^{efgh}	21.83±2.04^{e}	37.13±1.19^{d}	45.86±3.52^{h}	58.31±1.36^{f}
39	LW	9.33±1.2522^{a}	18.33±0.96231^{a}	25.593±1.252^{a}	31.13±2.5134^{a}	35.482±1.546^{ab}
40	RW	11.453±0.3028^{cdefg}	16.25±0.575^{bcd}	24.567±0.833^{a}	28.77±0.143^{bcd}	34.899±0.871^{ab}
41	RhW	10.833±0.956^{acdef}	16.043±1.48232^{bcd}	23.819±0.44^{a}	28.23±0.254^{f}	35.763±1.162^{ab}
42	LM	18.1533±0.957^{j}	29.067±0.208^{h}	36.267±0.738^{d}	40.61±0.64156^{bcd}	48.34±1.84637^{d}
43	RM	11.67±0.443^{cdefg}	16.283±1.0132^{bcd}	24.593±2.91^{a}	28.78±0.477^{bcd}	36.153±1.817^{b}
44	RhM	11.33±1.997^{bcdefg}	15.433±1.5423^{cd}	24.34±0.018^{a}	28.892±1.57974^{bcd}	34.897±2.54537^{ab}
45	Ascorbic acid	23.88±0.63^{k}	38.6933±0.62883^{j}	65.17±5.96278^{e}	78.7±0.5693^{g}	89.5067±0.96381^{e}

Table 55: FRAP activity by crude extracts of bacterial endophytes

No.	**Fungal isolates**	**20**	**40**	**60**	**80**	**100**
1	FLWW14	13.1433±0.98195^{cdefg}	22.78±0.67801^{cdef}	27.94±0.27185^{f}	36.7733±0.26407^{i}	43.0767±0.1193^{ef}
2	FRWS8	7.2967±0.86118^{b}	12.36±0.63095^{b}	15.7833±0.83811^{g}	22.0267±0.94448^{g}	26.4867±1.01673^{d}
3	FLWW5	19.78±0.63214^{h}	34.14±0.44933^{n}	48.6667±0.39273^{cef}	54.55±0.63238^{e}	59.3767±0.77797^{c}

4	FLWW15	12.7433±0.67002^{cdefg}	23.9667±0.50292^{efghi}	28.45±1.03523^{cef}	35.8933±0.54994^{i}	46.0533±0.6133^{ijk}
5	FLWW17	13.1033±0.39247^{cdefg}	25.41±0.52307^{ijk}	35.89±0.3629^{ab}	42.77±0.26665^{c}	47.7933±0.73283^{kl}
6	FLWW6	12.5±0.8271^{cdef}	25.7433±0.47543^{jk}	36.18±1.06672^{ab}	42.77±0.86435^{c}	48.1933±0.48758^{l}
7	FRSW7	11.9267±0.77925^{c}	26.7333±0.83032^{kl}	37.4367±0.77822^{b}	42.75±1.16735^{c}	47.5733±2.25445^{jkl}
8	FRWM12	19.9±0.31^{h}	33.96±0.29462^{n}	48.1167±0.54197^{cef}	54.88±1.43792^{e}	58.4733±0.55788^{bc}
9	FLWW12	18.26±0.66551^{a}	30.87±1.61397^{m}	44.5733±2.10039^{cef}	51.78±0.99378^{d}	57.48±1.15547^{ab}
10	FRWM1	12.13±0.64583^{cd}	23.6±0.38354^{defgh}	30.8133±0.71059^{cde}	36.29±1.16116^{i}	43.2067±0.86858^{ef}
11	FRWS4	12.5033±0.86558^{cdef}	25.7633±0.96043^{jk}	32.4333±0.66275^{d}	41.43±0.90416^{bc}	46.2333±0.48336^{ijk}
12	FLWW11	12.7833±0.29704^{cdefg}	23.1533±0.49369^{cdefg}	29.1367±0.51501^{cef}	36.59±0.39737^{i}	42.7067±0.71234^{e}
13	FRhWS1	12.79±0.79171^{cdefg}	21.5633±0.85524^{c}	28.9667±0.19858^{cef}	36.4133±1.18027^{i}	43.3667±0.39107^{efg}
14	FLWW18	12.3433±0.71396^{cde}	24.9367±0.82403^{hij}	36.1433±1.0289^{ab}	40.8133±0.35346^{b}	47.4067±0.68091^{jkl}
15	FLWS32	12.6733±0.3421^{cdef}	22±1.37884^{cd}	28.2167±0.91194^{ef}	36.9533±0.37647^{i}	43.73±0.64023^{efgh}
16	FRWW18	17.3367±0.89512^{a}	29.0733±0.7407^{a}	34.8867±1.5603^{a}	48.8033±0.58526^{a}	56.6267±1.71701^{a}
17	FRWM25	13.17±1.04288^{cdefg}	22.78±0.67801^{cdef}	27.94±0.27185^{f}	36.7733±0.26407^{i}	43.0767±0.1193^{ef}
18	FRWW30	13.5367±0.62549^{efg}	24.7433±0.90002^{ghij}	28.55±0.60233^{cef}	35.9367±0.08083^{i}	44.7767±1.18035^{fghi}
19	FLWM1	13.0233±0.37608^{cdefg}	22.3833±0.65851^{cde}	29.12±0.39038^{cef}	35.7667±0.62132^{i}	44.0767±0.6626^{efgh}
20	FLWW9	13.2±0.41905^{defg}	22.1367±0.3691^{cd}	28.97±0.22271^{cef}	36.2233±0.63736^{i}	45.1167±0.98287^{ghi}
21	FRWM27	13.03±0.41725^{cdefg}	22.4967±0.8188^{cde}	29.1567±0.41861^{cef}	36.4933±0.81746^{i}	43.9567±1.10074^{efgh}
22	FLWS1	13.3433±0.46608^{defg}	22.8033±0.80749^{cdef}	28.5333±1.31001^{cef}	36.67±0.31575^{i}	43.97±0.50388^{efgh}
23	FRhWW3	13.7767±0.48418^{fg}	22.6467±1.04882^{cde}	27.5467±1.5827^{f}	36.32±0.64583^{i}	45.3567±1.1607^{hi}
24	FRhWM2	13.9733±0.49571^{g}	23.48±1.3164^{defgh}	28.93±0.67816^{cef}	36.1433±0.82646^{i}	45.9133±0.41861^{ij}
25	FRhWS2	13.4567±0.6689^{efg}	23.8567±0.86396^{efghi}	29.2233±0.91446^{cef}	36.5367±0.74043^{i}	45.29±1.02504^{hi}
26	FRWM3	13.53±0.69864^{efg}	24.7233±1.085^{ghij}	28.57±0.77117^{cef}	36.7933±0.57457^{i}	43.3633±1.06275^{efg}
27	FLWS21	13.2133±0.93002^{defg}	23.1033±0.41669^{cdef}	28.7667±0.5428^{cef}	36.22±0.73736^{i}	42.94±0.84717^{e}
28	FLWS16	13.04±0.35791^{cdefg}	22.8133±0.57134^{cdef}	31.0967±1.41118^{cd}	36.93±0.79568^{i}	42.9533±0.18903^{ef}
29	FRWS24	12.81±0.52564^{cdefg}	23.4333±1.03055^{defgh}	28.77±0.50567^{cef}	36.63±1.04933^{i}	46.3033±1.0052^{ijk}
30	FRWW10	13.4733±0.84198^{efg}	23.2967±1.015^{defg}	28.8033±0.97552^{cef}	36.46±0.92065^{i}	45.4533±1.30799^{hi}

31	FRWW11	12.8067±0.30238^{cdefg}	27.4933±0.80903^{l}	36.64±0.69347^{ab}	40.8567±0.74809^{b}	47.3167±1.1387^{jkl}
32	FRhWW4	6.99±0.12288^{b}	11.3267±0.7259^{b}	15.7333±0.70501^{g}	20.63±0.54065^{h}	25.94±0.63695^{d}
33	FRWS9	13.2267±0.82081^{defg}	24.31±0.97535^{fghij}	28.8367±0.66161^{cef}	35.5167±0.87145^{i}	45.4433±1.17577^{hi}
34	FLWM11	12.6933±0.39929^{cdefg}	24.3267±1.01943^{fghij}	28.2967±1.14518^{cef}	36.4033±1.05168^{i}	46.4667±1.09038^{ijkl}
35	FLWW13	7.0667±0.14844^{b}	11.2733±0.30551^{b}	15.5667±0.4884^{g}	20.6333±0.45347^{h}	25.7333±0.42911^{d}
36	FLWS5	13.3533±0.45797^{defg}	23.9867±0.88189^{efghi}	28.5033±0.47035^{cef}	35.8567±0.89673^{i}	46.2367±1.06547^{ijk}
37	LS	19.48±1.24^{h}	30.73±1.22^{mn}	46.35±1.23^{ef}	57.44±1.82^{g}	66.06±1.62^{o}
38	RS	17.83±0.87^{a}	27.82±1.24^{l}	38.01±1.69^{b}	53.09±3.51^{e}	62.17±5.55^{n}
39	RhS	12.48±1.01^{cdefg}	21.83±2.04^{c}	37.13±1.19^{ab}	45.86±3.52^{h}	58.31±1.36^{bc}
40	LW	9.33±1.2522^{k}	18.33±0.96231^{o}	25.593±1.252^{h}	31.13±2.5134^{a}	35.482±1.546^{mn}
41	RW	11.453±0.3028^{l}	16.25±0.575^{p}	24.567±0.833^{h}	28.77±0.143^{j}	34.899±0.871^{n}
42	RhW	10.833±0.956^{kl}	16.043±1.48232^{p}	23.819±0.44^{i}	28.23±0.254^{j}	35.763±1.162^{mn}
43	LM	18.1533±0.957^{j}	29.067±0.208^{q}	36.267±0.738^{j}	40.61±0.64156^{b}	48.34±1.84637^{l}
44	RM	11.67±0.443^{l}	16.283±1.0132^{p}	24.593±2.91^{hi}	28.78±0.477^{j}	36.153±1.817^{n}
45	RhM	11.33±1.997^{l}	15.433±1.5423^{p}	24.34±0.018^{hi}	28.892±1.57974^{j}	34.897±2.54537^{n}
46	Ascorbic acid	23.88±0.63^{i}	38.6933±0.62883^{o}	65.17±5.96278^{cef}	78.7±0.5693^{f}	89.5067±0.96381^{m}

Table 56: FRAP activity by crude extracts of fungal endophytes

Discussion

Phytochemicals are crucial in the interactions between endophytic bacteria, endophytic fungi, and plant extracts. These chemical compounds, produced by plants, can influence the growth, behaviour, and colonization of endophytic microorganisms. In the case of endophytic bacteria, phytochemicals can act as signalling molecules, affecting bacterial colonization and behaviour. Some phytochemicals can inhibit or promote the growth of endophytic bacteria, depending on the compound and bacterial species (Basit et al., 2021). Phytochemicals also affect endophytic fungi, which can influence fungal colonization, growth, and reproduction. Certain phytochemicals can induce fungal defense mechanisms, while others can inhibit fungal pathogens. Additionally, endophytic fungi can produce phytochemicals, contributing to plant defense and health. The complex interactions between phytochemicals and endophytic fungi can impact plant-fungus relationships and ecosystem balance (Sharaf et al., 2022). Phytochemicals in plant extracts can also influence the growth and behavior of endophytic microorganisms. For instance, some phytochemicals can inhibit the growth of harmful microorganisms, while promoting the growth of beneficial ones. This selective inhibition can contribute to plant defense and health (Afzal et al., 2019).

In the present study, the phytochemical qualitative analysis of the 35 bacterial isolates and 36 fungal isolates revealed a diverse range of compounds, including alkaloids, flavonoids, saponins, sterols, tannins, terpenoids, cardiac glycosides, glycosides, coumarins, quinones, and proteins. According to the observation in this study, most fungal isolates showed the presence of phytochemical compounds than bacterial isolates. However, in the condition of phytochemical production, both of them showed more or less similar concentrations. This contrasts bacterial endophytes, which have been reported to produce a narrower range of phytochemicals, such as alkaloids, flavonoids, and phenols (Strobel et al., 2004; Singh et al., 2017a). In comparison to bacterial endophytes, the isolates in this study showed a higher diversity of phytochemicals, including

saponins, sterols, tannins, terpenoids, cardiac glycosides, glycosides, coumarins, quinones, and proteins. This suggests that the isolates may have a broader range of potential biological activities, including antioxidant, anti-inflammatory, and antimicrobial properties (Ruma et al., 2013; Sharaf et al., 2022).

In the case of the source of endophytes, root isolates hold more content of alkaloid, phenol, and terpenoid; leaf isolates have flavonoid, quinone, glycoside, and protein content; rhizome with high content of tannin and cardiac glycoside. The saponin, sterol, carbohydrate, and coumarin contents are evenly shown in isolates from all sources. *Rhizobium rhizophilum* BLWM1, *Bacillus anthracis* BLWW1, and *Bacillus albus* BLWW4 showed moderate alkaloid content. The bacterial isolate *Bacillus anthracis* BLWW1 shows the comparative amount of saponin. And *Bacillus anthracis* BLWW1 showed moderate to high terpenoid content. Basit et al., 2021 strongly validated these findings that the alkaloid, tannin, saponin, steroid, quinone, and terpenoid presence in endophytic bacterial isolates is notable. The presence of secondary metabolites in plants triggers the production of compounds in endophytes, creating a symbiotic relationship. Our study observed a high phytochemical composition in plant extracts, likely influencing various endophytic isolates to produce secondary metabolites. This finding is supported by a previous study, which demonstrated that endophytic bacteria, such as *Bacillus subtilis* and *Lactobacillus plantarum*, induced the production of secondary metabolites in kidney bean extracts during fermentation. This suggests that bacterial endophytes play a significant role in metabolite production, highlighting the complex interactions between plants and their endophytic microorganisms (Bonilla et al., 2014).

The phytochemical analysis of the fungal isolates in this study revealed a diverse range of compounds. This is consistent with previous studies that have reported the production of bioactive compounds by fungal endophytes (Singh et al., 2021; Kharwar et al., 2011). Isolates *Aspergillus aculeatus* FRWS8,

Aspergillus flavus FLWS32, and *Colletotrichum gloeosporioides* FRWS7 showed high alkaloid content. Isolates *Fusarium verticilliodes* FRWS4 and *Aspergillus japonicus* FRWW30 showed high phenol content. Isolates *Aspergillus aculeatus* FRWS8, *Rhizopus sp.* FLWW14 and *Paecilomyces variotii* FRhWM2 showed moderate to high terpenoid content. The presence of alkaloids, phenols, and terpenoids in several isolates suggests that these compounds may play a role in the biological activity of the fungal isolates. This is consistent with previous studies that have reported these compounds' antimicrobial and antioxidant activities (Strobel, 2018). It is noteworthy that cardiac glycosides and glycosides were found in many isolates because these substances have been shown to have antibacterial and antifungal properties (Kharwar et al., 2011; Singh et al., 2017a). Compared to fungal endophytes, the isolates in this study showed a higher diversity of phytochemicals, including coumarin and quinone, which have been reported to have antimicrobial and antioxidant activities (Kharwar et al., 2011). The presence of proteins in most of the isolates suggests that these compounds may play a role in the biological activity of the fungal isolates. This is consistent with previous studies that have reported the production of enzymes and other proteins by fungal endophytes (Strobel, 2018). As per Ebabhi et al., 2023 the presence of terpenoids and cardiac glycosides in *Aspergillus, Rhizopus, Fusarium* some isolates are particularly notable, as these compounds have been reported to have antioxidant and cardio-protective effects. The present study also supports this view. In addition, bacterial endophytes have been reported to produce mainly alkaloids and flavonoids, which have been shown to have antimicrobial and antioxidant activities (Strobel, 2018; Singh et al., 2017). However, the present study reveals that the endophytic bacterial isolates from *L. toxicaria* produced mainly saponin, sterols, and tannin, and phenol, glycoside, and carbohydrate contents are absent.

The study revealed that the presence and content of phytochemicals in plant parts vary depending on the season. Specifically, the highest levels of

phytochemicals were detected in summer, followed by winter, and the lowest levels were observed in the monsoon season. This seasonal variation in phytochemical content was corroborated by Vasantrao (2018), who found that the levels of phenol and tannin, two types of phytochemicals, decreased significantly across seasons, with the highest levels detected in summer and the lowest in the monsoon season. A study by Mallick and Chandra (2020) found that the concentrations of sugar, steroid, tannin, and phenol were significantly higher in winter than in the monsoon season. In contrast, the levels of alkaloids and flavonoids were found to be higher in summer than in winter. This suggests that seasonal variation affects the production of different phytochemicals in plants, with winter favouring the production of certain compounds and summer favouring the production of others. These studies all strongly stated that the profile of phytochemical compounds varies depending on the season. The diversity study of *L. toxicaria* revealed an intriguing phenomenon where the monsoon season exhibited reduced endophytic colonization. This observation can be attributed to the fluctuation of chemical compounds such as phenols, tannins, and alkaloids, which are known to influence endophytic populations in the host plant during this time. Some studies on the seasonal impact on endophytic diversity were also leading this concept (Zambell and White, 2017; El Mansy et al., 2020; Rampadarath et al., 2018).

The bacterial isolates in this study demonstrated notable antimicrobial properties, showcasing a wide range of activity against various pathogenic microorganisms. Notably, several isolates displayed potent inhibitory effects against Gram-positive and Gram-negative bacteria and fungi, highlighting their broad-spectrum antimicrobial potential. This is consistent with previous studies that have reported the production of bioactive compounds by bacterial endophytes, including antibiotics, bacteriocins, and other secondary metabolites (Singh et al., 2017a; Kharwar et al., 2011). However, the results of this study are distinct from previous reports in several ways. Firstly, the bacterial isolates in this study exhibited antimicrobial activity against a wider range of pathogenic

organisms than has been previously reported for bacterial endophytes. All isolates showed their inhibition on *S. typhi.* Secondly, the potency of the antimicrobial activity observed in this study was higher than that reported in previous studies, with several isolates exhibiting inhibition zones of 15 mm or greater. *Bacillus altitudinis* BLWS10, *Lysnibacillus pakistanensis* BRWS15, *Bacillus sp.* 4 BLWS6, and *Rhizobium rhizophilum* BLWM1 are some examples.

The antimicrobial activity of the fungal isolates in this study is comparable to that of fungal endophytes reported in previous studies. A study reported that fungal endophytes isolated from medicinal plants showed antimicrobial activity against a range of bacterial and fungal pathogens, with inhibition zones ranging from 10-25 mm (Kumari et al., 2018). Similarly, our study found that the fungal isolates showed significant antimicrobial activity against various bacterial test organisms and fungi, *Candida albicans*, with inhibition zones ranging from 15.73 mm to 22.07 mm. The broad-spectrum antimicrobial activity of the fungal isolates *Aspergillus terreus* FLWW5, *Diaporthe penetriteum* FLWS21, and *Fusarium* sp. 2 FRWW11 is particularly notable, as it suggests that these isolates may possess compounds with potential applications in the development of novel antimicrobial agents. This is consistent with previous studies that have reported the production of bioactive compounds by fungal endophytes, including polyketides, terpenes, and other secondary metabolites (Strobel et al., 2004; Singh et al., 2021; Singh and Kumar, 2023). The antimicrobial properties of Dark Septate Fungi (DSF) have been previously reported, with studies showing that they inhibit the growth of various pathogens, including bacteria, fungi, and nematodes, by producing a range of antimicrobial compounds such as phenolic acids, terpenoids, and alkaloids (Chepkirui and Stadler, 2017). Consistent with these findings, the present study observed that *Diaporthe penetriteum* FLWS21, a type of DSF, exhibited antimicrobial activity and produced a variety of bioactive compounds, including alkaloids, tannins, terpenoids, cardiac

glycosides, glycosides, and proteins, which contribute to its antifungal and antibacterial properties.

Compared to bacterial endophytes, the isolates in this study showed a higher proportion of antimicrobial activity, with 91.66% of isolates exhibiting activity against one or more test organisms. The fungal isolates in this study demonstrate significant potential as a rich source of novel antimicrobial compounds. This finding is consistent with previous research by Sharaf and Al-Zaidi (2021), who reported that ethyl acetate extracts of *Aspergillus nidulans*, *A. fumigatus*, and *A. flavus*, isolated from *Ocimum basilicum*, exhibited antimicrobial activity against various pathogens, including *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Candida albicans*. The antimicrobial potential of plant parts, similar to the phytochemical content, displayed varying activity levels depending on the season. This suggests that seasonal changes influence not only the phytochemical composition but also the antimicrobial properties of the plant parts, resulting in fluctuating levels of activity against microorganisms (Rampadarath et al., 2018). Bacterial isolates from the summer and fungal isolates from both summer and winter seasons exhibited enhanced antimicrobial potency against most of the tested microorganisms, following a similar pattern. This suggests that the summer season favors the production of antimicrobial compounds in bacteria, while fungi produce these compounds in both summer and winter, resulting in increased inhibitory activity against various pathogens.

A study by Akinsanya et al. (2015) isolated 29 culturable bacterial endophytes from *Aloe vera* tissues (root, stem, and leaf) and identified 13 genera through molecular characterization. The dominant genera were: Bacillus (20.7%), Pseudomonas (20.7%), Enterobacter (13.8%). Six isolates (*Pseudomonas, Bacillus, Chryseobacterium*, and *Shigella* sp.) demonstrated broad-spectrum antimicrobial activity against various pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus*

pyogenes, *Candida albicans.* Inhibition zones ranged from 6.0 ± 0.57 to 16.6 ± 0.57 mm. Additionally, 80% of the bacterial endophytes exhibited significant antioxidant activity, with DPPH scavenging properties exceeding 75% compared to ascorbic acid (92%). Specific endophytic bacteria, including *Pseudomonas hibiscicola*, *Macrococcus caseolyticus*, *Enterobacter ludwigii*, and *Bacillus anthracis,* produced bioactive compounds with high DPPH scavenging properties (75-88%). Others, such as *Bacillus tequilensis*, *Pseudomonas entomophila*, *Chryseobacterium indologenes*, *Bacillus aerophilus* produced compounds with antimicrobial activities against bacterial pathogens.

The DPPH assay leverages free radicals to gauge the antioxidant capacity of substances, specifically their ability to act as hydrogen donors or free radical scavengers, providing valuable insights into their potential to mitigate oxidative stress. The present study reports that the extracts with higher DPPH activity or higher antioxidant potential are *Bacillus sp.* 3 BLWS2, *Bacillus anthracis* BLWW1, *Bacillus amyloliquefaciens* BRWS3, *Lysnibacillus pakistanensis* BRWS15, and *Bacillus mycoides* BRWW6. Among fungal isolates, the highest antioxidant potential in *Rhizopus sp.* FLWW14. Ganguly et al., 2018 studied the antioxidant activity of *Bacillus anthracis* and revealed that this species could be potent for developing new therapeutic agents, especially for cancer. At the same time, the study on *Bacillus mycoides* to identify photosynthesis efficiency and capability in plant growth revealed that it has antioxidant activity and that this species can be used to promote plant growth and increase productivity. Hence the previous studies on these species expose the extended or advanced applications of these species in different fields like agriculture, phytochemistry, drug development, etc. Bacterial and fungal isolates have demonstrated notable ABTS activity, indicating their capacity to neutralize free radicals and protect against oxidative stress. This antioxidant potential is often attributed to the presence of bioactive compounds such as phenolic acids, flavonoids, and terpenoids. In the present study, bacterial isolate *Bacillus sp.* BLWS6 and fungal

isolate *Phanerochaete sordida* FLWS16, identified as potential endophytes, exhibited significant ABTS activity. *Bacillus sp.* BLWS6 showed the presence of phytochemicals like saponin and tannin, which are known to contribute to antioxidant activity. Similarly, *Phanerochaete sordida* FLWS16 contained a range of bioactive compounds, including flavonoid, phenol, terpenoid, coumarin, quinone, glycoside, and protein. These findings align with previous studies, which have reported high ABTS activity in bacterial isolates like *Bacillus* and *Pseudomonas*, as well as fungal isolates like *Aspergillus* and *Penicillium*. The ABTS activity of these isolates can be influenced by factors like growth conditions, temperature, and pH, highlighting the importance of optimizing these parameters to maximize antioxidant potential (Hamed et al., 2024).

Ferric Reducing Power Assay (FRAP) is another method to assess the antioxidant power of substances. This assay measures the ability of a sample to convert or reduce ferric ions (Fe 3+) to ferrous ions (Fe 2+). *Bacillus sp.* 3 BLWS2 bacterial sample portrayed 93.04 µg/ml with the highest antioxidant potential. The fungal samples that showed slightly higher IC50 values are *Phanerochaete sordida* FLWS16, *Aspergillus sp.* 4 FLWW12, and *Rhizopus sp.* FLWW14. The highest antioxidant potential sample's IC50 value is around 40 µg/ml more than the standard's. This may contain antioxidants that can scavenge free radicals. It needs further analysis and experimentation to find out the antioxidant molecule to develop a practical application of the samples. Sharaf et al., 2021 reported that the ethyl acetate extracts of fungal isolates, *Aspergillus nidulans*, *A. fumigatus*, and *A. flavus* isolated from *Ocimum basilicum* showed potential antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Candida albicans*. The comprehensive study on phytochemical production, antimicrobial, and antioxidant activities revealed that leaf isolates from the summer exhibited the highest potency, followed by root isolates and then winter season isolates. This suggests that the

summer season optimizes the production of bioactive compounds in leaves, making them a promising source for natural remedies. Notably, the rhizome isolates contained significant amounts of tannins and cardiac glycosides, which are known for their medicinal properties. Although few rhizome isolates demonstrated antimicrobial activity, their potential should not be overlooked, as they may possess unique compounds with therapeutic benefits. The seasonal variation in phytochemical production and antimicrobial activity highlights the importance of considering the timing of plant collection for optimal bioactive compound extraction.

Conclusion

Endophytes, microorganisms living within plant tissues, have garnered attention for their antimicrobial properties. They produce bioactive compounds inhibiting pathogenic bacteria and fungi, showcasing potential in developing new pharmaceutical agents. Research has demonstrated the potent antimicrobial activity of certain endophytes, particularly those found in medicinal plants. Extracts from endophytes have shown effectiveness against various bacteria and fungi, signaling their potential for drug development. In addition to antimicrobial effects, endophytes exhibit antioxidant properties, vital for combating oxidative stress. Studies have indicated that endophytic fungi and bacteria produce secondary metabolites with significant antioxidant activities, such as flavonoids and tannins. Phytochemical analysis reveals a rich diversity of compounds contributing to endophytes' bioactivity. Techniques like chromatography and mass spectrometry identify and quantify these phytochemicals, including alkaloids, terpenoids, and phenolic compounds responsible for antimicrobial and antioxidant activities.

Environmental conditions and host plants significantly influence the phytochemical profile of endophytic isolates. The interaction between the plant and its endophytes can lead to modulated metabolite production in response to stress factors, enhancing the endophytes' survival and benefiting the host plant. Recent biotechnological advancements, including genetic engineering and

synthetic biology, facilitate the exploration and utilization of endophytic metabolites. Researchers can now manipulate biosynthetic pathways to increase the yield of desired bioactive compounds, offering sustainable and cost-effective means to produce high-value compounds for medicinal use. The antimicrobial, antioxidant and phytochemical properties of endophytes present a promising frontier in the search for new natural products. Continued research is essential to unlock the potential benefits of endophytes for human health and agriculture, offering insights into sustainable practices for drug development and natural remedies against various pathogens and oxidative stress.

Chapter 5

Endophytic Enzyme Production and its Activity

Introduction

The protective potential of endophytic microorganisms in mitigating the impact of biotic and abiotic stresses on host plants is well-documented (Leitao & Enguita, 2016; White & Torres, 2010). These beneficial microorganisms mitigate stress impacts by producing essential biochemical resources, including phytohormones and extracellular enzymes (Khan et al., 2015). Although phytohormones are a recent discovery in endophytes, extracellular enzymes (exozymes) have garnered significant attention due to their industrial applications in food, fermentation, dye synthesis, and biotechnology. Endophytic microorganisms produce various extracellular enzymes, comprising hydrolases, lyases, oxidoreductases, and transferases (Traving et al., 2015). These enzymes are instrumental in establishing host-microbe symbiosis (Hallmann et al., 1997) and breaking down complex macromolecules – such as carbohydrates, lignin, organic phosphates, proteins, and sugar-based polymers – into easily transportable compounds, facilitating heterotrophic metabolism (Wingender et al., 2012). Furthermore, extracellular hydrolyases produced by endophytes counteract plant pathogenic infections (Leo et al., 2016).

Major endophytic extracellular enzymes, including protease, cellulase, and pectinase, play a vital role in plant-endophyte interactions, contributing to nutrient cycling, plant growth promotion, and defense against pathogens. These enzymes facilitate the breakdown of complex organic matter, releasing essential nutrients for plant uptake, and enabling endophytes to colonize and thrive within plant tissues. Protease, cellulase, and pectinase activities also allow endophytes to modulate plant cell wall structure, influencing plant development and stress responses. These enzymes have industrial applications in food processing, biofuel production, and textile manufacturing, highlighting endophytic enzymes' significance in ecological and biotechnological contexts. Various studies have observed protein production by endophytic isolates, showcasing their potential in decomposing protein-based substrates. Endophytic bacteria

and fungi have been found to produce proteases, which play a crucial role in nutrient cycling and plant-beneficial interactions. These enzymes facilitate the breakdown of complex proteins into simpler forms, making them available to plants, thereby promoting plant growth and development. Cellulase production by endophytic isolates is another significant aspect of their enzymatic repertoire. Cellulases are crucial in decomposing cellulose, a key component of plant cell walls, and endophytes have been found to produce these enzymes to varying degrees. This enzymatic activity enables endophytes to colonize plant tissues and facilitates the release of nutrients from cellulose-based substrates, benefiting both the endophyte and the host plant. Pectinase production by endophytic isolates has also been reported, highlighting their ability to degrade pectin, a complex carbohydrate in plant cell walls. Pectinases are essential for plant-beneficial interactions, as they facilitate the breakdown of pectin-based substrates, releasing nutrients and promoting plant growth. Endophytic fungi and bacteria have been found to produce pectinases, showcasing their potential in plant symbiosis and nutrient cycling. This chapter seeks to unlock the vast potential of endophytic resources by delving into the realm of extracellular enzymes derived from endophytic microorganisms.

Materials and Methods

Cell Wall Degrading Enzyme Production

The isolated endophytes were subjected to study for hydrolytic enzyme production. The three enzymes protease, cellulase, and pectinase were screened. The agar diffusion method was used to screen extracellular hydrolytic enzyme activity. The endophytes were cultured on selective indicator media tailored for each enzyme assay. Following incubation, the cultures were transferred to an incubator and maintained at optimal temperature and time conditions.

Protease Production

The proteolytic activity of endophytic bacteria and fungi was evaluated on skimmed milk agar plates. After spot inoculation and incubation (2-5 days, 36°C for bacteria and 30°C for fungi), the appearance of clear zones around the colonies confirmed protease production, as described by Chaiharn et al. (2008).

Cellulase Production

The endophytic bacterial isolates were evaluated for cellulase activity using Carboxy Methyl Cellulose (CMC) agar, following the method described by Ariffin et al. (2006). CMC agar plates were prepared and spot-inoculated with the isolates to accomplish this. Incubation was carried out for 5 days at optimal temperatures specific to each group of organisms. Cellulase production was indicated by the formation of a halo zone surrounding the colonies. To visualize cellulose degradation, the plates were subjected to a staining procedure. Initially, they were flooded with 1% (w/v) aqueous Congo red solution for 15 minutes. Subsequently, the Congo red solution was discarded, and the plates were treated with 1M NaCl for 15 minutes. The appearance of a clear zone around the colonies, resulting from hydrolysis, confirmed cellulase activity and cellulose degradation. The intensity of cellulase activity was quantified by measuring the diameter of the clear zone.

Pectinase Production

The isolates were evaluated for pectinase activity using pectin agar plates. Following spot inoculation, the plates were incubated for 7 days at specific temperatures. To visualize pectin degradation, the plates underwent a staining procedure involving a 15-minute treatment with 1% (w/v) aqueous Congo red solution, followed by a 15-minute treatment with 1M NaCl. The formation of a clear zone around the colonies, resulting from hydrolysis, indicated pectinase activity and pectin degradation. The diameter of the clear zone was measured to quantify the intensity of pectinase activity.

Submerged Fermentation

The isolates that showed positive results in primary screening were subsequently grown in a suitable liquid medium for enzyme production.

Pre-Inoculum Preparation

A pre-inoculum was prepared by inoculating 100 mL of liquid culture medium, identical in composition to the screening medium, with a loopful of the selected

isolates. The inoculated medium was then incubated on a rotary shaker at 120 rpm for 24 hours to develop the pre-inoculum.

Enzyme Source

Endophytic isolates were scaled up in batch cultures by transferring 5 mL of pre-inoculum to 95 mL of culture medium (pH 7), identical in composition to the pre-inoculum. Cultures were incubated on a rotary shaker at 120 rpm for 120 hours. Sampling occurred at 24, 48, 72, 96, and 120 hours. Collected samples underwent centrifugation at 10,000 rpm and 4°C for 20 minutes. The resulting cell-free supernatant served as the crude enzyme source.

Hydrolytic Enzyme Activity

Following initial screening, enzyme assays were conducted on isolates exhibiting positive results. Substrate-specific assays were employed, utilizing casein for protease, carboxymethyl cellulose (CMC) for cellulase, and pectin for pectinase activity. 24-hour-old cultures were centrifuged at 10,000 rpm for 20 minutes to prepare crude enzyme extracts. The resulting supernatant, obtained after discarding the cellular pellet, served as the crude enzyme source for subsequent assays.

Protease Assay

A protease assay was performed using casein as the substrate, based on the method described by Tuschida et al. (1986). The assay mixture consisted of 0.5 mL casein solution (2% w/v in phosphate buffer) and 0.5 mL enzyme preparation. After incubating at 40°C for 10 minutes, the reaction was terminated by adding 1 mL trichloroacetic acid (TCA) reagent, followed by centrifugation at 2000 rpm for 5 minutes. To quantify protease activity, 0.5 mL of the resulting supernatant was mixed with 5 mL of 0.44M Na_2CO_3 and incubated for 10 minutes. Then, 0.5 mL of two-fold diluted Folin-Ciocalteu reagent was added, and the mixture was incubated for an additional 20 minutes. Absorbance was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme releasing 1 µg tyrosine per mL per minute under these assay conditions. Enzyme blanks and reagent blanks were included to account for background tyrosine levels and potential errors.

Cellulase Assay

The CMCase assay utilized carboxymethyl cellulose (CMC) as the substrate. To prepare the reaction mixture, 1.0 g of CMC was dissolved in 100 mL of 0.2M phosphate buffer. The reaction mixture consisted of 0.5 mL of pre-incubated CMC solution and 0.25 mL of enzyme preparation. This mixture was incubated at 50°C for 10 minutes. To account for potential reducing sugars in the sample, enzyme blanks were prepared by adding DNS reagent before enzyme addition. Reagent blanks were also prepared by mixing 0.5 mL of CMC and 1.5 mL of DNS reagent. The reaction was terminated by adding 1.5 mL of dinitro salicylic acid reagent, followed by a 5-minute boiling water bath. Absorbance was measured at 540 nm, following the protocol established by Mandels et al. (1976).

Pectinase Assay

The pectinase assay utilized pectin as the substrate, prepared by dissolving 1 g of pectin in 100 mL of 0.2M phosphate buffer. The reaction mixture consisted of 0.9 mL of pre-incubated pectin solution and 0.1 mL of enzyme preparation. This mixture was incubated at 50°C for 10 minutes. To account for reducing sugars in the enzyme preparation, enzyme blanks were prepared by adding a DNS reagent before enzyme addition. Reagent blanks were also prepared by mixing 0.9 mL of substrate pectin solution, 0.1 mL phosphate buffer, and 3 mL of DNS reagent. The reaction was terminated by adding 3 mL of DNS reagent, followed by a 5-minute boiling water bath. Absorbance was measured at 540 nm, as described by Cao et al. (1992). Pectinase activity was expressed as μmol of glucose released per minute per milliliter (μmol min-1 mL-1) of culture filtrate or enzyme solution.

Optimization of Fermentation Conditions for Protease Production under Submerged Fermentation

Optimization of culture conditions and process parameters influencing protease production under submerged fermentation was carried out using a one-factor-at-a-time method (OFAT) (Sindhu et al., 2009)**.**

Optimal Influence of Agitation on Enzyme Production

The effect of different agitation conditions, such as 80, 100, 120, 140, and 160, on enzyme production by a bacterial isolate was investigated. Five ml of pre-inoculum culture was transferred to 95 ml of production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, enzyme activity in cell-free supernatant was determined.

Optimal Influence of Temperature on Enzyme Production

The effect of different temperature conditions, such as 32°C, 34°C, 36°C, 38°C, and 40°C on enzyme production by isolates was investigated. Five ml of pre-inoculum culture was transferred to 95 ml of production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, enzyme activity in cell-free supernatant was determined.

Optimal Influence of pH on Enzyme Production

Fermentation broth for isolate was formulated using previously optimized hours. Five ml of pre-inoculum were inoculated in fermentation broth with pH 4, 6, 8, 10, and 12. Enzyme activity in cell-free supernatant was determined.

Optimal Influence of Carbon Variables on Enzyme Production

Fermentation broth for isolate was formulated using optimized hours. The effect of different carbon sources, such as dextrose, maltose, starch, sucrose, and xylose, on enzyme production was investigated. These carbon sources were added individually in the fermentation broth at a constant concentration (1%, w/v). The original production medium's carbon source was substituted with alternative carbon sources. Following incubation, enzyme activity was assessed in the cell-free supernatant. Several concentration levels of the carbon source that showed the highest activity were considered for optimization (0.125%, 0.25%, 0.5%, 0.75%, and 1%).

Optimal Influence of Nitrogen Variables on Enzyme Production

Fermentation broth was optimized and inoculated with 5 mL of inoculum. The effects of different nitrogen sources (yeast extract, peptone, gelatin, sodium dihydrogen phosphate, and sodium carbonate) were investigated. Enzyme

activity in cell-free supernatant was determined post-incubation. Several concentration levels of the nitrogen source that showed the highest activity were considered for optimization (0.125%, 0.25%, 0.5%, 0.75%, and 1%).

Optimization of Fermentation Conditions for Cellulase Production under Submerged Fermentation

Optimization of culture conditions and process parameters influencing cellulase production under submerged fermentation was carried out using a one-factor-at-a-time method (OFAT) (Sindhu et al., 2009)**.**

Optimal Influence of Agitation on Enzyme Production

The effect of different agitation conditions, such as 80, 100, 120, 140, and 160, on enzyme production by a bacterial isolate was investigated. Five ml of pre-inoculum culture was transferred to 95 ml of production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, enzyme activity in cell-free supernatant was determined.

Optimal Influence of Temperature on Enzyme Production

The effect of different temperature conditions such as 32°C, 34°C, 36°C, 38°C, and 40°C on enzyme production by isolates was investigated. Five ml of pre-inoculum culture was transferred to 95 ml of production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, enzyme activity in cell-free supernatant was determined.

Optimal Influence of pH on Enzyme Production

Fermentation broth for isolate was formulated using previously optimized hours. Five ml of pre-inoculum were inoculated in fermentation broth with pH 4, 6, 8, 10, and 12. Enzyme activity in cell-free supernatant was determined.

Optimal Influence of Carbon Variables on Enzyme Production

A fermentation broth was formulated for the isolate, utilizing optimized incubation conditions. To investigate the impact of various carbon sources on enzyme production, the following substrates were individually added to the broth at a constant concentration of 1% (w/v): Carboxymethyl cellulose (CMC), fructose, maltose, starch, and sucrose. Alternative carbon sources replaced the carbon source of the original production medium. At the end of incubation,

enzyme activity in cell-free supernatant was determined. Several concentration levels of the carbon source that showed the highest activity were considered for optimization (0.125%, 0.25%, 0.5%, 0.75%, and 1%).

Optimal Influence of Nitrogen Variables on Enzyme Production

Fermentation broth for isolate was formulated using conditions optimized for an hour. The five ml inoculum was inoculated in the fermentation broth, and flasks were incubated to investigate the effect of different nitrogen sources such as yeast extract, peptone, casein, ammonium phosphate, and potassium nitrate. At the end of the incubation period, enzyme activity in cell-free supernatant was determined. Several concentration levels of the nitrogen source that showed the highest activity were considered for optimization (0.125%, 0.25%, 0.5%, 0.75%, and 1%).

Optimization of Fermentation Conditions for Pectinase Production under Submerged Fermentation

The culture conditions and process parameters influencing cellulase production under submerged fermentation were optimized using a one-factor-at-a-time method (OFAT) (Sindhu et al., 2009)**.**

Optimal Influence of Agitation on Enzyme Production

The effect of different agitation conditions, such as 80, 100, 120, 140, and 160, on enzyme production by a bacterial isolate was investigated. Five ml of pre-inoculum culture was transferred to 95 ml of production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, enzyme activity in cell-free supernatant was determined.

Optimal Influence of Temperature on Enzyme Production

The effect of different temperature conditions such as 30°C, 35°C, 40°C, 45°C, and 50°C on enzyme production by isolates was investigated. Five ml of pre-inoculum culture was transferred to 95 ml of production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, enzyme activity in cell-free supernatant was determined.

Optimal Influence of pH on Enzyme Production

Fermentation broth for isolate was formulated using previously optimized hours. Five ml of pre-inoculum were inoculated in fermentation broth with pH 4, 6, 8, 10, and 12. Enzyme activity in cell-free supernatant was determined.

Optimal Influence of Carbon Variables on Enzyme Production

Fermentation broth for isolate was formulated using optimized hours. The effect of different carbon sources such as fructose, maltose, glucose, galactose, and sucrose on enzyme production was investigated. These carbon sources were added individually in the fermentation broth at a constant concentration (1%, w/v). Alternative carbon sources replaced the carbon source of the original production medium. At the end of incubation, enzyme activity in cell-free supernatant was determined. Several concentration levels of the carbon source that showed the highest activity were considered for optimization (0.125%, 0.25%, 0.5%, 0.75%, and 1%).

Optimal Influence of Nitrogen Variables on Enzyme Production

Fermentation broth for isolate was formulated using conditions optimized for an hour. The five ml inoculum was inoculated in the fermentation broth, and flasks were incubated to investigate the effect of different nitrogen sources such as yeast extract, peptone, ammonium chloride, sodium nitrate, and potassium nitrate. At the end of the incubation period, enzyme activity in cell-free supernatant was determined. Several concentration levels of the nitrogen source that showed the highest activity were considered for optimization (0.125%, 0.25%, 0.5%, 0.75%, and 1%).

Screening of Significant Variables for Protease Production under Submerged Fermentation using Plackett-Burman (PB) Design

To identify key factors influencing protease production under submerged fermentation, a fractional factorial Plackett-Burman (PB) design was employed (Plackett and Burman, 1946). The experimental design matrix was generated using Design-Expert 10.0 software (Stat Ease Inc., Minneapolis, USA). In the present study, the effect of six assigned variables (incubation time, rpm, temperature, pH, maltose, and yeast concentration) was screened to identify the

components that significantly affected protease production. Each variable was tested at two levels: low (-1) and high (+1), as outlined in Table 57. The levels were determined based on preliminary studies under submerged fermentation. Protease production was expressed in Units per milliliter (U/mL). All experiments were conducted in triplicate to ensure reliability. Regression analysis of the experimental data was performed using statistical software.

Variable code	Variable	Unit	Low (-1) value	High (+1) value
X1	Incubation time	h	96	72
X2	Rpm	-	140	120
X3	Temperature	°C	38	36
X4	pH	-	10	8
X5	Maltose	%	0.5	1
X6	Yeast	%	0.5	1

Table 57: Variables and their experimental levels adopted in Plackett-Burman method for protease production

Based on results obtained from Plackett-Burman design, a first order polynomial design (equation 1) was fitted.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i \qquad (1)$$

where Y-predicted response, β_o, β_i - Constant coefficients, and X_i-Coded independent factors.

The impact of each variable on protease production was determined by calculating the effect (E) using the following formula:

E = (Total response at high level - Total response at low level) / (Number of trials)

This approach is consistent with previous studies (Gupta et al., 2004).

Optimization of Process Parameters for Protease Production using Response Surface Methodology

The variables selected based on the Plackett-Burman method were evaluated using a response surface Central Composite Design (CCD) to determine the individual and interactive effects of variables on enzyme production and to optimise their levels. The four variables, rpm, incubation time, temperature, and yeast selected based on the screening tests, were tested using Design-Expert

software 10.0 (Stat-Ease, Inc., Minneapolis, USA). CCD is a second-order design for the estimation of quadratic effect and two-parameter interactions. In the CCD, one numeric variable was tested at 5 levels: −α, −1, 0, +1 and +α (Table 58). Quadratic model chosen to represent the relationship fitted for the 4 variables was given in equation (2).

$$Y = \beta_0 + \sum_{i=1}^{k} X_i + \beta_{ii} \sum_{i=1}^{k} X_i^2 + \beta_i \beta_j \sum_{i=1}^{k} \sum_{j=1}^{k} X_i X_j \qquad (2)$$

Where Y is the predicted response, βo is the overall coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and X_iX_j is coded values.

The experiments were carried out in triplicate. Analysis of variance (ANOVA) of the experimental data was performed using the software.

Variables	Code	-α	Low level (-1)	Medium level (0)	High level (+1)	+α
Rpm	X_1	80	90	100	110	120
Incubation time	X_2	24	36	48	60	72
Temperature	X_3	32	33	34	35	36
Yeast	X_4	0.25	0.44	0.625	0.81	1

Table 58: Experimental range and levels of the independent variables in CCD

Experimental Validation of the Optimized Conditions

To verify the accuracy of the response surface model (RSM), a random set of experiments was conducted within the design space, testing all four variables. A random set of six combinations of variables was prepared and analyzed for protease production. A strong correlation between experimental and predicted values would confirm the model's authenticity and applicability for optimizing process variables.

Partial Purification of Protease

The protease isolated from the bacterial isolate through submerged fermentation was subjected to purification and characterization. The crude enzyme produced in the culture filtrate using the optimized conditions of isolate was purified employing standard purification protocols. All purification steps were

conducted at a controlled temperature of 4°C to maintain enzyme stability and optimize recovery.

Enzyme Precipitation using Ammonium Sulphate

The cultures were grown in the optimized media of the respective strain. After incubation, the cells were harvested by using centrifugation at 10000rpm for fifteen minutes. Fractional precipitation was performed using ammonium sulphate, from 0-80% according to Winarti et al., 2018. The supernatant was subjected to ammonium sulphate precipitation. To ensure efficient precipitation, the salt was slowly added over a 10-minute period, allowing for complete dissolution, while continually stirring at room temperature for an additional 25 minutes. The resulting precipitates were recovered through centrifugation at 10,000g for 10 minutes at 4°C. Following centrifugation, the precipitates were dissolved in 10 mM Tris-HCl buffer (pH 7.6). The ammonium sulphate fraction then underwent overnight dialysis against the same buffer (10 mM Tris-HCl, pH 7.6) at 4°C.

Dialysis

The sample is loaded onto the cellulose membrane dialysis tubing (14 kDa cut off, Sigma Aldrich) and both ends are tied to prevent leakage of sample. Then it is placed into an external chamber of dialysis buffer (10 mM Tris-HCl buffer (pH 7.6), with gentle stirring. The sample is dialyzed overnight at 4°C with four buffer changes. The protease activity and protease concentration were measured.

Characterization of the Enzyme

Molecular Weight Determination

The crude protein samples were run on SDS-PAGE gel prepared using 12% polyacrylamide separating gel and 4% stacking gel according to the method of Laemmli et al. (1970) using a Mini-PROTEAN electrophoresis apparatus. The protein was mixed with 5X sample loading buffer and about 20µl was loaded onto each well. A protein marker (Precision Plus Protein^{TM} Prestained Dual Colour Standard) was also run alongside the samples to be carried out at a

constant current of 40 mA in Tris-glycine SDS running buffer. The gel was fixed and stained using Coomassie Brilliant Blue R-250 staining solution for 45 minutes, then destained with a destaining solution and kept overnight. The bands obtained were observed and photographed.

Application Studies

Blood Stain Removal using Protease Enzyme

Blood samples were collected from the chicken stall in the sterilized polythene bag. The clean cotton fabric was soaked in blood and allowed to dry. The cloth was cut to equal sizes and incubated with the crude protease from endophytic bacteria. In one test tube, the enzyme was taken while the other tube was kept as a control (detergent). The dried cloth was put into each test tube containing 20 ml of enzyme and detergent and was incubated at 40°C for different time intervals. After 24 hrs incubation, each piece was rinsed with water for 2 min and then dried (Kanmani et al., 2011).

Results

Protease Production

Preliminary Screening of Bacterial Endophytes for Protease Production

All the selected bacterial endophytes were screened semi-quantitatively for their ability to produce protease enzyme by point inoculation on skimmed milk agar plates. The zone of hydrolysis produced, due to the lysis of casein after 96hr of incubation, was used to estimate the proteolytic activity of bacteria (Fig.102). The ratio of the zone's hydrolysis to fungi's growth diameter was calculated to determine the Relative Enzymatic Index (REA). The results also show that some isolates, such as BRWS3 and BLWW7, have high colony diameters but relatively low REA values, indicating they may have different enzymatic profiles. Further studies are needed to explore the enzymatic activities of these isolates. Among the 35 isolates, 26 isolates (74.28%) were found to be protease-positive. The REA values of all the positive isolates ranged from 1.042±0.014 to 2.285±0.278 (Table 59). The experiment was conducted in triplicates, and an average of the values were taken to determine the REA. Isolate BLWS10

showed the highest REA value of 2.285, indicating high enzymatic activity. Other isolates, such as BRWS15, BLWS7, and BLWS6, showed high enzymatic activity, with REA values above 1.4. The remaining 9 isolates showed no enzymatic activity. A conclusive inference about the most promising candidate for protease production could only be inferred from secondary screening results. Hence all the positive isolates were subjected to quantitative screening by submerged fermentation.

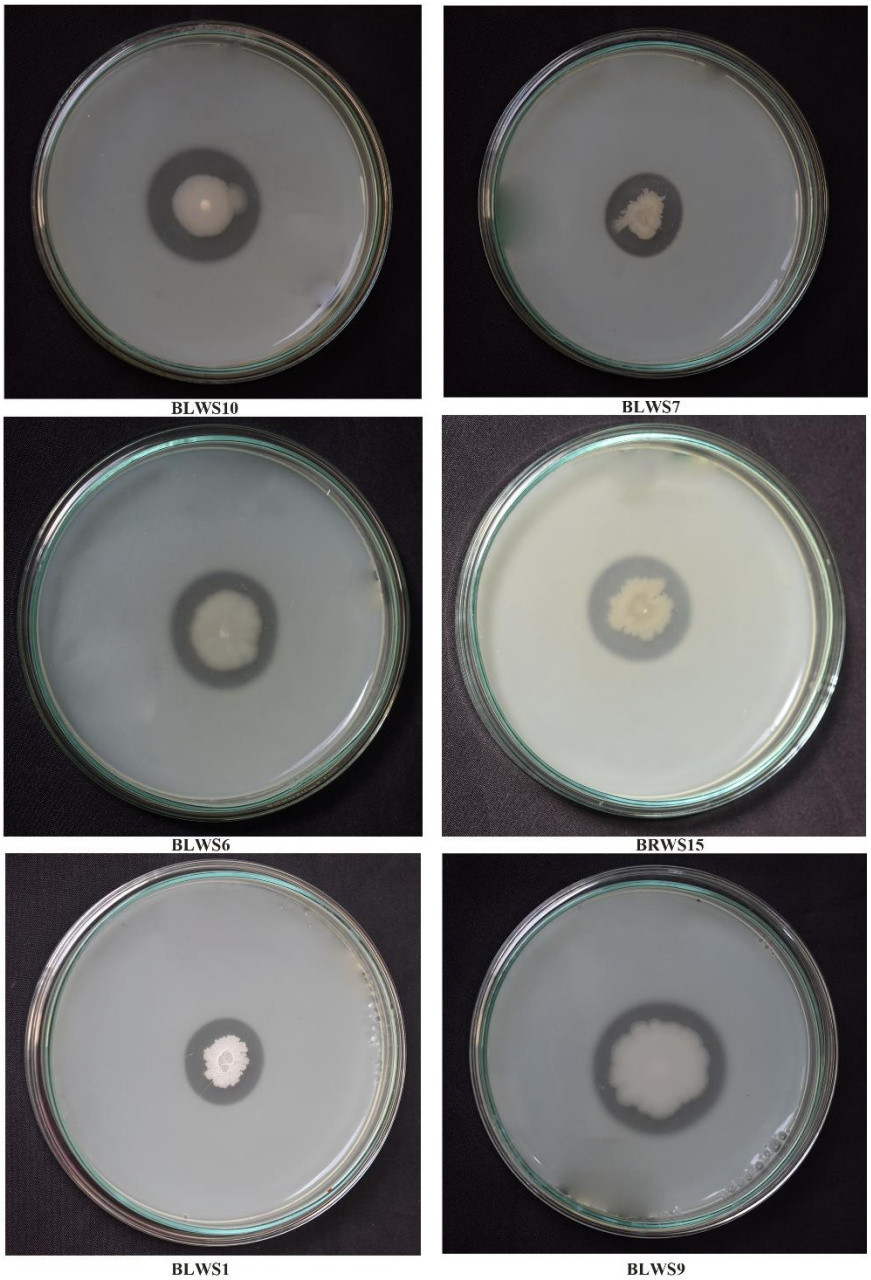


Fig. 102 Screening of protease production of bacterial isolates

Sl No.	Bacterial Isolates	Colony Diameter (mm)	Zone Diameter (mm)	Relative Enzymatic Index (REA)
1	BRWW12	7.28±0.583	7.883±0.722	1.083±0.056
2	BRWW6	29.07±0.947	33.473±1.23	1.151±0.012
3	BRWS13	0	0	0
4	BRWS15	16.273±0.673	26.58±0.61	1.634±0.102
5	BRWS1	0	0	0
6	BRWS3	69.89±1.398	73.093±1.036	1.046±0.031
7	BRWM2	2.413±0.417	3.08±0.156	1.298±0.193
8	BRWM3	31.04±3.238	38.057±0.976	1.237±0.139
9	BLWW1	35.223±1.134	40.683±1.947	1.156±0.067
10	BLWW4	40.17±1.204	49.623±2.675	1.235±0.032
11	BLWW2	34.193±1.738	42.637±1.541	1.248±0.059
12	BLWS11	0	0	0
13	BLWS6	21.113±2.32	29.993±1.1	1.428±0.105
14	BLWS7	16.443±1.934	28±0.22	1.75±0.182
15	BLWW5	0	0	0
16	BLWW7	60.667±4.596	63.237±5.469	1.042±0.014
17	BLWW8	30.073±2.92	34.873±2.812	1.161±0.034
18	BLWM4	26.717±1.164	30.133±0.868	1.13±0.075
19	BLWM1	52.707±4.851	56.397±5.03	1.07±0.005
20	BLWW10	0	0	0
21	BLWW9	57.143±4.398	62.033±4.31	1.086±0.017
22	BLWS10	5.53±0.551	12.54±0.537	2.285±0.278
23	BLWS9	25.823±1.292	34.593±3.647	1.339±0.107
24	BLWS4	34.373±3.059	40.343±2.394	1.184±0.18
25	BLWS2	32.667±4.443	40.1±2.865	1.243±0.198
26	BLWS1	22.893±1.591	30.697±1.78	1.349±0.175
27	BLWS3	0	0	0
28	BRhWS1	15.483±1.328	17.33±0.669	1.124±0.098
29	BRhWS8	24.863±1.865	31.403±3.164	1.269±0.179
30	BRhWW11	28.293±2.244	30.537±1.475	1.085±0.12
31	BRhWW7	0	0	0
32	BRhWM3	29.787±0.899	31.267±1.384	1.049±0.018
33	BRhWM10	4.423±0.368	5.31±0.357	1.203±0.072
34	BRhWM27	0	0	0
35	BRhWS22	0	0	0

Table 59: Primary screening of bacterial isolates for protease production

Secondary Screening for Protease Activity

All the protease-positive isolates, screened by semi-quantitative screening, were inoculated onto protease-specific fermentation broth and analyzed for protease production quantitatively for 24hr, 48hr, 72hr, 96hr, and 120hr. The enzyme production by each of the 26 isolates in submerged fermentation was conducted in triplicates, and activity was noted (Table 60). The isolate BLWS10 showed maximum protease activity yielding 69.547±1.707 U/ml at 72 hr followed by

BLWS7 (49.693±1.253 U/ml) at 72 hr and BRWS15 (47.247±0.867 U/ml) at 48 hr. This is significant commercially since BLWS10 isolate can produce maximum protease within a short period. Here, protein content was maximum at 72 hr of incubation.

Sl No.	Bacterial Isolates	Enzyme Activity (U/ml) (Mean±SD)
1	BRWW12	9.123±0.015
2	BRWW6	22.07±0.572
3	BRWS15	47.247±0.867
4	BRWS3	5.263±0.042
5	BRWM2	16.883±0.258
6	BRWM3	24.24±0.27
7	BLWW1	20.993±0.025
8	BLWW4	18.463±0.48
9	BLWW2	21.447±0.407
10	BLWS6	30.207±0.691
11	BLWS7	49.693±1.253
12	BLWW7	10.233±0.015
13	BLWW8	21.027±0.104
14	BLWM4	16.917±0.617
15	BLWM1	9.353±0.025
16	BLWW9	10.413±0.025
17	BLWS10	69.547±1.707
18	BLWS9	34.11±1.45
19	BLWS4	17.397±0.086
20	BLWS2	15.93±0.791
21	BLWS1	19.837±0.313
22	BRhWS1	21.05±0.265
23	BRhWS8	24.77±0.599
24	BRhWW11	8.353±0.047
25	BRhWM3	5.237±0.032
26	BRhWM10	16.357±0.462

Table 60: Secondary screening of bacterial isolates for protease production

Preliminary Screening of Fungal Endophytes for Protease Production

The enzymatic activity of the fungal isolates was evaluated using the Relative Enzymatic Index (REA), which considers the colony diameter and zone diameter (Table 61). Of the 36 fungal isolates, only 6 (16.6%) showed enzymatic activity, with REA values ranging from 1.132±0.084 to 2.09±0.147 (Fig.104). Isolate FLWS5 showed the highest REA value of 2.09, indicating high enzymatic activity. Isolates FRWS8, FLWW15, FRWM25, and FRWW30 also showed moderate to high enzymatic activity, with REA values above 1.1 (Fig.103). The remaining 30 isolates showed no enzymatic activity.

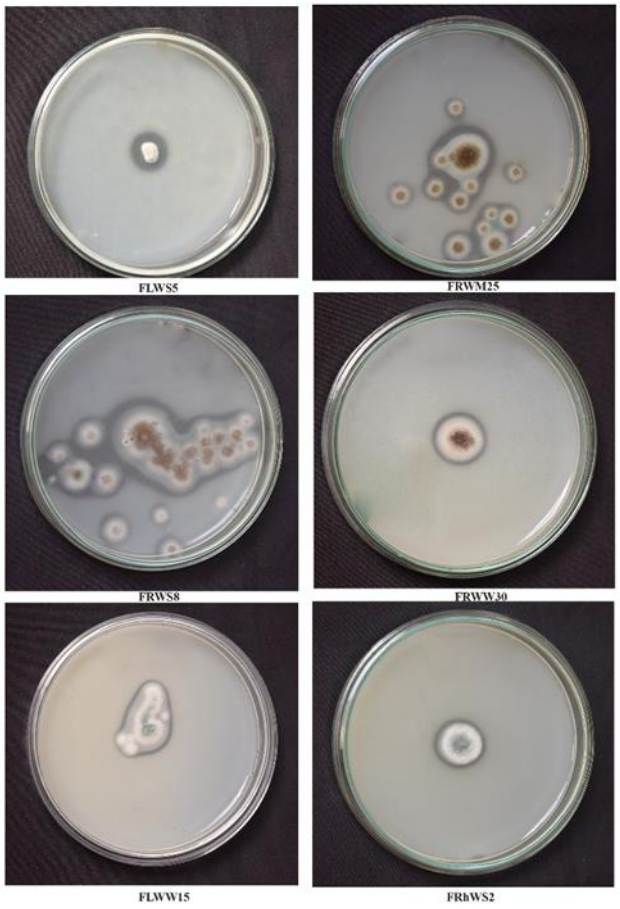


Fig. 103 Screening of protease production of fungal isolates

Sl No.	Fungal Isolates	Colony Diameter (mm)	Zone Diameter (mm)	Relative Enzymatic Index (REA)
1	FLWW14	0	0	0
2	FRWS8	21.177±0.35	25.287±0.705	1.194±0.014
3	FLWW5	0	0	0
4	FLWW15	18.253±0.753	21.247±0.627	1.164±0.014
5	FLWW17	0	0	0
6	FLWW6	0	0	0
7	FRWS7	0	0	0
8	FRWM12	0	0	0
9	FLWW12	0	0	0
10	FRWM1	0	0	0
11	FRWS4	5.12±0.61	0	0
12	FLWW11	0	0	0
13	FRhWS1	0	0	0
14	FLWW18	0	0	0
15	FLWS32	0	0	0
16	FRWW18	0	0	0
17	FRWM25	16.54±1.168	22.273±1.361	1.347±0.025
18	FRWW30	18.213±0.647	21.407±1.447	1.177±0.101
19	FLWM1	0	0	0
20	FLWW9	0	0	0
21	FRWM27	0	0	0

22	FLWS1	0	0	0
23	FRhWW3	0	0	0
24	FRhWM2	0	0	0
25	FRhWS2	14.62±0.997	16.5±0.527	1.132±0.084
26	FRWM3	0	0	0
27	FLWS21	0	0	0
28	FLWS16	0	0	0
29	FRWS24	0	0	0
30	FRWW10	0	0	0
31	FRWW11	0	0	0
32	FRhWW4	0	0	0
33	FRWS9	0	0	0
34	FLWM11	0	0	0
35	FLWW13	0	0	0
36	FLWS5	8.3±0.7	17.303±1.071	2.09±0.147

Table 61: Primary screening of fungal isolates for protease production

Secondary Screening for Protease Activity

Table 62 represents the enzyme activity of various fungal isolates, with values ranging from 18.3 to 38.913 units per milliliter (U/ml). The highest enzyme activity was observed in isolate FLWS5, while the lowest was in FRhWS2. The remaining isolates showed moderate enzyme activity, with some variation.

Sl No.	Fungal Isolates	Enzyme Activity (U/ml) (Mean±SD)
1	FRWS8	27.537±0.095
2	FLWW15	21.367±0.524
3	FRWM25	35.693±0.35
4	FRWW30	28.143±0.075
5	FRhWS2	18.3±0.115
6	FLWS5	38.913±1.171

Table 62: Secondary screening of fungal isolates for protease production

Submerged Fermentation Studies

Due to the diverse features of genetic, metabolic, and enzyme synthesis pathways, it is challenging to understand the catalytic and rate of reaction mechanism of protease in an unknown microbe. Thus, the traditional method of optimization studies using the one factor at a time (OFAT) approach has been undertaken in this study due to the necessity of understanding the influence of nutrients and physical conditions. A series of tests were carried out to determine an inexpensive and faster way to formulate media for protease production. In

addition to the nutritional elements necessary for their growth and metabolism, such as pH, temperature, agitation, and duration of fermentation. From the secondary screening studies, the optimal duration of fermentation was observed at 72 hr. Submerged fermentation studies were conducted only in the one topmost protease-producing isolate *Bacillus altitudinis* BLWS10.

Optimization of Fermentation Conditions for Protease Production under Submerged Fermentation

Optimal Influence of Agitation on Protease Enzyme Production

The effect of agitation speed (rpm) on protease activity and protein concentration is detailed in Fig. 104. As the agitation speed increases from 80 to 120 rpm, protease activity increases significantly from 23.78 to 69.81 U/ml, and protein concentration also rises from 1.47 to 6.83 mg/ml. However, further increases in agitation speed beyond 120 rpm led to a decline in protease activity and protein concentration. At 140 rpm, protease activity decreases to 50.94 U/ml, and protein concentration drops to 5.12 mg/ml. Similarly, at 160 rpm, protease activity falls to 47.23 U/ml, and protein concentration decreases to 4.88 mg/ml. The optimal agitation speed for protease production appears to be 120 rpm, which yields the highest protease activity and protein concentration. This suggests that excessive agitation beyond 120 rpm may be detrimental to protease production, possibly due to shear stress or enzyme denaturation.

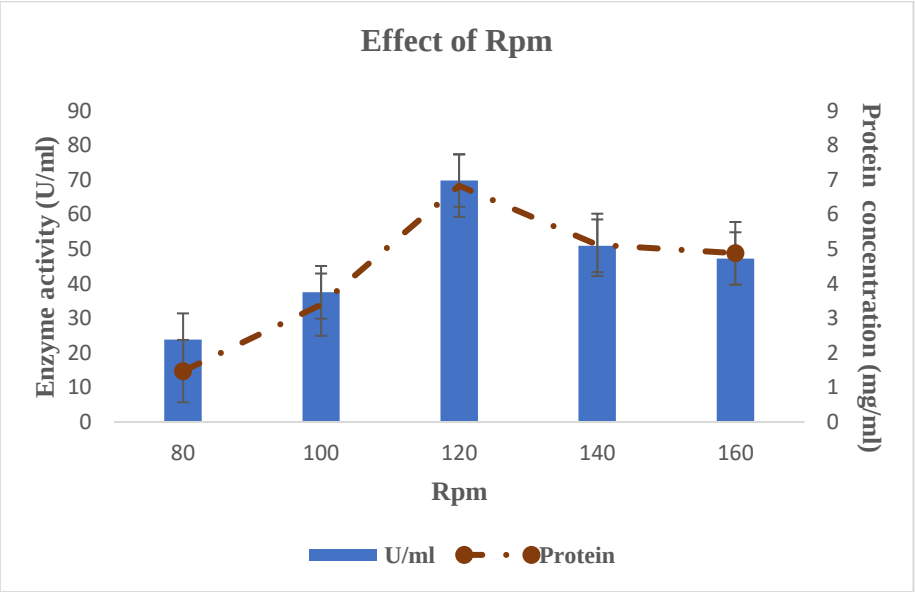


Fig. 104 Impact of different agitation (rpm) on the protease enzyme production

Optimal Influence of Temperature on Protease Enzyme Production

The strain BLWS10 was allowed to grow at different temperatures. As the temperature increases from 32°C to 36°C, protease activity and protein concentration rise significantly, with the highest values observed at 36°C (83.96 U/ml and 7.94 mg/ml, respectively). However, further increases in temperature beyond 36°C lead to a decline in protease activity and protein concentration. At 38°C, protease activity decreases to 68.36 U/ml, and protein concentration drops to 6.04 mg/ml. At 40°C, protease activity falls to 51.03 U/ml, and protein concentration decreases to 5.2 mg/ml. The optimal temperature for protease production appears to be 36°C, which yields the highest protease activity and protein concentration (Fig. 105). This suggests that temperatures above 36°C may be detrimental to protease production, possibly due to enzyme denaturation or protein degradation.

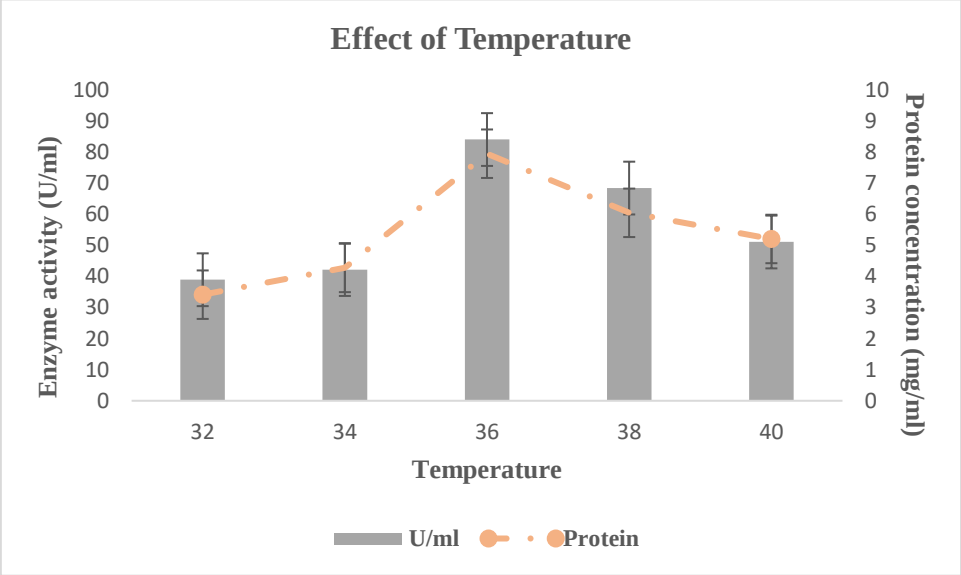


Fig.105 Impact of different temperatures on the protease enzyme production

Optimal Influence of pH on Protease Enzyme Production

As the pH increases from 4 to 8, protease activity and protein concentration rise significantly, with the highest values observed at pH 8 (107.41 U/ml and 9.12 mg/ml, respectively). This suggests that the enzyme is most active and stable at slightly alkaline conditions. However, further increases in pH beyond 8 lead to a decline in protease activity and protein concentration. At pH 10, protease activity decreases to 82.75 U/ml, and protein concentration drops to 7.9 mg/ml.

At pH 12, protease activity falls to 40.1 U/ml, and protein concentration decreases to 4.02 mg/ml, similar to the levels observed at pH 4. The optimal pH for protease production appears to be 8, which yields the highest protease activity and protein concentration (Fig. 106). This suggests that extreme pH conditions (acidic or basic) may be detrimental to protease production, possibly due to enzyme denaturation or protein degradation.

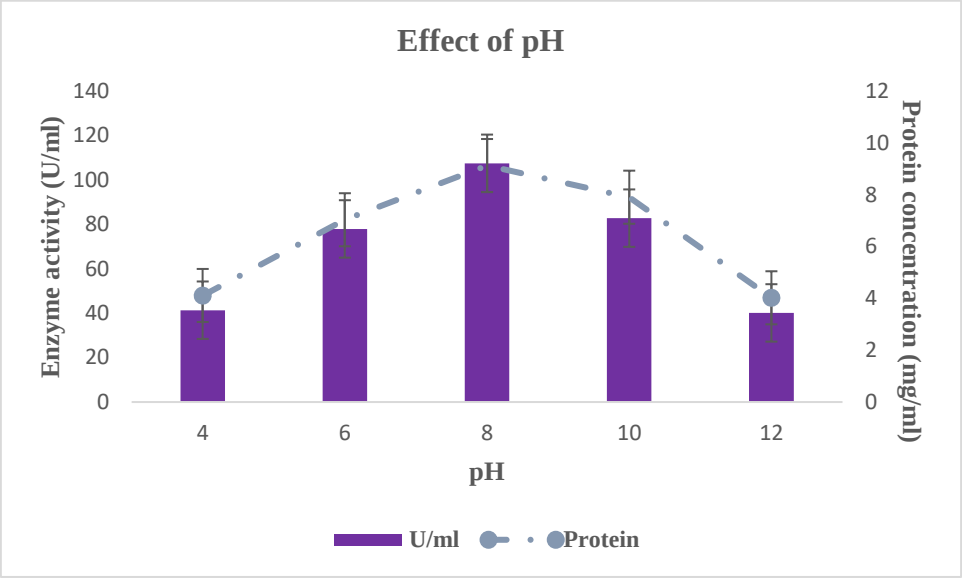


Fig.106 Impact of different pH on the protease enzyme production

Optimal Influence of Carbon Variables on Protease Enzyme Production

Maltose is the most effective carbon source, producing the highest protease activity (143.62 U/ml) and protein concentration (9.99 mg/ml). This suggests that maltose is the preferred carbon source for protease production. In contrast, dextrose, sucrose, and xylose are less effective carbon sources, resulting in lower protease activity and protein concentration. Starch shows moderate protease activity (68.9 U/ml) and protein concentration (6.58 mg/ml) (Fig. 107). The results indicate that the type of carbon source significantly impacts protease production, with maltose being the most suitable carbon source. The variation in protease activity and protein concentration among different carbon sources may be due to differences in their molecular structure, metabolism, or transport into the cell. Understanding the effect of carbon sources on protease production can help optimize fermentation conditions and improve enzyme yields.

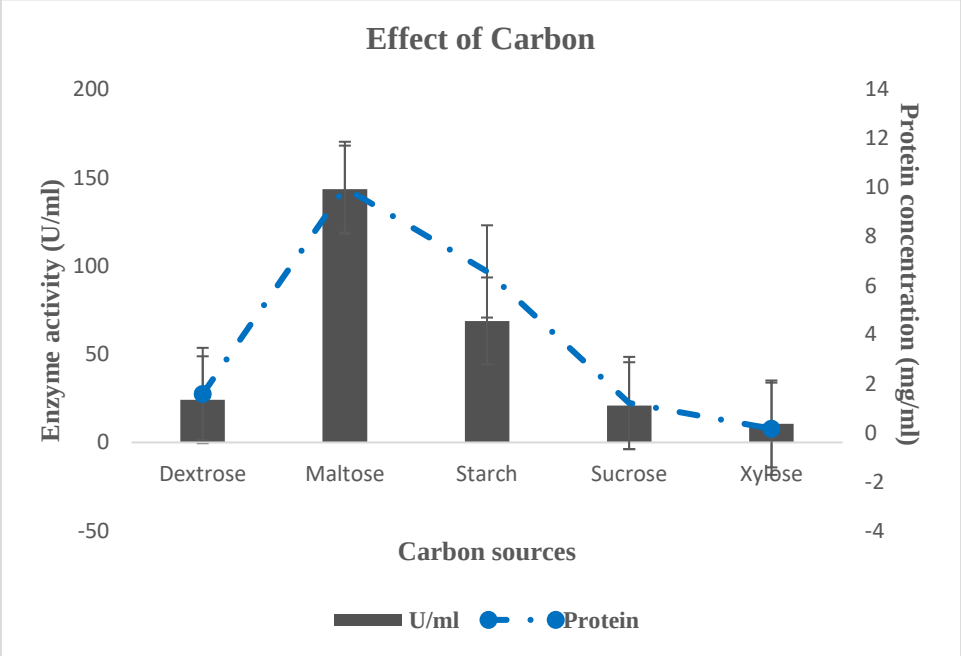


Fig.107 Impact of different carbon sources on the protease enzyme production

Optimal Influence of Nitrogen Variables on Protease Enzyme Production

Yeast extract is the most effective nitrogen source, resulting in the highest protease activity (168.13 U/ml) and protein concentration (10.74 mg/ml) (Fig. 108). This suggests that yeast extract provides the necessary nutrients and growth factors for optimal protease production. In contrast, other nitrogen sources such as peptone, gelatin, sodium dihydrogen phosphate, and sodium carbonate show lower protease activity and protein concentration. Peptone is the second most effective nitrogen source, but its protease activity and protein concentration are significantly lower than those of yeast extract. The variation in protease activity and protein concentration among different nitrogen sources may be due to differences in their nitrogen content, availability, and utilization by the microorganism. Yeast extract is a complex nitrogen source that provides a mix of amino acids, vitamins, and minerals, which may contribute to its superior performance.

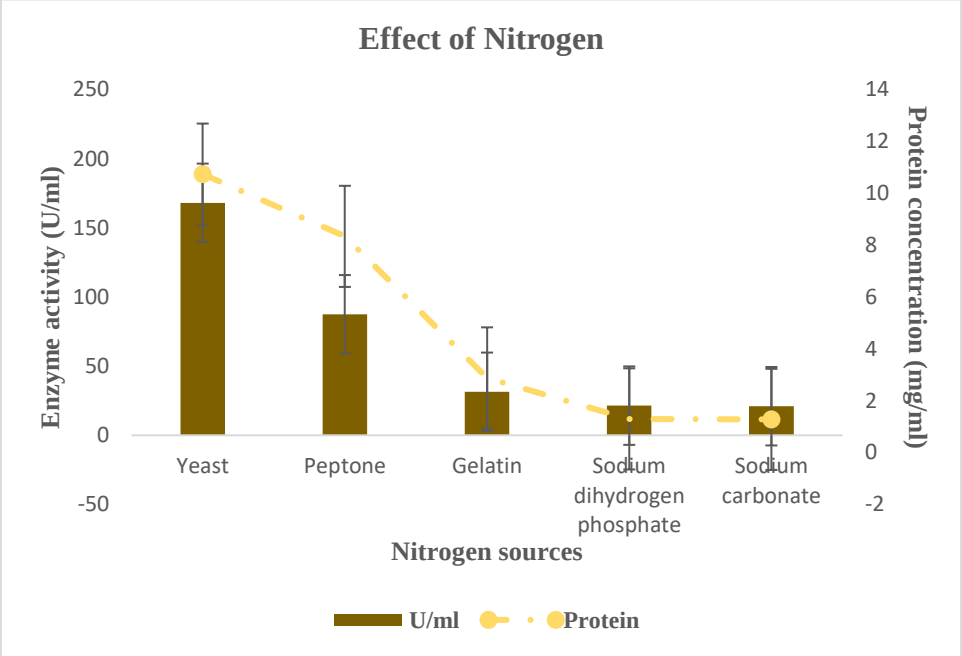


Fig 108 Impact of different nitrogen sources on the protease enzyme production

Optimal Influence of Maltose Variables on Protease Enzyme Production

As the concentration of maltose increases from 0.125% to 1%, protease activity and protein concentration also increase. The highest protease activity (143.62 U/ml) and protein concentration (9.99 mg/ml) are observed at 1% maltose concentration (Fig 109). The results suggest that maltose positively affects protease production, and increasing its concentration enhances enzyme activity and protein yield. The incremental increase in protease activity and protein concentration with increasing maltose concentration indicates that the microorganism can efficiently utilize maltose as a carbon source. The optimal concentration of maltose for protease production appears to be 1%, beyond which further increases may not be necessary. The results also suggest that maltose concentration can be fine-tuned to achieve desired protease activity and protein concentration levels.

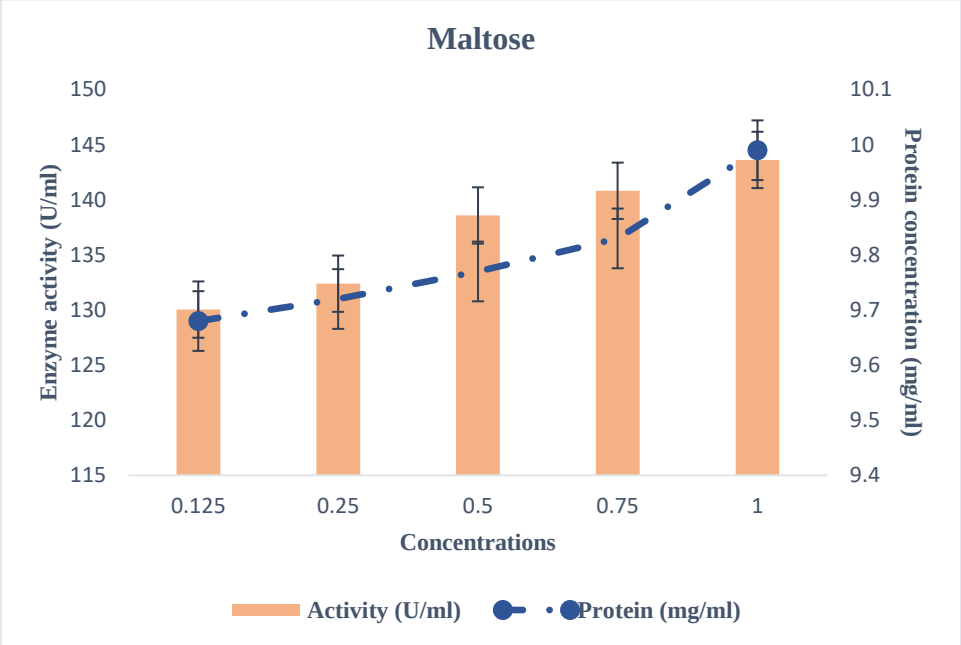


Fig.109 Impact of different concentrations of maltose on the protease enzyme production

Optimal Influence of Yeast Variables on Protease Enzyme Production

Protease activity and protein concentration also increase as the yeast extract concentration increases from 0.125% to 1%. The highest protease activity (168.13 U/ml) and protein concentration (10.74 mg/ml) are observed at 1% yeast extract concentration (Fig. 110). The results suggest that yeast extract positively affects protease production, and increasing its concentration enhances enzyme activity and protein yield. The incremental increase in protease activity and protein concentration with increasing yeast extract concentration indicates that the microorganism can efficiently utilize yeast extract as a nitrogen source. The optimal yeast extract concentration for protease production appears to be 1%, beyond which further increases may not be necessary. The results also suggest that yeast extract concentration can be fine-tuned to achieve desired protease activity and protein concentration levels.

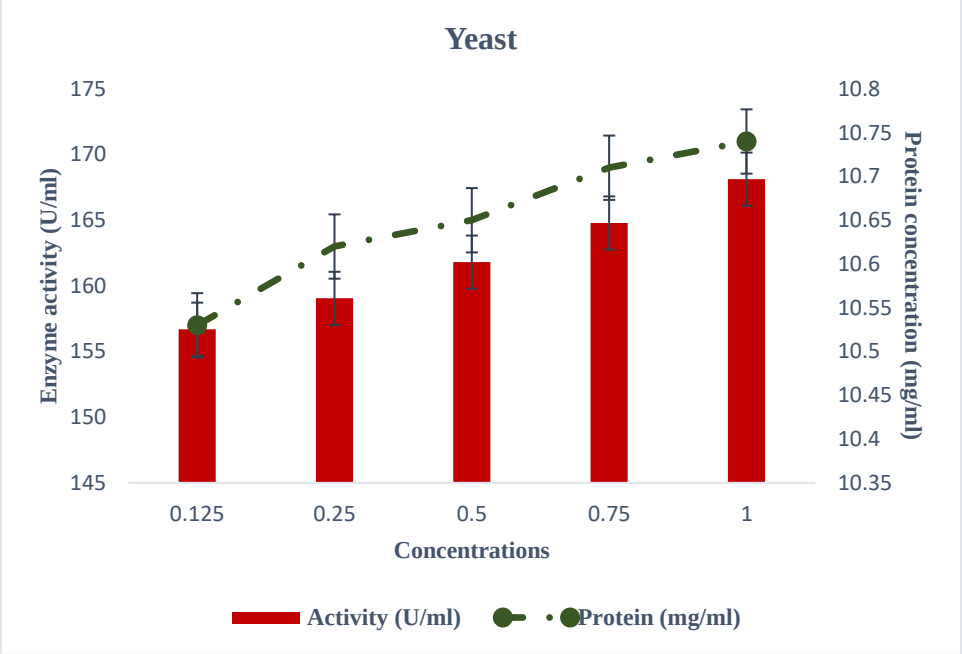


Fig.110 Impact of different concentrations of yeast on the protease enzyme production

Protease Production from *Bacillus altitudinis* BLWS10 under Submerged Fermentation (SmF) with Optimized Parameters

Protease production by BLWS10 in SmF was monitored under the conditions optimized through one variable approach. These conditions included production medium containing maltose, yeast extract, pH 8.0, incubation time 72 hr, temperature 36°C, and agitation rate 120 rpm. All the optimized conditions suggested for protease production confirmed that the highest variation showed in enzyme activity from protease production 69.54 U/ml to 168.13 U/ml.

Optimization of Fermentation Conditions for Protease Production under Submerged Fermentation using Statistical Methods

Statistical measures were undertaken to evaluate the optimization of protease production by *Bacillus altitudinis* BLWS10 using the package Design-Expert® version 10.0 (Stat Ease Inc., Minneapolis, USA). Initially, a fractional factorial Plackett-Burman design was used to screen and identify the significant parameters affecting enzyme production. Later, response surface methodology was employed to understand these significant variables' individual and interaction effects.

Screening of Significant Variables for Protease Production using Submerged Fermentation by *Bacillus altitudinis* BLWS10 using PB Design

A Plackett-Burman design was employed to identify the significant variables affecting protease production. This design analyzed eight variables, comprising six assigned variables and two unassigned dummy variables. The six assigned variables included incubation time, agitation speed (rpm), temperature, pH, maltose concentration, and yeast concentration. The design matrix and corresponding responses for screening these significant variables influencing enzyme production are presented in Table 63.

Run Order	Experimental Values						Protease Activity (U/ml)	
							Experimental	Predicted
Run	A	B	C	D	E	F		
1	-1	-1	+1	+1	+1	-1	386.25	407.83
2	-1	+1	-1	-1	+1	+1	410.25	422.47
3	+1	-1	-1	+1	+1	+1	415.56	415.36
4	+1	+1	-1	+1	-1	-1	452.3	452.19
5	+1	-1	+1	-1	-1	+1	471.52	458.31
6	-1	-1	-1	-1	-1	-1	465.87	455.84
7	+1	+1	+1	-1	+1	-1	499.47	499.36
8	-1	+1	+1	+1	-1	+1	489.32	488.97

Table 63: Eight-trial PB design matrix for six variables with coded values along with the observed and predicted protease activity

The statistical significance of the model equation was evaluated using f-test ANOVA (Table 64). The results revealed that the obtained regression is statistically significant. The model's F-value of 96.5 confirmed its statistical significance. Notably, there is only a 0.05% chance that an F-value of this magnitude could occur due to random noise. The Prob > F values less than 0.05 indicated that model terms are significant. Specifically: X1 (p-value: 0.007), X2 (p-value: 0.032), X3 (p-value: 0.041), and X6 (p-value: 0.024) demonstrated significant influence on protease production. These results confirm the reliability of the model in predicting protease production based on the selected variables.

Model F value	**96.5**
Prob > F	>0.05
Mean	90.8
R - squared	0.998
Adj R - squared	0.987
Pred R- squared	0.99
Adeq Precision	19.86
Coefficient of variance (%)	2.87

Table 64: ANOVA for the experiments with PB design for protease production by BLWS10

The influence of tested variables on protease production was calculated based on their effect estimates given in the Pareto chart (Fig.111). It may be noted that incubation time, rpm, temperature, maltose, and yeast concentration exerted significant influence on protease production by BLWS10. Temperature showed a positive effect on protease production i.e. with an increase in them, protease activity also increased. But other factors showed a negative effect i.e. with an increase there was a decrease in protease activity.

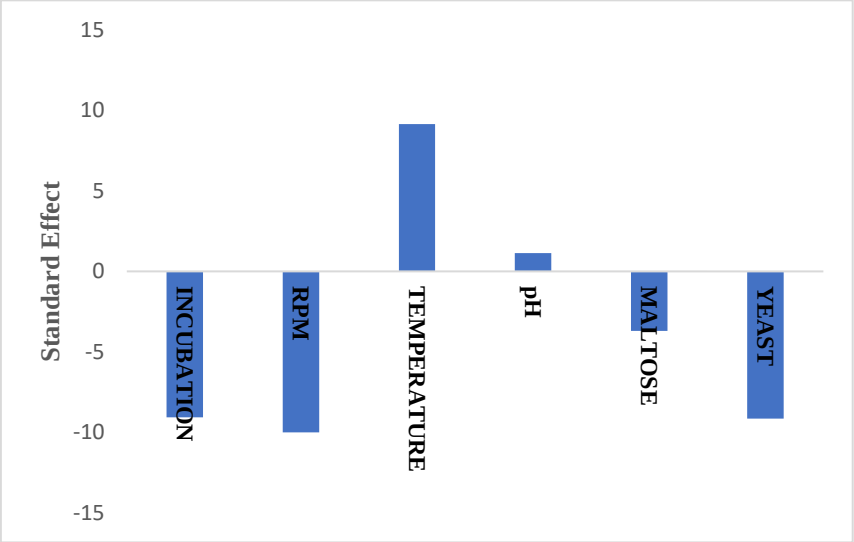


Fig.111 Pareto chart showing the effects of individual variables on protease production by BLWS10 based on PB experimental results

Experimental validation confirmed the accuracy of the PB design. The model's predictions closely matched the experimental results, demonstrating its reliability for predicting protease production (Fig.112).

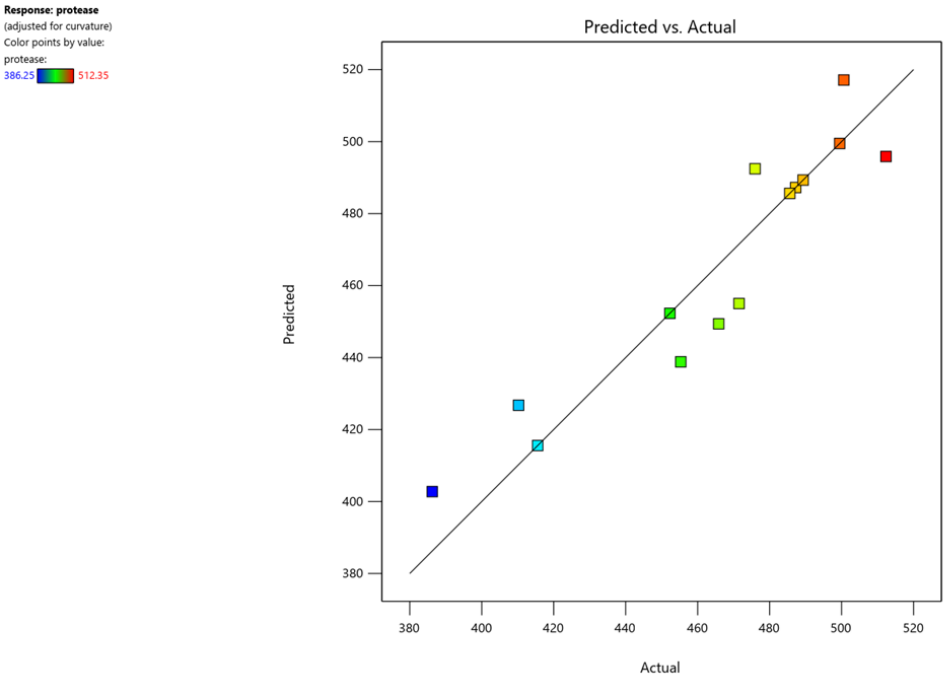


Fig.112 Validation of Plackett-Burman Design

The results obtained for the PB design showed that, among the six variables tested, four *viz.* rpm, temperature, yeast, and incubation time were statistically significant for protease production. The first-order model equation representing the response relation to independent variables was derived as below.

First-Order Model Equation:

Protease, Y (U/ml) = 67.17 + 9.145* Temperature – 9.992* rpm – 9.05* Incubation time – 9.132* Yeast

Optimization of Process Parameters for Protease Production using Response Surface Methodology

Response Surface Central Composite Design was used to determine the optimum levels of variables found to be significant based on the PB experimental design *viz.* rpm, incubation time, temperature, and yeast. The range and the levels of these variables used are given in the table. A total of 30 experiments were performed, and their corresponding results are shown in Table 65. The minimum and maximum protease activity obtained was 254.16 U/ml and 656.21 U/ml respectively. The highest protease activity was obtained in run no. 4, where the rpm was 100, incubation time 72hr, temperature 34°C, and

yeast 0.625%. The lowest activity was obtained in run no. 2, where the rpm was 90, incubation time 36hr, temperature 35°C, and yeast 0.44%.

Run no.	Rpm	Incubation time	Temperature	Yeast	Enzyme activity (U/ml)	
					Experimental	Predicted
1	-1	1	1	1	552.01	544.11
2	-1	-1	1	-1	254.16	293.45
3	-1	1	1	-1	379.58	438.20
4	0	2	0	0	656.21	587.95
5	1	-1	1	-1	591.36	544.58
6	0	0	0	2	605.38	628.82
7	-1	1	-1	-1	646.15	573.52
8	0	0	2	0	601.11	573.55
9	-1	-1	1	1	567.25	490.84
10	1	1	1	-1	617.85	633.61
11	0	0	0	0	546	544.80
12	0	0	0	0	545.25	544.11
13	0	0	0	0	546.19	545.01
14	0	-2	0	0	319.54	371.89
15	0	0	0	-2	572.15	532.80
16	0	0	0	0	580.14	544.80
17	-1	1	-1	1	482.65	558.86
18	0	0	0	0	471.12	544.80
19	-1	-1	-1	-1	354.89	355.00
20	0	0	-2	0	512.36	524.02
21	0	0	0	0	580.14	544.80
22	1	1	1	1	623.48	652.80
23	-1	-1	-1	1	461.13	431.83
24	1	-1	-1	1	499.62	470.42
25	2	0	0	0	614.23	584.39
26	1	-1	-1	-1	485.95	480.83
27	1	1	-1	-1	537.25	643.094
28	1	-1	1	1	596.18	655.27
29	-2	0	0	0	336.45	350.39
30	1	1	-1	1	594.56	655.26

Table 65: Central composite design (CCD) of factors in coded levels with protease activity as a response

The results obtained for the Central Composite experimental design were analyzed by a second-order regression model using Design Expert software and the following regression equation for protease production level.

Protease activity (U/ml) = 544.81 + 58.50 * rpm + 54.01 * incubation time + 12.38 * temperature + 24.01 * yeast – 13.93 * rpm * incubation time + 31.46 * rpm * temperature – 21.68 * rpm * yeast –18.44 * incubation time * temperature – 22.87 * incubation time * yeast + 30.14 * temperature * yeast – 19.35 * rpm^2 – 16.22 * incubation $time^2$ + 0.9947 * $temperature^2$ + 9 * $yeast^2$

Analysis of Variance (ANOVA) revealed that the model is statistically significant (Table 66), with a model F-value of 4.11 and a p-value of 0.005. The model terms are considered significant since the p-value is less than 0.0500. Specifically, X1 and X2 emerged as significant model terms, indicating their substantial influence on the response variable.

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**P-value**
Model	2.454E+05	14	17528.82	4.11	0.0050
A-rpm	82132.83	1	82132.83	19.28	0.0005
B-incubation time	70019.64	1	70019.64	16.44	0.0010
C-temperature	3679.58	1	3679.58	0.8638	0.3674
D-Yeast	13831.20	1	13831.20	3.25	0.0917
AB	3106.11	1	3106.11	0.7292	0.4066
AC	15832.56	1	15832.56	3.72	0.0730
AD	7518.19	1	7518.19	1.76	0.2039
BC	5440.91	1	5440.91	1.28	0.2762
BD	8369.96	1	8369.96	1.96	0.1813
CD	14535.32	1	14535.32	3.41	0.0845
A^2	10274.19	1	10274.19	2.41	0.1413
B^2	7216.42	1	7216.42	1.69	0.2127
C^2	27.14	1	27.14	0.0064	0.9374
D^2	2222.79	1	2222.79	0.5218	0.4812
Residual	63898.06	15	4259.87		
Lack of fit	55967.91	10	5596.79	3.53	0.0883
Pure error	7930.15	5	1586.03		

Cor Total	3.093E+05	29

Table 66: ANOVA for Response Surface Quadratic model for protease production by BLWS10

Three-dimensional response surface curves were plotted to investigate the interaction effects of variables on protease production and optimize individual factors. These curves facilitated the identification of maximum protease production by BLWS10. The model predicted a maximum protease activity of 655.27 U/ml, validated experimentally with an activity of 596.18 U/ml.

To analyze the interactive effects of variables, 3D surface curves were generated by plotting two independent variables against each other while maintaining the other variables at their central (0) level. Figures 113-118 illustrate the dependency of protease production on predicted factors, providing valuable insights into the optimization process.

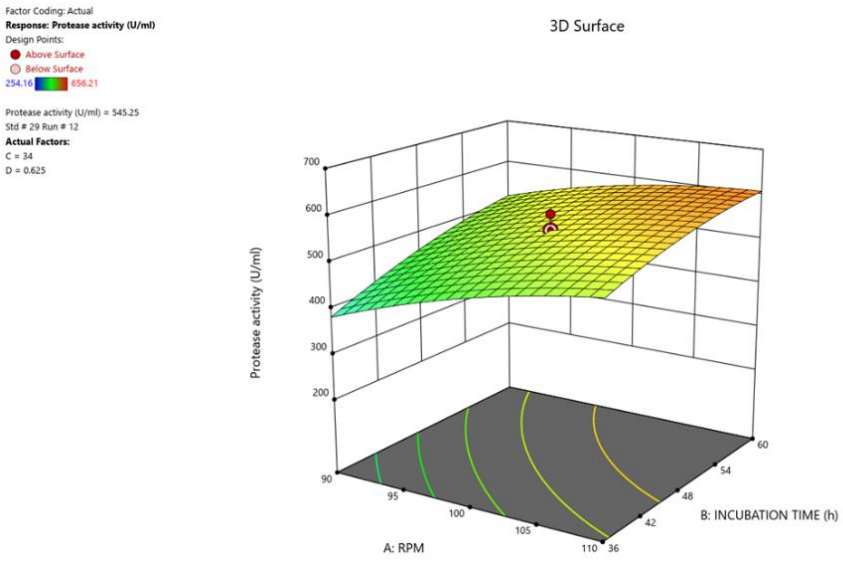


Fig.113 Three-dimensional response surface plot for protease production showing the interactive effects of the rpm and incubation time

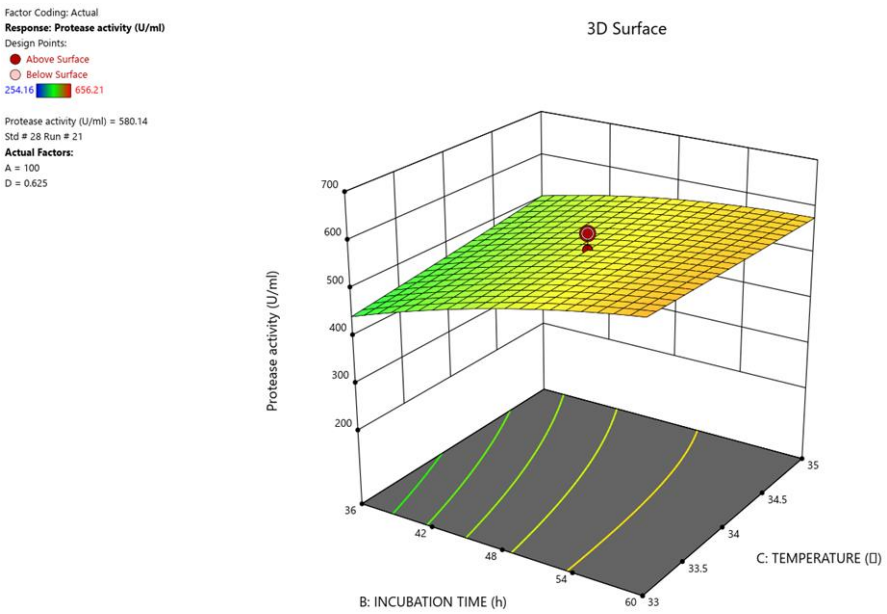


Fig.114 Three-dimensional response surface plot for protease production showing the interactive effects of the incubation time and temperature

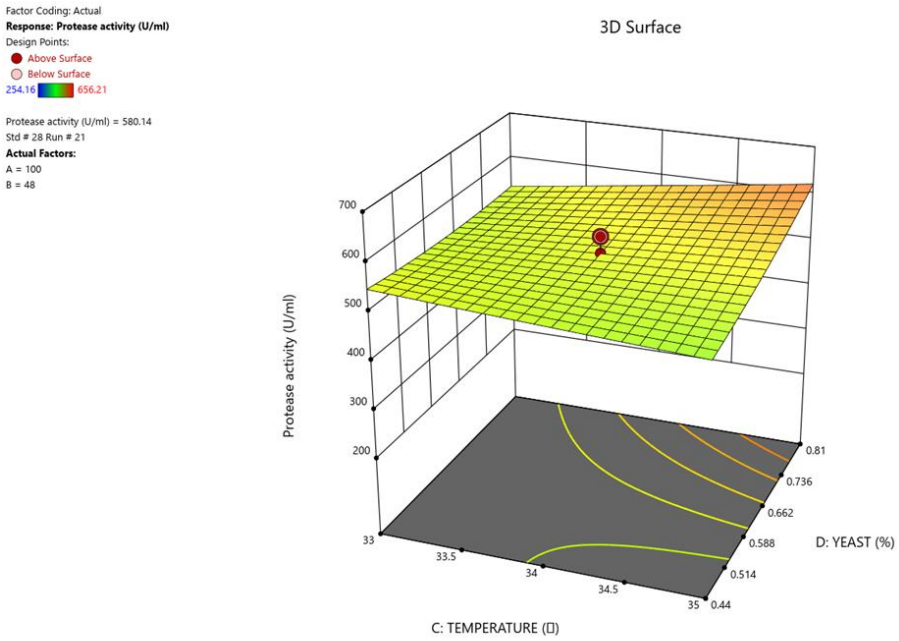


Fig.115 Three-dimensional response surface plot for protease production showing the interactive effects of the temperature and yeast

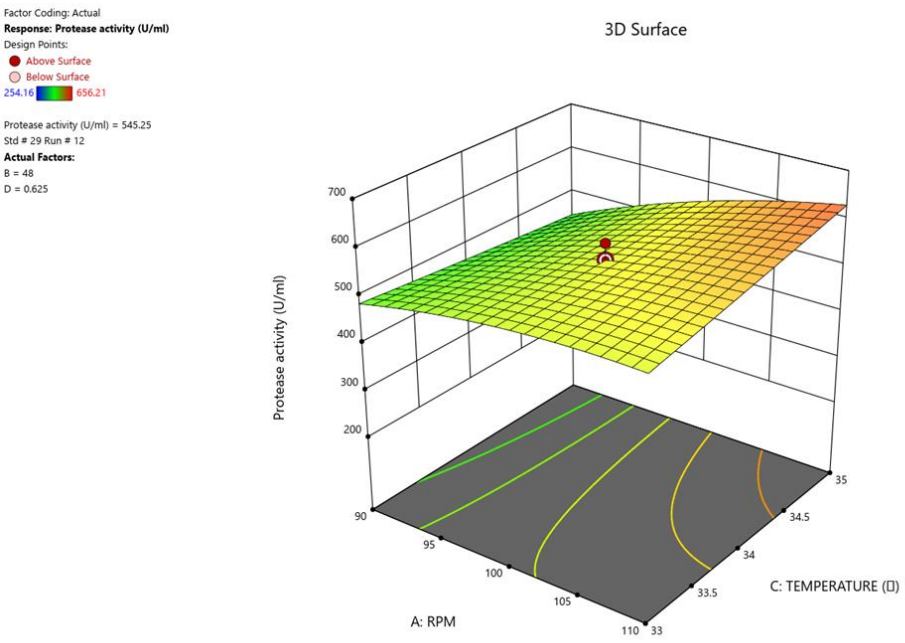


Fig.116 Three-dimensional response surface plot for protease production showing the interactive effects of the rpm and temperature

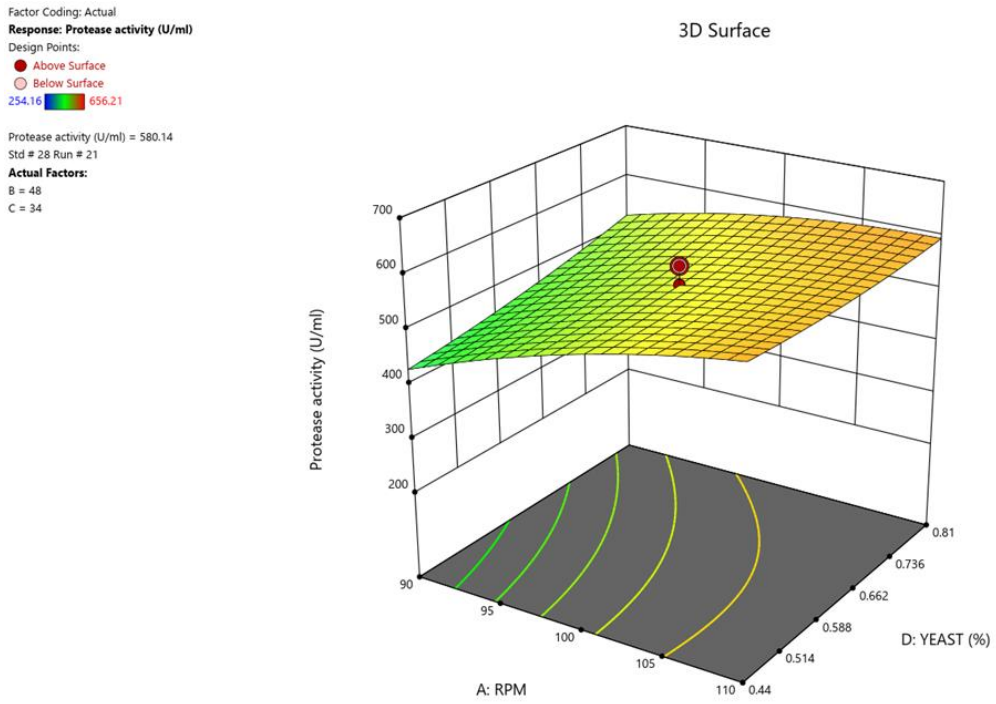


Fig.117 Three-dimensional response surface plot for protease production showing the interactive effects of the rpm and incubation time

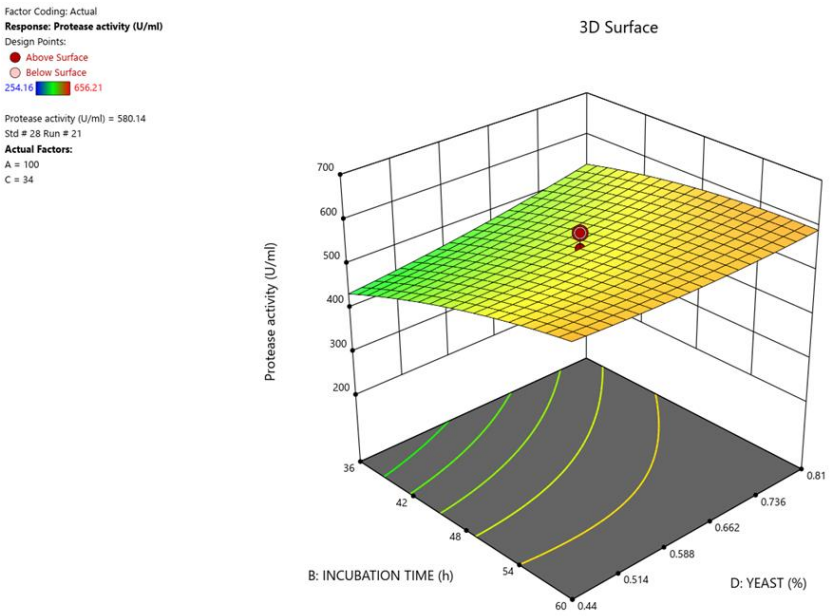


Fig.118 Three-dimensional response surface plot for protease production showing the interactive effects of the incubation time and yeast

Validation of the Response Model

To validate the predictive accuracy of the Response Surface Methodology (RSM) model, six random checkpoint formulations were analyzed for protease production. The results showed that all dependent variable values fell within the predicted limits. Table 67 presents the obtained and predicted values of the checkpoint formulations, along with the percentage prediction error. A visual representation of the correlation between observed and predicted values is provided in Figure 119. The strong linearity between experimental and predicted values is evidenced by a high R-squared value (R2) of 0.924. Furthermore, the percentage prediction error ranged from 0.21% to 4.58%, demonstrating the high prognostic capability of the RSM model. This confirms the reliability of the model for predicting protease production within the tested parameters.

Run No.	Checkpoint formulation X_1:X_2:X_3:X_4	Experimental value	Predicted value	Percentage error
1	90:60:35:0.81	552.01	544.11	-1.43%
2	100:48:36:0.625	601.11	573.55	-4.58%

3	100:48:32:0.625	512.36	524.02	2.28%
4	100:48:34:0.625	471.12	484.80	2.91%
5	100:48:34:0.625	545.25	544.11	-0.21%
6	110:36:33:0.44	485.95	480.83	-1.05%

Table 67: Checkpoint formulations, the predicted and experimental values of Response Variables, and percentage prediction error

Upon comprehensive evaluation of grid searches, formulation no. 5, as shown in Table 67, was the best media formulation for protease production as the error for the response of the dependable variables was low. The optimized media formulation was 100 rpm, 48hr incubation, 34°C temperature, and yeast 0.625%.

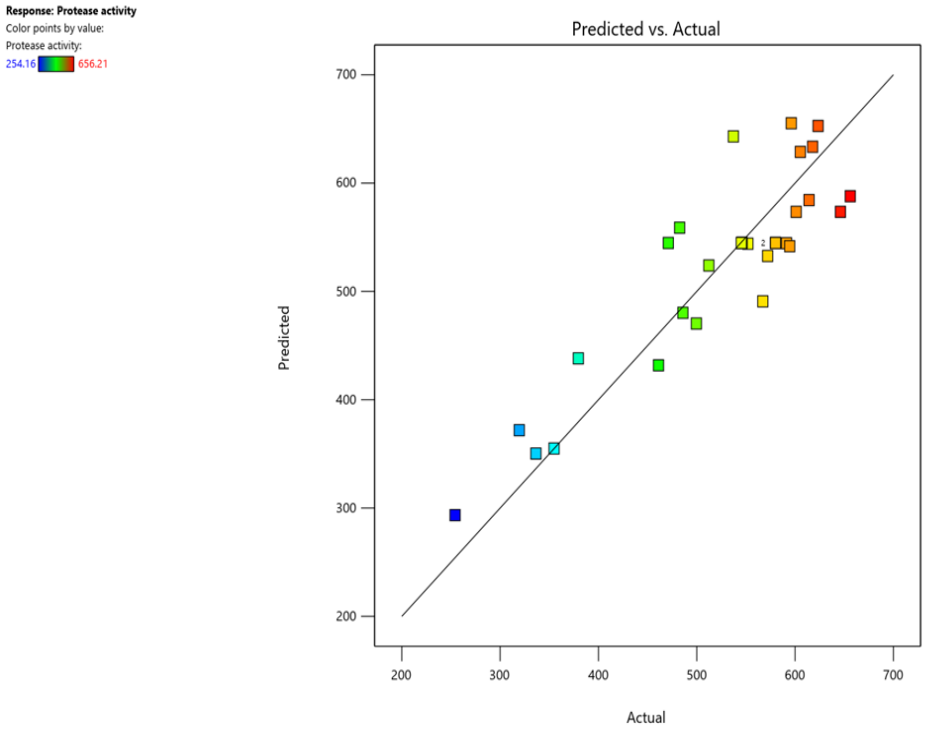


Fig.119 Linear correlation plots between observed and predicted values for protease production

Partial Purification of Protease

The enzyme from *Bacillus altitudinis* BLWS10 was produced using optimized media conditions. The crude enzyme obtained after centrifugation was subjected to further purification steps, which were carried out at 4°C.

Ammonium Sulphate Precipitation

Fractional precipitation with ammonium sulphate was carried out from 0-80%. The crude enzyme of BLWS10 employing both submerged fermentations was precipitated at 60% (w/v) saturation of ammonium sulphate, whereas the crude enzyme. The precipitate was dissolved in a minimum quantity of 10 mM tris buffer pH 7.6 and dialyzed against several volumes of the same buffer for 24 hr. The enzyme activity and protein content were determined.

Molecular Weight Determination of the Enzymes

The partially purified enzyme samples were loaded onto a 12% polyacrylamide gel to determine their molecular weight. The protein bands were visualized by Coomassie Brilliant Blue. The partially purified enzyme showed a band of approximately 27kDa (Fig.120).

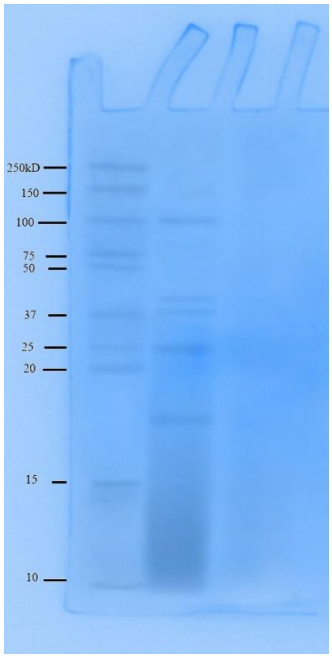


Fig.120 SDS-PAGE analysis of partially purified protease
(Lane 1: Protein ladder, Lane 2: Ammonium sulphate precipitate of crude enzyme)

Cellulase Production

Preliminary Screening of Bacterial Endophytes for Cellulase Production

The primary screening of cellulase production by endophytic bacteria revealed that out of the 35 bacterial isolates, 24 (68.57%) showed cellulase activity, with Relative Enzymatic Index (REA) values ranging from 1.054±0.061 to

8.111±1.644 (Table 68). Isolate BLWW2 showed the highest REA value of 8.111, indicating high cellulase activity. Other isolates, such as BRWM2, BLWS4, and BLWS3, also showed high cellulase activity, with REA values above 2.9 (Fig.121). The remaining 11 isolates showed no cellulase activity. This suggests that the bacterial isolates have diverse cellulase production capabilities, which could be useful for biotechnological applications, such as biofuel production and waste management. The results also show that some isolates, such as BRhWW11, and BRhWM3, have high colony diameters but relatively low REA values, indicating that they may have different cellulase production profiles.

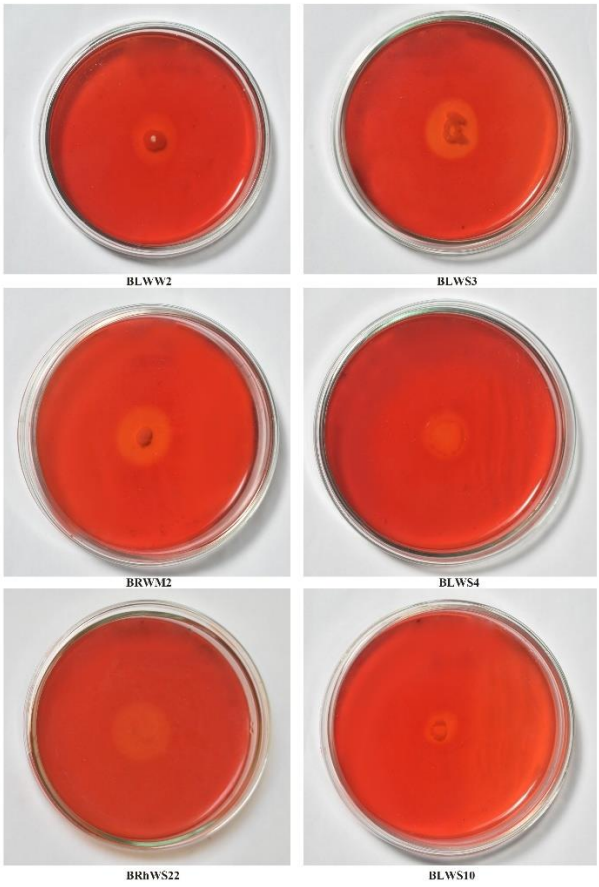


Fig. 121 Screening of cellulase production of bacterial isolates

Sl No.	Bacterial Isolates	Colony Diameter (mm)	Zone Diameter (mm)	Relative Enzymatic Index (REA)
1	BRWW12	0	0	0
2	BRWW6	0	0	0
3	BRWS13	0	0	0
4	BRWS15	0	0	0
5	BRWS1	0	0	0

6	BRWS3	12.333±1.528	18.333±1.528	1.511±0.302
7	BRWM2	10.667±0.577	32.667±2.517	3.07±0.302
8	BRWM3	20.333±2.517	27.333±2.517	1.348±0.042
9	BLWW1	19±1	24±1	1.266±0.101
10	BLWW4	15.333±0.577	18±1	1.176±0.104
11	BLWW2	2.667±0.577	21±1	8.111±1.644
12	BLWS11	0	0	0
13	BLWS6	13±1	18±1	1.392±0.166
14	BLWS7	3.333±0.577	4.667±0.577	1.417±0.22
15	BLWW5	0	0	0
16	BLWW7	16±1	19.667±1.528	1.236±0.171
17	BLWW8	21±1	23±1	1.098±0.1
18	BLWM4	25±1	26.333±1.528	1.054±0.061
19	BLWM1	9.667±0.577	11.333±0.577	1.174±0.065
20	BLWW10	0	0	0
21	BLWW9	16.333±1.528	20±1	1.233±0.15
22	BLWS10	7±1	11.333±1.528	1.663±0.465
23	BLWS9	12±2	15±2	1.263±0.176
24	BLWS4	7.667±0.577	22.333±2.517	2.94±0.561
25	BLWS2	13.333±0.577	16.667±1.528	1.249±0.083
26	BLWS1	11.667±1.528	17±2	1.486±0.36
27	BLWS3	4.667±1.528	19.333±2.082	4.611±2.15
28	BRhWS1	20±2.646	30±2	1.528±0.317
29	BRhWS8	24.667±3.055	28±3.606	1.135±0.009
30	BRhWW11	35±2	39±2.646	1.114±0.025
31	BRhWW7	0	0	0
32	BRhWM3	27±2	36.333±2.082	1.353±0.161
33	BRhWM10	0	0	0
34	BRhWM27	0	0	0
35	BRhWS22	10.333±1.528	24.333±1.528	2.374±0.196

Table 68: Primary screening of bacterial isolates for cellulase production

Secondary Screening for Cellulase Activity

Table 69 represents the enzyme activity of 24 bacterial isolates, measured in units per milliliter (U/ml). The enzyme activity values range from 13.39 to 68.617±3.821 U/ml, indicating significant variation among the isolates. The highest enzyme activity was observed in isolate BLWW2 (68.617±3.821 U/ml), followed by BRWM2 (60.633 ± 0.397 U/ml) and BLWS3 (66.72 ± 0.814 U/ml). In contrast, the lowest enzyme activity was seen in isolate BLWW9 (13.39 ± 0.448 U/ml). Most isolates exhibited moderate enzyme activity, ranging from 20 to 50 U/ml. However, some isolates showed relatively high or low enzyme activity.

Sl No.	Bacterial Isolates	Enzyme Activity (U/ml) (Mean±SD)
1	BRWS3	54.293±0.756
2	BRWM2	60.633±0.397
3	BRWM3	33.687±3.861
4	BLWW1	42.583±0.415
5	BLWW4	20.833±0.258
6	BLWW2	68.617±3.821
7	BLWS6	19.353±0.29
8	BLWS7	20.507±0.24
9	BLWW7	13.39±0.448
10	BLWW8	36.613±0.249
11	BLWM4	30.423±0.376
12	BLWM1	39.49±0.352
13	BLWW9	17.063±0.035
14	BLWS10	57.51±0.599
15	BLWS9	16.24±0.435
16	BLWS4	54.18±0.476
17	BLWS2	18.74±0.246
18	BLWS1	21.43±0.316
19	BLWS3	66.72±0.814
20	BRhWS1	14.81±0.131
21	BRhWS8	20.84±0.652
22	BRhWW11	23.377±0.225
23	BRhWM3	16.34±0.826
24	BRhWS22	55.607±2.094

Table 69: Secondary screening of bacterial isolates for cellulase production

Preliminary Screening of Fungal Endophytes for Cellulase Production

The primary screening of cellulase production by endophytic fungi revealed that out of the 36 fungal isolates, 13 (36.1%) showed cellulase activity, with Relative Enzymatic Index (REA) values ranging from 1.055±0.018 to 1.486±0.249 (Table 70). Isolate FRWM1 showed the highest REA value of 1.486, indicating high cellulase activity. Other isolates, such as FLWW5, FRhWS2, and FLWM1, also showed high cellulase activity, with REA values above 1.2. The remaining 23 isolates showed no cellulase activity. The results also show that some isolates, such as FRWW18

Fig. 122 Screening of cellulase production of fungal isolates

and FRWW11, have high colony diameters but low cellulase activity, indicating that they may have different enzymatic profiles (Fig.122).

Sl No.	Fungal Isolates	Colony Diameter (mm)	Zone Diameter (mm)	Relative Enzymatic Index (REA)
1	FLWW14	0	0	0
2	FRWS8	0	0	0
3	FLWW5	10.667±0.577	13.333±1.528	1.258±0.215
4	FLWW15	9.667±1.528	11±1	1.147±0.089
5	FLWW17	0	0	0
6	FLWW6	6.667±1.528	8±1	1.223±0.154
7	FRWS7	0	0	0
8	FRWM12	11±1	13.333±1.528	1.227±0.253
9	FLWW12	0	0	0
10	FRWM1	12±1	17.667±1.528	1.486±0.249
11	FRWS4	0	0	0
12	FLWW11	0	0	0
13	FRhWS1	0	0	0
14	FLWW18	0	0	0
15	FLWS32	0	0	0
16	FRWW18	40±2	46.333±1.528	1.162±0.096
17	FRWM25	0	0	0
18	FRWW30	10.667±1.155	12±1	1.133±0.153
19	FLWM1	12±1	15±1	1.26±0.189
20	FLWW9	0	0	0
21	FRWM27	0	0	0
22	FLWS1	11±1	12±1	1.094±0.091
23	FRhWW3	0	0	0
24	FRhWM2	0	0	0
25	FRhWS2	10.667±0.577	14.667±0.577	1.379±0.114
26	FRWM3	27±1.732	30.333±1.528	1.129±0.132
27	FLWS21	0	0	0
28	FLWS16	0	0	0
29	FRWS24	0	0	0
30	FRWW10	0	0	0
31	FRWW11	30±1	31.667±1.528	1.055±0.018
32	FRhWW4	0	0	0
33	FRWS9	0	0	0
34	FLWM11	0	0	0
35	FLWW13	0	0	0
36	FLWS5	9±1	10.667±0.577	1.191±0.08

Table 70: Primary screening of fungal isolates for cellulase production

Secondary Screening for Cellulase Activity

Table 71 represents the enzyme activity of 13 fungal isolates ranging from 13.163 to 46.817 U/ml, indicating variation among the isolates. The

highest enzyme activity was observed in isolate FRWM1 (46.817 ± 0.129 U/ml), followed by FRWW11 (29.18 ± 0.113 U/ml) and FLWW5 (33.7 ± 0.149 U/ml). In contrast, the lowest enzyme activity was seen in isolate FRWW18 (13.163 ± 0.248 U/ml). Most isolates exhibited moderate enzyme activity, ranging from 20 to 30 U/ml.

Sl No.	Fungal Isolates	Enzyme Activity (U/ml) (Mean±SD)
1	FLWW5	33.7±0.149
2	FLWW15	26.243±0.582
3	FLWW6	27.703±0.793
4	FRWM12	25.87±2.75
5	FRWM1	46.817±0.129
6	FRWW18	13.163±0.248
7	FRWW30	15.437±0.103
8	FLWM1	18.413±0.304
9	FLWS1	14.64±1.074
10	FRhWS2	25.393±0.571
11	FRWM3	16.893±0.021
12	FRWW11	29.18±0.113
13	FLWS5	25.613±0.309

Table 71: Secondary screening of fungal isolates for cellulase production

Submerged Fermentation Studies

The optimization of culture was done through a conventional method, 'one-variable-at-a-time'. The parameters optimized for maximum cellulase production were fermentation period, agitation, incubation temperature, pH, carbon sources, and nitrogen sources. From the secondary screening studies, the optimal duration of the fermentation period was observed at 24 hr. Submerged fermentation studies were conducted only in the one topmost cellulase-producing isolate *Bacillus sp*. BLWW2.

Optimization of Fermentation Conditions for Cellulase Production under Submerged Fermentation

Optimal Influence of Agitation on Cellulase Enzyme Production

As the agitation speed increases from 80 to 160 rpm, cellulase activity and protein concentration also increase. The highest cellulase activity (68.645 U/ml) and protein concentration (0.58 mg/ml) are observed at 160 rpm (Fig. 123). The results suggest that agitation speed positively affects cellulase production, and

increasing the speed enhances enzyme activity and protein yield. The incremental increase in cellulase activity and protein concentration with increasing agitation speed indicates that the microorganism can efficiently utilize the nutrients and oxygen supplied at higher agitation speeds. The optimal agitation speed for cellulase production appears to be 160 rpm, beyond which further increases may not be necessary. The results also suggest that agitation speed can be fine-tuned to achieve desired levels of cellulase activity and protein concentration.

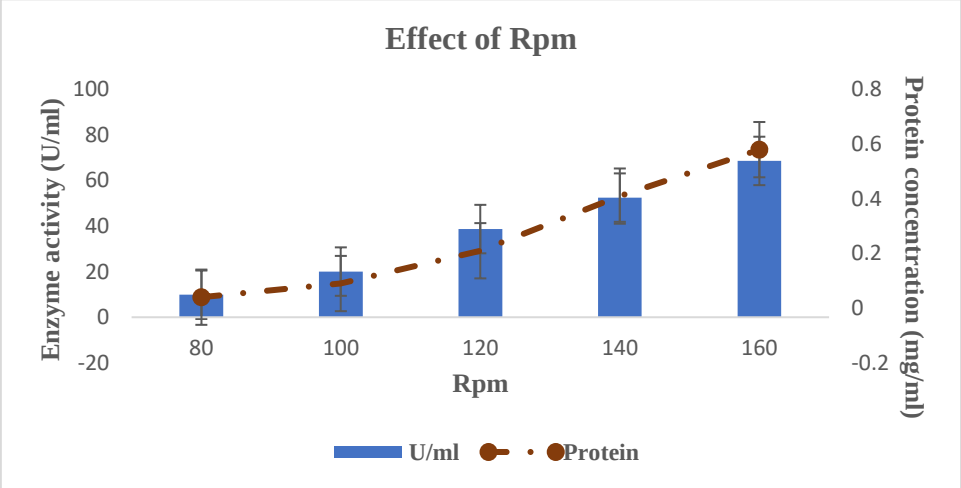


Fig.123 Impact of different agitation (rpm) on the cellulase enzyme production

Optimal Influence of Temperature on Cellulase Enzyme Production

As the temperature increases from 32°C to 38°C, cellulase activity, and protein concentration also increase, with the highest values observed at 38°C (68.69 U/ml and 0.63 mg/ml, respectively). However, further increases in temperature beyond 38°C lead to a decline in cellulase activity and protein concentration, as seen at 40°C (56.91 U/ml and 0.42 mg/ml, respectively) (Fig. 124). This suggests that 38°C is the optimal temperature for cellulase production. The results indicate that temperature has a significant impact on cellulase production, and optimal temperatures need to be identified to maximize enzyme activity and protein concentration. Temperatures above or below the optimal range may lead to reduced enzyme activity and protein yield.

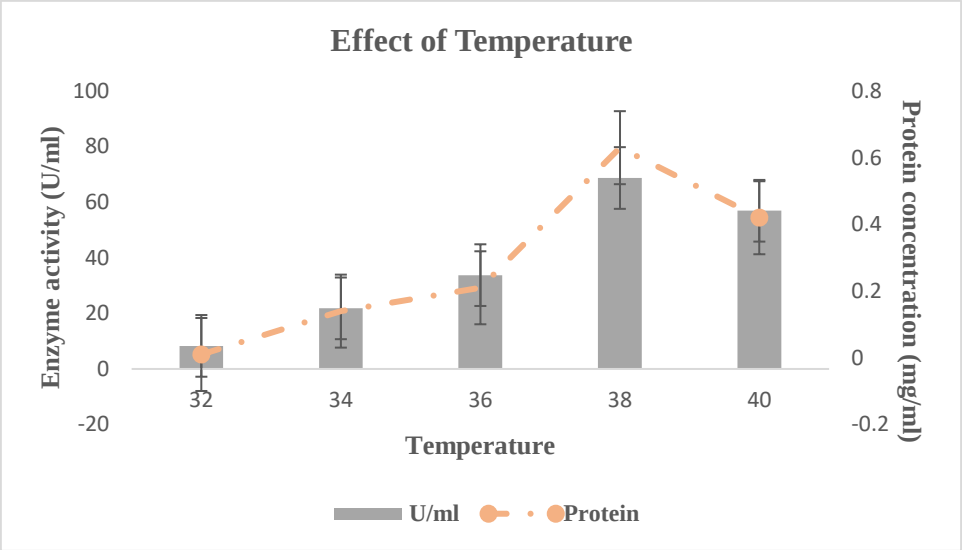


Fig.124 Impact of different temperatures on the cellulase enzyme production

Optimal Influence of pH on Cellulase Enzyme Production

As the pH increases from 4 to 8, cellulase activity and protein concentration vary, with the highest values observed at pH 8 (72.54 U/ml and 0.68 mg/ml, respectively) (Fig. 125). However, at pH 10 and 12, cellulase activity and protein concentration decrease significantly. The optimal pH for cellulase production appears to be 8, which yields the highest enzyme activity and protein concentration. pH values outside this range may lead to reduced enzyme activity and protein yield. The results indicate that pH has a significant impact on cellulase production, and optimal pH conditions need to be identified to maximize enzyme activity and protein concentration. pH affects the enzyme's structure, function, and stability, making it a critical parameter in fermentation processes.

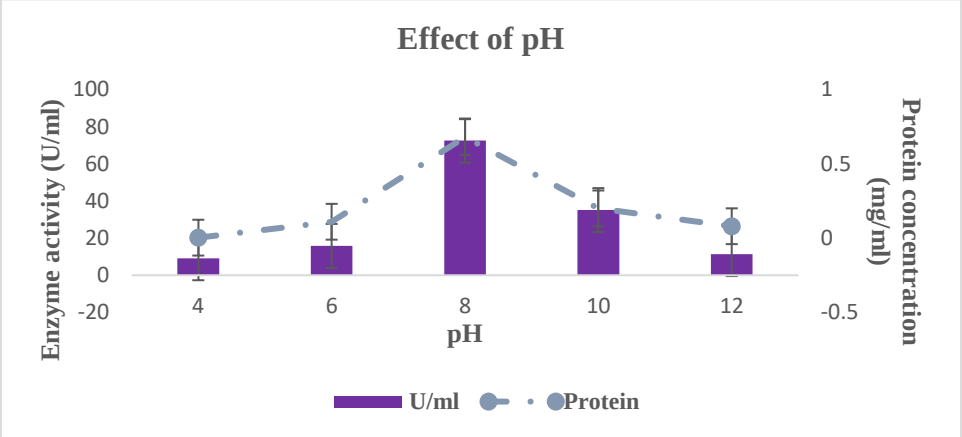


Fig.125 Impact of different pH on the cellulase enzyme production

Optimal Influence of Carbon Variables on Cellulase Enzyme Production

Carboxymethyl cellulose (CMC) is the most effective carbon source, resulting in the highest cellulase activity (72.83 U/ml) and protein concentration (0.71 mg/ml) (Fig. 126). This suggests that CMC is the preferred carbon source for cellulase production. In contrast, other carbon sources such as fructose, maltose, starch, and sucrose show lower cellulase activity and protein concentration. Fructose and starch are particularly ineffective, resulting in negligible enzyme activity and protein yield. Maltose and sucrose show moderate activity, but still significantly lower than CMC. The variation in cellulase activity and protein concentration among different carbon sources may be due to differences in their molecular structure, metabolism, or transport into the cell. CMC, being a cellulose derivative, may be more readily utilized by the microorganism for cellulase production.

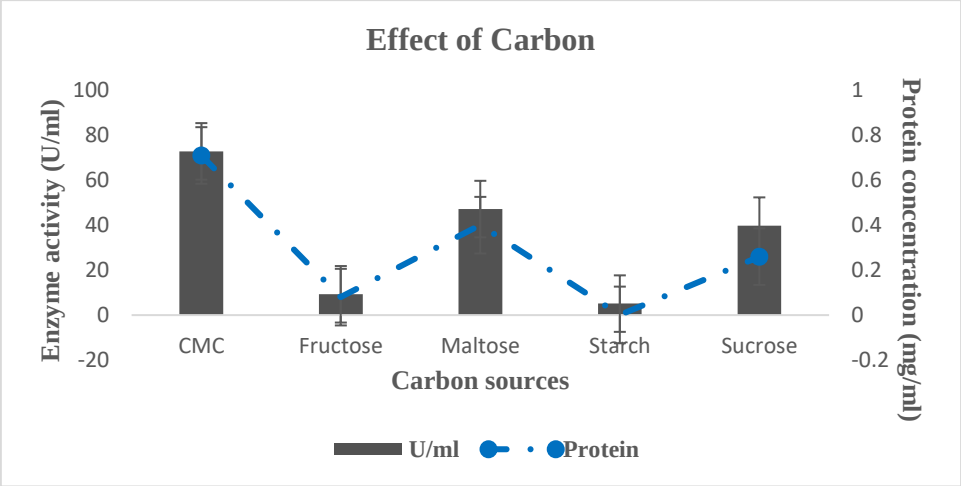


Fig.126 Impact of different carbon sources on the cellulase enzyme production

Optimal Influence of Nitrogen Variables on Cellulase Enzyme Production

Peptone is the most effective nitrogen source, resulting in the highest cellulase activity (75.98 U/ml) and protein concentration (0.79 mg/ml) (Fig. 127). This suggests that peptone provides the necessary nutrients for optimal cellulase production. In contrast, other nitrogen sources such as yeast extract, casein, ammonium phosphate, and potassium nitrate show lower cellulase activity and protein concentration. Yeast extract and casein, organic nitrogen sources, result in moderate enzyme activity, while inorganic nitrogen sources (ammonium

phosphate and potassium nitrate) result in significantly lower activity. The variation in cellulase activity and protein concentration among different nitrogen sources may be due to differences in their nitrogen content, availability, and utilization by the microorganism. Peptone, a rich source of amino acids and peptides, may provide the necessary building blocks for cellulase production.

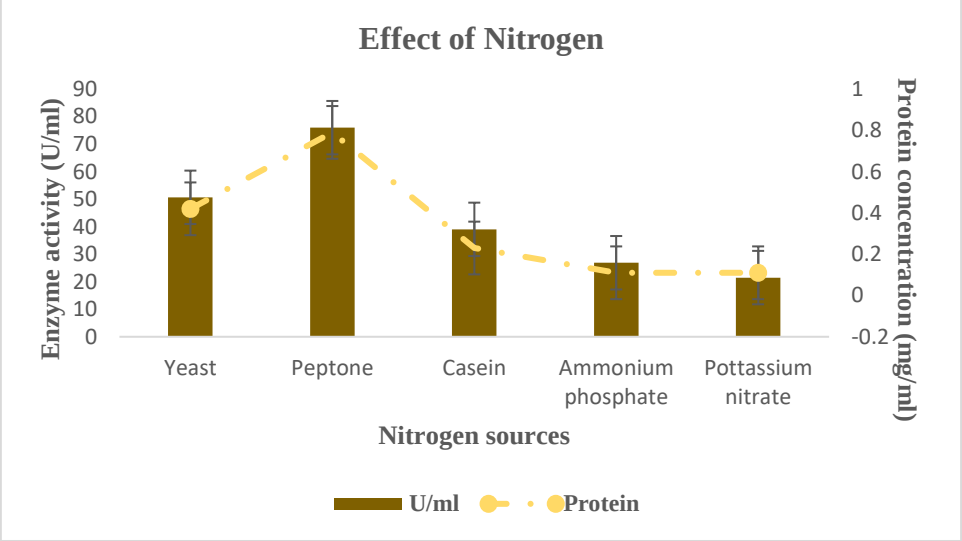


Fig.127 Impact of different nitrogen sources on the cellulase enzyme production

Optimal Influence of CMC Variables on Cellulase Enzyme Production

As the CMC concentration increases from 0.125% to 1%, cellulase activity and protein concentration also increase, with the highest values observed at 1% CMC (72.83 U/ml and 0.71 mg/ml, respectively) (Fig. 128). The results suggest that CMC has a positive effect on cellulase production, and increasing its concentration enhances enzyme activity and protein yield. The significant increase in cellulase activity and protein concentration between 0.5% and 1% CMC indicates that the microorganism can efficiently utilize CMC as a carbon source. The optimal CMC concentration for cellulase production appears to be 1%, beyond which further increases may not be necessary. The results also suggest that CMC concentration can be fine-tuned to achieve desired levels of cellulase activity and protein concentration.

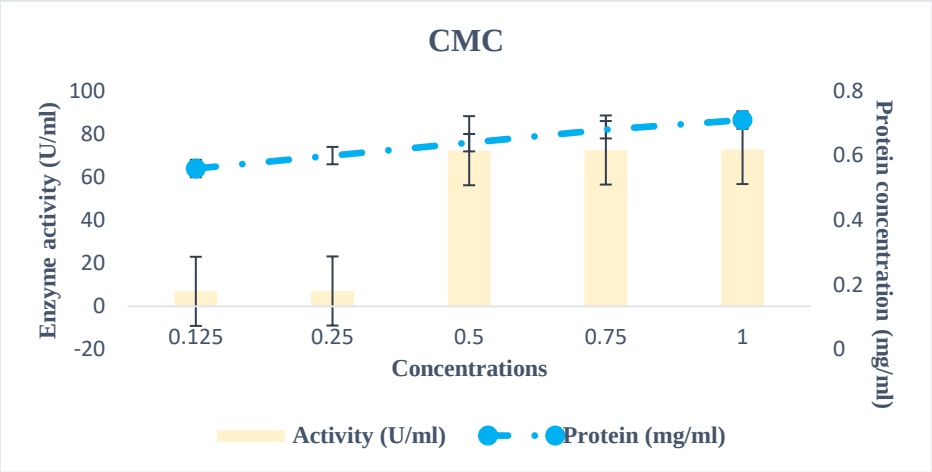


Fig.128 Impact of different concentrations of CMC on the cellulase enzyme production

Optimal Influence of Peptone Variables on Cellulase Enzyme Production

As the peptone concentration increases from 0.125% to 1%, cellulase activity and protein concentration increase slightly, with the highest values observed at 1% peptone (75.98 U/ml and 0.79 mg/ml, respectively) (Fig. 129). The results suggest that peptone positively affects cellulase production, but the effect of increasing peptone concentration is minimal beyond 0.125%. This indicates that the microorganism can efficiently utilize peptone as a nitrogen source, even at low concentrations. The optimal peptone concentration for cellulase production appears to be 0.125%, as further increases in concentration do not significantly enhance enzyme activity or protein yield. The results also suggest that peptone concentration can be fine-tuned to achieve desired cellulase activity and protein concentration levels.

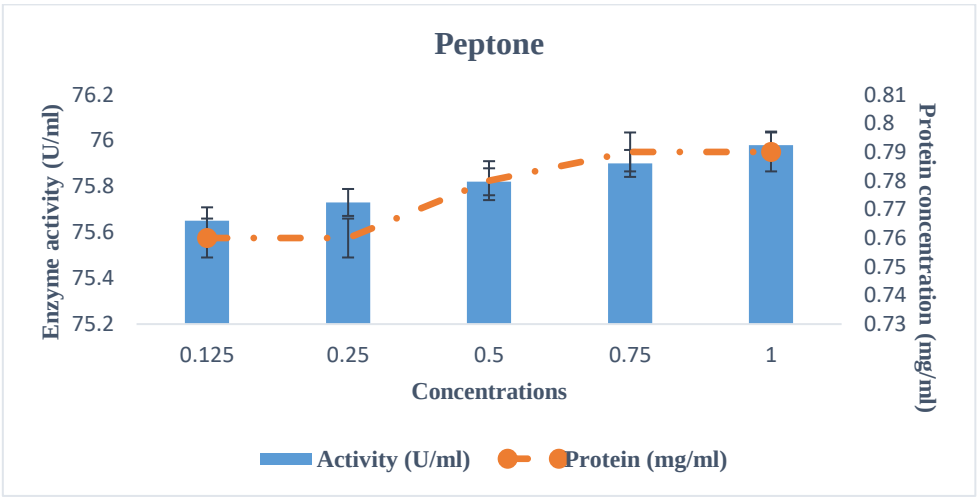


Fig.129 Impact of different concentrations of peptone on the cellulase enzyme production

Cellulase Production from *Bacillus sp.* BLWW2 under Submerged Fermentation (SmF) with Optimized Parameters

Cellulase production by BLWW2 in SmF was monitored under the conditions optimized through one variable approach. These conditions included production medium containing CMC, peptone, pH 8.0, incubation time 24 hr, temperature 38°C, and agitation rate 160 rpm. All the optimized conditions suggested for cellulase production confirmed that the highest variation showed in enzyme activity from cellulase production 68.645 U/ml to 75.98 U/ml.

Pectinase Production

Preliminary Screening of Bacterial Endophytes for Pectinase Production

The primary screening of pectinase production by endophytic bacteria revealed that out of the 35 bacterial isolates, 28 (80%) showed pectinase activity, with Relative Enzymatic Index (REA) values ranging from 1.114±0.118 to 3.444±0.139 (Table 72). Isolate BRWW12 showed the highest REA value of 3.444, indicating high pectinase activity (Fig.130). Other isolates, such as BLWS6, BRhWS1, and BLWS9, also showed high pectinase activity, with REA values above 2.0. The remaining 10 isolates showed no pectinase activity. This suggests that the bacterial isolates have diverse pectinase production capabilities, which could be useful for various biotechnological applications, such as food processing and textile manufacturing. The results also show that some isolates, such as BRWW6 and BLWS7, have high colony diameters but low pectinase activity, indicating that they may have different enzymatic profiles.

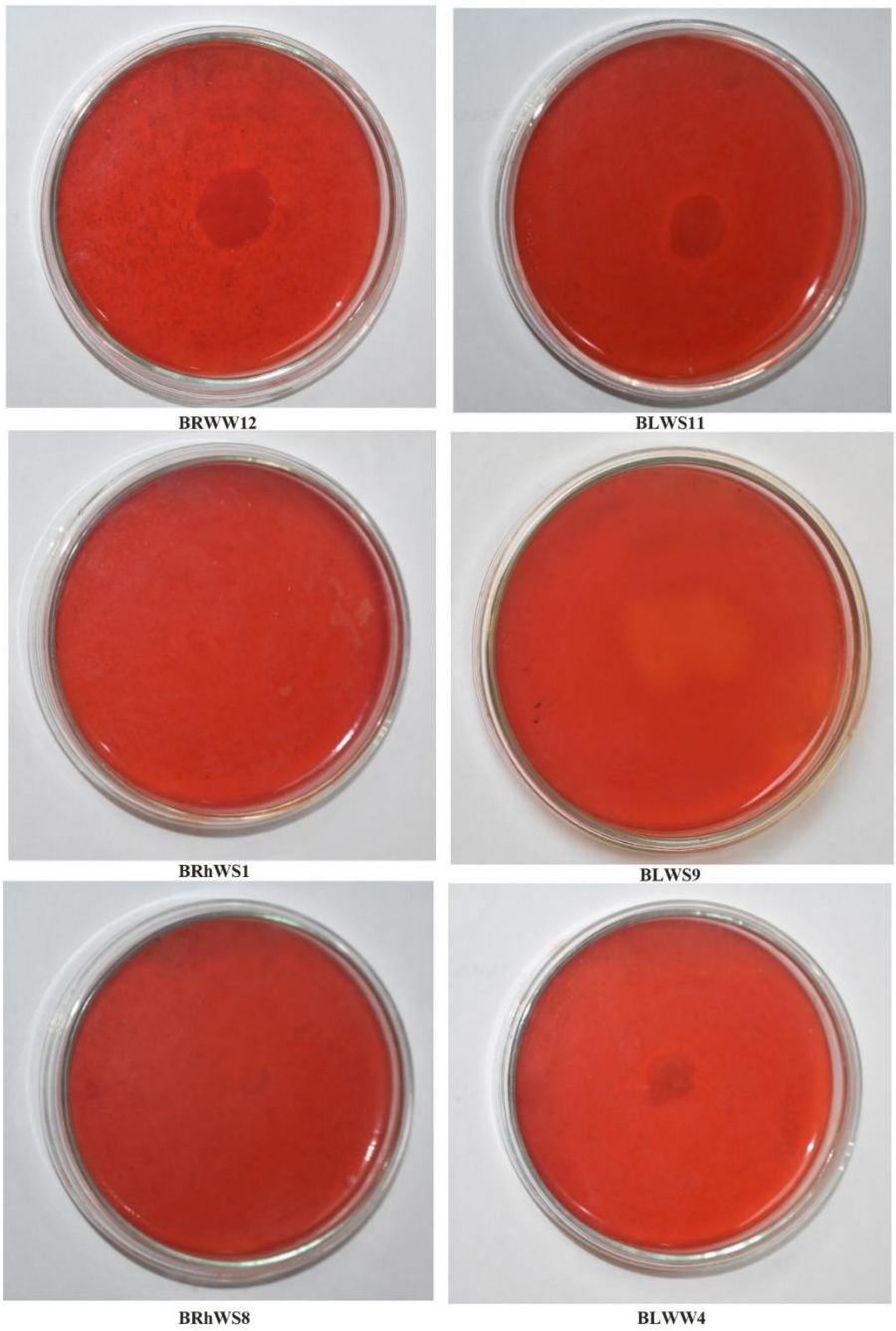


Fig. 130 Screening of pectinase production of bacterial isolates

Sl No.	Bacterial Isolates	Colony Diameter (mm)	Zone Diameter (mm)	Relative Enzymatic Index (REA)
1	BRWW12	5.333±0.577	18.333±1.528	3.444±0.139
2	BRWW6	21.333±1.528	23.667±1.528	1.114±0.118
3	BRWS13	19.333±1.528	22.333±2.517	1.155±0.09
4	BRWS15	0	0	0
5	BRWS1	0	0	0
6	BRWS3	16.667±1.155	26.333±2.082	1.583±0.144
7	BRWM2	5.333±0.577	11±1	2.089±0.379

8	BRWM3	13.333±1.528	29.667±2.517	2.237±0.23
9	BLWW1	13.667±1.528	24.333±2.082	1.802±0.32
10	BLWW4	8.667±0.577	21.333±1.528	2.468±0.215
11	BLWW2	21.667±1.528	30.333±0.577	1.406±0.128
12	BLWS11	6±1	11±1	1.879±0.453
13	BLWS6	5.667±0.577	19±1	3.367±0.219
14	BLWS7	23.667±2.517	27.667±1.528	1.177±0.136
15	BLWW5	0	0	0
16	BLWW7	17.333±1.528	24.333±2.082	1.412±0.185
17	BLWW8	11±1	18.667±1.528	1.715±0.292
18	BLWM4	11.667±1.528	19±1	1.654±0.3
19	BLWM1	17.667±1.155	21.667±2.082	1.233±0.18
20	BLWW10	0	0	0
21	BLWW9	13±1.732	25.667±1.528	2.006±0.354
22	BLWS10	0	0	0
23	BLWS9	8.667±1.528	22.333±1.528	2.615±0.334
24	BLWS4	22.667±2.517	33.667±3.512	1.495±0.198
25	BLWS2	6.667±1.528	15.333±2.517	2.456±1.024
26	BLWS1	0	0	0
27	BLWS3	0	0	0
28	BRhWS1	11±1	31.667±2.082	2.884±0.101
29	BRhWS8	8±1	20.333±1.528	2.585±0.521
30	BRhWW11	0	0	0
31	BRhWW7	0	0	0
32	BRhWM3	15±1	23±2	1.544±0.237
33	BRhWM10	9±1	11±1	1.228±0.118
34	BRhWM27	0	0	0
35	BRhWS22	8.667±1.528	15.333±1.528	1.832±0.534

Table 72: Primary screening of bacterial isolates for pectinase production

Secondary Screening for Pectinase Activity

Table 73 represents the enzyme activity of various bacterial isolates, with values represented as mean ± standard deviation (SD). The bacterial isolates' enzyme activity values range from 5.817 to 56.96 U/ml. The enzyme activity values vary significantly among the bacterial isolates. Some isolates, such as BRWW12 and BLWS6, exhibit high enzyme activity (56.96 and 40.45 U/ml, respectively), while others, like BLWM4 and BLWS7, show relatively low activity (5.817 and 7.643 U/ml, respectively). The standard deviation values indicate the variability of enzyme activity within each isolate, with some isolates (e.g., BRWM3 and BLWW2) showing higher variability (SD > 1) than others (e.g., BRWS3 and BLWS9, with SD < 0.2).

Sl No.	Bacterial Isolates	Enzyme Activity (U/ml) (Mean±SD)
1	BRWW12	56.96±1.032
2	BRWW6	20.267±0.137
3	BRWS13	24.85±0.07
4	BRWS3	18.607±0.146
5	BRWM2	25.993±0.195
6	BRWM3	22.32±1.075
7	BLWW1	10.197±0.607
8	BLWW4	30.993±0.1
9	BLWW2	27.347±1.106
10	BLWS11	25.487±0.344
11	BLWS6	40.45±0.235
12	BLWS7	7.643±1.681
13	BLWW7	17.657±0.22
14	BLWW8	15.36±0.282
15	BLWM4	5.817±0.127
16	BLWM1	11.667±0.306
17	BLWW9	20.68±0.288
18	BLWS9	35.09±0.044
19	BLWS4	14.353±0.48
20	BLWS2	31.867±0.56
21	BRhWS1	37.727±0.633
22	BRhWS8	35.457±0.315
23	BRhWM3	32.797±0.964
24	BRhWM10	22.16±0.171
25	BRhWS22	24.47±0.09

Table 73: Secondary screening of bacterial isolates for cellulase production

Preliminary Screening of Fungal Endophytes for Pectinase Production

The primary screening of pectinase production by endophytic fungi revealed that out of the 36 fungal isolates, only 8 (22.2%) showed pectinase activity, with Relative Enzymatic Index (REA) values ranging from 0.985±0.173 to 1.72±0.131 (Table 74). Isolate FRWM12 showed the highest REA value of 1.72, indicating high pectinase activity (Fig.131). Other isolates, such as FRWW18, FLWW12, and FLWM1, showed moderate to high pectinase activity, with REA values above 1.2. The remaining 28 isolates showed no pectinase activity.

Sl No.	Fungal Isolates	Colony Diameter (mm)	Zone Diameter (mm)	Relative Enzymatic Index (REA)
1	FLWW14	12.333±1.528	12±1	0.985±0.173
2	FRWS8	0	0	0
3	FLWW5	0	0	0
4	FLWW15	0	0	0
5	FLWW17	0	0	0
6	FLWW6	5±1	8±1	1.617±0.126
7	FRWS7	0	0	0
8	FRWM12	12.667±1.528	21.667±1.528	1.72±0.131
9	FLWW12	13.667±0.577	21.667±1.528	1.59±0.171
10	FRWM1	0	0	0
11	FRWS4	0	0	0
12	FLWW11	0	0	0
13	FRhWS1	0	0	0
14	FLWW18	0	0	0
15	FLWS32	24±1.732	28.333±1.155	1.182±0.039
16	FRWW18	21±2	26.667±1.528	1.274±0.087
17	FRWM25	0	0	0
18	FRWW30	0	0	0
19	FLWM1	22±2	28±1	1.277±0.071
20	FLWW9	0	0	0
21	FRWM27	0	0	0
22	FLWS1	0	0	0
23	FRhWW3	0	0	0
24	FRhWM2	0	0	0
25	FRhWS2	0	0	0
26	FRWM3	0	0	0
27	FLWS21	0	0	0
28	FLWS16	0	0	0
29	FRWS24	0	0	0
30	FRWW10	0	0	0
31	FRWW11	0	0	0
32	FRhWW4	0	0	0
33	FRWS9	0	0	0
34	FLWM11	0	0	0
35	FLWW13	0	0	0
36	FLWS5	5.667±0.577	7.333±0.577	1.3±0.12

Table 74: Primary screening of fungal isolates for pectinase production

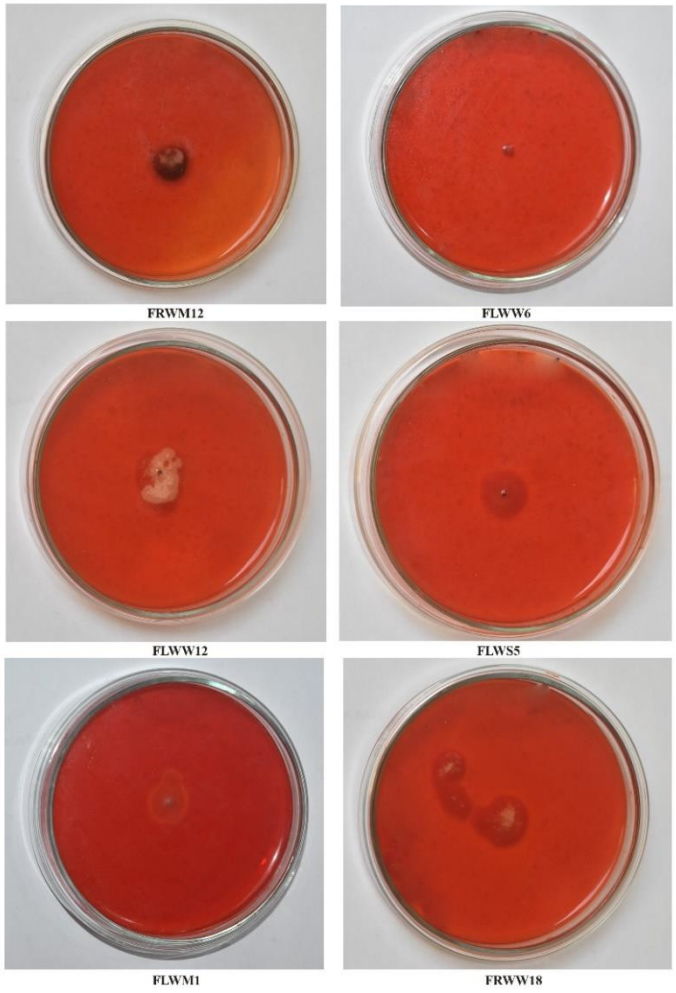


Fig. 131 Screening of pectinase production of fungal isolates

Secondary Screening for Pectinase Activity

Table 75 represents the enzyme activity of eight fungal isolates, with values represented as mean ± standard deviation (SD). The enzyme activity values vary among the bacterial isolates, with some isolates (e.g., FRWM12 and FLWW6) exhibiting high enzyme activity (39.383 and 35.74 U/ml, respectively), while others (e.g., FLWS32 and FLWW14) show relatively lower activity (14.19 and 15.453 U/ml, respectively). The standard deviation values indicate the variability of enzyme activity within each isolate, with some isolates (e.g., FLWS5) showing higher variability (SD = 0.736) than others (e.g., FLWW12 and FLWM1, with SD < 0.3). Overall, this table suggests that the fungal isolates have distinct enzyme

activity profiles, which could be relevant for understanding their metabolic capabilities or potential applications.

Sl No.	Bacterial Isolates	Enzyme Activity (U/ml) (Mean±SD)
1	FLWW14	15.453±0.242
2	FLWW6	35.74±0.44
3	FRWM12	39.383±0.516
4	FLWW12	26.993±0.255
5	FLWS32	14.19±0.44
6	FRWW18	18.23±0.382
7	FLWM1	19.527±0.237
8	FLWS5	21.063±0.736

Table 75: Secondary screening of fungal isolates for pectinase production

Submerged Fermentation Studies

The culture was optimized using the conventional "one-variable-at-a-time" approach. The incubation temperature, pH, carbon and nitrogen sources, agitation, and fermentation time were all optimized for maximum pectinase production. Based on the secondary screening investigations, 72 hours was the ideal fermentation period length. Only one of the best pectinase-producing isolates, *Bacillus ferrooxidans* BRWW12, was the subject of submerged fermentation investigations.

Optimization of Fermentation Conditions for Pectinase Production under Submerged Fermentation

Optimal Influence of Agitation on Pectinase Enzyme Production

As the agitation speed increases from 80 to 160 rpm, pectinase activity, and protein concentration increase, with the highest values observed at 160 rpm (57.01 U/ml and 0.71 mg/ml, respectively) (Fig. 132). The results suggest that agitation speed has a positive effect on pectinase production, and increasing the speed enhances enzyme activity and protein yield. The incremental increase in pectinase activity and protein concentration with increasing agitation speed indicates that the microorganism can efficiently utilize the nutrients and oxygen supplied at higher agitation speeds. The optimal agitation speed for pectinase production appears to be 160 rpm, beyond which further increases may not be necessary. The results also suggest that agitation speed can be fine-tuned to achieve desired levels of pectinase activity and protein concentration.

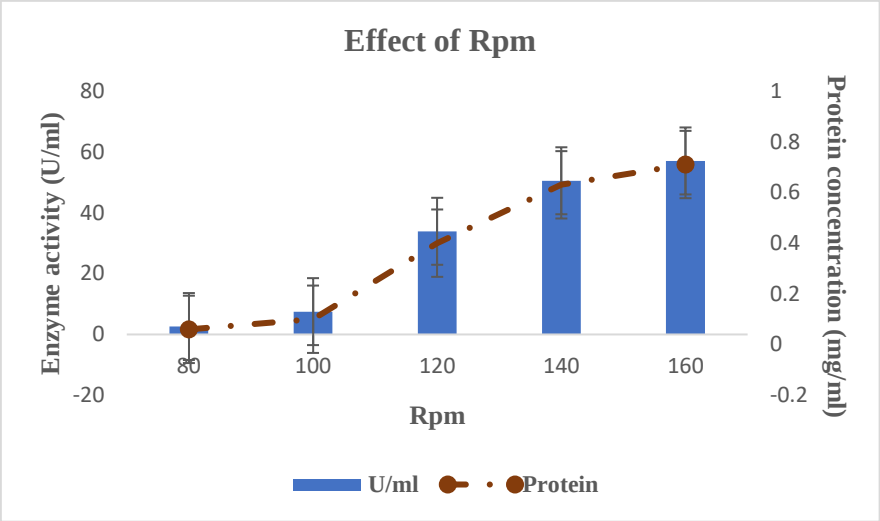


Fig.132 Impact of different agitation (rpm) on the pectinase enzyme production

Optimal Influence of Temperature on Pectinase Enzyme Production

As the temperature increases from 30°C to 50°C, pectinase activity and protein concentration increase, with the highest values observed at 50°C (57 U/ml and 0.68 mg/ml, respectively) (Fig. 133). The results suggest that temperature has a positive effect on pectinase production, and increasing the temperature enhances enzyme activity and protein yield. The increment in pectinase activity and protein concentration with increasing temperature indicates that the microorganism can efficiently utilize the nutrients and oxygen supplied at higher temperatures. The optimal temperature for pectinase production appears to be 50°C, beyond which further increases may not be necessary. The results also suggest that temperature can be fine-tuned to achieve desired levels of pectinase activity and protein concentration.

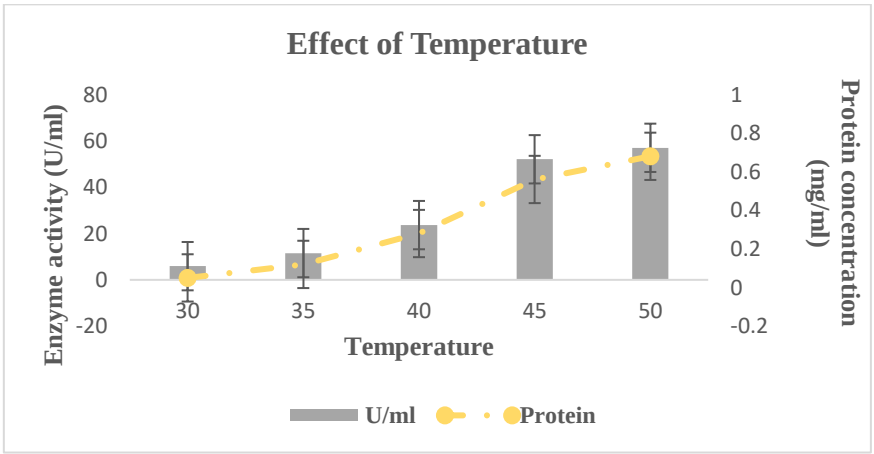


Fig.133 Impact of different temperatures on the pectinase enzyme production

Optimal Influence of pH on Pectinase Enzyme Production

As the pH increases from 4 to 6, pectinase activity and protein concentration also increase, with the highest values observed at pH 6 (58.88 U/ml and 0.75 mg/ml, respectively) (Fig. 134). However, further increases in pH beyond 6 result in a significant decrease in pectinase activity and protein concentration. The optimal pH for pectinase production appears to be 6, which suggests that the microorganism produces pectinase optimally at slightly acidic conditions. pH values outside this range may lead to reduced enzyme activity and protein yield. The results highlight the importance of optimizing pH for pectinase production and demonstrate that pH has a significant impact on enzyme activity and protein concentration.

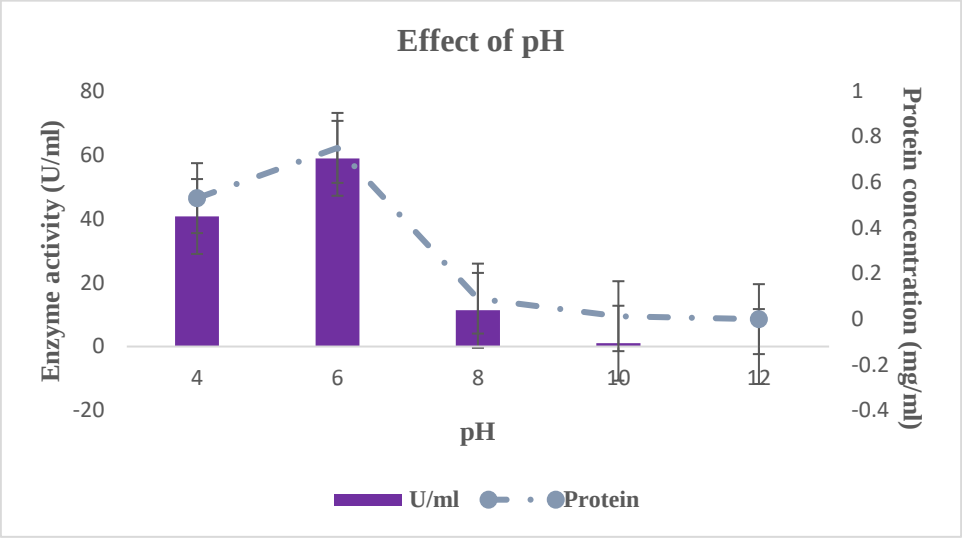


Fig.134 Impact of different pH on the pectinase enzyme production

Optimal Influence of Carbon Variables on Pectinase Enzyme Production

Sucrose is the most effective carbon source, resulting in the highest pectinase activity (59.1 U/ml) and protein concentration (0.78 mg/ml) (Fig. 135). In contrast, other carbon sources such as fructose, maltose, glucose, and galactose show lower pectinase activity and protein concentration. Fructose and galactose result in moderate enzyme activity, while maltose and glucose show significantly lower activity. The variation in pectinase activity and protein concentration among different carbon sources may be due to differences in their

molecular structure, metabolism, or transport into the cell. Sucrose, being a disaccharide, may be more readily utilized by the microorganism for pectinase production.

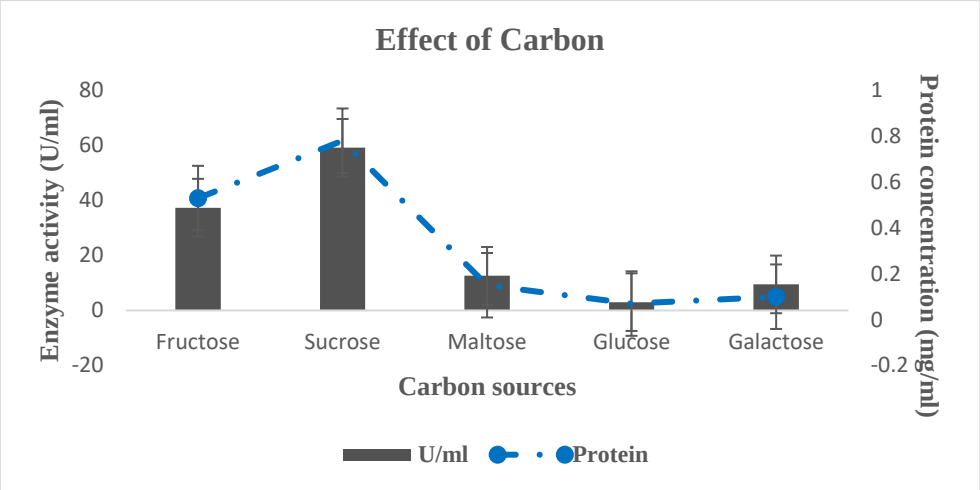


Fig.135 Impact of different carbon sources on the pectinase enzyme production

Optimal Influence of Nitrogen Variables on Pectinase Enzyme Production

It is clear from the data that different nitrogen sources have a significant impact on pectinase activity and protein concentration. Ammonium chloride is the most effective nitrogen source, resulting in the highest pectinase activity (61.09 U/ml) and protein concentration (0.82 mg/ml) (Fig.136). In contrast, other nitrogen sources such as yeast extract, peptone, sodium nitrate, and potassium nitrate show lower pectinase activity and protein concentration. Yeast extract and peptone result in moderate enzyme activity, while sodium nitrate and potassium nitrate show significantly lower activity. The variation in pectinase activity and protein concentration among different nitrogen sources may be due to differences in their nitrogen content, availability, and utilization by the microorganism. Ammonium chloride, being a readily available nitrogen source, maybe more easily utilized by the microorganism for pectinase production.

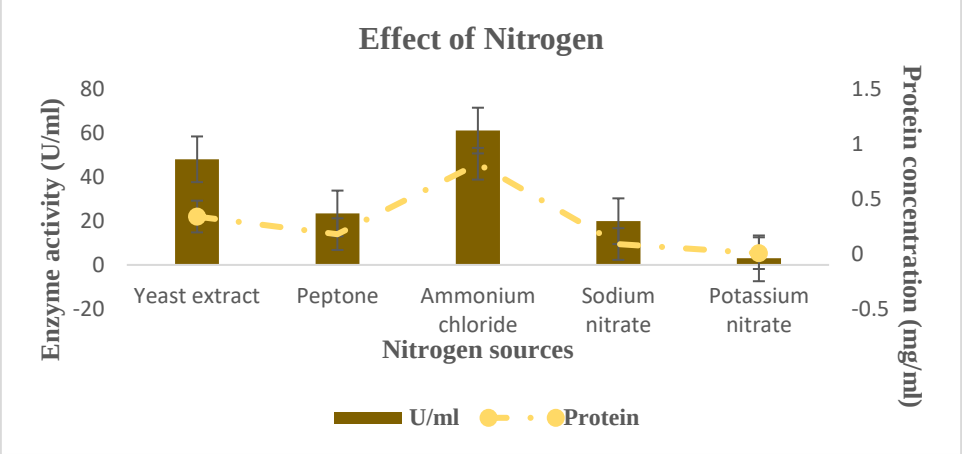


Fig.136 Impact of different nitrogen sources on the pectinase enzyme production

Optimal Influence of Sucrose Variables on Pectinase Enzyme Production

As the sucrose concentration increases from 0.125% to 1%, pectinase activity and protein concentration also increase, with the highest values observed at 1% sucrose (59.1 U/ml and 0.78 mg/ml, respectively) (Fig. 138). The results suggest that sucrose has a positive effect on pectinase production, and increasing the sucrose concentration enhances enzyme activity and protein yield. The incremental increase in pectinase activity and protein concentration with increasing sucrose concentration indicates that the microorganism can efficiently utilize sucrose as a carbon source. The optimal sucrose concentration for pectinase production appears to be 1%, beyond which further increases may not be necessary. The results also suggest that sucrose concentration can be fine-tuned to achieve desired levels of pectinase activity and protein concentration.

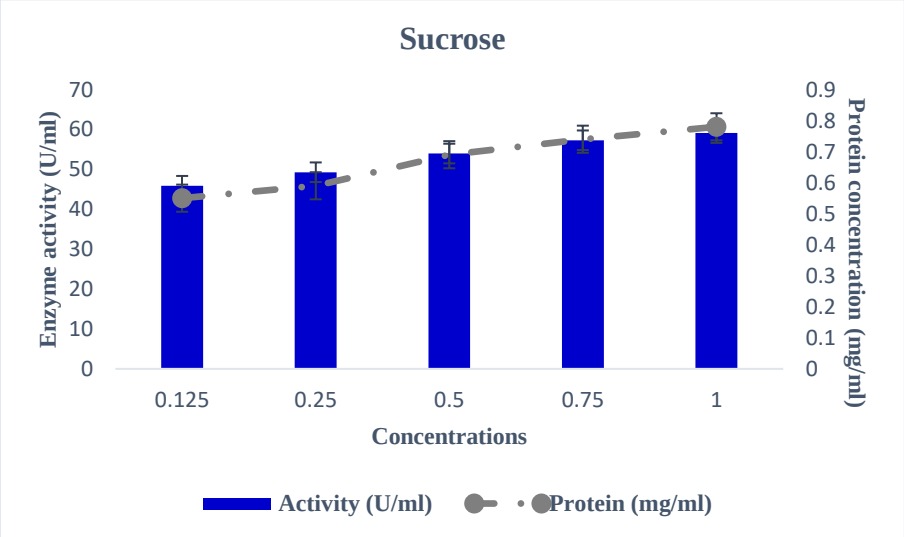


Fig.137 Impact of different concentrations of sucrose on the pectinase enzyme production

Optimal Influence of Ammonium Chloride Variables on Pectinase Enzyme Production

As the ammonium chloride concentration increases from 0.125% to 1%, pectinase activity and protein concentration also increase, with the highest values observed at 1% ammonium chloride (61.09 U/ml and 0.82 mg/ml, respectively) (Fig. 138). The results suggest that ammonium chloride has a positive effect on pectinase production, and increasing the ammonium chloride concentration enhances enzyme activity and protein yield. The incremental increase in pectinase activity and protein concentration with increasing ammonium chloride concentration indicates that the microorganism can efficiently utilize ammonium chloride as a nitrogen source. The optimal ammonium chloride concentration for pectinase production appears to be 1%, beyond which further increases may not be necessary. The results also suggest that ammonium chloride concentration can be fine-tuned to achieve desired levels of pectinase activity and protein concentration.

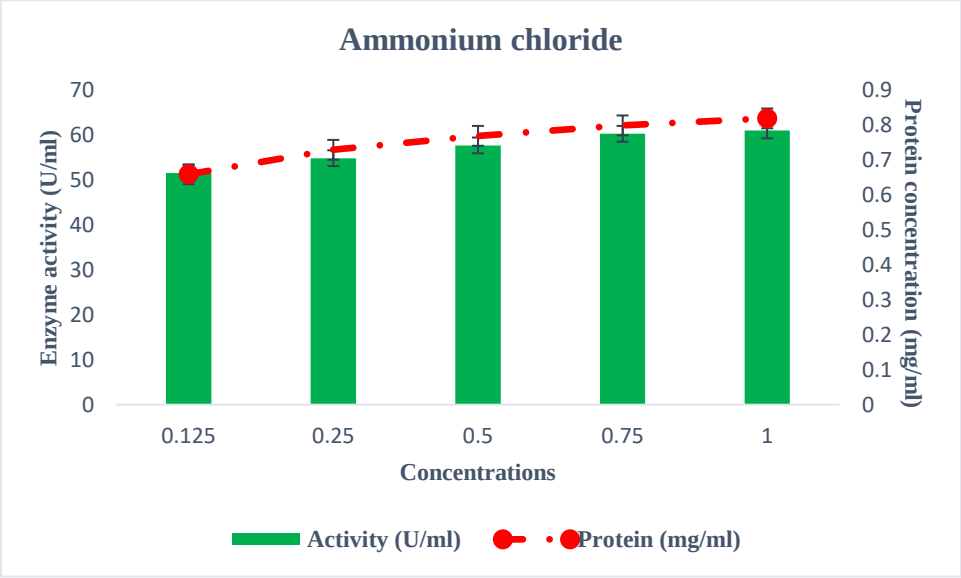


Fig.138 Impact of different concentrations of ammonium chloride on the pectinase enzyme production

Pectinase Production from *Bacillus ferrooxidans* BRWW12 under Submerged Fermentation (SmF) with Optimized Parameters

Pectinase production by BRWW12 in SmF was monitored under the conditions optimized through one variable approach. These conditions included production

medium containing sucrose, ammonium chloride, pH 6, incubation time 72 hr, temperature 50°C, and agitation rate 160 rpm. All the optimized conditions suggested for pectinase production confirmed that the highest variation in enzyme activity from 56.96 U/ml to 61.09 U/ml.

Application Studies

Blood Stain Removal using Protease Enzyme

The protease enzyme extracted from BLWS10 demonstrated exceptional efficiency in removing blood stains from cotton fabric. Under optimized conditions, the enzyme was able to break down the protein-based stains, resulting in a significant destaining effect. Observations made at 3-hour intervals showed a gradual decrease in stain intensity, with the fabric becoming increasingly cleaner (Fig.139). After 12 hours of soaking in the enzyme solution, the stain had faded to a faint mark, indicating a substantial reduction in stain intensity. The destaining intensity can be described as follows:

- 0-3 hours: Moderate stain intensity, with a noticeable decrease in color density
- 3-6 hours: Significant reduction in stain intensity, with the stain becoming lighter and less prominent
- 6-9 hours: Further decrease in stain intensity, with the stain appearing as a faint discoloration
- 9-12 hours: Minimal stain intensity, with the fabric appearing almost completely clean

The effectiveness of the protease enzyme in removing blood stains suggests its potential as a valuable additive in detergent solutions. Its ability to break down protein-based stains makes it an ideal candidate for tackling tough stains, and its efficiency under optimized conditions highlights its potential for industrial applications.

Fig. 139 Blood stain removal using crude enzyme. a. stained cloth; b. stained cloth treated with detergent; c. stained cloth treated with crude enzyme after 3 hr; d.treated after 6hrs; e. treated after 9hrs; f. treated after 12hrs.

Discussion

Enzymes are widely used biocatalysts in many industrial domains and are in great demand for bioprocesses. Current studies concentrate on finding new enzymes that meet the necessary specifications for commercial use (Hirata et al., 2013). Proteases are essential in many industrial applications such as food processing, detergents, and medicines because they break down the protein peptide bonds. Proteases are mostly found in *Bacillus* species, including *B. subtilis* (Fayek and El-Sayed, 1980), *B. cereus* (Vijayaraghavan et al., 2014), *B. polymyxa* (Fayek and El-Sayed, 1980), and others. Proteases have been recovered from a variety of bacterial species.

In this study, *B. altitudinis* BLWS10 demonstrated significant protease production under optimized conditions. This is in line with previous studies. For instance, Yang et al., 2020 reported that *B. altitudinis* produced high yields of protease when cultured in a medium containing casein as the primary nitrogen source. Similarly, our study confirmed that casein supplementation-initiated protease activity, achieving an enzyme activity of 69.54 U/ml. Moreover, the optimal pH for protease activity in the present study was found to be pH 8.

These values are consistent with the findings of Kumar et al., 2012, who reported the optimum condition of pH ranging from 8.5 to 12.5 for *B. altitudinis* and the molecular weight of 28kDa. The steadiness of the enzyme under these conditions suggests its potential for industrial applications where such environmental parameters are prevalent. The previous studies of Kumar and Borah, 2012; Kotb et al., 2023 are somewhat similar to the present study in the conditions of temperature, pH, incubation time, and carbon source (Maltose). Reddy et al., 2022 reported that *Bacillus sp.* attained their maximum protease production at 48 hr. The Plackett-Burman design is a widely employed statistical technique for identifying the most significant factors among multiple parameters, streamlining the experimentation process (Plackett and Burman, 1946). This is typically followed by Response Surface Methodology (RSM), which helps determine the optimal levels of key variables to enhance protease production (Romsomsa et al., 2010). By applying these statistical methods, researchers can significantly reduce the number of experiments, time, and costs involved, making them essential tools for optimizing enzyme production. In a study on protease enzyme production, four Bacillus isolates (*B. subtilis, B. amyloliquefaciens, B. megaterium*, and *B. licheniformis*) were optimized with different conditions and confirmed that yeast extract was the best nitrogen source (Boominadhan et al., 2009).

A study on batch fermentation of *Bacillus sp.* using response surface methodology achieved high yields of alkaline protease (1939 U/ml). This study demonstrates the importance of incubation time in maximizing protease production and highlights the potential for optimized fermentation conditions to enhance enzyme yields. From their observations, inoculum density had no significant impact, while incubation time significantly affected protease yields across all carbon and nitrogen concentrations. Optimal protease production (1939 U/ml) was achieved after 96 hours of incubation. The model equation was validated experimentally, resulting in a 2.6-fold increase in protease production (from 750 U/ml to 1939 U/ml) when compared to the mean observed response

at the zero level of all variables (Puri et al., 2002). A similar methodology was followed in the present study also supported by the statement that the incubation time and temperature showed a significant effect in protease production of 580.14 U/ml. The optimized protease production was formulated at 110 rpm, 48hr incubation, 34°C temperature, yeast 0.625%.

Chauhan and Gupta (2004) optimized culture conditions for producing a widely suited detergent protease from *Bacillus sp.* RGR-14 using a two-step statistical experimental design. They identified key factors through a screening experiment and then used response surface design to further optimize production. The study found that complex carbon and nitrogen sources like starch, casamino acid, and soybean meal favored protease production, while phosphate ions played a significant role. A 12.85-fold increase in protease production was achieved, but high casamino acid concentrations repressed production. The optimized model was validated in shake flasks, achieving 3914 U ml−1 protease production, and can be applied to cost-effective media for economic protease production.

Protease enzyme is a highly effective solution for removing blood stains due to its ability to break down protein-based substances, including hemoglobin. By hydrolyzing blood's protein components, the protease enzyme makes it easier to remove stubborn stains from various surfaces, including fabrics, carpets, and hard surfaces. Bacillus proteases boast exceptional characteristics that make them highly sought after for various industrial applications, contributing to around 60% of global enzyme sales. Their remarkable stability and activity across a wide pH and temperature range make them an ideal choice for the detergent industry, where they play a crucial role in effectively removing stubborn stains from fabrics (Annamalai et al., 2013). The benefits of using protease enzyme for blood stain removal include its gentle nature, which reduces the risk of surface damage or discoloration. Additionally, protease enzyme is biodegradable and can be used in combination with other enzymes or cleaning agents for enhanced effectiveness. While protease enzyme may not

completely remove all blood stains, especially those that have had time to set, it is a valuable tool in the cleaning arsenal, offering a safe and effective solution for removing protein-based stains. The crude enzyme extracted from *B. altitudinis* demonstrated effective blood stain removal capabilities, consistent with previous findings on proteases from various *Bacillus* and other species. This suggests that the enzyme shares similar properties and applications with proteases from other sources. Comparative studies revealed that the crude enzyme from *B. altitudinis* exhibited similar characteristics and uses as proteases from *Bacillus sp. Cab44* (Hamza, 2017), *Pseudomonas aeruginosa* (Najafi et al., 2005), *Spilosoma obliqua* (Anwar and Saleemuddin, 1997), and *Bacillus licheniformis* U1 (Dudhagara et al., 2014). These findings highlight the potential of the crude enzyme for various applications, including blood stain removal, and warrant further investigation into its industrial and commercial uses.

Cellulases and pectinases are the group of enzymes that break down cellulose into glucose units and pectin respectively playing a vital role in biofuel production, textile processing, and paper industries, fruit juice clarification, and wastewater treatment. *Bacillus sp.* has been widely studied for its cellulase production capabilities. In the present study, *Bacillus sp*. showed average cellulase activity, with an enzyme yield of 75.98 U/ml. Malik et al., 2021 studied cellulase production from *B. subtilis* revealing that CMC is a good carbon source for enzyme production. The present study nearly aligns well with the findings of Ibrahim et al., 2024, who reported the optimization of cellulase activity under similar fermentation conditions of temperature 40°C, pH 8.5 by following OFAT. Bhutani et al., 2021 revealed that the enzymatic index is a rapid method for estimating enzyme production, with more than 1.0 representing the enzyme secretion. From their study, a notable discrepancy exists between the enzymatic indices and enzyme activity for cellulase (2.79), ranging from 38.1±2.1 to 574.63±27.8 U/ml and pectinase (2.6) from 430±43.5 to 672.6±22.3 U/ml and those found in the present study. However, in the case

of proteases, the enzymatic index highlights the similarity. In the current study, *Bacillus ferooxidans* exhibited substantial pectinase activity, with an enzyme yield of 39.38 U/ml. This is comparable to the 33.15 U/ml reported by Kaur and Gupta, 2017 for pectinase production by *Bacillus subtilis* under similar conditions. The present study identified the optimal temperature and pH for pectinase activity as 50°C and pH 6 respectively. While according to Kumari et al., 2022, the optimum pH and temperature for the pectinase enzyme production were pH 7 and 37°C, respectively, which contradicts the findings of the present study.

Conclusion

The enzyme production capabilities of endophytic bacteria and fungi have far-reaching implications for agriculture, medicine, and biotechnology. These microorganisms produce diverse enzymes that enhance plant growth, health, and resilience. By producing enzymes like cellulases, ligninases, and proteases, endophytes facilitate nutrient uptake, augment stress tolerance, and bolster resistance to pathogens. Endophytes' enzymatic capabilities also present opportunities for biotechnological applications in sustainable waste management and biofuel production. They can break down lignocellulose, enabling more efficient degradation of plant biomass and reducing biofuel production costs. This makes biofuels a more sustainable and economically viable alternative to fossil fuels. Endophytic microorganisms have been discovered to produce novel enzymes with remarkable stability and activity under extreme conditions. These enzymes have potential applications in food production, pharmaceuticals, and beyond. Unraveling the mechanisms of enzyme production in endophytes can lead to the development of more efficient biocatalysts, driving green chemistry practices and innovative solutions. The relationships between endophytes and host plants have significant implications for agriculture, particularly in addressing global food security challenges.

Endophytic bacteria, specifically *Bacillus* species, are promising producers of various enzymes, including protease, cellulase, and pectinase. These enzymes

have significant industrial applications, such as in the detergent, textile, and food processing industries. Protease, for instance, is used in detergent formulations to remove protein-based stains, while cellulase and pectinase are used in textile and food processing to break down cellulose and pectin, respectively. The use of endophytic *Bacillus* species for enzyme production offers several advantages, including their ability to thrive in a wide range of environments, their genetic diversity, and their capacity to produce high levels of enzymes. Additionally, endophytic bacteria can be easily isolated from plant tissues and cultivated in vitro, making them a sustainable source of enzymes. By optimizing culture conditions and using genetic engineering techniques, the production of protease, cellulase, and pectinase by endophytic Bacillus species can be further enhanced, leading to more efficient and cost-effective enzyme production processes. Future research should focus on understanding endophyte-plant interactions and identifying specific endophytic strains that can enhance crop resilience and yield. Additionally, endophytic enzymes can be harnessed for bioremediation, promoting ecosystem health and mitigating ecological damage.

Chapter 9
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List of Publications and Presentations

Publications during the research period

Pooja, P., & Pradeep N. S. (2024). Bioprospecting of endophytic fungi for secondary metabolites and enzyme production. In. Advances in Environmental Research. Nova Science Publishers. Volume 103. pp. 197-218.

Pooja, P., Gopika, O., Gayathri, M. R., & Pradeep, N. S. (2023). Antimicrobial and Phytochemical Analysis of *Lagenandra toxicaria* Dalz. and its Fungal Endophytes. National Academy Science Letters, 46(4), 357-360.

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Presentations during the research period

Pooja, P. & Pradeep N. S. 2024. Plant Growth Promoting, Phytochemical, and Antioxidant Properties of Endophytes from *Lagenandra toxicaria* Dalz. In: 36th Kerala Science Congress, Kasaragod. Kerala State Council for Science, Technology and Environment, Kerala, 167-168.

Pooja, P. & Pradeep N. S. 2023. Optimization and Enzyme production of Endophytic Bacteria from *Lagenandra nairii* Ramam. & Rajan. In: 35th Kerala Science Congress, Idukki. Kerala State Council for Science, Technology and Environment, Kerala, 265-266.

Pooja, P. & Pradeep N. S. 2022. Comparative Study of Antimicrobial Activity and Phytochemical Analysis of *Lagenandra toxicaria* Dalz. and its Endophytic Fungus. In: 34th Kerala Science Congress, Trivandrum. Kerala State Council for Science, Technology and Environment, Kerala, 359-360.

Pooja, P. & Pradeep N. S. 2021. Screening of antimicrobial activity of endophytic fungus isolated from *Lagenandra toxicaria* Dalz., 2nd International Conference on Climate Change and its Effect on Environment, Food and Society, Arts, Commerce and Science College, Sonai, 193-194

Chapter 6

Characterization of Endophytes for Plant Growth Promotion Traits

Introduction

Endophytic microorganisms, including bacteria and fungi, have generated considerable interest in plant growth promotion due to their remarkable ability to colonize the internal tissues of plants. These microorganisms contribute to enhanced growth, increased biomass, and improved resilience against environmental stressors, making them invaluable in sustainable agricultural practices (Santos et al., 2019). In particular, evidence indicates that bacterial endophytes possess unique traits that facilitate the colonization of plant tissues, allowing them to access nutrients and interact synergistically with the plant, ultimately enhancing overall growth and yield (Chaturvedi and Singh, 2016). Furthermore, the presence of fungal endophytes can also play a crucial role by contributing to plant health and vigour through mechanisms such as nutrient assimilation and the enhancement of the plant's antioxidative defense systems, thereby promoting growth under various stress conditions (Sturz and Nowak, 2000; Santos et al., 2019; Verma et al., 2022; Mei and Flinn, 2010).

The association between endophytic microbes and their host plants is characterized by a multifaceted and adaptive interplay, where both partners benefit from the relationship. Endophytic bacteria, for instance, play a crucial role in regulating plant morphogenesis through phytohormone production. These include auxins, cytokinins, and gibberellins, which stimulate cytokinesis and cellular extension, ultimately leading to enhanced plant fitness and biomass (Sturz and Nowak, 2000; Chaturvedi and Singh, 2016; Santos et al., 2018).

Furthermore, these microbes can enhance the plant's resilience to environmental stresses, such as pathogens, pests, and extreme environmental conditions. Endophytic bacteria can deter herbivores and pathogens by producing secondary metabolites, promoting plant health and survival. For instance, certain endophytes have been shown to secrete bioactive substances with antimicrobial potency against fungal pathogens (Perez-Garcia et al., 2011).

They can induce systemic resistance within the plant, resulting in an augmented defence response.

The mutualistic relationship between endophytes and plants results in the biosynthesis of VOCs, modulating plant growth and defence, which can facilitate communication between different plant species or even attract beneficial insects. This aspect of endophytic microbial activity emphasizes the ecological significance of these interactions in natural ecosystems and indicates their potential applications in sustainable agriculture practices (Karamanoli Lindow, 2006).

Materials and Methods

The isolated endophytes were further analyzed for optimal plant development promotional traits using the following analyses:

Indole 3-Acetic Acid Production

Qualitative Analysis of Indole 3-Acetic Acid (IAA) Production of Endophytes

The production of indole-3-acetic acid (IAA) by endophytic isolates was first observed by Gordon and Weber (1951). To detect IAA production, employing a method involving inoculation of bacterial and fungal isolates into nutrient-rich broths supplemented with 0.2% L-tryptophan. Specifically, bacterial isolates were grown in 10ml of nutrient broth at 36°C for 6-10 days, while fungal isolates were cultivated in potato dextrose broth at 30°C. Following incubation, the cultures were centrifuged at 3000 rpm for 20 minutes. A distinct red colouration indicated the presence of IAA. To quantify IAA production, the researchers utilized Salkowski's reagent, comprising 0.5ml FeCl3 in 50ml of 35% HClO4. This reagent was added to 1ml of culture supernatant, and the resulting pink colour was measured spectroscopically at 535nm after 20 minutes. Uninoculated broth served as a reference, allowing for accurate quantification of IAA concentrations. By comparing absorbance values to a standard curve (Patten and Glick, 1996), the researchers were able to determine the exact amount of IAA produced by each endophytic isolate.

Quantitative Analysis of Indole 3-Acetic Acid (IAA) Production of Endophytes

To confirm indole-3-acetic acid (IAA) production, positively responding isolates were cultured in 200ml of broth supplemented with 0.2% L-tryptophan. After 6-10 days of incubation, the cultures were centrifuged, and the supernatant was acidified to pH 2.5-3.0 using 1N HCl. Ethyl acetate extraction followed, and the resulting extract was vacuum-dried. The dried powder was reconstituted in 1 ml of methanol and analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC). The HPLC system consisted of a quaternary pump, fraction collector, column oven, and degasser, coupled to a triple quadrupole mass spectrometer. Chromatographic separation was achieved on a Phenomenex Gemini NX C18 column at 40°C, employing a gradient elution method with water and acetonitrile (with 0.1% formic acid) at a flow rate of 0.3 mL/min. The 11.4-minute run time included a series of ramps and holds. The instrument was operated in multiple reaction monitoring mode, with targeted transitions for indole-3-acetic acid (IAA). Data acquisition and analysis were performed using Xcalibur and Trace Finder software. A calibration curve was generated for quantitative analysis using a series of IAA standards (0.05-5 µg/ml) prepared in methanol.

Optimization of Fermentation Conditions for IAA Production under Submerged Fermentation

The culture conditions and development constraints influencing IAA production under submerged fermentation were optimized using a one-factor-at-a-time method (OFAT) (Sindhu et al., 2009)**.**

Optimal Influence of Agitation on IAA Production

The effect of different agitation conditions, such as 120, 140, 160, 180, and 200, on IAA production by a selected isolate was investigated. A 5 ml aliquot of pre-inoculum culture was used to inoculate 95 ml of production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, IAA production in cell-free supernatant was determined.

Optimal Influence of Temperature on IAA Production

The effect of different temperature conditions such as 32°C, 34°C, 36°C, 38°C, and 40°C on enzyme production by isolates was investigated. The pre-inoculum culture (5 ml) was inoculated into 95 ml production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, enzyme activity in cell-free supernatant was determined.

Optimal Influence of pH on IAA Production

Fermentation broth for isolate was formulated using previously optimized hours. Five ml of pre-inoculum were inoculated in fermentation broth with pH 4, 6, 8, 10, and 12. Enzyme activity in cell-free supernatant was determined.

Optimal Influence of Tryptophan on IAA Production

The effect of different temperature conditions, such as 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%, on enzyme production by isolates was investigated. The pre-inoculum culture (5 ml) was inoculated into 95 ml production medium, and the flasks were placed in a shaker incubator for optimized hours. At the end of incubation, enzyme activity in cell-free supernatant was determined.

Bioactivity Test of IAA Extracts on Chilli Seeds

The experiment utilized MS medium with 0.03g/ml sucrose supplementation in a sterile petri dish (pH adjusted to 5.8 with NaOH), chilli seeds were planted to investigate the impact of the extract of an IAA-producing endophytic fungal isolate on root growth. After being surface sterilized for one minute with 50% ethanol, the seeds were treated with 0.1% $HgCl_2$ and then rinsed with sterile distilled water. The seeds were put on the agar plates, 5 on each plate. After germination 4 seeds in each plate were treated by the fungal extract. Seeds were grown at 25°C with alternating 12-hour light and dark periods. Following germination, plates were positioned at a 50° angle to facilitate plant growth. Sterile cork burrs were used to drill 5 mm holes, 0.5 cm from the capsicum seedlings' root tips, and the holes were then filled with 20 µl of methanolic solutions containing fungal extracts. The seed with conditions of untreated, treated nutrient broth with tryptophan, and methanol were used for the study.

Every 3-day interval after germination, the primary root growth, adventitious root, and shoot were measured. The experiment was done in triplicate.

Statistical Analysis of Data

Statistical analyses were executed using GraphPad Prism version 7.0 for Windows. They utilized a one-way analysis of variance (ANOVA) model with Bonferroni's correction for multiple comparisons to determine significant differences in plant fitness parameters.

Phosphate Solubilization

Qualitative Analysis of Phosphate Solubilization of Endophytes

To evaluate the phosphate solubilization ability of endophytic isolates, a qualitative assessment was conducted using Pikovaskaya's medium (Verma et al., 2011). Spot inoculation was performed, and the isolates were incubated at 36°C (bacteria) and 30°C (fungi) for 5-7 days. Phosphate solubilization was indicated by the appearance of a clear yellow zone surrounding microbial growth. The solubilization index (PSI) was calculated to quantify the isolates' ability to solubilize tricalcium phosphate (TCP) on Pikovaskaya's agar medium. The PSI was determined using the formula:

PSI index = (Total diameter of colony + halo zone) / Colony diameter

This method allowed for the identification of phosphate-solubilizing isolates and the estimation of their solubilization efficiency.

Quantitative Estimation of Phosphate Solubilization of Endophytes

Phosphate solubilizers were analyzed further to find out more about quantitative phosphate solubilization. The phosphomolybdate blue colour technique was used to determine the amount of accessible phosphorus. 250 ml flasks were filled with 100 ml of Pikovaskaya’s broth (adjusted to pH 7) and 0.4 g of tricalcium phosphate per 100 ml. For 20 minutes, the flasks were autoclaved at 121°C. One milliliter of each phosphate-solubilizing bacterial strain was injected into each autoclaved flask, and the flasks were then shaken at 10,000 rpm for ten days. For fifteen minutes, the suspension was centrifuged at 10,000 rpm to extract any insoluble materials and germs. A spectrophotometer was used

to measure the available phosphorous (P) at 882 nm, and a reference KH_2PO_4 curve was used to calibrate the results (Venkateswarlu et la., 1984).

ACC (1-Aminocyclopropane-1-Carboxylate) Deaminase Production

Qualitative Estimation of ACC Deaminase Production of Endophytes

ACC deaminase production was qualitatively evaluated using the method of Jasim et al. (2015). Isolates were grown on DF minimal medium with ACC as the nitrogen source. Growth observed after incubation at 28°C for 5-7 days indicated ACC deaminase activity.

Quantitative Estimation of ACC Deaminase Production of Endophytes

The selected isolates were cultivated on DF minimal medium individually containing $(NH_4)_2SO_4$, with ACC and without ACC in intervals of 2 to 10 days. The growth of endophytic isolate in different media was measured by dry weight (Shahzad et al., 2010).

Results

Indole 3-Acetic Acid Production

Qualitative Analysis of Indole 3-Acetic Acid (IAA) Production of Endophytic Bacteria

The most significant auxin found naturally in higher plants is IAA. It is among the auxins with the highest physiological activity. It encourages plants to grow in reaction to light and gravity. Its main purpose is to promote plant growth. It encourages the elongation of plant cells. Plant orientation can be influenced by it as it stimulates cell division in response to gravity and sunlight. It essentially affects plants in four main ways: it encourages fruit development, controls seedling orientation, stimulates root branching, and elongates shoots. It enhances root growth, length, and surface area, boosting water and nutrient uptake. When colonizing plant tissues, the bacteria that produce IAA have an advantage over their competitors. Bacterial IAA production causes plant cell walls to relax and encourages root exudation. Contrarily, this offers a variety of nutrients that promote bacterial growth. IAA can also play a very significant role as a signal molecule by controlling the host plant's root development and improving the relationship between the plant and the microbe. A two-step auxin

biosynthesis pathway in plants converts tryptophan to IAA by first converting it to indole-3-pyruvate (IPA) and then back to IAA. The primary pathway for auxin biosynthesis consists of this two-step conversion of tryptophan to IAA.

The qualitative production of indole-3-acetic acid (IAA) was investigated in 35 isolates using the Salkowski reagent, yielding positive results in bacterial isolates as evidenced by pink to deep red coloration. (Fig.140). The bacterial isolates exhibited a wide range of IAA concentrations, varying from 0 to 243.45 µg/ml. The highest IAA concentration was observed in isolate BRhWW7, with a value of 243.452 ± 0.007 µg/ml on the 10^{th} day of incubation (Table 76). Other isolates with high IAA concentrations included BRWS15 (110.096 ± 0.064 µg/ml), BRWS1 (95.766 ± 0.006 µg/ml), and BRWM3 (94.951 ± 0.046 µg/ml). In contrast, several isolates showed relatively low IAA concentrations, including BLWW5 (4.561 ± 0.008 µg/ml), BLWS10 (10.349 ± 0.02 µg/ml), and BLWS9 (12.03 ± 0.03 µg/ml). Two isolates, BLWS11 and BLWW10, showed no detectable IAA production. Most isolates exhibited moderate IAA concentrations, ranging from 20 to 70 µg/ml. Examples of these isolates include BRWW6 (43.844±0.051 µg/ml), BRWS13 (51.936 ± 0.005 µg/ml), and BLWW1 (55.935 ± 0.017 µg/ml).

Fig. 140 Screening of Indole 3- Acetic Acid production of bacterial isolates

Sl No.	Bacterial isolates	Concentration (µg/ml)
1	BRWW12	3.25±0.038
2	BRWW6	43.84±0.051
3	BRWS13	51.93±0.005
4	BRWS15	110.09±0.064
5	BRWS1	95.76±0.006
6	BRWS3	29.71±0.024
7	BRWM2	92.86±0.017
8	BRWM3	94.95±0.046
9	BLWW1	55.93±0.017
10	BLWW4	51.36±0.014
11	BLWW2	53.65±0.029
12	BLWS11	0
13	BLWS6	31.45±0.015
14	BLWS7	60.37±0.016
15	BLWW5	4.56±0.008
16	BLWW7	47.81±0.016
17	BLWW8	46.96±0.03
18	BLWM4	38.35±0.021
19	BLWM1	89.23±0.022
20	BLWW10	0
21	BLWW9	64.48±0.051
22	BLWS10	10.34±0.02
23	BLWS9	12.03±0.03
24	BLWS4	62.65±0.04
25	BLWS2	47.55±0.007
26	BLWS1	68.14±0.029
27	BLWS3	48.31±0.015
28	BRhWS1	19.87±0.01
29	BRhWS8	65.70±0.009
30	BRhWW11	21.32±0.012
31	BRhWW7	243.45±0.007
32	BRhWM3	26.69±0.008
33	BRhWM10	22.16±0.013
34	BRhWM27	63.82±0.011
35	BRhWS22	17.57±0.09

Table 76: Qualitative analysis of Indole 3-Acetic Acid (IAA) production of endophytic bacteria

Qualitative Analysis of Indole 3-Acetic Acid (IAA) Production of Endophytic Fungi

The qualitative IAA production of 36 isolates was examined. Using the Salkowki reagent to produce a pink to deep red colour, the fungal isolates demonstrated their capacity to produce IAA (Fig.141). The fungal isolates exhibited a wide range of IAA concentrations, varying from 0 to 153.264 µg/ml (Table 77). The highest IAA concentration was observed in isolate FLWW6, with 153.264 ± 0.015 µg/ml values. Other isolates with high IAA concentrations

included FLWS1 (129.291 ± 0.026 µg/ml), FLWS21 (121.28 ± 0.01 µg/ml), and FLWW15 (91.862 ± 0.017 µg/ml). In contrast, several isolates showed relatively low IAA concentrations, including FRhWM2 (5.855 ± 0.005 µg/ml), FLWW13 (5.334 ± 0.006 µg/ml), and FRhWW3 (9.959 ± 0.012 µg/ml) (Fig.142). Notably, four isolates, FRWM12, FRWM1, FRWM25, and FRWM27, showed no detectable IAA production. Most isolates exhibited moderate IAA concentrations, ranging from 20 to 70 µg/ml. Examples of these isolates include FLWW14 (15.761 ± 0.008 µg/ml), FLWW5 (51.077 ± 0.016 µg/ml), and FRWS7 (61.775 ± 0.008 µg/ml). These findings suggest that the fungal isolates have varying capacities for IAA production, which could impact plant growth-promoting and environmental interaction abilities. Some isolates showed IAA concentrations between 60 and 100 µg/ml, such as FRWS8 (69.659 ± 0.025 µg/ml), FRWS7 (61.775±0.008 µg/ml), and FRWM27 (64.88 ± 0.01 µg/ml). Due to their moderate to high IAA production, these isolates may have potential applications in agriculture and environmental remediation.

Sl No.	Fungal isolates	Concentration (µg/ml)
1	FLWW14	15.76±0.008
2	FRWS8	69.65±0.025
3	FLWW5	51.07±0.016
4	FLWW15	91.86±0.017
5	FLWW17	36.98±0.01
6	FLWW6	153.26±0.015
7	FRWS7	61.77±0.008
8	FRWM12	0
9	FLWW12	47.74±0.002
10	FRWM1	0
11	FRWS4	57.62±0.031
12	FLWW11	33.51±0.007
13	FRhWS1	12.25±0.012
14	FLWW18	49.44±0.012
15	FLWS32	24.73±0.028
16	FRWW18	60.53±0.015
17	FRWM25	0
18	FRWW30	59.13±0.025
19	FLWM1	11.15±0.013
20	FLWW9	15.64±0.025
21	FRWM27	64.88±0.01
22	FLWS1	129.29±0.026
23	FRhWW3	9.95±0.012

24	FRhWM2	5.85±0.005
25	FRhWS2	29.47±0.005
26	FRWM3	0
27	FLWS21	121.28±0.01
28	FLWS16	17.60±0.009
29	FRWS24	39.85±0.012
30	FRWW10	37.70±0.009
31	FRWW11	42.47±0.009
32	FRhWW4	42.33±0.456
33	FRWS9	38.04±0.037
34	FLWM11	12.56±0.012
35	FLWW13	5.33±0.006
36	FLWS5	19.91±0.01

Table 77: Qualitative analysis of Indole 3-Acetic Acid (IAA) production of endophytic fungi

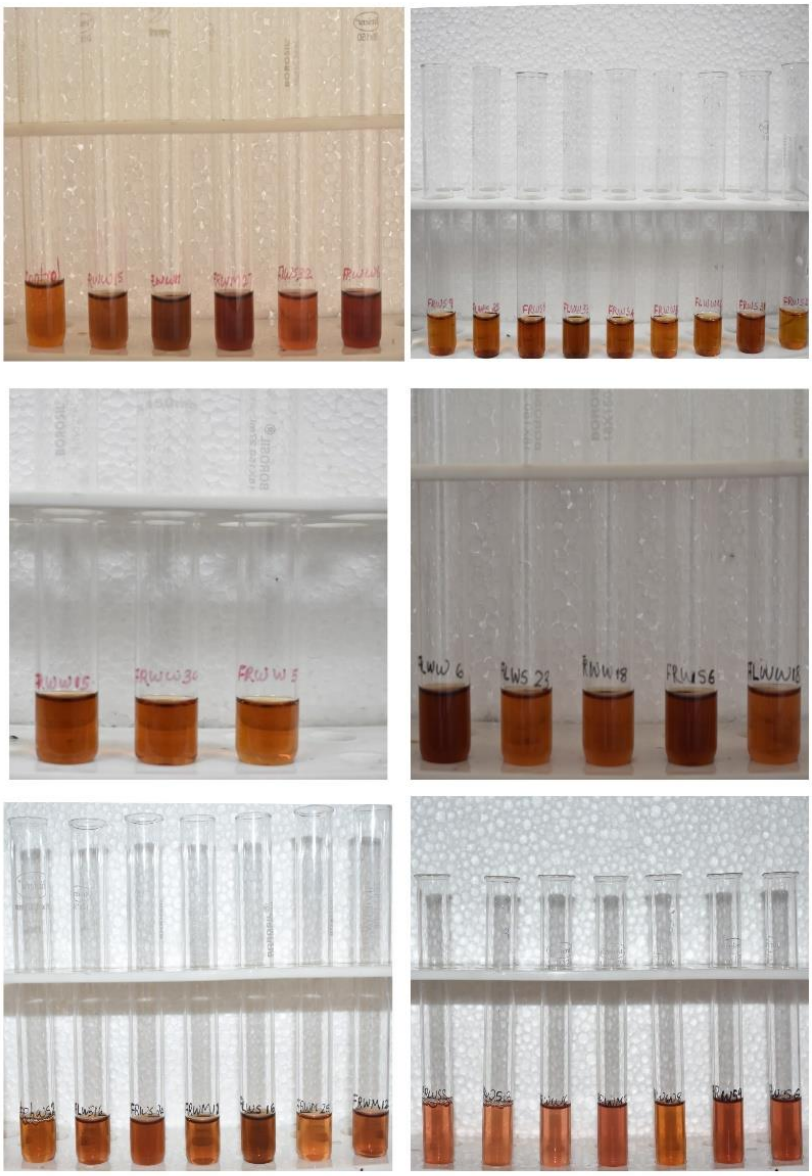

Fig. 141 Screening of Indole 3- Acetic Acid production of fungal isolates

Optimization for the Production of IAA from *Enterobacter sp.* BRhWW7 Isolated from *L. toxicaria*

To find out the supporting conditions for the enhanced growth in the production of IAA by selected endophytic *Enterobacter sp.* BRhWW7, the process optimization study, was carried out. Different parameters like agitation, temperature, pH, and different tryptophan concentrations were optimized concerning crude biomass and IAA production in shake culture conditions. The final extract biomass was recorded in mg/100mL. Effects of agitation (120, 140, 160, 180, 200), temperature (32°C, 34°C, 36°C, 38°C, 40°C), pH (4.0, 6.0, 8.0, 10.0, 12.0), and tryptophan (0.1%, 0.2%, 0.3%, 0.4%, 0.5%) were studied by inoculating the bacteria in Nutrient Broth (NB) and the impact on IAA production was investigated.

Optimal Influence of Agitation on IAA Production

Fig. 142 shows how agitation speed affects Indole-3-Acetic Acid (IAA) production and biomass by a microorganism. As the agitation speed increases, both biomass production and IAA synthesis also increase. At the lowest agitation speed of 120 rpm, biomass production is 813 mg and IAA concentration is 161.07 µg/ml. As the speed increases to 200rpm, biomass production rises to 1630 mg and IAA concentration reaches 252.33 µg/ml. This indicates a direct relationship between agitation speed and IAA production. The optimal agitation speed for IAA production is 200rpm, which yields the highest IAA concentration. This suggests that optimal agitation promotes efficient oxygen transfer, nutrient mixing, and cell growth, leading to enhanced IAA production.

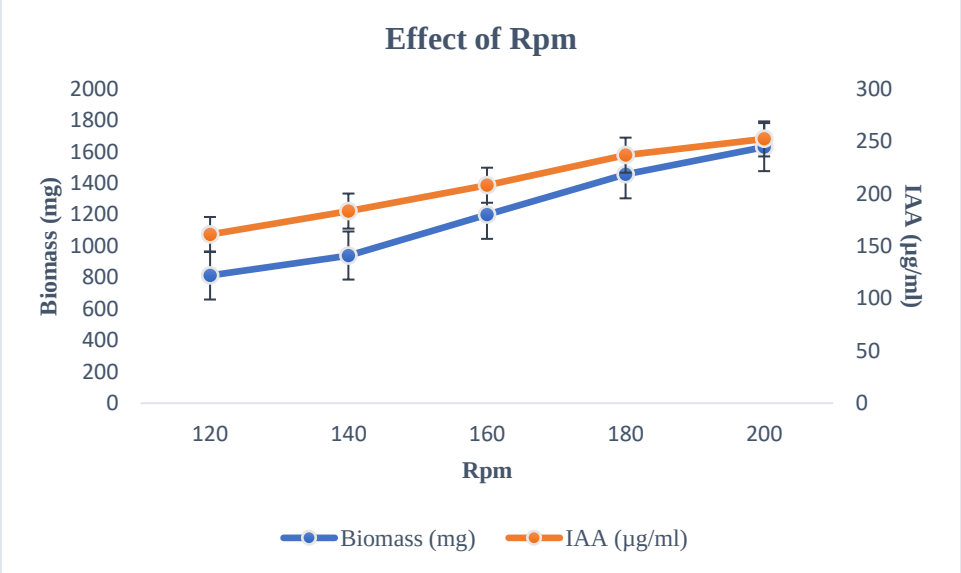

Fig.142 Effect of rpm variables in the IAA production

Optimal Influence of Temperature on IAA Production

Fig. 143 shows the impact of different temperatures on biomass production and Indole-3-Acetic Acid (IAA) synthesis by a microorganism. By way of the temperature increases from 32°C to 40°C, biomass production and IAA synthesis also increase, but with a slight decrease at 40°C. At 32°C, biomass production is 965 mg and IAA concentration is 196.71 µg/ml. As the temperature rises to 38°C, biomass production increases significantly to 1840 mg and IAA concentration reaches its peak at 276 µg/ml. However, at 40°C, biomass production slightly decreases to 1871 mg and IAA concentration drops to 259.63 µg/ml. The optimal temperature for IAA production appears to be 38°C, which yields the highest IAA concentration. This suggests that temperatures above 38°C may be detrimental to IAA production, while temperatures between 32°C and 38°C promote optimal growth and IAA synthesis.

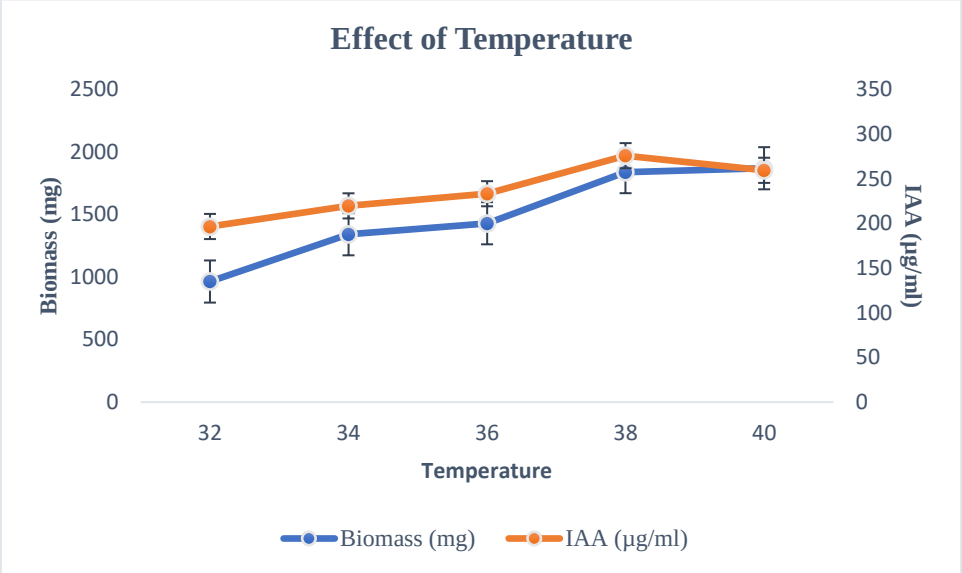

Fig.143 Effect of temperature variables in the IAA production

Optimal Influence of pH on IAA Production

Fig. 144 shows the effect of pH on biomass production and Indole-3-Acetic Acid (IAA) synthesis by a microorganism. The results indicate that the microorganism grows well and produces IAA optimally at a slightly acidic to neutral pH range. At pH 4, biomass production is 1847 mg and IAA concentration is 278.35 µg/ml. The highest IAA concentration (279.06 µg/ml) is observed at pH 6, with biomass production (1851 mg). As the pH increases to 8 and above, biomass production and IAA synthesis decrease, with the lowest values observed at pH 12 (1601 mg biomass and 245.9 µg/ml IAA). The optimal pH for IAA production appears to be around 6, which is close to neutral. This suggests that the microorganism is sensitive to extreme pH values and thrives best in a slightly acidic to neutral environment, leading to optimal IAA production.

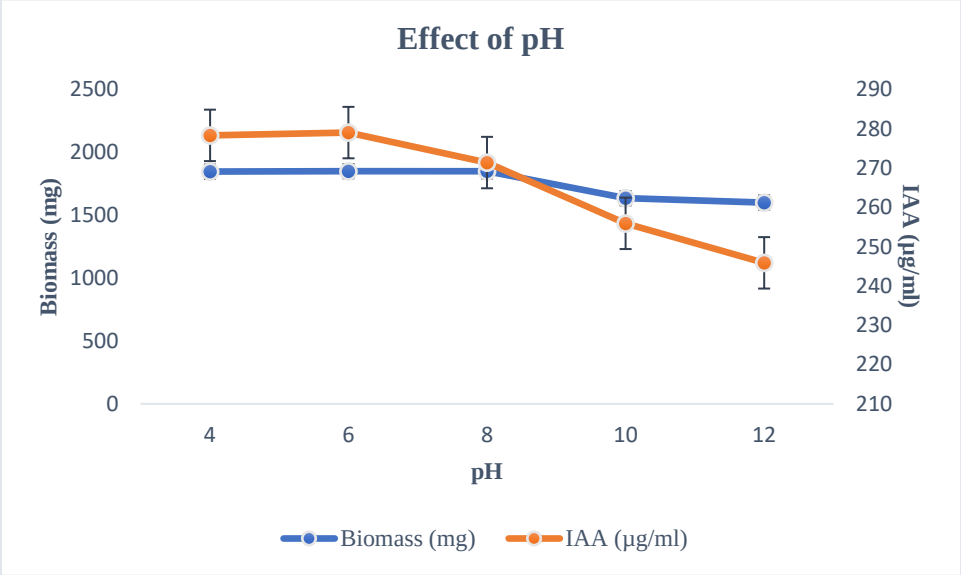

Fig.144 Effect of pH variables in the IAA production

Optimal Influence of Tryptophan on IAA Production

The impact of tryptophan concentration on biomass production and Indole-3-Acetic Acid (IAA) synthesis by a microorganism is shown in Fig. 145. Tryptophan is an amino acid that aids as a precursor for IAA production. As the tryptophan concentration increases from 0.1 to 0.3%, biomass production and IAA synthesis also increase, with the highest IAA concentration (317.08 µg/ml) observed at 0.3% tryptophan. This suggests that tryptophan is essential for IAA production, and increasing its concentration enhances IAA synthesis. However, further increases in tryptophan concentration (0.4 and 0.5%) lead to a slight decrease in IAA production, indicating that excessive tryptophan may be inhibitory to IAA synthesis. The optimal tryptophan concentration for IAA production appears to be 0.3%, which balances biomass growth and IAA synthesis.

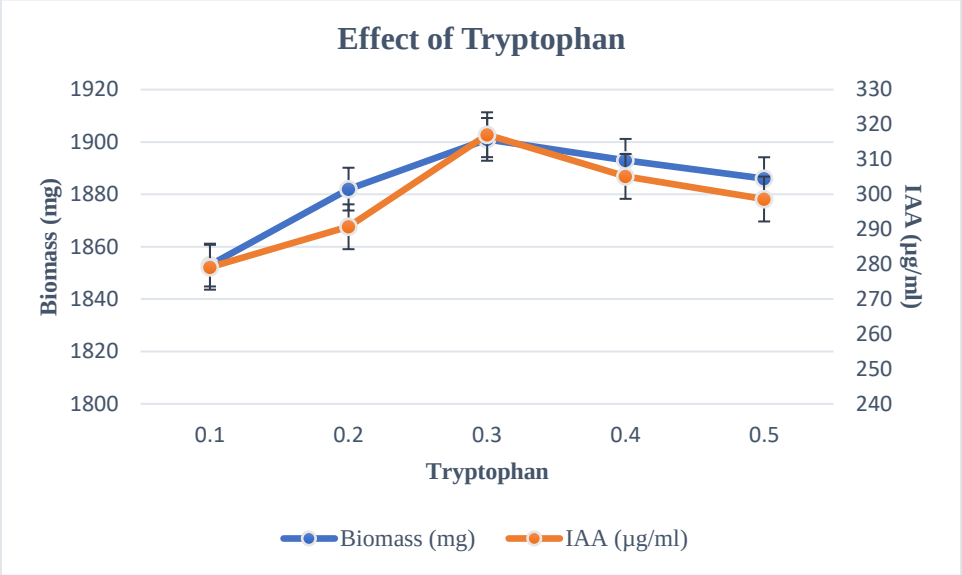

Fig.145 Effect of tryptophan variables in the IAA production

Confirmation of IAA by HPLC Analysis

IAA was identified and verified for synthesis using High-Performance Liquid Chromatography. The standard concentration and IAA's HPLC responses (retention time) were shown to be substantially positively correlated. Both before and after the secondary metabolite optimization, the plate displays a typical chromatogram of the fungal extract. IAA in the pre-optimized sample had a retention time of 15.889 minutes, which coincided with the genuine IAA's retention duration of 15.956 minutes at 280 nm. An appropriate standard curve was used to quantify IAA (Fig.146). With a peak height of 114586 and an area of 5472943, the chemical contained in the pre-optimization extract was 147.697 mg/l (Fig.147). Following optimization, the compound's concentration was raised to 255.618 mg/l with a retention time of 16.234 in the 9525810 region and a peak height of 196132 according to Fig.148.

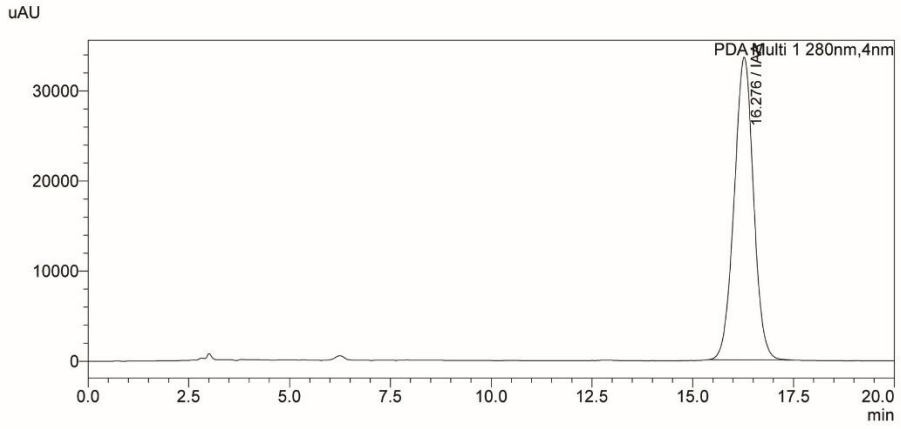

Fig. 146 Standard graph of IAA

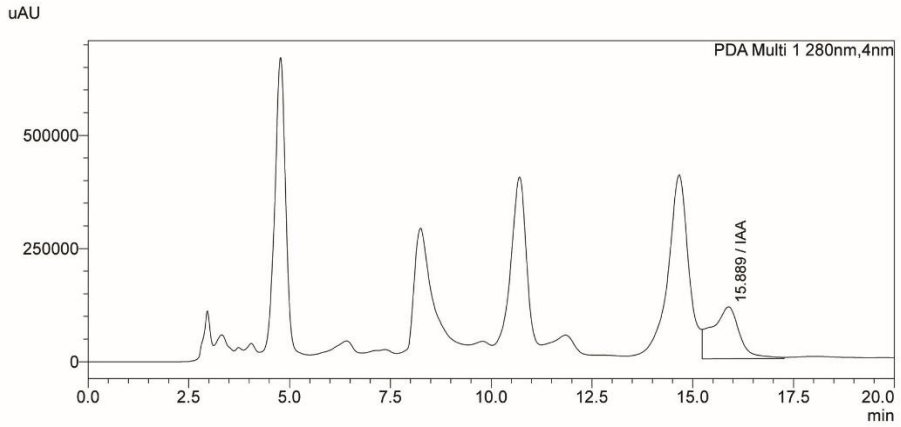

Fig. 147 HPLC analysis of pre-optimized IAA extract

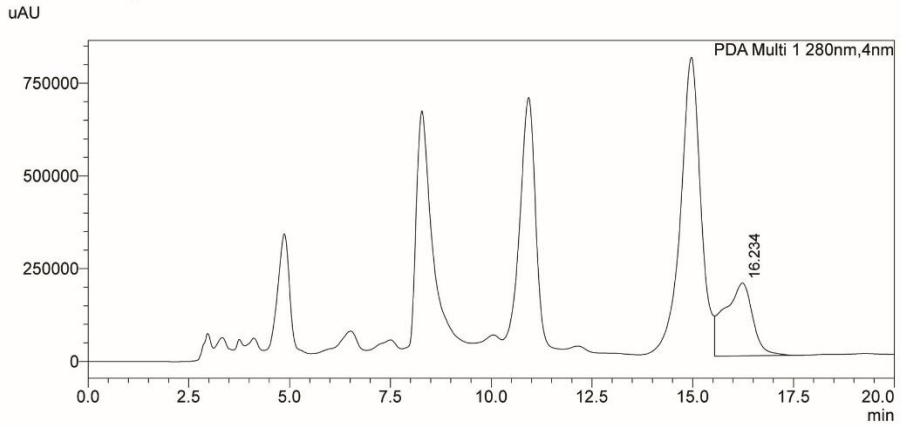

Fig. 148 HPLC analysis of optimized IAA extract

Bioactivities of Endophyte Extracts on *Capsicum annuum*

This study examined the growth-promoting effects of endophytic indole-3-acetic acid (IAA) produced by Enterobacter sp. BRhWW7 on *Capsicum annuum*, utilizing ferment broth extracts with enhanced IAA production in response to tryptophan (Fig. 149). The impacts of these extracts were evaluated

against standard IAA solutions, pure methanol solvent, and untreated plant controls. Notably, the solvent exhibited minimal inhibition of primary root growth compared to untreated controls. The experiment revealed significant differences in tap root development between control and methanol-treated plants. Over 3-day intervals from the 5th to 20th day, control plants displayed tap root growth ranging from 1 ± 0.00 mm to 4.67 ± 0.577 mm, whereas methanol-treated plants developed tap roots up to 1 ± 0.00 mm to 16 ± 1 mm (Fig. 150). Furthermore, the activities of methanolic ferment broth extracts were compared to IAA solutions diluted with the same organic solvent. The development of adventitious roots also varied between control and methanol-treated plants. Control plants exhibited adventitious root growth from the 10th to 20th day after inoculation, whereas methanol-treated plants developed adventitious roots between the 10th and 15th day. Fig. 149 showed the different stages of seedlings' emergence in the in-vitro culture method.

Seeds treated with the microorganism showed rapid germination, starting on the 2nd day of inoculation. The radicle emerged quickly, followed by the plumule and adventitious roots. The treated seeds exhibited enhanced root growth, with prominent elongation of the root tip and emergence of adventitious roots from the tap root. However, the number of adventitious roots decreased over time, possibly due to inadequate substrate or the presence of methanol. By day 20, growth slowed, and the seedlings were ready for transfer to suitable environmental conditions (Fig. 151). The microorganism treatment promoted early and robust seedling growth, setting the stage for healthy plant development.

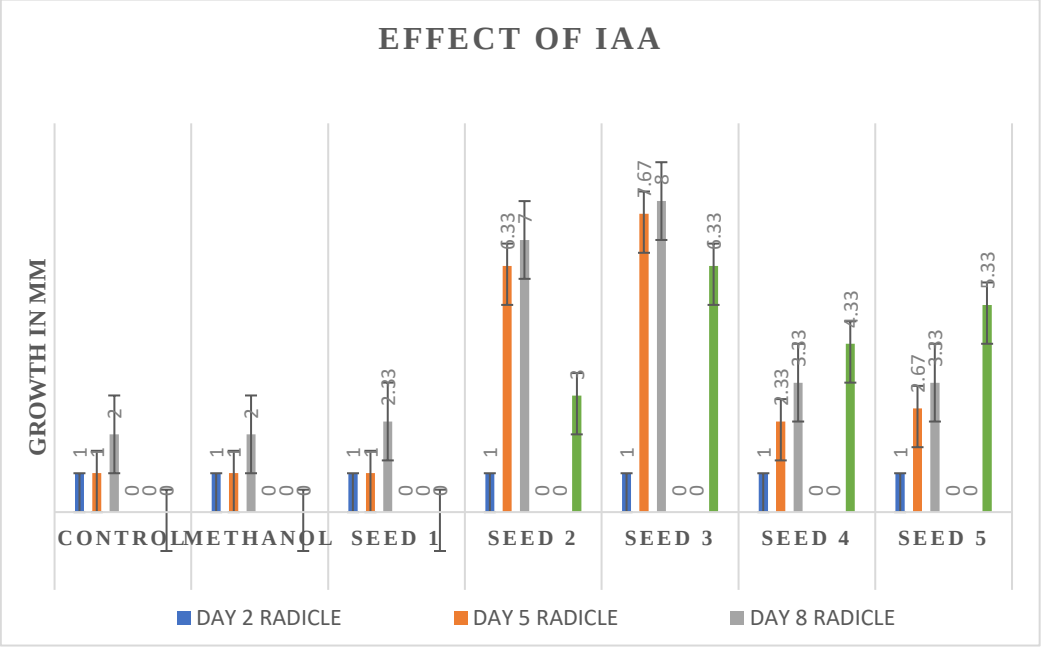

Fig 150 Effect of IAA for the development of germination

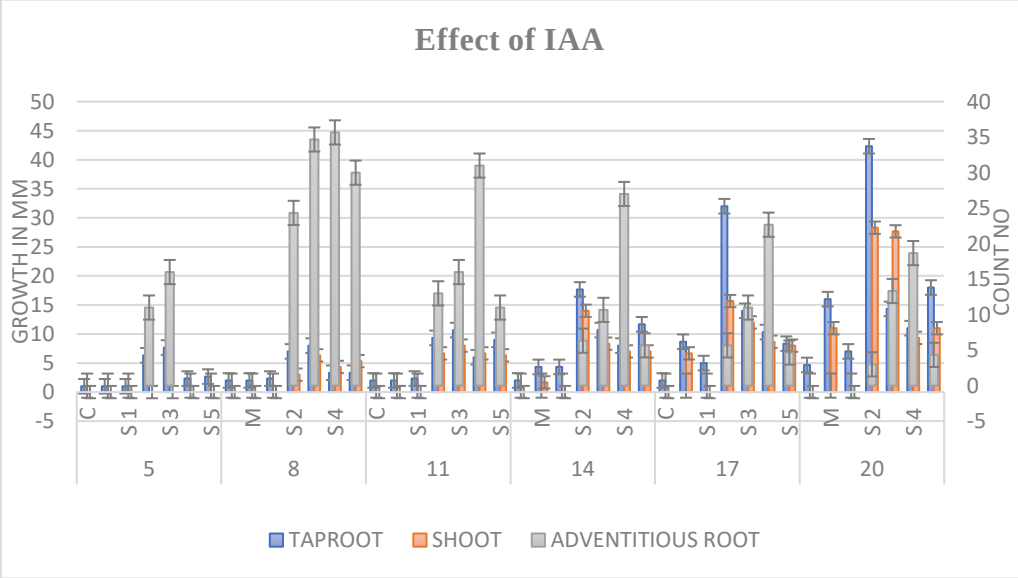

Fig 151 Effect of IAA on the growth of seedlings

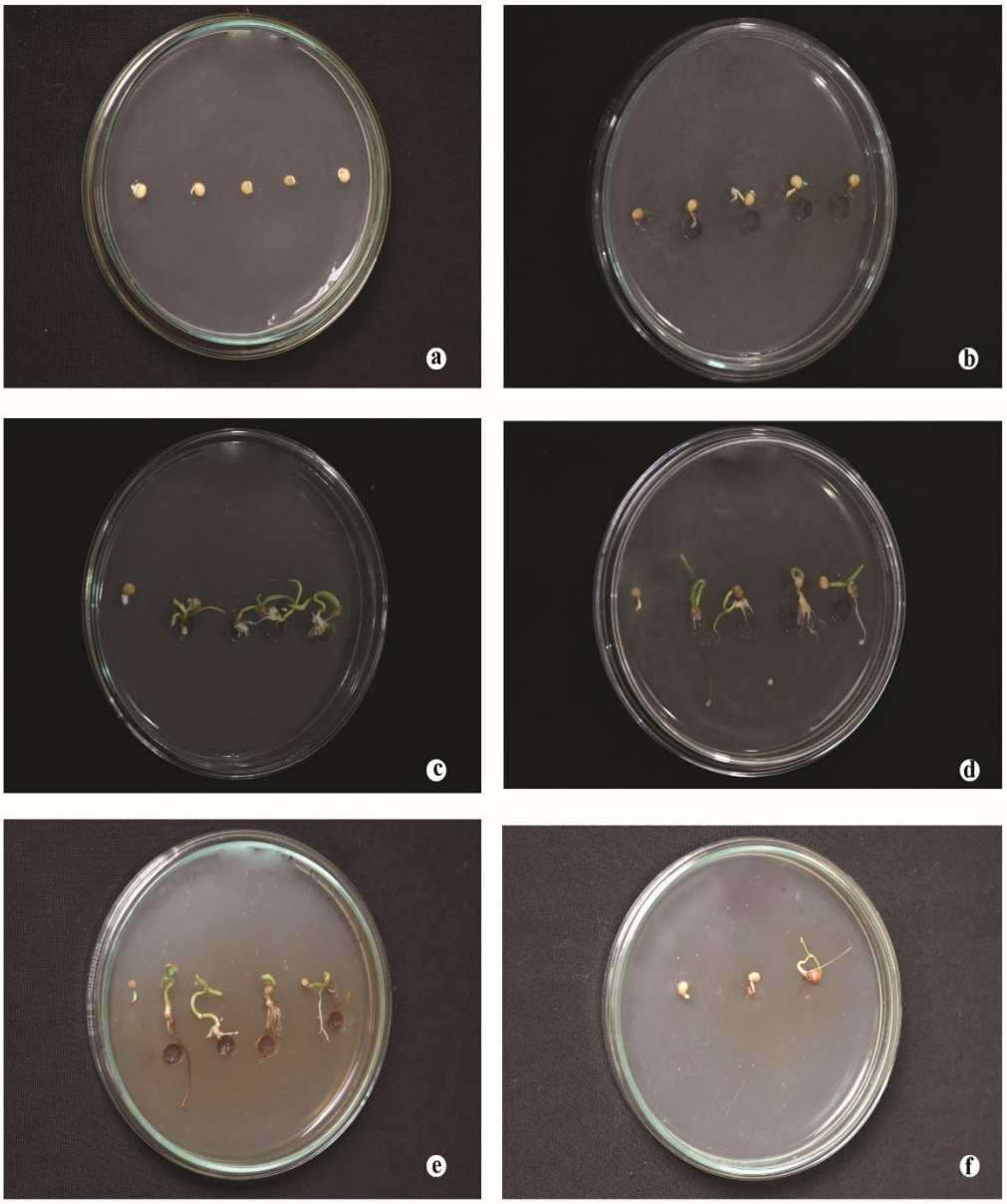

Fig. 149 Effect of *Enterobacter sp.* BRhWW7 extract on the growth of *C. annuum* seeds (a-e) different stages of the tap root, adventitious root, and shoot emergence (f) Control plate

Phosphate Solubilization

Qualitative Estimation of Phosphate Solubilization of Endophytic Bacteria

One of the most important macronutrients for plants is phosphorus (P). It is extremely crucial to the growth of plants. Additionally, it is essential for many physiological processes, including respiration, the transfer and storage of energy, cell division, cell enlargement, photosynthesis, and root system development. Furthermore, for plants to successfully finish their typical production cycle, they require phosphorous. Phosphorite anions (H_2PO_4 and

HPO_4^-) are the form in which plants absorb phosphorus from the soil. Therefore, the capacity of bacteria to solubilize insoluble phosphate increases the availability of phosphorus for plant growth by making it easier to access.

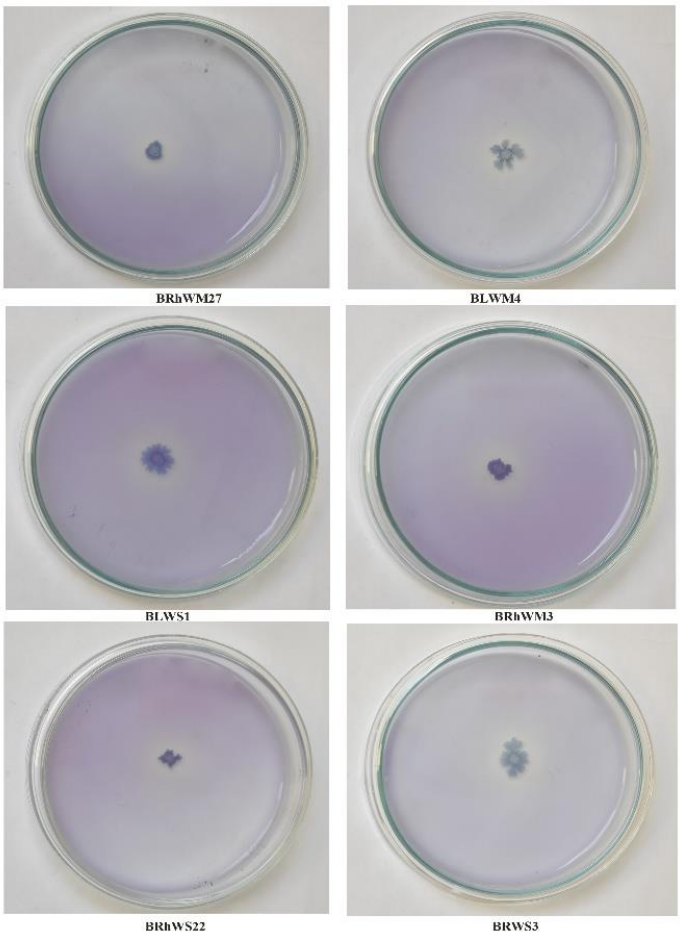

Fig. 152 Screening of phosphate solubilization of bacterial isolates

The bacterial isolates exhibited varying degrees of phosphate solubilization, as indicated by the Phosphate Solubilization Index (PSI) (Fig.152). The PSI values ranged from 0 to 3.067, with most isolates showing moderate to high phosphate solubilization capabilities. Several isolates demonstrated high PSI values, including BRhWM27 (3.067 ± 0.115), BRhWM3 (2.821 ± 0.062), and BLWM4 (2.939 ± 0.052) (Table 78). These isolates have potential applications in agriculture and environmental remediation due to their capability to solubilize phosphates and make them available to plants. In contrast, some isolates showed low or no phosphate solubilization, including BRWW12, BRWW6, BRWS15, BLWW10, etc., all with PSI values 0. These isolates may not be effective in solubilizing phosphates, but could potentially have other beneficial effects on plant growth. Many isolates exhibited moderate PSI values, ranging from 2 to 2.5. Examples include BRWS3 (2.553 ± 0.026), BLWW7 (2.167 ± 0.058), and BRhWS1 (2.429 ± 0.143). These isolates may have moderate phosphate solubilization capabilities, which could still benefit plant growth.

Sl No.	Bacterial isolates	Colony diameter (mm)	Zone diameter (mm)	PSI
1	BRWW12	0	0	0
2	BRWW6	0	0	0
3	BRWS13	2±0	3±0	2.5±0
4	BRWS15	0	0	0
5	BRWS1	0	0	0
6	BRWS3	12.67±0.577	19.67±0.577	2.55±0.026
7	BRWM2	6±0	7.67±0.577	2.28±0.096
8	BRWM3	3±0	4±0	2.33±0
9	BLWW1	0	0	0
10	BLWW4	9±0	10.67±0.577	2.18±0.064
11	BLWW2	0	0	0
12	BLWS11	0	0	0
13	BLWS6	0	0	0
14	BLWS7	0	0	0
15	BLWW5	0	0	0
16	BLWW7	10±0	11.67±0.577	2.17±0.058
17	BLWW8	8.33±0.577	12±0	2.44±0.096
18	BLWM4	10.67±0.577	20.67±0.577	2.94±0.052
19	BLWM1	15±0	18.33±0.577	2.22±0.038
20	BLWW10	0	0	0
21	BLWW9	6±0	11.33±0.577	2.89±0.096
22	BLWS10	11±0	16.67±0.577	2.51±0.052
23	BLWS9	0	0	0
24	BLWS4	10±0	13±0	2.3±0
25	BLWS2	12±0	18±0	2.5±0
26	BLWS1	2±0	3.67±0.577	2.83±0.289
27	BLWS3	0	0	0
28	BRhWS1	7±0	10±1	2.43±0.143
29	BRhWS8	7±0	10±0	2.43±0
30	BRhWW11	7±0	9±0	2.29±0
31	BRhWW7	0	0	0
32	BRhWM3	7.33±0.577	13.33±0.577	2.82±0.062
33	BRhWM10	0	0	0
34	BRhWM27	5±0	10.33±0.577	3.07±0.115
35	BRhWS22	6±0	10±2	2.67±0.333

Table 78: Qualitative estimation of phosphate solubilization of endophytic bacteria

Qualitative Estimation of Phosphate Solubilization of Endophytic Fungi

Phosphate solubilization capabilities of 36 endophytic fungi were investigated using a modified Pikovaskaya medium, where tri-calcium phosphate served as the phosphorus source. The fungal isolates exhibited varying degrees of phosphate solubilization, as indicated by the Phosphate Solubilization Index (PSI) (Fig.153). The PSI values ranged from 0 to 2.909, with most isolates showing moderate to high phosphate solubilization capabilities. Several isolates demonstrated high PSI values, including FLWW14 (2.909 ± 0.091), FRhWS1 (2.272 ± 0.038), and FLWW18 (2.392 ± 0.014) (Table 79). In contrast, many isolates (FLWW5, FLWW15, FLWW17, FLWW6, FRWS7, FRWM12, FLWW12, FRWM1, FRWS4, FLWW11, etc.) showed no phosphate solubilization, with PSI values of 0. These isolates may not be effective in solubilizing phosphates, but could potentially have other beneficial effects on plant growth. Some isolates exhibited moderate PSI values, ranging from 2 to 2.5. Examples include FRhWW4 (2.41 ± 0.044), FLWS5 (2.567 ± 0.058), and FRhWW3 (2.31 ± 0.041). These isolates may have moderate phosphate solubilization capabilities, which could benefit plant growth. A few isolates showed high zone diameters but low PSI values, such as FRWM25 (35 ± 0, PSI = 2.207 ± 0).

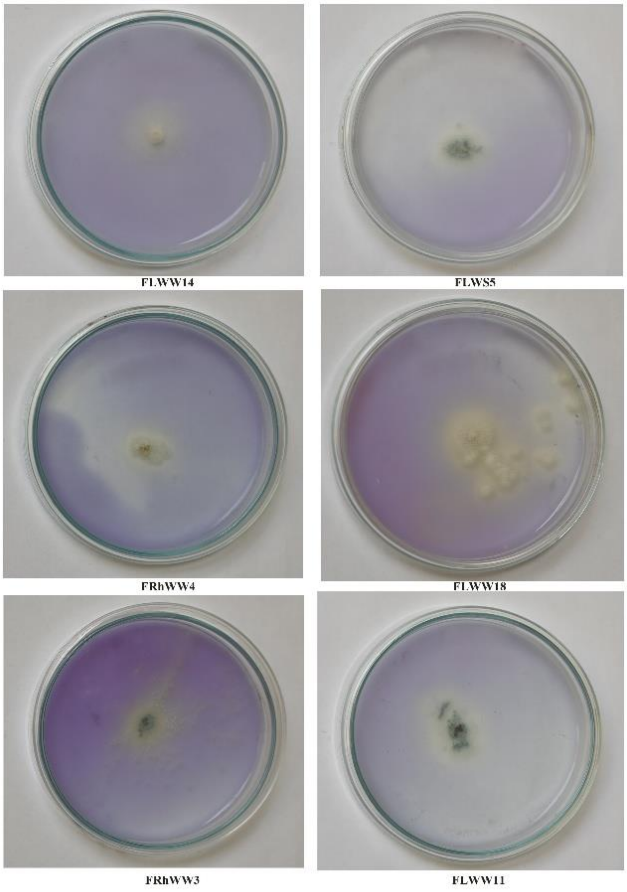

Fig. 153 Screening of phosphate solubilization of fungal isolates

Sl No.	Fungal isolates	Colony diameter (mm)	Zone diameter (mm)	PSI
1	FLWW14	11±0	21±1	2.91±0.091
2	FRWS8	20±0	23.67±0.577	2.18±0.029
3	FLWW5	0	0	0
4	FLWW15	0	0	0
5	FLWW17	0	0	0
6	FLWW6	0	0	0
7	FRWS7	0	0	0
8	FRWM12	0	0	0
9	FLWW12	0	0	0
10	FRWM1	0	0	0
11	FRWS4	0	0	0
12	FLWW11	0	0	0
13	FRhWS1	19.67±0.577	25±0	2.27±0.038
14	FLWW18	15.33±0.577	21.33±0.577	2.39±0.014
15	FLWS32	0	0	0
16	FRWW18	0	0	0
17	FRWM25	29±0	35±0	2.21±0
18	FRWW30	0	0	0
19	FLWM1	0	0	0
20	FLWW9	0	0	0
21	FRWM27	0	0	0
22	FLWS1	0	0	0
23	FRhWW3	14±0	18.33±0.577	2.31±0.041
24	FRhWM2	0	0	0
25	FRhWS2	0	0	0
26	FRWM3	0	0	0
27	FLWS21	0	0	0
28	FLWS16	0	0	0
29	FRWS24	0	0	0
30	FRWW10	0	0	0
31	FRWW11	0	0	0
32	FRhWW4	13±0	18.33±0.577	2.41±0.044
33	FRWS9	0	0	0
34	FLWM11	0	0	0
35	FLWW13	0	0	0
36	FLWS5	10±0	15.67±0.577	2.57±0.058

Table 79: Qualitative estimation of phosphate solubilization of endophytic fungi

Quantitative Estimation of Phosphate Solubilization of *Enterobacter asburiae* BRhWM27

Enterobacter asburiae BRhWM27 showed a high PSI of 3.07±0.115 and was then subjected to quantitative analysis of phosphorous solubilization in Pikovaskaya's broth. The broth was supplemented with tri-calcium phosphate,

i.e. TCP (0.1%), as an inorganic substrate at neutral pH (7.0). The quantitative estimation of phosphorous solubilization was observed for 10 days. The amount of phosphorous released by the isolate and the pH of each interval was recorded. The fig.154 shows the effects of phosphate solubilization by a microorganism over 10 days. The activity of phosphate solubilization increases steadily from 4.2±0.1 on day 2 to 12.43±0.32 on day 10, indicating enhanced solubilization of phosphates over time. Concurrently, the pH decreases from 6.67±0.25 on day 2 to 2.83±0.25 on day 10, suggesting that the microorganism's phosphate solubilization activity is accompanied by acidification of the environment. This is a common phenomenon, as many phosphate-solubilizing microorganisms produce organic acids that lower the pH and enhance phosphate solubilization. The optimal phosphate solubilization activity is observed on day 10, with a corresponding pH of 2.83±0.25. This indicates that the microorganism's ability to solubilize phosphates is maximized, making it a potential candidate for applications in agriculture and biotechnology, such as biofertilizers or soil amendments.

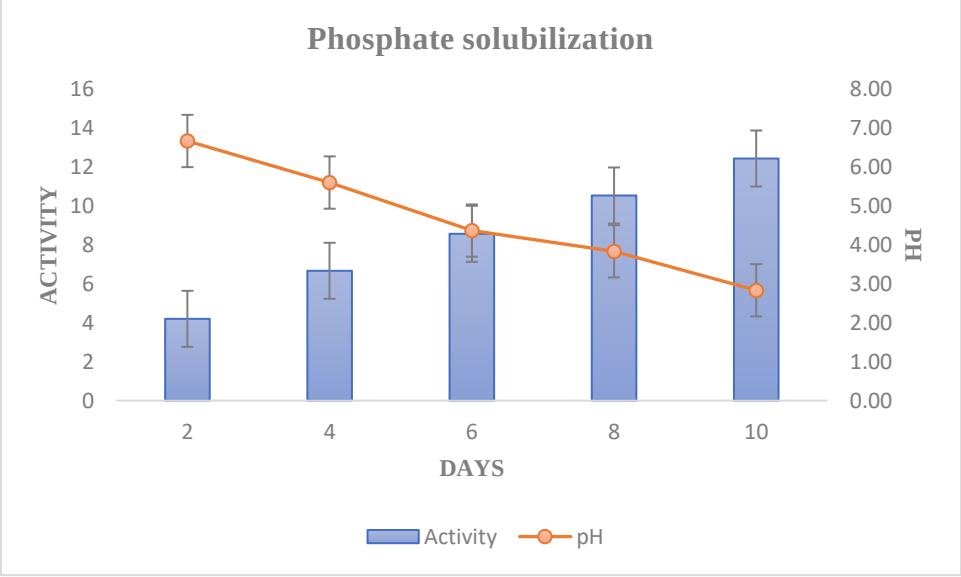

Fig.154 Effects of phosphate solubilization in activity and pH

ACC (1-Aminocyclopropane-1-Carboxylate) Deaminase Production

Qualitative Estimation of ACC Deaminase Production of Endophytic Bacteria

The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase plays a crucial role in plant stress tolerance by catalyzing the degradation of ACC, a key

precursor to ethylene, into α-ketobutyrate and ammonia. Ethylene, a plant hormone regulating various vegetative and developmental processes, is produced in response to biotic and abiotic stress. ACC deaminase mitigates stress-induced ethylene production, promoting plant growth and development under adverse conditions. 35 endophytic bacterial isolates were cultured on Dworkin and Foster (DF) minimal medium for investigating ACC deaminase production. The isolates' ability to utilize ACC as a sole nitrogen source was assessed by monitoring bacterial growth on plates containing ACC and (NH4)2SO4 separately.

Based on qualitative tests for ACC deaminase production, 17 isolates were found positive for ACC deaminase production (Fig.155). The bacterial isolates showed varying levels of ACC deaminase production, with differences in diameter ranging from 0 to 12.667 mm. The highest ACC deaminase production was observed in isolates BRWS3 (12±1 mm) and BLWS7 (12.667±0.577 mm), indicating their potential to degrade ACC and promote plant growth (Table 80). In contrast, several isolates showed no ACC deaminase production, as indicated by a difference of 0 mm.

Sl No.	Bacterial isolates	Initial diameter (mm)	Final diameter (mm)	Difference (mm)
1	BRWW12	0	0	0
2	BRWW6	0	0	0
3	BRWS13	4.67±0.577	7.33±0.577	2.67±0.577
4	BRWS15	0	0	0
5	BRWS1	0	0	0
6	BRWS3	1.33±0.577	13.33±1.528	12±1
7	BRWM2	0	0	0
8	BRWM3	4.33±0.577	6.33±0.577	2±0
9	BLWW1	2±0	2.33±0.289	0.33±0.289
10	BLWW4	0	0	0
11	BLWW2	2.67±0.577	3.67±0.577	1±0
12	BLWS11	0	0	0
13	BLWS6	0	0	0
14	BLWS7	1±0	13.67±0.577	12.67±0.577
15	BLWW5	0	0	0
16	BLWW7	5±0	5±0	0
17	BLWW8	0	0	0
18	BLWM4	0	0	0

19	BLWM1	0	0	0
20	BLWW10	4±0	7.67±0.577	3.67±0.577
21	BLWW9	5±0	6.33±0.577	1.33±0.577
22	BLWS10	5±0	6.67±0.577	1.67±0.577
23	BLWS9	0	0	0
24	BLWS4	9.67±0.577	12.67±0.577	3±1
25	BLWS2	2±0	10.33±0.577	8.33±0.577
26	BLWS1	0	0	0
27	BLWS3	4±0	4±0	0
28	BRhWS1	3±0	3±0	0
29	BRhWS8	4±0	4±0	0
30	BRhWW11	3.67±0.577	5.67±0.577	2±1
31	BRhWW7	3±0	5.33±0.577	2.33±0.577
32	BRhWM3	2.67±0.577	9.33±0.577	6.67±0.577
33	BRhWM10	4±0	5±0	1±0
34	BRhWM27	2±0	7.67±0.577	5.67±0.577
35	BRhWS22	3.67±0.577	8.33±0.577	4.67±1.155

Table 80: Qualitative estimation of ACC deaminase production of endophytic bacteria

Qualitative Estimation of ACC Deaminase Production of Endophytic Fungi

The qualitative analysis of ACC deaminase production by 36 fungal isolates, with values ranging from 0 to 67±3.61, is presented in Table. 81 & Fig.156. The highest ACC deaminase production was observed in isolate FRWW10, with a value of 67±3.61, followed by FLWS32, with a value of 39.5±3.775. In contrast, several isolates, including FLWW6 and FLWM11, showed no ACC deaminase production, with values of 0. Most isolates exhibited moderate levels of ACC deaminase production, ranging from 1±0 to 27±1 (Table 81). Notably, 14 isolates showed high levels of production, with values above 10.

Sl No.	Fungal isolates	Initial diameter (mm)	Final diameter (mm)	Difference (mm)
1	FLWW14	1.4±0.1	15±1	13.6±0.954
2	FRWS8	2±0	8.33±0.577	6.33±0.577
3	FLWW5	3±0	15.33±0.577	12.33±0.577
4	FLWW15	1±0	11.67±1.528	10.67±1.528
5	FLWW17	2±0	3±0	1±0
6	FLWW6	0	0	0
7	FRWS7	3±0	18.67±0.577	15.67±0.577
8	FRWM12	1±0	12.33±0.577	11.33±0.577
9	FLWW12	1.17±0.289	11±1	9.83±1.258
10	FRWM1	2.33±0.577	27.67±1.528	25.33±1.155
11	FRWS4	1±0	20±4	19±4

12	FLWW11	1.33±0.577	19.33±0.577	18±1
13	FRhWS1	1.67±0.577	18.67±3.215	17±3.46
14	FLWW18	2.33±0.577	15.67±0.577	13.33±0.577
15	FLWS32	1.17±0.289	40.67±4.041	39.5±3.775
16	FRWW18	1±0	15.67±0.577	14.67±0.577
17	FRWM25	1.33±0.577	26±1	24.67±0.577
18	FRWW30	5±0	30±0	25±0
19	FLWM1	2±0	29±1	27±1
20	FLWW9	2±0	21±1	19±1
21	FRWM27	1±0	7.33±1.528	6.33±1.528
22	FLWS1	2.33±0.577	8±1	5.67±0.577
23	FRhWW3	3±0	10.33±0.577	7.33±0.577
24	FRhWM2	1±0	18±0	17±0
25	FRhWS2	1±0	21±1	20±1
26	FRWM3	2.67±0.577	16±1	13.33±1.528
27	FLWS21	1.33±0.577	12±2	10.67±1.528
28	FLWS16	1.67±0.577	10.33±0.577	8.67±1.155
29	FRWS24	2±0	6±1	4±1
30	FRWW10	2.67±1.155	69.67±4.509	67±3.61
31	FRWW11	2±0	10.33±2.517	8.33±2.517
32	FRhWW4	3±1	11.67±2.887	8.67±3.055
33	FRWS9	8.67±0.577	23±2	14.33±2.082
34	FLWM11	0	0	0
35	FLWW13	1±0	27±1	26±1
36	FLWS5	1.33±0.577	11.67±2.082	10.33±1.528

Table 81: Qualitative estimation of ACC deaminase production of endophytic fungi

Quantitative Estimation of ACC Deaminase Production in *Fusarium chlamydosporum* FRWW10

The graph shows the growth of a fungal isolate in response to ACC deaminase production over 10 days. The control treatment exhibits steady growth, increasing from 96.33±0.57 to 193.00±2.64. In contrast, the ACC treatment shows enhanced growth, increasing from 147.33±0.67 to 251.37±1.79, indicating a positive effect of ACC deaminase on fungal growth. The ammonium sulfate treatment also shows increased growth compared to the control but to a lesser extent than the ACC treatment. This suggests that ACC deaminase has a more significant impact on fungal growth than ammonium sulfate. The growth patterns indicate that ACC deaminase production stimulates fungal growth, with the most significant increases observed between days 6 and 10 (Fig.157).

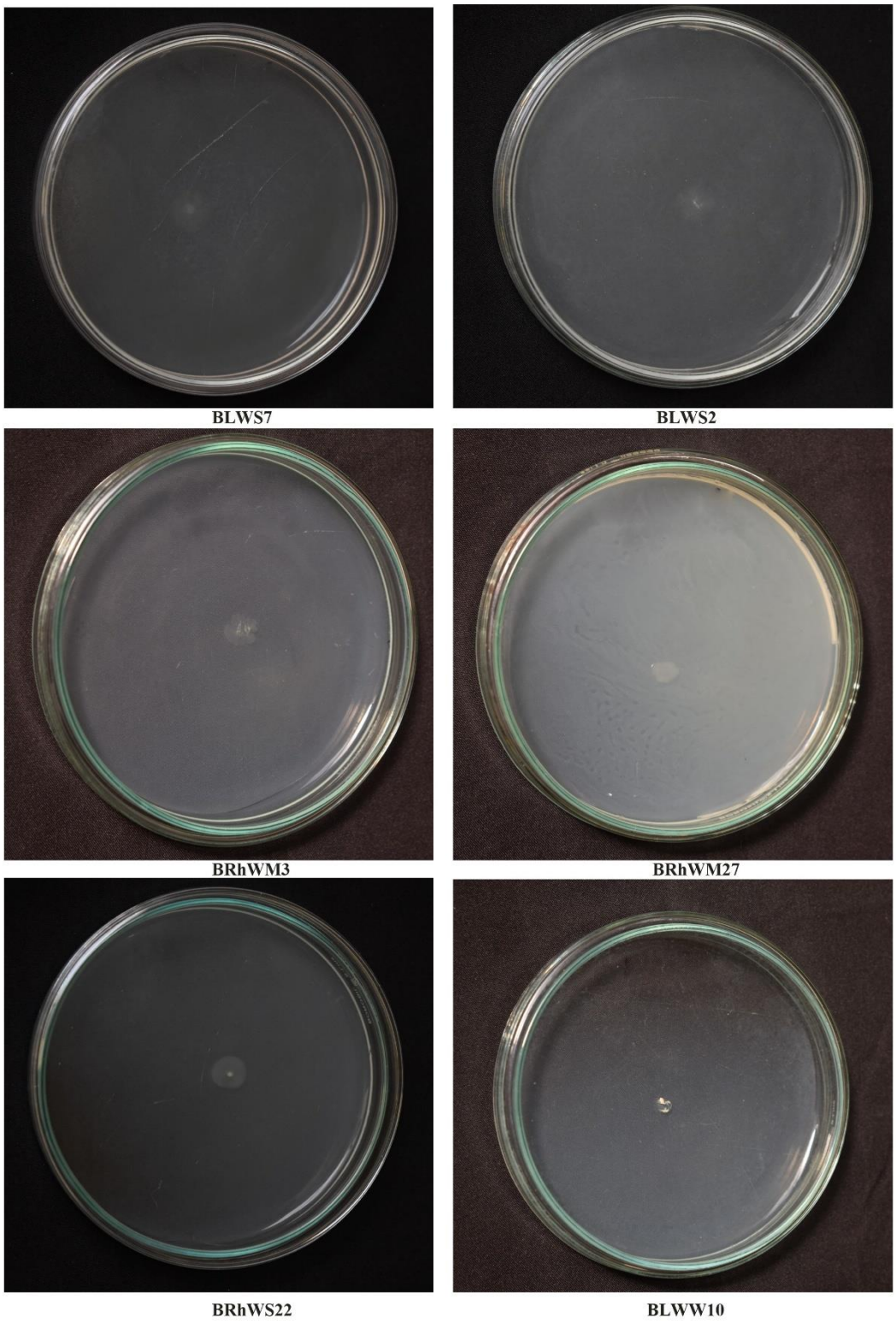

Fig. 155 Screening of ACC deaminase production of bacterial isolates

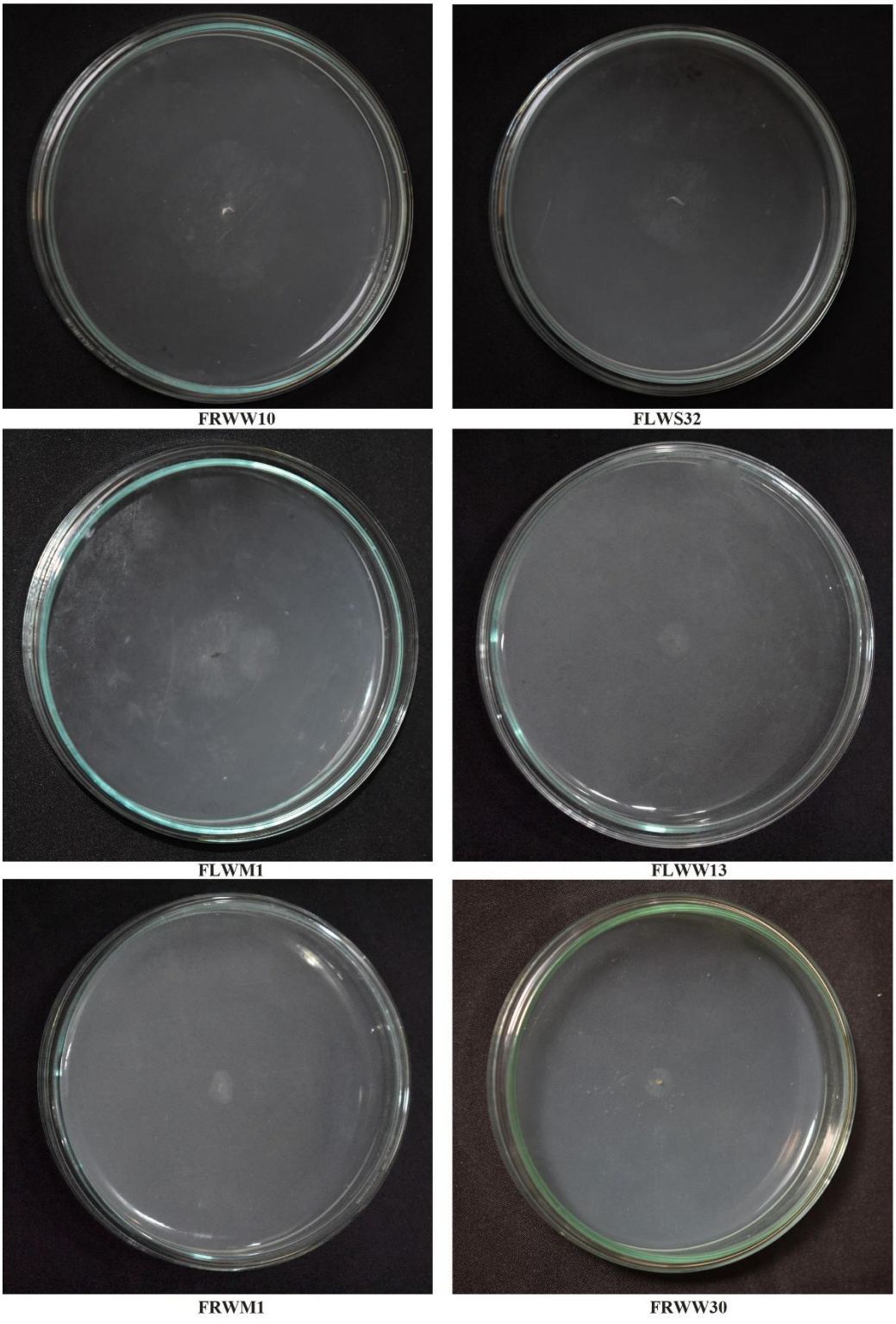

Fig. 156 Screening of ACC deaminase production of fungal isolates

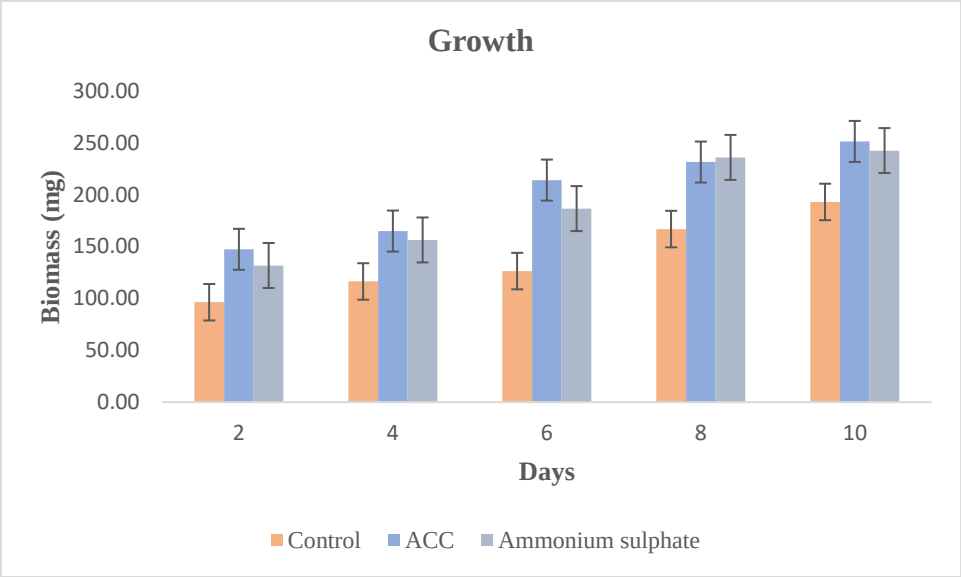

Fig.157 Biomass production of *Fusarium chlamydosporum* FRWW10 in different conditions

Discussion

Plant growth promotion (PGP) studies in endophytic organisms are crucial for understanding the symbiotic relations between plants and microorganisms (Batra et al., 2018). Endophytes, living within plant tissues, can enhance plant growth and productivity by producing phytohormones, solubilizing minerals, and suppressing pathogens (Eid et al., 2021). PGP studies in endophytes have significant implications for sustainable agriculture, as they offer eco-friendly alternatives to chemical fertilizers and pesticides (Mishra et al., 2016). For instance, endophytic bacteria like *Enterobacter* and *Bacillus* species have been shown to produce IAA, a phytohormone that promotes plant growth (Khianngam et al., 2023). Furthermore, endophytes can improve plant stress tolerance and improve soil health, making them valuable tools for mitigating climate change impacts on agriculture (Shaffique et al., 2022). Exploring endophytic organisms for PGP activities can lead to the discovery of novel bioactive compounds and innovative approaches for plant growth promotion (Gouda et al., 2016).

Enterobacter is a genus of bacteria that has been found to produce Indole-3-Acetic Acid (IAA), a beneficial trait for plant growth promotion. This bacterium is a promising tool for agricultural applications due to its ability to thrive in

various environments and produce high levels of IAA. Enterobacter's IAA production capabilities make it a valuable microorganism for developing biofertilizers and other agricultural products. Zhang et al., 2021 isolated and identified a series of predominant *Enterobacter* strains with remarkable Indole-3-acetic acid (IAA)--producing capabilities. These strains, primarily dependent on tryptophan, exhibited significantly high IAA yields, reaching up to 3477 μg mL−1 and 3378 μg mL−1. The study found that these IAA-producing *Enterobacter* strains significantly promoted maize growth, increasing root length, plant height, fresh weight, and dry weight.

The present study also reveals that *Enterobacter sp*. has a remarkable capacity to produce 255.61 mg/ml of IAA, a phytohormone with significant plant growth-promoting properties. These results align with work of Khianngam et al. (2023), who identified and optimized IAA production in bacterial species isolated from the roots of *Chrysopogon zizanioides* (L.). Notably, *Enterobacter hormaechei* and *Bacillus aryabhattai* produced substantial amounts of IAA, reaching 246 μg/ml and 195.55 μg/ml, respectively. These bacterial species effectively enhanced rice seed germination, with significant increases in root and shoot growth, providing strong evidence for their potential as plant growth promoters. Bessai et al., 2022 stated the optimal pH of the IAA production in *Enterobacter* sp. DMKU-RP206 was 6 which supported the present study, and the IAA production of *E. cloacae* was maximum at 37°C.

Consistent with our findings, previous studies reported that *B. subtilis* DR2 (KP455653) exhibits optimal growth at pH 7 (Kumari et al., 2018). Bhutani et al., 2021 studied the plant growth promotion activities of IAA production and phosphate solubilization. In their observation, 11.83±0.54 to 35.83±2.2 μg/ml was the production range of IAA i.e., much less than the current study. However, their study also successfully implemented the pot experiment to analyze the efficiency of the plant growth promotion properties of *B. megaterium* NAP8. In the present study, a bioactivity test was demonstrated to confirm that the endophytic bacterial IAA production enhances root growth. This experimental

data was strongly supported by the studies of Li et al., 2008. *Enterobacter sp.,* with high IAA production, shows great promise for improving crop yields and industrial efficiency, offering a promising avenue for sustainable plant growth promotion. *Enterobacter sp.* DMKU-RP206, isolated from Thai rice leaves, showed plant growth-promoting traits, particularly high indole-3-acetic acid (IAA) production. Optimized conditions using Box-Behnken design identified l-tryptophan as crucial, with optimal levels of lactose, yeast extract, NaCl, pH, temperature, and shaking speed. Scaling up production resulted in a 13.4-fold increase, reaching 5561.7 mg l−1, the highest reported for the *Enterobacter* genus, showcasing the potential for agricultural applications (Nutaratat et al., 2017).

Research has shown that endophytic bacteria in the root zone can increase the bioavailability of soil phosphorus, boosting plant productivity (Duangpaenga et al., 2013). This fact was strongly supported by the current study and agreed with the evidence that the bacterial isolate from the rhizome showed as a good phosphate solubilizer. Sanjotha and Manawadi, 2016 studied phosphate-solubilizing bacteria. They reported that *Bacillus, Pseudomonas, Rhizobium, Azatobacter* are some species that showed the highest percentage of phosphate solubilization and the optimum temperature seen between 35°C and 40°C in which the present study also supported this statement. Enterobacter is another group of bacteria having the potential to solubilize phosphorous from the soil and uptake the phosphate ions to plants. Mohamed et al., 2019 reported that the bacteria from the root zone of wheat plants showed potential phosphate solubilization ability and the species identified are *Pantoea sp., Enterobacter aerogenes*, and *Enterobacter sp.*

Researchers isolated 21 bacterial strains exhibiting phosphate-solubilizing activity in a 2020 study led by Mendoza-Arroyo. Notably, strain ITCB-09, identified as *Enterobacter sp*., demonstrated exceptional phosphate solubilization capability, releasing 865.98 µg/mL of inorganic phosphate through organic acid production. Furthermore, ITCB-09 produced extracellular

polymeric substances and siderophores, enhancing its phosphate solubilization efficiency. When immobilized in beads, ITCB-09 was applied to *Capsicum chinense* Jacq. seedlings, yielding promising results. The treatment positively impacted seedling growth, underscoring the strain's potential as a biofertilizer. This sustainable solution offers an innovative approach to plant growth promotion and phosphate management, addressing environmental concerns associated with chemical fertilizers.

Enterobacter sp. Fs-11, isolated from the rhizosphere of the sunflower that produces indole acetic acid, solubilizes tri-calcium phosphate and colonizes sunflower roots. It utilizes various substrates, produces malic and gluconic acid, and has a high homology with *E. cloacae* pqqE gene. Fs-11 strongly colonizes sunflower roots, and its inoculation increases plant height, fresh and dry weight, and total phosphorus content. This rhizobacterium, with its exceptional phosphate-solubilizing and plant growth-promoting capabilities, offers tremendous promise as a bio-inoculant for enhancing sunflower productivity in phosphorus-deprived environments, offering a sustainable solution for plant growth promotion and phosphorus management (Shahid et al., 2012).

Fusarium species have been found to produce 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase, an enzyme that breaks down ACC, a precursor to ethylene production in plants. This enzyme production by Fusarium can help mitigate plant stress responses, such as those caused by drought, salinity, and heavy metals, by reducing ethylene levels. ACC deaminase-producing Fusarium strains have potential agricultural applications as biofertilizers or bioinoculants to enhance plant growth and stress tolerance. Rehman et al., 2022 cocultured *Trichoderma gamsii* and *Fusarium proliferatum* on *Moringa oleifera.* They found that the significant enhancement in growth traits, including biomass, chlorophyll content, soluble sugars, secondary metabolites, IAA, salicylic acid, etc. leads to the reduction in ACC level, hydrogen peroxidase, ABA. *Fusarium graminearum,* a plant-pathogenic fungus, surprisingly lacks the gene responsible for ethylene-forming enzyme, yet possesses two potential

genes encoding ACC deaminase. One of these genes encodes an active ACC deaminase enzyme with kinetic properties similar to those found in plant growth-promoting bacteria, which can reduce ethylene stress. Although ACC deaminase production might be expected to impact fungal virulence, knockout mutants of both genes showed no significant effect on fungal spread or mycotoxin content in infected wheat, suggesting that ACC deaminase production is dispensable for full virulence in *F. graminearum* (Svoboda et al., 2019).

Conclusion

Endophytic microorganisms, residing within plant tissues without causing harm, play a vital role in promoting plant growth and development. These beneficial bacteria and fungi employ various biochemical mechanisms to enhance nutrient availability, improve plant resilience against stresses, and stimulate growth. Notably, they produce Indole-3-Acetic Acid (IAA), solubilize phosphates, and exhibit ACC deaminase activity. IAA, a crucial plant hormone, regulates cell elongation, root formation, and fruit development, while its synthesis by endophytic bacteria contributes to increased root and shoot growth. Endophytic microorganisms also solubilize inorganic phosphates, making them more available for plant uptake. This process involves secreting organic acids and phosphatases that break down complex phosphorus forms, supporting energy transfer, photosynthesis, and nucleic acid synthesis. Furthermore, ACC deaminase activity in endophytes mitigates ethylene-related stress by lowering ACC levels, reducing ethylene production, and promoting growth, especially under stressful conditions like drought or salinity.

Enterobacter and *Fusarium* are two microorganisms with plant growth-promoting abilities. *Enterobacter* produces Indole-3-Acetic Acid (IAA), solubilizes phosphate, and produces ACC deaminase, reducing ethylene stress in plants. *Fusarium* also produces IAA, although its role in plant interactions is unclear, and produces ACC deaminase, but its impact on fungal virulence is dispensable. While Enterobacter has a broader range of plant growth-promoting

abilities, both microorganisms have potential applications in agriculture and plant growth promotion. By forming a symbiotic alliance, endophytes and host plants exhibit heightened resistance to biotic and abiotic stresses, translating into enhanced growth, biomass production, root development, and plant hardiness. This relationship promotes carbon sequestration and soil health, as enhanced growth contributes organic matter and nutrients back to the soil ecosystem. By understanding the mechanisms of endophytic isolates, researchers and agricultural practitioners can harness their potential in sustainable agriculture, exploring and validating these relationships in various plant species and environmental conditions.

Chapter 7

Summary & Conclusion

The research on Lagenandra toxicaria, an aquatic plant, has unveiled a fascinating world of endophytic microorganisms that play a crucial role in its growth and development. Our comprehensive study, which involved the isolation and diversity analysis of endophytic bacteria and fungi from three plant parts (leaves, roots, and rhizomes) across three locations (Pakkom, Kottavayal, and Mattilayam) and three seasons, has revealed a rich diversity of microorganisms with varied potential activities. The findings, such as the root tissues yielding more endophytes during the summer season in Pakkom and Kottavayal, and the impact of location and season on the endophytic community, are significant and enlightening.

Our comprehensive analysis of diversity indices and the effects of individual and pairwise variables—such as season, location, and tissue type—on endophyte diversity was conducted using MANOVA and bi-plot analysis. The results were statistically significant. We found notable differences in endophytic bacterial diversity at the 1% level concerning location, season, and plant type, which provides a solid basis to our conclusions.

Location significantly influenced the Shannon Diversity Index (SDI), the Standardized Weighted Diversity Index (SWDI), and Margalef richness, while seasonal variations had greater significance than the other factors. Principal Component Analysis (PCA) identified two primary components driving variability in the bacterial data, accounting for 85.6% and 12.2% of the total variance, respectively. Additionally, we identified *Bacillus amyloliquefaciens* as the dominant species, with the highest colonization rate of 11.96%, further reinforcing the robustness of our findings.

The analysis of endophytic fungal diversity revealed statistically significant differences at the 1% level. The location factor significantly impacted several diversity indices, including Species Diversity Index (SDI), Shannon-Wiener Diversity Index (SWDI), evenness, and Margalef richness, with significance

levels ranging from 1% to 5%. Additionally, all factors had statistically significant effects on the Community Richness (CR) and Margalef richness. However, other dependent variables, such as SDI and evenness, exhibited less significant effects.

In the case of SWDI, the interaction effects of location and season were significant. Pairwise comparisons showed significant differences in CR among the three locations at the 5% level, although no significant difference was found between Kottavayal and Pakkom. Significant differences were also observed in SDI and SWDI between Mattilayam and the other two locations, Kottavayal and Pakkom. In contrast, evenness did not show any significant differences among the locations.

Furthermore, location-wise comparisons indicated no significant differences in any characteristics, except CR, between Kottavayal and Pakkom, suggesting similarities in endophytic fungal diversity at these locations. The principal component (PC) analysis focused on the standardized data set of fungi to reduce dimensionality and identify the key components explaining the variance in the data. Over 99% of the variance was attributed to the first two principal components. The loading for the first principal component was quite uniform across all original variables, indicating that each variable contributed similarly and positively to the variance captured by the first PC. In the second PC, CR contributed highly positively, followed by SDI, while evenness had a significant negative contribution. *T*he most dominant genus identified for further study was *Aspergillus aculeatus*.

Lagenandra toxicaria and its endophytes can produce a variety of bioactive compounds, including alkaloids, steroids, terpenoids, quinones, and phenolic acids. These compounds possess antimicrobial and antioxidant properties, helping to protect the plant from pathogens and oxidative stress, which in turn enhances plant vitality and biomass production. Fungal endophytes are the main sources of these secondary metabolites. Notably, *Diaporthe penetriteum*

FLWS21, a fungal isolate from leaf samples, has demonstrated significant antimicrobial activity.

Further analysis using High-Performance Liquid Chromatography (HPLC) identified diethyl phthalate, an antimicrobial compound, in both the fungal isolate and the plant's leaf extract. The presence of diethyl phthalate in both the endophyte and the plant suggests a potential symbiotic relationship that contributes to the plant's resistance mechanisms. This emphasizes the role of endophytes in improving plant vitality and biomass production, highlighting the potential for discovering novel bioactive compounds with antimicrobial and antioxidant properties.

To maximize the production of these crude metabolites, optimization techniques were applied for the specific species. After optimization, the antimicrobial potential of the selected isolate extract was evaluated at various concentrations, confirming that the isolate is a rich source of antimicrobial compounds.

Additionally, it was noted that the presence and concentration of phytochemical compounds, along with the antimicrobial potential of plant extracts, vary seasonally. Notably, summer showed a higher concentration of these compounds, indicating that seasonal factors can influence the production and accumulation of bioactive compounds in the plant. This underscores the importance of considering seasonal variations when investigating the antimicrobial properties of aquatic plant extracts.

The antioxidant potential of endophytic isolates from *Lagenandra toxicaria* was evaluated using various assays. The results indicated that leaf isolates of both bacteria and fungi exhibited significant DPPH scavenging activity, while root isolates demonstrated notable ABTS activity. Additionally, both leaf and root isolates of bacteria and fungi showed reducing power. Notably, the bacterial isolate *Bacillus sp.* BLWS6 and the fungal isolate *Phanerochaete sordida* FLWS16 exhibited promising antioxidant activity, which can be attributed to the presence of flavonoids, phenols, terpenoids, and glycosides. Overall, fungal isolates displayed a more pronounced antioxidant potential compared to

bacterial isolates. These findings suggest that endophytic isolates from *Lagenandra toxicaria*, especially the fungal ones, could serve as a valuable source of natural antioxidants. Further exploration and investigation into the potential applications of these isolates in the pharmaceutical and nutraceutical sectors are warranted. Moreover, the various bioactive compounds present in these isolates underscore the importance of conserving and studying the endophytic diversity of *Lagenandra toxicaria*.

Lagenandra toxicaria endophytes play a crucial role in plant defense and produce extracellular enzymes that influence nutrient uptake and utilization. Research shows that most bacterial and fungal species exhibit high protease production, with *Bacillus* species being the predominant genera that produce all studied enzymes, including protease, cellulase, and pectinase.

Using a central composite design (CCD) within response surface methodology (RSM), researchers optimized enzyme production to model the relationship between enzyme activity and various factors such as temperature, pH, incubation time, rotation per minute (rpm), carbon source, and nitrogen source. RSM analysis identified the most influential factors, determined the optimal conditions for maximum enzyme activity, and predicted enzyme activity under different conditions. This approach reduces experimentation time and costs, improves accuracy, and enhances the understanding of enzyme mechanisms and kinetics, ultimately optimizing enzyme production and application conditions.

Additionally, the endophytes from *Enterobacter* and *Fusarium* species exhibit plant growth-promoting traits, including phosphate solubilization, indole-3-acetic acid (IAA) production, and ACC deaminase activity. These microorganisms enhance seed emergence, root elongation, and overall plant growth, making them suitable candidates for bioinoculants. By solubilizing phosphates, producing plant growth-promoting hormones like IAA, and mitigating ethylene-related stress through ACC deaminase activity, these endophytes significantly improve plant growth, increase biomass production,

and enhance nutrient use efficiency. Their potential as bioinoculants offers a promising approach to boosting plant productivity and sustainability.

The endophytic approach in *Lagenandra toxicaria* offers significant potential for advancing sustainable agriculture and plant biotechnology. By understanding how endophytes interact with plants, researchers can create innovative strategies to enhance crop productivity, improve resilience, and reduce reliance on chemical inputs. Further investigation of the endophytes associated with *Lagenandra toxicaria* could reveal new opportunities for promoting plant growth, managing diseases, and supporting environmental sustainability. This knowledge could contribute to the development of eco-friendly agricultural practices, minimizing ecological impact and promoting long-term sustainability.

Chapter 8

Recommendations

Elucidation of the Molecular Mechanisms Underlying Plant-Endophyte Interactions:

Further research is essential to unravel the signalling pathways and molecular mechanisms governing the interactions between *Lagenandra toxic area* and its endophytes. This is particularly true given the observed decline in plant vigour and habit size when the plant is transferred from its natural habitat to a controlled pot environment. This highlights the need to understand the complex relationships between the plant and its associated microorganisms.

Enhance the Production and Application of Diethyl Phthalate:

Further optimization is needed to improve the yield, purification, and isolation of Diethyl phthalate, as well as predict its structural properties, to unlock its full potential in pharmaceutical applications, where it can be used as a plasticizer, solvent, or active ingredient, and potentially lead to the development of new drugs and treatments.

Maximize the Plant Growth-Promoting Potential of Endophytes:

To develop *Lagenandra toxicaria* endophytes as effective biofertilizers or bioinoculants, further optimization using statistical tools like Response Surface Methodology (RSM) will be employed. This will enhance their ability to promote plant growth and development under diverse environmental conditions, ensuring their potential is fully harnessed for sustainable agriculture practices.

Assess the Bioremediation Potential of Endophytes:

Building on the known ability of *Lagenandra toxicaria* rhizomes to purify water and remove heavy metals, further research should explore the capacity of its endophytes to degrade pollutants and heavy metals, unlocking their potential as a natural solution for environmental remediation and sustainable water management.

Unlock the Biocontrol Potential of Endophytes:

Given the known nematicidal activity of *Lagenandra toxic area*, further research should investigate the biocontrol potential of its endophytes against a broader range of plant pathogens and pests to discover novel, eco-friendly solutions for managing plant diseases and promoting sustainable agriculture practices.

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Dr. N.S. Pradeep
Principal Scientist
KSCSTE - Malabar Botanical Garden
and Institute for Plant Sciences
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