

**DEVELOPMENT OF A MICROBIAL REMEDIATION
SYSTEM FOR THE ENHANCED BIODEGRADATION
OF POLYETHYLENE BAGS**

*Thesis submitted to the
University of Calicut, in partial fulfilment of the
requirements for the award of the degree of*

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY
under the faculty of science

By

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Under the guidance of
Prof (Dr). Denoj Sebastian



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CERTIFICATE

This is to certify that the thesis entitled “**DEVELOPMENT OF A MICROBIAL REMEDIATION SYSTEM FOR THE ENHANCED BIODEGRADATION OF POLYETHYLENE BAGS**” is a bonafide research work done by **Mrs NITHYA JAYAN.**, under my supervision and guidance in the Department of Life Sciences, University of Calicut for the award of the degree of Doctor of Philosophy in Microbiology, under the faculty of Science of the University of Calicut. I also certify that the same has not been submitted for any other degree, diploma, or associateship in any other University. Both the adjudicators have not recommended any modifications or suggestions and therefore the original thesis is resubmitted as such. The soft copy is attached is the same as that of resubmitted copy.

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Nithya Jayan

Dedicated to

*My Father, Mother, Daughter and all those who
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ABSTRACT

DEVELOPMENT OF A MICROBIAL REMEDIATION SYSTEM FOR THE ENHANCED BIODEGRADATION OF POLYETHYLENE BAGS

The global escalating ecological burden of persistent polyethylene waste necessitates sustainable mitigation strategies. This study presents an innovative biodegradation approach utilising indigenous bacterial strains isolated from long-term plastic landfills, namely Alathur, Laloore, and Bhramapuram in Kerala, India. Soil samples in triplicate were collected from each dump site after recording physicochemical parameters such as pH and temperature and enriched in carbon-free liquid mineral salt medium (CFLMSM) for one month. These were exposed to non-pre-treated low-density polyethylene (LDPE) films under static incubation for 120 days. Preliminary screening with Coomassie Brilliant Blue staining followed by repeated quadrant streaking identified potent degraders, whose biodegradation potential was subsequently validated through weight reduction assays, BATH tests, FDA assays, and CO₂ evolution analysis.

The most efficient strains, OBA5 ($42.73 \pm 1.105\%$, Alathur), OLA2 ($39.7 \pm 1.27\%$, Laloore), and OBM3 ($40.22 \pm 2.04\%$, Bhramapuram) exhibited weight loss of LDPE films were identified by biochemical tests, 16S rDNA sequencing, phylogenetic tree construction, and MALDI-TOF analysis, and identified as *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, and *Micrococcus luteus* NJD3. Strong biofilm formation on LDPE films was demonstrated by total cell count, EPS production, protein estimation, pH shifts of media, and FDA assays. Enzymatic quantifications of biofilms formed on the LDPE films further revealed the catalytic activities of laccases, lipases, proteases, lignin peroxidases, manganese peroxidases, and esterases. Moreover, biosurfactant production, contributing to the reduction of LDPE hydrophobicity, was demonstrated through oil displacement and emulsification index assays, and substantiated by FTIR analysis.

Advanced validation of bacterial adherence and polymer surface alterations was conducted using FE-SEM, SEM-EDAX, AFM, roughness parameter calculation, and water contact angle analysis, all of which confirmed enhanced hydrophilicity. Mechanical degradation of LDPE films was verified by tensile testing, while chemical modifications were assessed through FTIR, ATR-FTIR-FPA, and GC-MS analyses, with carbonyl index calculations highlighting strain-specific degradation efficiencies.

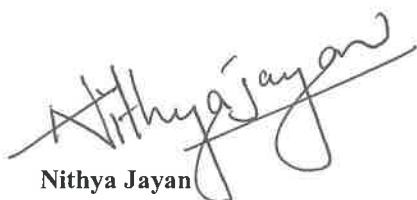
To enhance biodegradation, a laboratory-formulated artificial consortium of *B. cereus* NJD1, *P. aeruginosa* NJD2, and *M. luteus* NJD3 was developed. Its performance was evaluated through biofilm assays, weight loss experiments, and enzymatic profiling, revealing superior synergistic activity compared to individual strains. Further, the potency of the strains on the polymer films was authenticated through sophisticated analytical techniques such as FE-SEM, SEM-EDAX, AFM, roughness parameter calculations, WCA analysis, tensile testing, FTIR, ATR-FTIR-FPA and GC-MS analyses.

Finally, four soil microcosm setups amended with LDPE films were established to evaluate bioremediation potential under bioaugmentation and biostimulation approaches. Physicochemical

properties of the garden soil and heterotrophic counts in it were measured, while LDPE degradation was assessed via biomass quantification, FDA assays, CO₂ evolution, and weight loss analysis. SEM, AFM, FTIR, and GC-MS further confirmed the degradation of LDPE films buried in soil for four months.

This multidisciplinary investigation demonstrates that indigenous bacterial strains and their formulated consortium can significantly accelerate LDPE degradation under both laboratory and soil conditions. These findings lay the foundation for eco-sustainable strategies to mitigate plastic waste accumulation and combat white pollution globally.

Keywords: LDPE films, CFLMSM, Indigenous bacterial strains, static cultures, biofilm formation, extracellular enzyme profiling, artificial microbial consortium, soil augmentation.



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സംഗ്രഹം

പോളിയെത്തിലീൻ ബാഗുകളുടെ ത്വരിത നശീകരണത്തിനാവശ്യമായ സൂക്ഷ്മജീവികളിലൂടെയുള്ള പരിഹാര സംവിധാനത്തിന്റെ വികസനം

അനുദിനം വർദ്ധിച്ചുവരുന്ന പോളിയെത്തിലീൻ മാലിന്യങ്ങളുടെ ശരിയായ രീതിയിലുള്ള സുസ്ഥിരമായ നശീകരണം ലോകവ്യാപകമായി നമ്മൾ നേരിടുന്ന ഒരു പ്രതിസന്ധിയാണ്. ഈ ലക്ഷ്യത്തിലൂന്നിക്കൊണ്ടാണ് ഈ ഗവേഷണപഠനം പൂർത്തീകരിച്ചിട്ടുള്ളത്. കേരളത്തിലെ ആലത്തൂർ, ലാലൂർ, ബ്രഹ്മപുരം എന്നീ മാലിന്യ നിക്ഷേപ പ്രദേശങ്ങൾ കേന്ദ്രീകരിച്ചാണ് ഈ പഠനം നടത്തിയത്. ഇത്തരം പ്രദേശങ്ങളിൽ നിന്നും ലഭിച്ച തദ്ദേശീയ ബാക്ടീരിയ വകഭേദങ്ങൾ ഉപയോഗിച്ചുള്ള ഒരു പുതിയ ജൈവ നശീകരണ സമീപനം ആണ് ഈ ഗവേഷണത്തിലൂടെ പരിശോധിക്കുന്നത്. ഇത്തരം പ്രദേശങ്ങളിലുള്ള മണ്ണ് ശാസ്ത്രീയ മാർഗങ്ങളിലൂടെ ശേഖരിക്കുകയും അവയുടെ പിഎച്ച് മൂല്യം, താപനില തുടങ്ങിയ ഭൗതിക രാസപരമായ ഘടകങ്ങൾ രേഖപ്പെടുത്തുകയും ചെയ്തു. ഇവയെ കാർബൺ രഹിത ലിക്വിഡ് മിനറൽ സാൾട്ട് മീഡിയത്തിൽ ഒരു മാസത്തോളം സസ്പെന്ഷനായിരിക്കാൻ ശേഷം സ്റ്റാറ്റിക് ഇൻകുബേഷനിൽ 120 ദിവസത്തോളം തുടർന്ന് നിരീക്ഷിച്ചു. മുൻകൂട്ടി പരിവർത്തിതമാക്കിയ ഫിലിമുകൾ ആണ് ഈ മീഡിയത്തിൽ പരീക്ഷിച്ചത്. ക്യുമാസ്സി ബ്രിഡ്ജിയൻ ബ്ലൂ എന്ന സ്റ്റെയിനിംഗ് ഉപയോഗിച്ച് ഉള്ള പ്രാഥമിക സ്ക്രീനിങ്ങിനു ശേഷം ക്യാപ്രൈൻ സ്ട്രീക്കിങ് രീതി പലതവണ ആവർത്തിച്ച് LDPE നശീകരണശേഷിയുള്ള ബാക്ടീരിയ വകഭേദങ്ങളെ തിരിച്ചറിഞ്ഞു. ഭാരം കുറയ്ക്കുന്ന പരീക്ഷണങ്ങൾ, ബാത്ത് ടെസ്റ്റുകൾ, എഫ്ഡിഎ അസ്സേകൾ, കാർബൺ ഡൈയോക്സൈഡ് പുറപ്പെടുവിക്കൽ വിശകലനങ്ങൾ എന്നീ പരീക്ഷണങ്ങളിലൂടെ ജൈവ നശീകരണശേഷി സ്ഥിരീകരിക്കുകയും ചെയ്തു.

പോളിയെത്തിലീൻ ഫിലിമുകളുടെ ഭാരം ഗണ്യമായി കുറയ്ക്കാൻ കഴിഞ്ഞു മുൻ ബാക്ടീരിയ വകഭേദങ്ങളെയാണ് മാലിന്യ നിക്ഷേപ കേന്ദ്രത്തിൽ നിന്നും ലഭിച്ചത്. ഇവയിൽ ആലത്തൂരിൽ നിന്നും ലഭിച്ച OBA5 എന്ന ബാക്ടീരിയയും (ഏകദേശം 43% തോളും ഭാരക്കുറവ്) ലാലൂരിൽ നിന്നും കിട്ടിയ OLA2 എന്ന ബാക്ടീരിയയും (ഏകദേശം 40% തോളും) ബ്രഹ്മപുരത്തു നിന്നും ലഭിച്ച OBA5 എന്ന ബാക്ടീരിയയും (ഏകദേശം 42% തോളും) ഉൾപ്പെടുന്നു. ജൈവരാസ പരിശോധനകൾ, 16S rDNA സീക്വൻസിംഗ്, ഫൈലോജെനെറ്റിക് ടീ നിർമ്മാണം, MALDI-TOF വിശകലനം എന്നിവ വഴി ഈ നശീകരണ ശേഷിയുള്ള ബാക്ടീരിയകൾ ബാസിലസ് സിറസ്സ് NJD1, സൂഡോമോണാസ്സ് എയറോജിനോസ്സാ NJD2, മൈക്രോകോക്കസ് ലൂട്ടിയസ്സ് NJD3 എന്നിവയാണെന്ന് തിരിച്ചറിഞ്ഞു. എൽഡിപിഇ ഫിലിമുകളുടെ ഉപരിതലത്തിൽ പറ്റിപ്പിടിച്ച് ഉള്ള മൊത്തം കോശങ്ങളുടെ എണ്ണം അളന്നും ഇ പി എസ് ഉൽപാദനം, പ്രോട്ടീൻ അളവ് മുതലായവ കണക്കാക്കിയും ഫിലിമുകൾ ഇൻകുബേറ്റ് ചെയ്ത മീഡിയത്തിന്റെ pH മൂല്യ മാറ്റങ്ങൾ പഠിച്ചും എഫ്ഡിഎ അസ്സേകൾ നടത്തിയും, എൽഡിപിഇ ഫിലിമുകളിൽ ശക്തമായ രീതിയിൽ ബാക്ടീരിയയുടെ ബയോഫിലിം രൂപീകരണമുണ്ടെന്നു കണ്ടെത്തി. ലക്കേസ്, ലിപ്പേസ്, പ്രോട്ടീസ്, ലിഗ്സിൻ പെറോക്സിഡേസ്, മാംഗനീസ് പെറോക്സിഡേസ്, എസ്റ്ററേസ് എന്നീ എൻസൈമുകളുടെ അളവ് കണക്ക് കൂട്ടുന്നതിലൂടെ ഈ ബാക്ടീരിയകൾക്ക് LDPE ഫിലിമുകളെ വിഘടിപ്പിക്കുവാനുള്ള ശേഷി തെളിയിക്കപ്പെട്ടു. കൂടാതെ, LDPE ഫിലിമുകളുടെ ഹൈഡ്രോഫോബിസിറ്റി കുറയ്ക്കുന്നതിന് കാരണമാകുന്ന ബയോസർഫക്റ്റുകളുടെ ഉൽപാദനം, ഓയിൽ ഡിസ്പേഴ്സന്റ്, എമൾസിഫിക്കേഷൻ ഇൻഡക്സ് പരീക്ഷണങ്ങൾ, FTIR വിശകലനം എന്നിവ വഴി സ്ഥിരീകരിക്കാനായി.

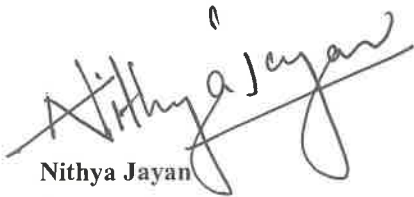
FE-SEM, SEM-EDAX, AFM, റഫ്സ് പാരാമീറ്റർ കണക്കുകൂട്ടൽ, വാട്ടർ കോൺടാക്ട് ആംഗിൾ വിശകലനം എന്നിവ വഴി പോളിമർ പ്രതലത്തിൽ ഒട്ടി നിൽക്കാനുള്ള ബാക്ടീരിയയുടെ ചേർപ്പ് ശേഷിയും പോളിമർ ഉപരിതലത്തിൽ തദാരാ ഉണ്ടാകുന്ന മാറ്റങ്ങളും കണ്ടെത്തുവാൻ കഴിഞ്ഞു. ഫിലിമുകളുടെ മെക്കാനിക്കൽ ഡിഗ്രഡേഷൻ ടെൻസൈൽ ടെസ്റ്റിങ്ങ് വഴി തെളിയിച്ചു. പ്രസ്തുത ഫിലിമുകളിൽ സംഭവിച്ച രാസപരിഷ്കരണങ്ങളാകട്ടെ FTIR, ATR-FTIR-FPA, GC-MS വിശകലനങ്ങളിലൂടെയാണ് കണ്ടെത്തിയത്.

LDPE ഫിലിമുകളുടെ മീതെയുള്ള ജൈവനശീകരണ ശേഷി വർദ്ധിപ്പിക്കുന്നതിനായി ബാസിലസ് സിറസ്സ് NJD1, സൂഡോമോണാസ്സ് എയറോജിനോസ്സാ NJD2, മൈക്രോകോക്കസ് ലൂട്ടിയസ്സ് NJD3 എന്നീ ബാക്ടീരിയകളെ പരീക്ഷണശാലയിൽ സംയോജിപ്പിക്കുകയും മൈക്രോബിയൽ കൺസോർഷ്യം കൃത്രിമമായി വികസിപ്പിക്കുകയും ചെയ്തു. ബയോഫിലിം അസ്സേകൾ, ഭാരം കുറയ്ക്കുന്ന പരീക്ഷണങ്ങൾ, എൻസൈമാറ്റിക് പ്രൊഫൈലിംഗ് എന്നിവ വഴി ബാക്ടീരിയ കൺസോർഷ്യത്തിന്റെ നശിപ്പിക്കൽപ്രകടനം വിലയിരുത്തി. ഒറ്റയ്ക്ക് ബാക്ടീരിയകൾ നടത്തുന്ന വ്യക്തിഗത നാശപ്രവർത്തനത്തേക്കാളും മികച്ച സിനർജിസ്റ്റിക് പ്രവർത്തനശേഷി ഈ കൂട്ടുചേർന്ന ബാക്ടീരിയകളുടെ കൺസോർഷ്യത്തിനുണ്ടെന്ന് ഇതിലൂടെ തെളിഞ്ഞു. പോളിമർ ഫിലിമുകളിൽ ഇവയുടെ പ്രവർത്തന ശേഷി FE-SEM, SEM-EDAX, AFM, റഫ്സ് പാരാമീറ്റർ കണക്കുകൂട്ടൽ, വാട്ടർ കോൺടാക്ട് ആംഗിൾ, വിശകലനം, ടെൻസൈൽ ടെസ്റ്റിങ്ങ്, FTIR, ATR-FTIR-FPA, GC-MS എന്നീ ഉന്നത സാങ്കേതിക വിദ്യകൾ ഉപയോഗിച്ച് വീണ്ടും സ്ഥിരീകരിച്ചു.

അവസാനമായി, ബയോ ഓഗ്മെന്റേഷൻ, ബയോ സ്റ്റിമുലേഷൻ സമീപനങ്ങൾ ഉപയോഗിച്ചുള്ള എൽഡിപിഇ ഫിലിമുകളുടെ നശീകരണ സാധ്യതാ പഠനമാണ് ഗവേഷക നടത്തിയത്. ഇതിനായി നാലു വ്യത്യസ്ത രീതിയിൽ സജ്ജീകരിച്ച മണ്ണും എൽഡിപി ഫിലിമുകളും ചേർന്ന അതിസൂക്ഷ്മ മാതൃകകൾ തയ്യാറാക്കി. ഇതിനായി മണ്ണിന്റെ ഭൗതികരാസ ഗുണങ്ങളും അതിലെ സൂക്ഷ്മജീവികളുടെ എണ്ണവും അളന്നു. ഈ സമീപനങ്ങളിലെ എൽഡിപിഇ നശീകരണം, ബയോമാസ് കണക്കാക്കൽ, എഫ്ഡിഎ അസേകൾ, CO₂ പുറപ്പെടുവിക്കൽ പരീക്ഷണം, ഫിലിമിന്റെ ഭാരം കുറയുന്നത് അളക്കുന്ന സമീപനങ്ങൾ എന്നിവ ഉപയോഗിച്ച് നിരീക്ഷിക്കപ്പെട്ടു. മണ്ണിൽ നാല് മാസം കഴിച്ച് എൽഡിപിഇ ഫിലിമുകളുടെ നാശം, SEM, AFM, FTIR, GC-MS എന്നീ സാങ്കേതിക വിദ്യകൾ ഉപയോഗിച്ച് തെളിയിച്ചു.

തദ്ദേശീയ ബാക്ടീരിയകളുടെ വകഭേദങ്ങളും അവ ഒന്നുചേർന്ന കൺസോർഷ്യങ്ങളും ലബോറട്ടറി സാഹചര്യത്തിലായാലും പ്രകൃത്യാ ഉള്ള മണ്ണിലായാലും എൽഡിപിഇ നശീകരണം വേഗത്തിലാക്കാൻ കഴിവുള്ളവയാണെന്ന് ഗവേഷകയുടെ ഈ മൾട്ടിഡിസിപ്ലിനറി ഗവേഷണപഠനം തെളിയിച്ചു. പ്ലാസ്റ്റിക് മാലിന്യ ശേഖരണം കുറയ്ക്കുന്നതിനും ലോകവ്യാപകമായ വൈറ്റ് പൊല്യൂഷനെ ചെറുക്കുന്നതിനുമായുള്ള പരിസ്ഥിതി-സുസ്ഥിരമായ മാലിന്യ പ്രതിരോധതന്ത്രങ്ങൾക്ക് ഈ കണ്ടെത്തലുകൾ അടിത്തറയിടുന്നു.

താക്കോൽ വാക്കുകൾ: എൽഡിപിഇ ഫിലിമുകൾ, CFLMSM, തദ്ദേശീയ ബാക്ടീരിയ വകഭേദങ്ങൾ, സ്റ്റാറ്റിക് കൾച്ചർ കണ്ടീഷൻ, ബയോഫിലിം രൂപീകരണം, എൻസൈം പ്രൊഫൈലിംഗ്, കൃത്രിമ മൈക്രോബിയൽ കൺസോർഷ്യം, ബയോ ഓഗ്മെന്റേഷൻ, ബയോ സ്റ്റിമുലേഷൻ സമീപനങ്ങൾ.


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ABBREVIATIONS

LDPE	- Low-Density Polyethylene
CFLMSM	- Carbon-free Liquid Mineral Salt Medium
TGA-DTA	- Thermogravimetric Analysis-Differential Thermal Analysis
MSB	- Mineral Salt Broth
SDS	- Sodium dodecyl sulphate
R ₂ A	- Reasoner's medium
rpm	- revolutions per minute
CBB	- Coomassie Brilliant Blue
CFU/g	- Colony Forming Unit/gram
t _{1/2}	- Half-life
OD	- Optical Density
ASTM	- American Society for Testing and Materials
BATH	- Bacterial Adhesion to Hydrocarbon test
CSH	- Cell Surface Hydrophobicity
UV	- VIS Spectrophotometer – Ultraviolet-visible Spectrophotometer
FDA	- Fluorescein Diacetate Hydrolysis Assay
FDSPB	- Fluorescein Diacetate Sodium Phosphate Buffer
TSI	- Triple Sugar Iron
ONPG	- Orthonitrophenol-β-D-galactopyranoside
NCBI	- National Centre for Biotechnology Information
PCR	- Polymerase Chain Reaction
BLAST	- Basic Local Alignment Search Tool
MEGA 11	- Molecular Evolutionary Genetics Analysis Version 11

MUSCLE	– Multiple Sequence Comparison by Log-Expectation
MSP	- Main Spectrum Profiling
PMF	- Protein Mass Fingerprint
MALDI TOF-MS	- Matrix Assisted Laser Desorption Ionisation-Time of Flight Mass
BSA	- Bovine Serum Albumin
EPS	- Extracellular Polysaccharide
ABTS	- 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
pNPP	- p-nitrophenyl palmitate
LiP	- Lignin Peroxidase
MnP	- Manganese Peroxidase
μL	- Microliter
mM	- Milli molar
CMC	– Carboxy Methylcellulose
U/mL	- Units per Millilitre
μg/mL	- Microgram per Millilitre
FESEM	- Field Emission Scanning Electron Microscopy
SEM-EDAX	- Scanning Electron Microscopy with Energy Dispersive X-ray Analysis.
Wt%	- Weight Percentage
3D	- Three Dimensional
2D	– Two Dimensional
AFM	- Atomic Force Microscopy
S _a	- Surface Roughness Mean Data
S _z	- Maximum Surface Height
S _q	- Roughness in Root Mean Square
S _p	- Maximum Height of Peak
S _v	- Maximum Depth of Pit

WCA	- Water Contact Angle
UTM	- Universal Testing Machine
UMTS	- Universal Mechanical Testing System
MPa	- Megapascal
N/m	- Newtons per meter
KN	- Kilo Newton
FTIR	- Fourier Transform Infrared Spectroscopy
KI	- Keto Carbonyl Indices
ESI	- Ester Carbonyl Indices
VBI	- Vinyl Bond Indices
IDBI	- Internal Double Bond Indices
FPA	- Focal plane array
ATR	- Attenuated Total Reflectance
nm	- Nanometer
GC-MS	- Gas Chromatography-Mass Spectroscopy
PTFE	- Polytetrafluoroethylene
PAEs	- Phthalic Acid Esters
DBP	- Dibutyl Phthalate
DEHP	- di-(2-ethyl hexyl)-phthalate
PA	- Phthalic acid
KMNO ₄	- Potassium permanganate
H ₃ BO ₃	- Boric acid
NaHCO ₃	- Sodium bicarbonate
K ₂ Cr ₂ O ₇	- Potassium dichromate
H ₃ PO ₄	- Phosphoric acid
FeSO ₄ (NH ₄) ₂ SO ₄ .6H ₂ O	- Ferrous ammonium sulphate
PO ₄ -P	- Inorganic orthophosphate
NH ₄ OAC	- Ammonium acetate
OC%	- Organic Carbon Percentage
SOC	- Soil Organic Carbon

SIC	- Soil Inorganic Carbon
Kg/Ha	- Kilograms per Hectare
SEM Value	- Standard Error of Mean Value
ANOVA	– Analysis of Variance

CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1. Plastic - a wonder material

Plastic is a synthetic polymer derived from petroleum by-products via chemical modification. Their complex macromolecular structure comprises repeating units of monomers (Chanda, 2021). The word plastic was adopted from the Greek word 'plastikos,' which means moldable to different shapes (Du & Wang, 2021). Its features, such as lightweight, high mechanical strength, flexibility, durability, resistance to moisture, and cost-effectiveness, have increased its global popularity (Joseph et al., 2021). Due to the immense versatility and broad applicability of plastics, conventional materials such as cloth, paper, wood, and metals have been quickly replaced (Nayanathara Thathsarani Pilapitiya & Ratnayake, 2024). Moreover, the use of plastic materials has extended to all fields that directly influence human life, for instance, the agricultural, industrial, automotive, infrastructure, textile, household, and even medical care sectors (Andrady & Neal, 2009; Singh et al., 2017).

Since 1950, global plastic output has increased dramatically—from 1.5 million tonnes to 359 million tonnes by 2018 (Shanmugam et al., 2020). This hike represents a 239-fold increase since 1950, driven by industrial demand and consumer convenience. However, the plastic industries worldwide expect to double from 464 million tonnes in 2020 to 884 million tonnes by 2050, potentially leading to a total stock accumulation of 4,725 million tonnes since 2000 (Ali et al., 2021).

Rapid industrialisation, urbanisation, and the use-and-throw habits of modern society eventually increase the production and waste in our Ecosystem (Siddiqua et al., 2022). Plastic materials are mainly categorised based on the polymer's thermal behaviour and rigidity as thermosetting plastics and thermoplastics (Bîrcă et al., 2019). Thermosetting plastics undergo chemical changes during curing and are converted to rigid cross-linked structures. Polyester, polyurethanes, epoxy resin, and melamine formaldehyde are examples of thermosetting plastics. On the other hand, thermoplastics are highly flexible and linear structures that can be melted and reshaped multiple times without significant chemical changes. The largest market for plastic materials is for thermoplastics, which includes polyethylene (PE), polyethylene terephthalate (PET), polyvinyl chloride (PVC), and polypropylene (PP) (Kibria et al., 2023; Taurozzi et al., 2023). Among the thermoplastics, polyethylenes (LDPE, LLDPE, and HDPE) are widely used as single-end-use plastics. These thermoplastic materials are broadly classified into two classes by the nature of their internal structure: semicrystalline (e.g., polyethylenes, PET, nylons, etc.) and amorphous (e.g., polystyrenes and polycarbonates).

Based on their backbone structures, synthetic polymers are again categorised into two. They are C-C and C-O polymers (Jiang et al., 2021). (Illustrated in Fig.1.1) Most C-C polymers have been prepared by vinyl polymerisation and are non-hydrolysable. It shares 77% of the global market. In contrast, C-O backbone polymers are hydrolysable and contribute only 18% of total plastic production. C-C polymers are generally more hydrophobic and may exhibit elevated melting

temperatures due to robust C-C bonds. In contrast, C-O polymers can be more hydrophilic depending on their structure. Examples of C-C polymers include polyethylene (PE), polypropylene (PP), and polystyrene (PS). C-O polymers comprise polyether (PEO), polyurethanes (PUR or PU), and polylactic acid (PLA). Almost all plastic polymers are either aliphatic (Polyethylenes) or aromatic (Polyurethanes) and their derivatives (Polyvinyl chloride) (Dey et al., 2020). Polymers may consist of single monomeric units or be formed as copolymers. Copolymers can contain alternating sequences of distinct monomers or random distributions of repeating units. Depending on their architecture, they may also occur as block, graft, linear, or branched structures. (Politakos & Avgeropoulos, 2023).

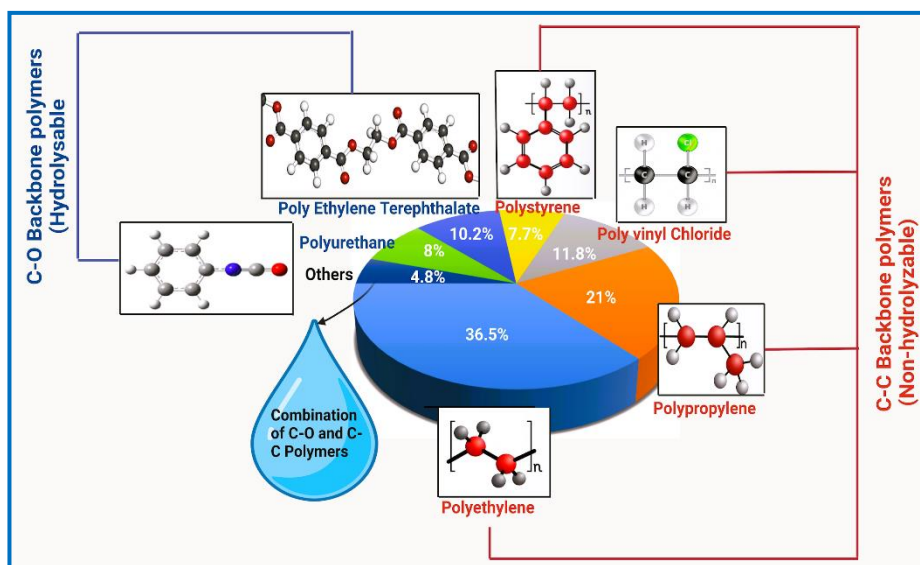


Fig.1.1 Global market shares of C-C and C-O plastic polymers with their molecular structures.

1.1 Polyethylenes (PE)

Polyethylenes, also known as polyethenes, are highly stable and versatile C-C synthetic resins within the polyolefin family (Sauter et al., 2017). They are made of ethylene monomers with a chemical formula of $(C_2H_4)_n$ (Somoza-Tornos et al., 2020). Polyethylene accounts for approximately 25-34% of the global plastics market for making goods like wrapping materials, water bottles and tanks. Due to their high hydrophobicity, crystallinity, molecular weight, and chemical resistance, polyethylene materials are considered recalcitrant (Law & Narayan, 2022). Compared to other plastic types, the global production and consumption of polyethylenes are incredibly high. The annual increase in polyethylene production is projected to be 12% (Siracusa, 2019).

Polyethylenes can be categorised into two general groups: branched (LDPE and LLDPE) and linear (HDPE and UHMWPE) (Salakhov et al., 2021). The degree of branching in polyethylene affects various polymer structural properties, including crystallinity, opacity, melting temperature, and surface hardness. Each type of polyethylene has its unique density and functional groups (Ronca, 2017) (see Table 1).

Besides the main polyethylene types, the following are also considered polyethylene types.

1. Cross-linked polyethylenes(XPE) are analogous to HDPE and MDPE, a thermosetting type of polyethylene used in plumbing and as insulators of medium—to high-voltage cables (Sakellariou et al., 2013).

2. Very Low-Density Polyethylenes(VLDPE) are formed by co-polymerising ethylene with short alpha-olefin chains. They are linear polymers interspersed with numerous short chains (Okamura, 2015).
3. Ultra-low-molecular-weight Polyethylenes (ULMWPE) are by-products of crude oil cracking into ethylene. They are also known as PE-WAX and are utilised in the lubrication and additive industries (Pattnaik & Sutar, 2022).

The type and number of branches on the chain significantly influence the properties of polyethylene (Roiron et al., 2021). Type of process: High-pressure or low-pressure processes affect the branching of the chain and the branch itself. However, the high-pressure process generates it through radical polymerisation, resulting in both short—and long-chain branches in its structure. It is highly moldable (Froese et al., 2024).

Polyethylene production is increasing daily in all countries (Khan et al., 2023; OECD, 2022; Statista, 2025). Polyethylene is the largest synthetic polymer, with an annual production exceeding 100 million tons, accounting for approximately one-third of overall plastic material production (Schwab et al., 2024). Rapid industrialisation and urbanisation have led to significant polyethylene markets in Asia (57.1 Mt/year) and North America (33.8 Mt/year) (Mufarrij et al., 2023). In the world, China is the largest producer and consumer of all plastic materials, particularly single-use plastics (Sun & He, 2023).

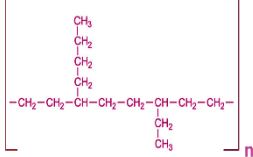
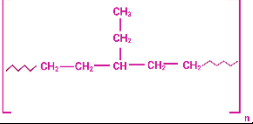
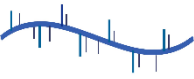
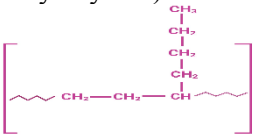
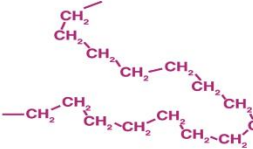
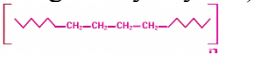
S. No	Polymer Type	Density (g/cm ³)	Branching Nature	Characteristics
1	LDPE (Low-Density Polyethylene) 	0.920–0.925	Long and short branches 	Lower tensile strength; used for packaging (carry bags, films, food wraps).
2	LLDPE (Linear Low-Density Polyethylene) 	0.923–0.928	Short branches, more linear 	Higher tensile strength than LDPE; used for toys, bubble wraps, and containers.
3	MDPE (Medium-Density Polyethylene) 	0.930–0.943	Moderate branching 	Good shock/drop resistance; used for plumbing, piping, sacks, and shrink film.
4	HDPE (High-Density Polyethylene) 	0.948–0.965	Low branching 	High tensile strength, low melting point; used for medical devices, bottles, and tubs.
5	UHMWPE (Ultra High Molecular Weight Polyethylene) 	>0.966	Linear 	Abrasion/corrosion resistant; used for vehicle armour, suspension lines, cables.

Table 1.1 Major polyethylene categories are specified by the American Society for Testing and Materials (ASTM) (Paxton et al., 2019).

1.2 Single-use polyethylenes

Single-use polyethylenes, particularly LDPE, are major contributors to plastic pollution. Plastic bags, food containers, and other packaging materials are the major constituents of such polyethylenes. Although it comprises various grades of polyethylenes, such as LDPE, LLDPE, VLDPE, PET, PP, and HDPE, a significant portion of single-use polyethylene waste consists of LDPE-based packaging materials, including films or sheets, which are highly resistant to microbial attack for an extended period (Karmakar, 2022). The toxic additives and microplastics released from single-use plastics into the surrounding environment have a severe impact on the balance of our biosystem (Yu et al., 2024). As a result, there is a rising global effort to curb its usage. Several government agencies and public health organisations have started promoting alternatives (such as reusable bags, containers, and other eco-friendly materials), implementing bans, encouraging industries to use take-back programs, launching awareness programs, and providing financial assistance to waste-to-energy models to mitigate the colossal amount of waste (Kumar et al., 2021).

1.2.1 LDPE- a widely used polymer compound

Low-density polyethylenes (LDPE) are characterised by low density, which results in their flexibility and toughness. It is extensively used in the production of packaging materials, consumer goods, construction materials, and mulching films for agricultural purposes (Mansoor et al., 2022). The LDPE compound was discovered accidentally by Imperial Chemical Industries (ICI) in 1933 during high-pressure ethylene polymerisation experiments (LeNoir, 2022). Worldwide, China and India

are the two countries experiencing the most rapid growth in their production (Shaikh et al., 2023).

1.2.2 Basic Structure of Low-Density Polyethylene

LDPE materials are polymerised forms of gaseous ethylene under high pressure (150-350 MPa) and temperature (80–300°C), depending on the process conditions, in the presence of oxide initiators (Liyana Bekri et al., 2023). Branched chains (short and long) in their structure (in 2% of carbon atoms) make the compound flexible and recyclable (Kumar Sen & Raut, 2015) (Fig. 1.2). Its durability (short span at 95°C, long span at 80°C), ductility, 30-50% crystallinity, water impermeability, microbial resistance and cheap production rate made the compound highly acceptable and invincible in the packaging and transporting industries (Yuan & Xu, 2023). They are the chief component in plastic carry bags, agricultural mulch films, and polyhouse sheets. LDPE film coatings are frequently used in papers, clothing, toys, and electronics.

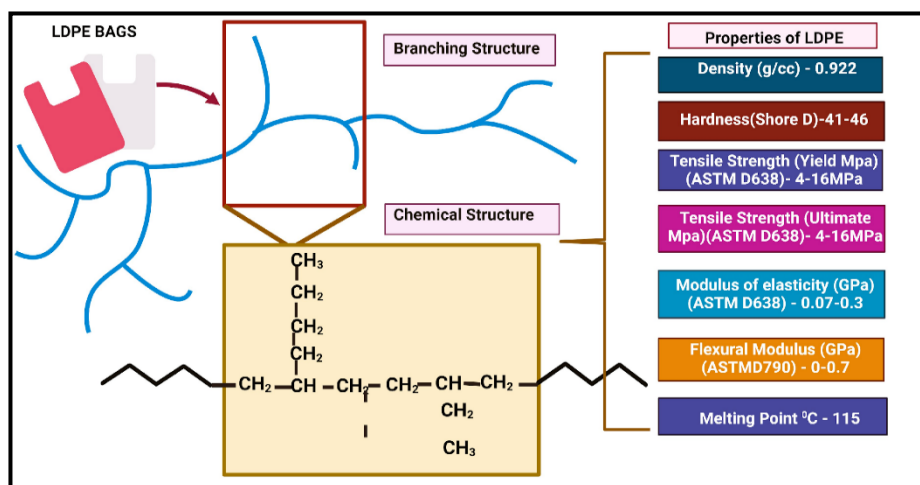


Fig.1.2: Molecular structure of LDPE, showing short and long branching patterns.

1.2.3 Distribution of LDPE trash in our Ecosystem

The widespread accumulation of polyethylene waste worldwide is primarily attributed to its inconspicuous mass production, low cost, indiscriminate disposal, inadequate infrastructure, lack of scientific degradation methods, insufficient regulatory frameworks, and ineffective waste management strategies (Geyer et al., 2017; Schultz & Reinhardt, 2023). Furthermore, poor plastic waste handling practices worldwide have consistently contributed to environmental issues, including water pollution, soil degradation, urban flooding, and other ecological disturbances (Carpenter & Wolverton, 2017).

Under prevailing conditions, commonly used LDPE-based plastic bags are estimated to persist in the environment for approximately 100 to 1,000 years, depending on environmental conditions such as UV exposure and microbial activity (Welden, 2020). Alarmingly, a substantial portion of the plastic carry bags produced annually by global plastic industries are discarded after a single use (Suleman et al., 2022).

Low-density polyethylene materials hurt the ecosystem throughout their life cycle, from resource extraction to final disposal. It is estimated that nearly 72% of all synthetic polymers produced globally ultimately accumulate in terrestrial and marine landfills (Dimassi et al., 2022). Their uncontrolled disposal and rapid weathering release harmful substances, including phthalates, plasticisers, heavy metals, toxic volatile organic compounds (VOCs), microplastics, dyes, fillers, and hazardous gases such as methane and carbon dioxide (Nzimande et

al., 2024). These emissions degrade the aesthetic and environmental quality of urban and rural landscapes, disrupt wildlife, damage aquatic ecosystems, and diminish agricultural fertility (Mir et al., 2023; Shetty et al., 2023). The bioaccumulation of these chemicals ultimately leads to immune system malfunction, cancer, lung diseases, neurological problems, and gastrointestinal illnesses in humans.

1.3 Conventional plastic eradication mechanisms

Landfilling, incineration, recycling, and pyrolysis are other conventional methods for disposing of synthetic polymers from our ecosystem (Skariyachan et al., 2018). However, all these traditional methods for eradicating plastic face several detrimental environmental challenges. Consequently, scientists concentrated on using microbes to decompose polyethylene compounds in an environmentally safer manner.

1.4 Degradation mechanisms of low-density polyethylenes

Most environmental degradation methods, such as photo-degradation (UV rays-assisted degradation) and thermo-oxidative and hydrolytic degradations of LDPE materials, require prolonged time and high energy (Akan et al., 2021). Moreover, the mechanical degradation method releases a significant amount of persistent organic pollutants (Niaounakis, 2017) into the environment, and chemical degradation leads to the improper disposal of chemicals (Moharir & Kumar, 2019) used in the process. Microbial degradation, insect-based degradation, and combined degradation (an amalgamation of the actions of bioagents and pre-modifications of polymers using temperature, UV,

gamma rays, steaming, and chemical treatments) are the three significant methods for the safe degradation of the compound (Chamas et al., 2020). However, the complete mineralisation of this polymer compound within a short span is still being researched.

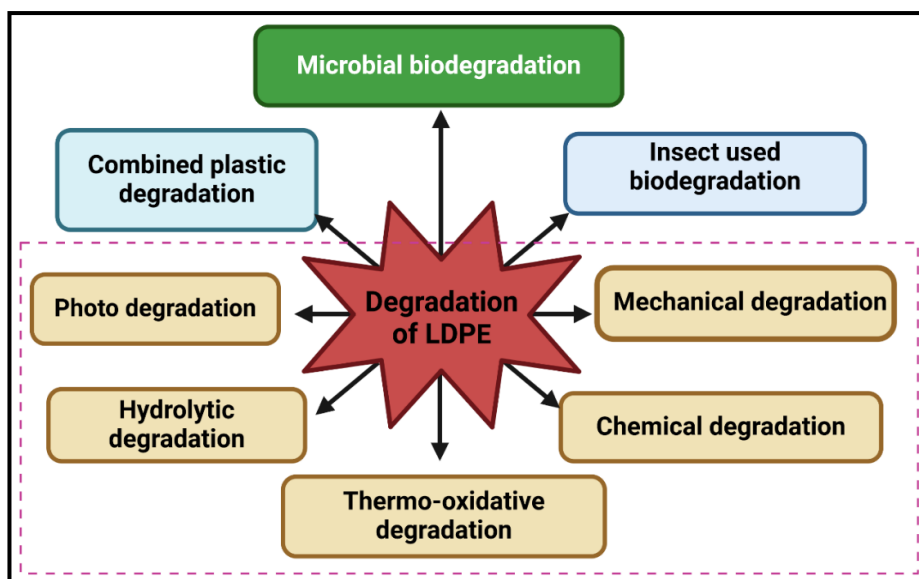


Fig.1.3 Various possible methods of degradation of the LDPE compounds

1.4.1 Microbial biodegradation

Recently, numerous studies have opened new avenues for the safe degradation of LDPE materials using natural or engineered microbes with designed enzyme action (Okal et al., 2023; Tao et al., 2021; Yan et al., 2016). However, only a few could validate significant degradation using a microbial strain (Gao & Sun, 2021). In addition, very few studies have assessed the biodegradation of pretreated LDPE films using natural individuals or consortia.

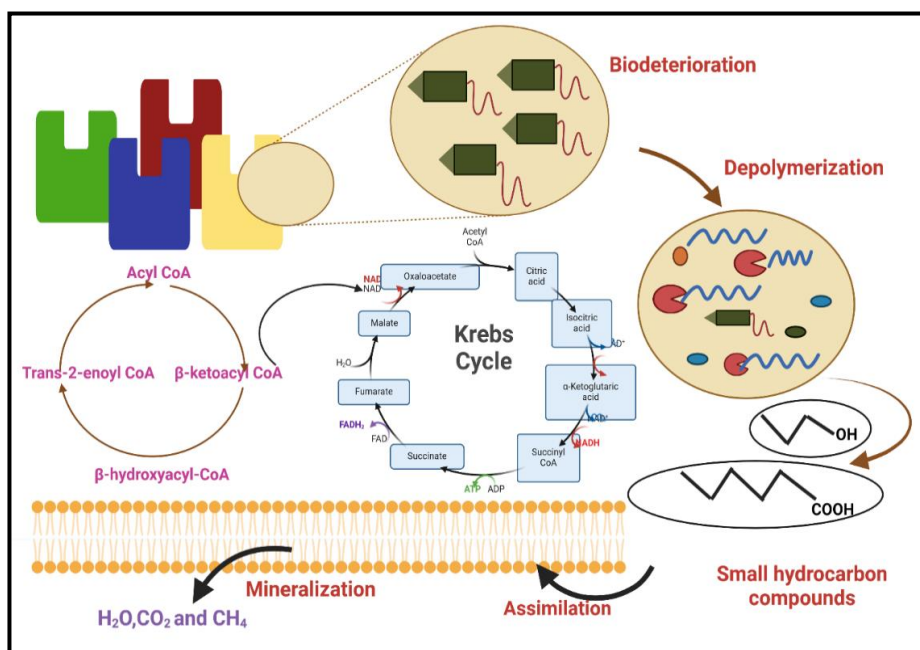


Fig. 1.4 Overall reactions in microbial degradation

1.4.1.1 Structural peculiarities for biofilm formation

The colonisation of microbes on the LDPE films may be due to the potential strains' extracellular matrix production or biosurfactant production. Most microbial surfactants comprise intricate compounds, including lipids, proteins, carbohydrates, and complex structures. They may allow microbes to aggregate and cling to hydrophobic polymer surfaces, facilitating biofilm formation (Song et al., 2020). It severely disrupts the surface structure, along with the micro utilisation to use the compound to establish chemical changes (Ji et al., 2024). Many earlier researchers have proven that surface erosion on polymer films may be a prerequisite for effective biodegradation (Bhagwat et al., 2021; Gupta et al., 2016; McFall et al., 2024).

1.4.1.2 Enzymes for the biodegradation of LDPE compounds

The enzymes produced by microbial strains are the most potent weapons for the biodegradation of LDPE materials. They cut, hydrolyse, oxidise, reduce, and esterify the polymer's interior molecular structure (Morales Ramos et al., 2024). Many studies have reported that the potentialities of bacterial enzymes, such as laccases, lipases, proteases, hydroxylases, manganese peroxidases, lignin peroxidases, and esterases, are responsible for LDPE biodegradation (Kavitha & Bhuvaneswari, 2021; Mohanan et al., 2020). Linear alkane-degrading enzymes, such as alkane hydroxylases produced by *Pseudomonas aeruginosa* E7, are efficient in degrading LDPE compounds (Jeon & Kim, 2015). Cytochrome P-450 from *Bacillus* sp. has also been shown to be an enzyme involved in the degradation of polyethylene strips (Khandare et al., 2022). It will initially produce several crevices, pits, and cracks on LDPE films, leading to form degradation in the internal layers of the polymeric film (Thakur et al., 2018). The enzymes can produce metabolic products such as acids, alcohols, and alkalis by reacting with polyethylene films (Zhang et al., 2023).

1.5 Soil augmentation of potential bacterial strains for curtailing LDPE pollution

Bioaugmentation and stimulation of microbial strains using nutrients are the most cost-effective mechanisms for accelerating bioremediation in natural environments. These in-situ bioremediation mechanisms, which result in the production of non-toxic end products, emerge as a

promising technology for removing white pollution from the environment. Several recent studies have demonstrated that microbes can be modified to adhere to LDPE films when mixed in soil samples and utilised as an energy source (Esmaeili et al., 2013; Sajjad et al., 2022).

1.6 Study Objectives

Concerns about escalating plastic pollution are growing daily in the modern world. The prospect of developing an effective microbial process to eliminate the accumulation of LDPE-like polymers in our environment still faces numerous challenges. However, several microbial species have been demonstrated to be capable of biodegradation, and an innate bacterial strain or consortium consistently adheres to non-pre-treated LDPE films. It degrades efficiently for a prolonged period in a static culture environment at room temperature, but its application is limited to a few studies. Another shortcoming in research in this domain is the insufficient insight into the enzymatic roles of the strain and the microbial structural features that support strong biofilm formation on polymeric surfaces. Although several recent researchers have demonstrated biofilm formation on different hydrocarbon surfaces, their investigations have shown a meagre amount of weight reduction in polymer pieces. An effective natural consortium or in-vitro stable condition under natural conditions is still under research. To date, the demonstration of metabolic products, structural alterations, reductions in mechanical strength, and chemical changes has not been fully explored. To elucidate the exact metabolic path and genomic activities

requires much research. The field application of potentiality has proven beneficial for microbial species, and scientists have not extensively explored its efficacy in recent years.

We conducted analyses focusing on the following primary objectives to address these research gaps.

- Screen and identify bacterial species from long-run plastic dumps without anthropogenic activities.
- Evaluate robust biofilm formation on non-pre-treated LDPE films under strenuous conditions in a carbon-free mineral salt medium maintained at room temperature for 120 days.
- Assess the catalytic activities of isolated strains in biofilm by quantitatively and qualitatively demonstrating the presence of extracellular enzymes.
- Assess structural peculiarities of efficient strain that are helpful for their adherence to the LDPE films.
- Validate the potential activities of strain for LDPE biodegradation using sophisticated analytical techniques.
- Formulate an artificial consortium consistent in room temperature and static conditions for the degradation of LDPE film.
- Investigate the efficacies of potential bacterial strains or consortia in LDPE mixed garden soil samples using augmentation and stimulation strategies.

CHAPTER 2

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REVIEW OF LITERATURE

2.1 History of plastic production

The manufacturing of synthetic polymers began in 1862 with the introduction of Parkesine (celluloid), a chemical derived from chloroform and castor oil, which was showcased at an exhibition in London (Strom & Rasmussen, 2011). However, the initial synthetic plastic, Bakelite, was synthesised by interacting with phenol and formaldehyde under heat and pressure. It was discovered by a Belgian chemist, Leo Baekeland, in 1907, who took out a U.S. patent in 1909 and introduced the term 'Plastics' (Geyer et al., 2017). The discovery of polyethylenes may have signalled the start of the plastic age, but its mass adoption took off only following World War II period (1939-1945) (Stanley et al., 2025). Plastics have become indispensable in modern society due to their versatility and widespread applications (Horton, 2022). It has outperformed all other artificial and natural compounds in the market for packaging and other daily life applications (Kounina et al., 2024; A. T. Williams & Rangel-Buitrago, 2022).

Most plastic polymers are by-products of petrochemical products. The significant steps in manufacturing plastic compounds include extracting raw materials, refining the process, polymerisation (addition or condensation polymerisation), and compounding or processing (Speight, 2020).

Significant discoveries in the plastic age of the 20th century

- Whinfield and Dickson of Calico Printer, a UK-based Company, synthesised PET resin in 1941 (Rex & Tennant, 1949). However, the trademark registration for the compound was first received by the DuPont Company in the U.S. in 1952. After extensive research, Nathaniel Wyeth of the DuPont Company patented PET-based bottles in 1973 (Le et al., 2019).
- High-density polyethylene compounds were discovered by German chemist Karl Ziegler in 1953 (Ziegler et al., 1955) and received the Nobel Prize for the discovery in 1963.
- Polyvinyl chloride, the polymerised form of vinyl chloride, was first manufactured by Eugen Baumann in 1872. In 1926, Waldo Semon of Goodrich Company started using additives in PVC. Dibutyl phthalates were used in PVC in 1933 (Semon & Stahl, 1981).
- Low-density polyethylene compounds were produced in 1933 by Dr John Swallow and Perrin of ICI (Imperial Chemical Industries) (Achilias et al., 2007).
- Paul Hogan and Robert Banks conducted the initial demonstration of polypropylene synthesis. Montecatini, which began commercial production of this product in 1951 (Corradini, 2004), initially produced it.
- Polystyrene was first discovered by Eduard Simon, a Berlin physician, in 1839 (Ojo & Bugarin, 2021; Simon, 1839). In 1937, Robert Dreisbach and his colleagues at Dow Chemical Company in Michigan purified styrene monomer through the dehydrogenation

of ethylbenzene, initiating worldwide production in 1938 (Boyer, 1981). In 1954, they also began producing expanded polystyrene foams (EPS) (Q. Liu et al., 2005).

- Polycarbonates were discovered in 1898 by a German scientist, Alfred Einhorn (Fox, 1986). Later, in 1953, German researcher Hermand Schnell of Bayer received a patent for the discovery of linear polycarbonates, and he started a brand named Makrolon in 1955 for global commercialisation. A week after Hermand's discovery in 1953, Daniel Fox at General Electric Company in Massachusetts, USA, discovered branched polycarbonates and began their massive production in 1960 (Kyriacos, 2017).
- Polylactic acid (PLA) was first manufactured commercially by DuPont scientist Wallace Carothers in 1932 through a process of heating lactic acid under vacuum and ring-opening polymerisation (de França et al., 2022).
- Nylons: Nylon 66 was first synthesised by DuPont's research facility in 1935 (Deopura, 2008). In response, Paul Schlack at I. G. Farben developed nylon 6 (Veit, 2022).
- Acrylic polymer: This polymer was synthesised in 1880 by G. W. A. Kahlbaum and extensively studied by Otto Rohm in 1991 (Matsuo et al., 1999).

2.2 Types of synthetic plastic resins in daily life

Although fifty different families of plastic materials exist, all are classified into seven major groups, designated by resin codes 1-7, depending on the polymer constituent and nature. They are as follows,

2.2.1 Polyethylene terephthalate (PET) (Resin code 1): This thermoplastic polymer is amorphous or semicrystalline (Sonnendecker et al., 2022). It is a polymer composed of ethylene glycol and terephthalic acid, exhibiting anti-air and anti-inflammatory properties (Pang et al., 2016). It is beneficial in the packaging and textile industries.

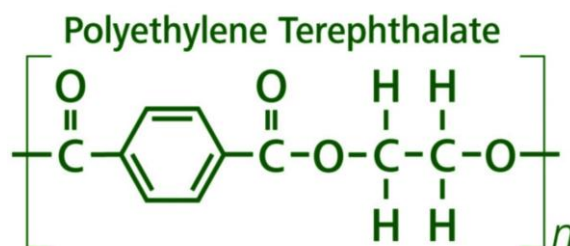


Fig. 2.1 Chemical structure of PET polymer with empirical formula $(C_{10}H_8O_4)_n$.

2.2.2 High-density polyethylenes(HDPE) (Resin code 2): The polymer is made up of ethylene monomer, which differs from LDPE compounds in the degree of branching. Catalysts like Ziegler-Natta ensure a lower degree of branching in the chemical structure of HDPE. HDPE compounds are manufactured without adding phthalates and BPA (Y. A. Liu & Sharma, 2023).



Fig. 2.2 Chemical structure and branching nature of HDPE with empirical formula $(C_2H_4)_n$

2.2.3 Polyvinyl chloride (PVC) (Resin code 3): PVC is a heat-resistant and highly flexible synthetic polymer containing heavy metals, phthalates, and bisphenol A. The compound's toxic leachates may cause chronic bronchitis, congenital disabilities, genetic mutations, cancer, deafness, vision impairments, and internal organ dysfunction (Campanale et al., 2020).

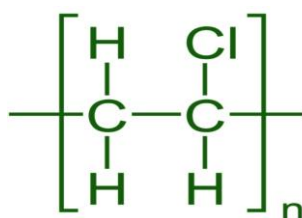


Fig. 2.3 Chemical structure of PVC with empirical formula $(C_2H_3Cl)_n$

2.2.4 Low-Density Polyethylenes (LDPE) (Resin code 4): This polymeric form of ethylene monomer is synthesised under high-pressure conditions and is widely used in the packaging industry. LDPEs are highly crystalline, hydrophobic, branched polymers that are highly resistant to acids, bases, and esters. This synthetic plastic is renowned for its low-temperature corrosion resistance, hardness, and flexibility (Ram & Miltz, 1972).



Fig 2.4 Chemical structure and branching nature of LDPE $(C_2H_4)_n$

2.2.5 Polypropylenes (PP) (Resin code 5): They are semitransparent polymers, heavier and more robust than polyethylenes due to a methyl side chain. It is beneficial in manufacturing medicine covers, bottles, and food packs. Zeiger-Natta catalysts link propylene monomers to form a specific orientation, resulting in a rigid structure, and are widely used in the manufacture of pipes (Cui et al., 2017).

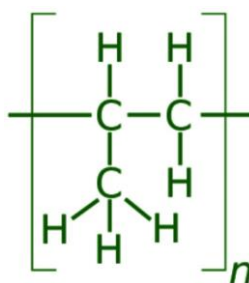


Fig. 2.5 Chemical structure of polypropylenes with empirical formula $(\text{C}_3\text{H}_6)_n$

2.2.6 Polystyrenes (P.S.) (Resin code 6): They are a highly neurotoxic, reproductive disorder, and a carcinogenic polymer comprising aromatic hydrocarbon units of styrene (Saeed et al., 2023). It is an inexpensive and transparent plastic-type, solid at room temperature and liquid at higher temperatures. It is used to manufacture packaging materials, insulators, laboratory equipment, household items, and medical products (Hwang et al., 2020).

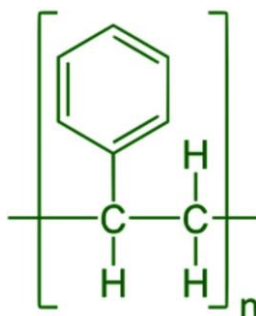


Fig. 2.6 Chemical Structure of polystyrenes with empirical formula $(C_8H_8)_n$

2.2.7 Others (Resin code 7): This category includes polycarbonates, polylactic acid fibres (bioplastics), nylons, acrylics, and plastic types not included in resin codes 1-6.

- a. Polycarbonates are thermoplastic compounds with bisphenol A (BPA). They mainly produce containers and other food packaging (Floudas et al., 1993).

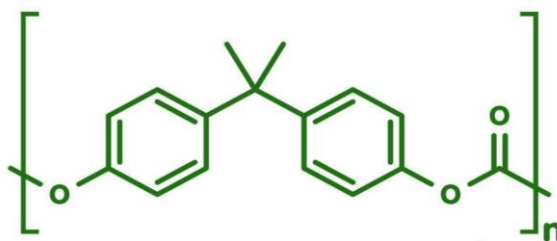


Fig. 2.7.a The chemical structure of polycarbonates with the empirical formula $(C_{16}H_{14}O_3)_n$.

- b. Polylactic acid (PLA) is a primarily biodegradable plastic synthesised using corn, cassava, and sugar cane starch. It is widely used in food packaging, vehicle upholstery, 3D printing filaments, and feminine hygiene products (Z. Yang et al., 2024).

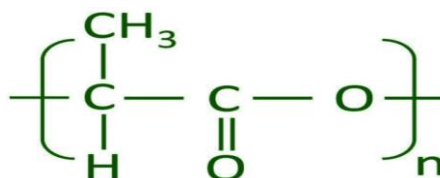


Fig. 2.7.b Chemical structure of polylactic acid ($\text{C}_3\text{H}_4\text{O}_2$)_n

- c. Nylons: They are synthetic fibres composed of polyamides. It is instrumental in the textile and food packaging industries (Ozger et al., 2013).

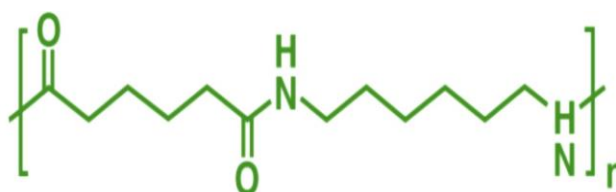


Fig. 2.7.c Chemical structure of Nylon 66 with empirical formula ($\text{C}_{12}\text{H}_{22}\text{N}_2\text{O}_2$)_n

- d. Acrylic polymers are widely used in paints, nail polishes, and superglue formulations. They are transparent, elastic, and breakage-resistant compounds (Lam et al., 2021)

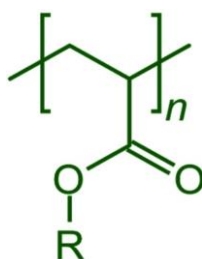


Fig. 2.7.d Chemical structure of the acrylic polymer ($\text{C}_3\text{H}_4\text{O}_2$)_n

RESIN CODES	1	2	3	4	5	6	7
RESIN NAME	PET POLYETHYLENE TEREPHTHALATE	HDPE HIGH DENSITY POLYETHYLENE	PVC POLYVINYL CHLORIDE	LDPE LOW DENSITY POLYETHYLENE	PP POLYPROPYLENE	PS POLYSTYRENE	OTHERS POLY CARBONATES,POLY LACTIC ACID (BIO PLASTICS), NYLON AND ABS
Recyclability (in percentage per annum)	36%	30 - 35%	< 1%	6%	3%	34%	very low
Time period to decompose	5-10 years	100 years	Not determined	100-1000 years	20-30 years	50 years	Not determined
Toxicity level	 Highly toxic  less toxic						
Microbial leachate	Antimony Oxide, Bromine, Diazomethane Lead Oxide, Nickel, Ethylene Oxide and Benzene	Chromium oxide, Benzoyl peroxide, hexane and cyclohexane	Benzene,CCl ₄ , Phthalate, Ethylene oxide, Mercury, Cadmium and Bisphenol	Benzene, Chromium oxide, Tert- butyl hydroperoxide and phthalate	Methanol,2-6 di tert-butyl-4- methyl phenol and Dibutyl Dithiocarbamate	Styrene,Ethyl benzene ,Ehylene,CC ₄ ,, Polyvinyl alcohol, Antimony oxide, Tert-butyl hydroperoxide	BPA, BPS and all toxins mentioned for all other plastic types
Major Products	Bottles	Shampoo bottles, Milk and grocery bags	Plumbing materials	Carry bags,sheets	Plastic containers, furniture, Hangers,Auto mobile parts	Plastic cups, Containers, Super Market trays	Baby bottles, CDs,Car parts

Table 2.1 Plastic materials are categorised into seven global standard resin codes (ASTM D7611) depending on their recyclability, biodegradability, and toxicity level (Shamsuyeva & Endres, 2021).

2.3 Plastic additives

Except for hydrocarbon components, a small number of plastic additive categories are added to nearly every form of plastic to modify its bulk characteristics and ensure its durability (Hahladakis et al., 2018; Wiesinger et al., 2021). Plasticising agents, stabilising agents, antioxidants, flame retardants, colouring agents, filler substances, and processing aids are common plastic additives (Maddela et al., 2023) (Table 2.3). Various environmental and health risks are associated with the migration of these chemicals into the ecosystem (N. S. Bolan et al., 2020). Additionally, several studies have reported that the release of

plastic additives from food packaging can alter the taste, aroma, and safety of the items (Alamri et al., 2021).

S. No	Category of Additives	Amount Range (% w/w)	Chemical Nature	Explanations
1	Plasticisers	25% (semi-rigid PVC); >86% (flexible PVC)	Chlorinated paraffin, phthalates, adipates	Used in all types of plastics.
2	Flame retardants	12–18%	Antimony, phosphorus, and hexabromocyclohexane compounds	3 types: Organic non-reactive, inorganic non-reactive, reactive.
3	UV stabilisers and antioxidants	Up to 3%	Biphenyl A, heavy metals (Cd, Pb)	UV stabilisers absorb/deflect UV rays; antioxidants prevent oxidation.
4	Inorganic pigments	0.001–10%	Zinc oxide, zinc sulfide, iron oxide, and titanium dioxide	Invisible with low insolubility.
5	Fillers (reinforcing/non-reinforcing)	Up to 55%	Calcium carbonates, clay, talc, glimmers, metal powders, glass fibres	Leaching can cause health hazards.
6	Biocides	Up to 1%	Arsenic (As), tin (Sn) compounds	Primarily in soft PVC and polyurethanes.
7	Slip agents	0.1–3%	Fatty acid esters/amides, metal stearates	Added based on polymer type.

Table 2.2: Brief description of the main types of plastic additives.

2.4 Use of plastic materials

Single-use packaging materials account for a sizable portion of the plastic market worldwide (Phelan et al., 2022) (Fig. 2.8). This is largely due to the widespread use of 'use-and-throw' habits. The switch from reuse to single-use has accelerated plastic material production (R. Kumar et al., 2021). The rapid manufacture of plastic materials has rendered them an inescapable component of trash repositories.

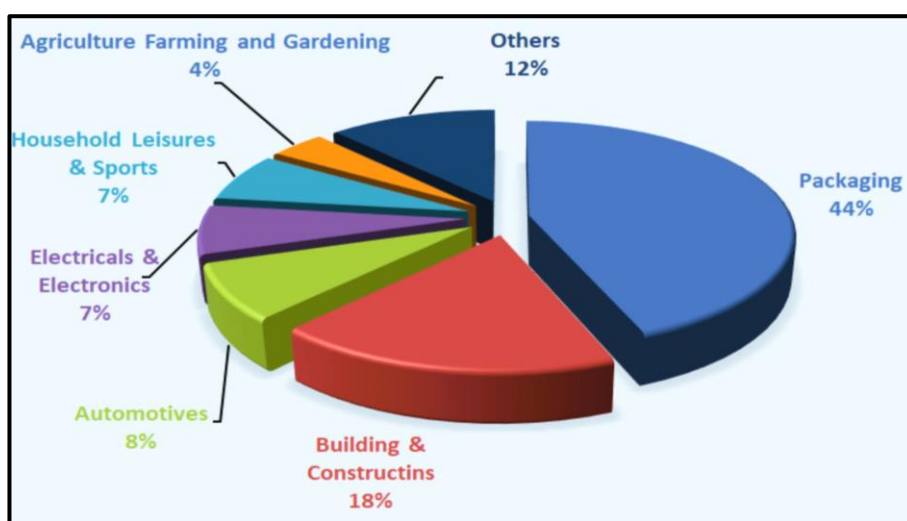


Fig. 2.8. Global share of the plastic market as per the data published by (Dokl et al., 2024).

2.5. Rate of Plastic Production and Disposal

2.5.1 Global scenario of plastic production

Plastic was a revolutionary anthropogenic discovery in the 20th century (Klotz & Haupt, 2022). Tremendous population growth, faster urbanisation, and industrialisation are the key factors behind its massive production (Siddiqua et al., 2022). The entire life cycle of the compound contributes to climate change and biodiversity loss

(Jambeck & Walker-Franklin, 2023). No discernible pattern in the plastic output was observed until 1990, after which a variable but steady trend was noted until 2005, followed by a sharp rise in usage after 2018 (Y. Wang et al., 2023). According to the OECD report, in 2019, nearly 22 metric tonnes of mismanaged plastic materials were released into the environment. Of that total, 88% were macroplastics, and 12% were microplastics, severely affecting ecological harmony. Moreover, within 30 years, the overall yearly production of plastic quadrupled. Thus, excessive plastic production and waste generation have led to a global plastic catastrophe (Walker & Fequet, 2023).

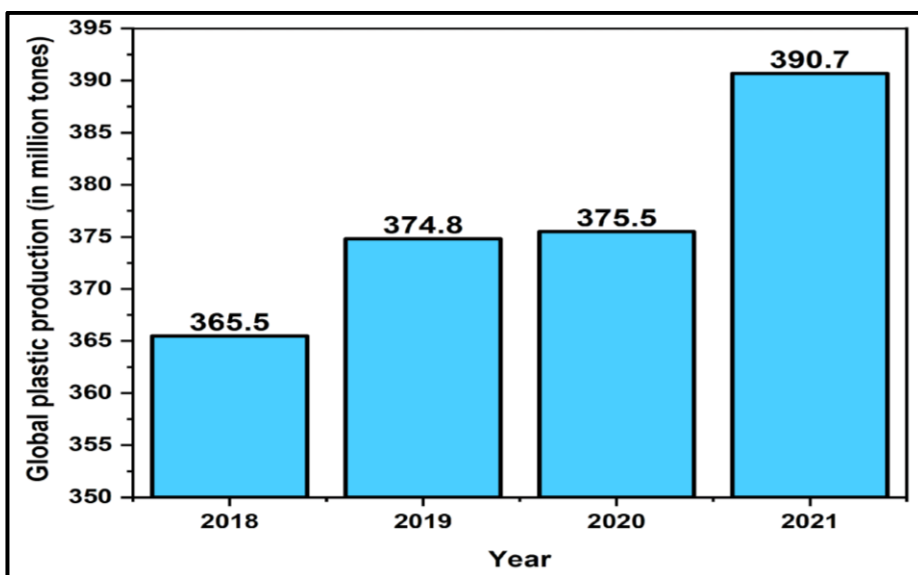


Fig.2.9 Global plastic production from 2018-2021 (It is a rounded estimate of the total count of fuel-based plastics (90-91%), Recycled plastics (8%), and bio-attributed plastics (1-2%) (X. Liu et al., 2022).

In 2021, China topped the world in debris accumulation and plastic manufacturing (Sun et al., 2022). In that year, they generated 80.1 million tons of plastic items (Fürst & Feng, 2022). However, China

banned the import of twenty-four types of plastic waste materials (Brooks et al., 2018). Before their stringent ban, they mainly imported plastic scraps from the USA, Japan, Europe, and Southeast Asia (55.7% of global plastic scraps). Hong Kong Port was the central transshipment hub for imports and exports (Z. Yang et al., 2019). Unfortunately, most plastic waste imported is incinerated or landfilled (Wen et al., 2021). The Chinese dumps in marine and aquatic environments mainly consist of Polyethylene (PE) (37%), Polypropylene (PP) (23%), Polyethylene terephthalates (PET) (12%), and mixed types (14%).

North America is the second-largest producer of plastic, behind China. Nearly 18% of the world's plastic production occurs annually. Scientists have reported that the rate of plastic littering in the U.S. in coastal areas, especially within 50 kilometres of shore, is substantial (Law et al., 2020). Following China's stringent ban, plastic scrap imports shifted to other Asian countries, including India, Malaysia, Indonesia, Thailand, and Pakistan (Bidashimwa et al., 2023). Recent reports on plastic garbage exports from the U.S. to other countries indicate that Canada and Mexico have become leading destinations (Hendrickson et al., 2024).

In 2021, the European Environment Agency reported that plastic production in European countries reached 57.2 Metric tons, mainly due to the enormous production of single-use plastic materials used in medical packaging and food packaging (C. Lee et al., 2024). Germany is the European Union's top producer and exporter of plastic packaging waste materials (Strobel et al., 2023). In contrast, Ireland produces 65

kg or more of plastic packaging waste per person annually (Somlai et al., 2023). Germany, the Netherlands, Norway, and Spain have plastic recycling rates exceeding 40% (Tumu et al., 2023).

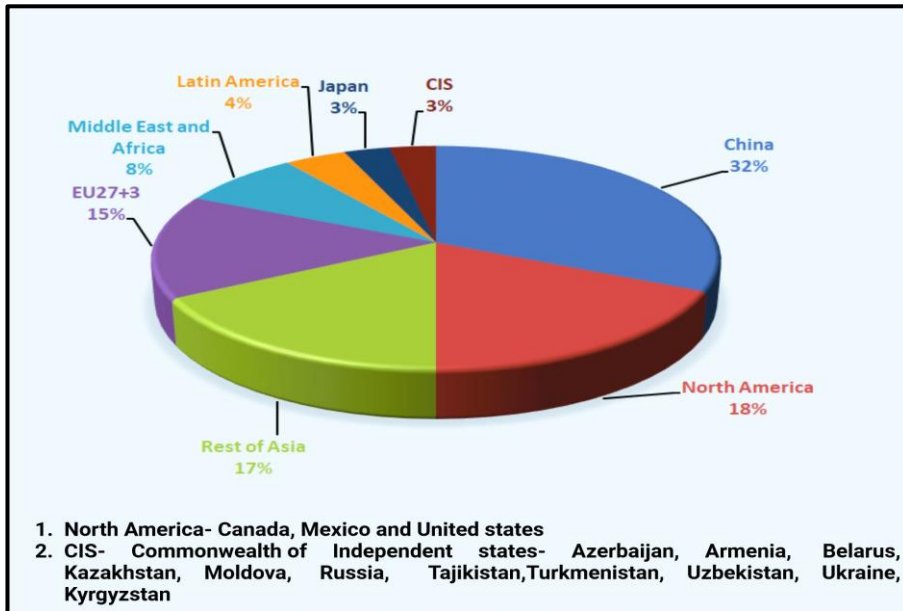


Fig. 2.10 Major plastic-producing regions worldwide (Melchor-Martínez et al., 2022).

On July 28, 2023, the Swiss-based research consultancy Earth Action (EA) announced the first International Plastic Overshoot Day, marking a point when plastic waste exceeded its management capacity (Chen et al., 2022). It is projected that by 2024, almost 70 million tonnes of plastic will be deposited into the environment due to an imbalance between the quantities of plastics used and the inability to handle plastic disposal effectively. According to an EA report in 2023, 60% of the world's population resides in areas where plastic waste is already out of control (Jiao et al., 2024).

However, the value of plastic waste generation per capita (Kg/cap) differs worldwide. According to the OECD report, in 2019, the highest per capita values were reported in developed countries, such as the USA (220.5 Kg/cap) and Canada (177.9 Kg/cap)(OECD, 2022).

2.5.2 Recent rise in the production of Polyethylene materials around the globe

Polyethylene production is growing in all countries and is expected to reach 157 million tonnes annually by 2023 (Nayanathara Thathsarani Pilapitiya & Ratnayake, 2024). China is the global leader in both the production and consumption of single-use plastics (Sun & He, 2023).

The COVID-19 epidemic significantly exacerbated global plastic production. It was mainly due to the exigency of polyethylene-lined personal protective equipment (PPE), such as masks, PPE kits, and head shields, as well as the widespread use of single-use food packets (Benson et al., 2021). It compelled all countries to either lift their bans or relax their waste management policies, particularly on single-use plastic materials such as LDPE bags with a thickness of less than 50µm and plastic bottles. It resulted in a massive increase in plastic accumulation in oceans and rivers (Y. Peng et al., 2021).

2.5.3 Waste Disposal around the globe

LDPE is an invincible plastic polymer used in manufacturing packaging materials, extrusion coating, films, and adhesives (Liyana Bekri et al., 2023). The production of LDPE materials surpassed 51 billion globally in 2023, primarily in North America and Asia, with a yearly growth rate of 9.6% (Mendoza et al., 2025). LDPE carrier bags

are estimated to comprise over 60% of all plastic bags produced due to their outstanding lifespan and mechanical strength (Horodytska et al., 2018). Thus, LDPE-based shopping bags and other packaging films have become integral to Landfills sprouting in our surroundings (Dybka-Stepień et al., 2021).

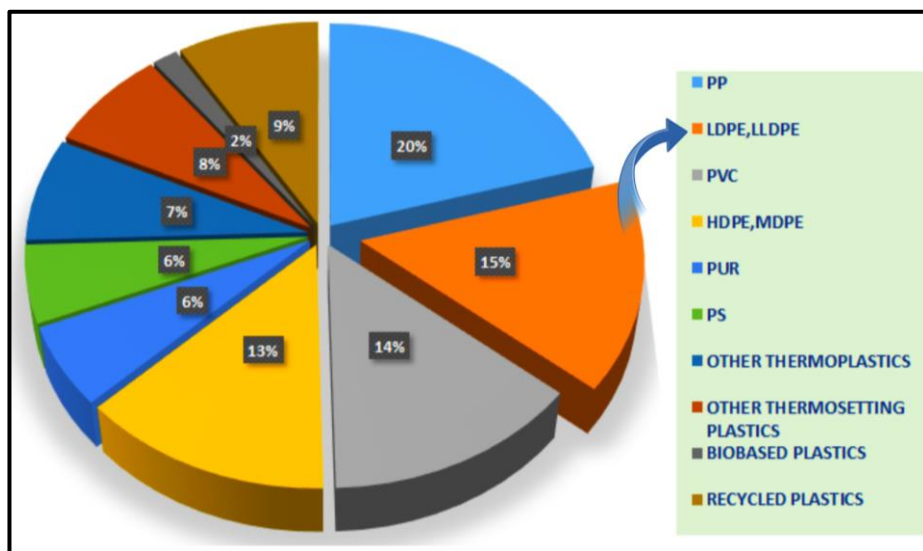


Fig. 2.11 Global production of LDPE and other plastic types (K. Li et al., 2024).

2.5.4 Indian scenario of plastic production and waste generation

Plastic manufacturing in India has increased substantially over the past five years, reaching approximately 1.5 million metric tonnes annually, with a recycling rate of 9 per cent of the total 7 billion tonnes of plastic produced (Tiwari et al., 2023). Inadequate waste management policies, improper plastic imports, a lack of awareness, and ineffective regulations in a heavily populated and diverse country are the reasons behind waste accumulation (S. Kumar et al., 2017). According to the Swedish agency's 2023 excess plastic accumulation report (E. A. report

2023), India is portrayed as having 16.7 days of plastic overshoot and a significant mismanaged plastic waste index of 98.55% (Soares et al., 2024). In India, major urban centres such as Delhi and Mumbai are substantial contributors to plastic waste, primarily due to the use of single-use plastics (Yadav et al., 2022).

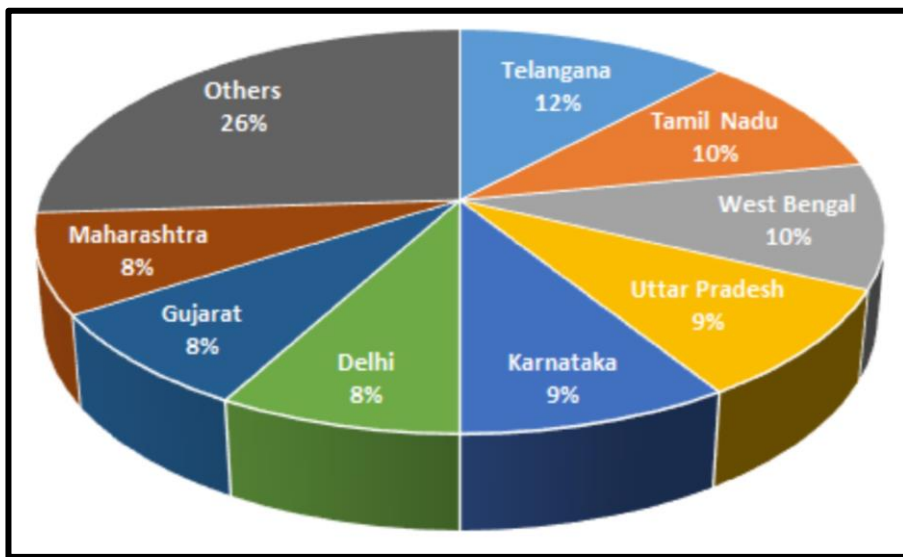


Fig. 2.12 Mismanaged plastic waste in Indian states (P. P. Gupta et al., 2023)

2.6 Enforcement of bans and implementation of robust international policies to tackle the plastic pollution crisis

The current trajectory of plastic accumulation and incineration compelled all international leaders and organisations, such as the UN, EU, UNWTO, EPA, etc., to propose effective management policies, campaigns, and stringent actions, such as bans on identified single-use plastic materials or reducing several packaging materials from daily

use (Fayshal, 2024). A few essential global efforts to fight against such concerns are,

- India spearheaded a campaign during the United Nations' fourth Environment Assembly, held in India in 2019, to prohibit pollution caused by single-use plastics, recognising the pressing global necessity to address the problem (Mentis et al., 2022).
- In 2022, the Nairobi Environmental Assembly of the United Nations approved a resolution to reduce plastic pollution and progressed towards developing a definitive agreement by 2024. Its significant objectives encompass all stages of plastic's lifecycle, including production, utilisation, and disposal (Stoett et al., 2024).
- The global tourism industry has committed to popularising a circular economy of plastics by 2025 (Strippoli et al., 2024).
- In 2015, the EU developed an action plan to establish a circular economy. The agenda aims to enhance the research-based nature of the legislation and strengthen regulations regarding the recycling of plastic products (Di Vaio et al., 2023)
- Bangladesh was one of the first nations to take the initiative and ban plastic bags in 2002, paving the way for future plastic regulations (UNEP, 2018). Many other countries followed Bangladesh's lead (Muposhi et al., 2022).
- Numerous nations have implemented legal prohibitions on single-use plastic items, including straws, utensils, plastic stirrers, and packaging materials (Kiessling et al., 2023; Krantzberg et al., 2023).

- In the East African region, Rwanda took the initial step toward banning the manufacture, import, and use of plastic bags through legislation enacted in 2008 (Hakuzimana, 2021).

Following pioneering efforts, many countries have implemented bans and additional taxes on plastic products (Behuria, 2021; Desalegn & Tangl, 2022). In addition, global leaders have begun encouraging recycling by adhering to ASTM standards and implementing extended producer responsibility laws (Sakao et al., 2024).

2.7 Plastic management policies and campaigns by the Government of India

- To address concerns about plastic management, India held World Environment Day in 2018 under the theme "Beat plastic pollution " (Ghosh et al., 2021).
- Keeping the adverse effects in mind and adopting the resolution of UNEA-4, the Government of India declared a ban on plastic carry bags with a thickness of less than 50 μm , effective from September 30, 2021. Then, the carry bag thickness for the ban rose to 75 microns and finally to 120 microns from December 31, 2022 (Mondal et al., 2023).
- The Plastic Waste Management Regulation 2021 was issued by the Ministry of Environment, Forest and Climate Change (MoEFCC) and implemented on July 1, 2022 (MoEFCC, 2023). It prohibited several one-time plastic materials with low usage and high potential for landfilling (Dhiman et al., 2023).
- In compliance with the PWM amendments, the MoEFCC published Guidelines on Extended Producer Responsibility (EPR)

(the producer's responsibility to decompose eco-friendly plastic materials) effective February 16, 2022 (Bhadra & Mishra, 2021).

Amid all these stringent regulations, the production of plastic materials is increasing alarmingly every minute due to their wide acceptability. It, in turn, ends up in landfills of terrestrial, marine, and other aquatic ecosystems. Despite regulatory efforts, India struggles to enforce plastic bans due to the persistent influence of informal markets operating in remote and unregulated areas (Knoblauch & Mederake, 2021). This challenge is compounded by limited infrastructure, the absence of affordable substitutes, the economic entrenchment of plastic usage, and minimal public awareness and participation (Nøklebye et al., 2023).

2.8 Plastic pollution - an evil threat to the ecosystem

Plastic materials in various forms cause white pollution worldwide. They are macroplastics (> 5 cm), mesoplastics (5 mm-5 cm), microplastics (0.1 μm -5 mm), and nanoplastics (< 0.1 μm) (Taurozzi & Scalici, 2024). Recently, many researchers have identified that the presence of microplastics and nanoplastics poses numerous challenges to our ecosystems (Allen et al., 2022) (Fig. 2.13). Microplastics can be classified into two categories: primary and secondary. The Primary microplastics come from industrial waste, textile applications, and body and skincare items and are released into natural habitats. Secondary microplastics are created through exposure to UV light, chemical weathering, physical weathering (such as washing machines), and mechanical weathering (like tidal waves) (Lehtiniemi et al., 2018). Recently, scientists demonstrated the toxic nature of LDPE-based

microplastics on the marine microalga *Chaetoceros calcitrans* (Senousy et al., 2023).

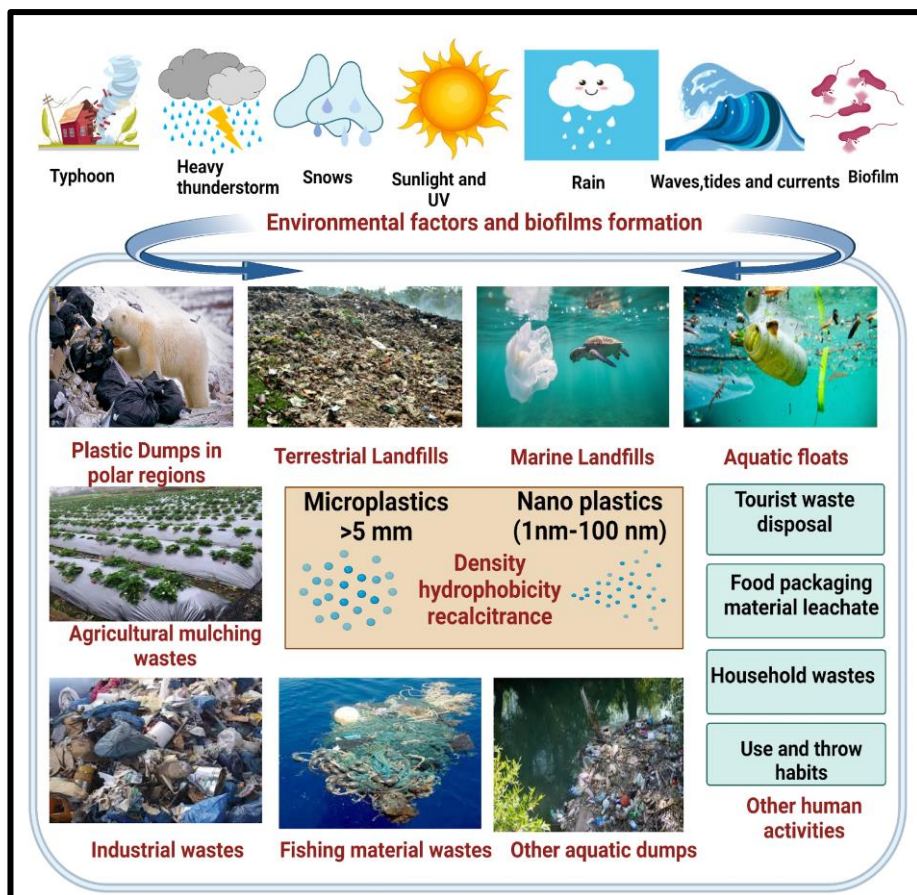


Fig. 2.13 The environmental factors affecting the release of harmful microplastic and nanoplastic particles from various natural plastic dumps in our ecosystem.

2.8.1 Sources of Plastic Pollution

The primary sources of plastic pollution in our ecosystem include the long-term dumpsites of terrestrial, marine, and atmospheric ecosystems. It causes severe damage to flora and fauna, disrupts the trophic balance, and contributes to climate change.

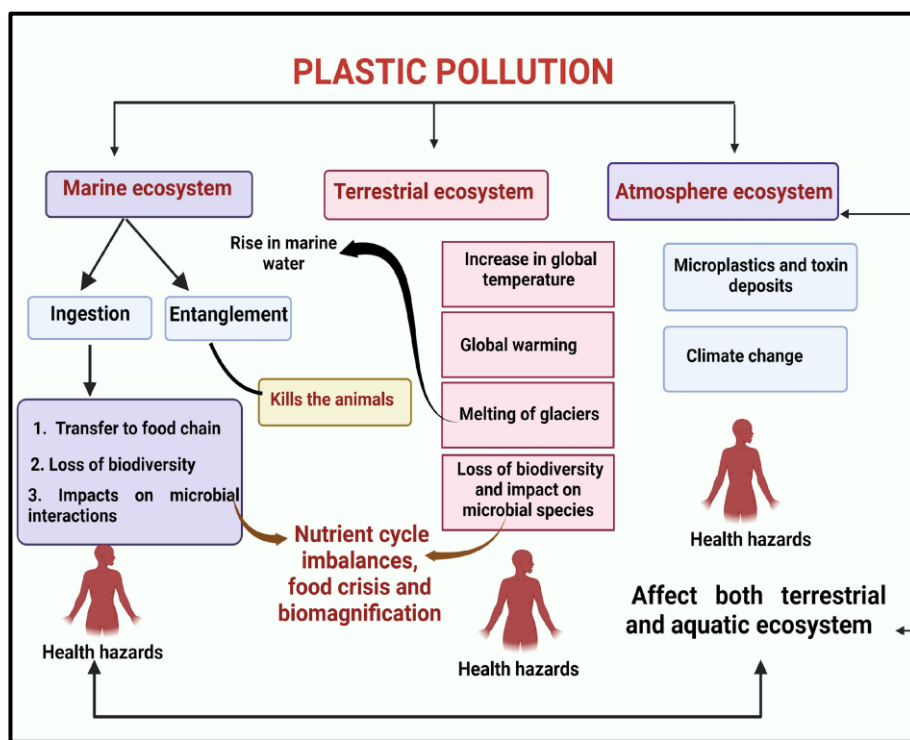


Fig.2.14 Pestilence of plastic pollution in different types of ecosystems

2.8.1.1. Landfills in terrestrial ecosystems

Terrestrial landfilling is a highly significant and ecologically sound approach to contemporary urban solid waste management (Zhu et al., 2021). A landfill is a designated terrestrial area for the disposal and containment of solid waste, typically engineered with layers to avert environmental contamination, where waste decomposes over time, generating methane gas that can be harnessed as an energy source. In the twenty-first century, 79% of plastic waste is released into landfills (Rhodes, 2018). Terrestrial landfills severely damage soil sustainability by altering microbial interactions and physical parameters (Rai et al., 2023). The terrestrial landfilling not only causes the macro-level

accumulation but also leads to microplastic (plastic with 5 mm- 1 mm in size) or nano-plastic accumulation (size ranges from 1 nm- 1000 nm) due to weathering, exposure to sunlight/UV, microbial biodegradation and other disintegration processes (Yu et al., 2022). These microplastics (MPs) and nanoplastics (NPs) can persist in soil for decades, causing even more detrimental effects on our ecosystem (Hurley & Nizzetto, 2018). HDPE, LDPE, Polypropylene (PP), and PET are the major constituents in terrestrial landfills (O'Rourke et al., 2022). Due to physical, biological, and chemical processes, landfills release toxic gases such as methane, greenhouse gases such as CO₂, CO, etc., volatile organic compounds, plastic additives, pigments, potentially harmful elements, and malodorous gases into our bio-system, resulting in ecological imbalances and health hazards such as cancer, mental and physical imbalances, and neuronal damages (H. Yang et al., 2018).

2.8.1.2 Marine dumpsites

Since 1972, plastic waste in the marine environment has been considered a serious concern (Hoseini & Bond, 2022). Plastic particles floating in the oceans have reached over 170 trillion, accounting for 60-80% of total polluting materials in the marine environment (Eriksen et al., 2023). The adverse impacts of plastic pollution in marine ecosystems include entanglement of aquatic fauna, widespread distribution of microplastics in oceanic and coastal waters, chemical leachate release, emission of greenhouse gases, and discharge of harmful toxicants (Oliveira et al., 2020). Plastic waste enters the marine atmosphere through rivers and beaches, illicit sea dumping,

fishing net entanglement, and shore activities (van Emmerik & Schwarz, 2020). Nowadays, the ingestion of macroplastics by sea turtles (Eastman et al., 2020) and seabirds (Rivers-Auty et al., 2023) is a common scenario worldwide. *Fulmarus glacialis* (Northern fulmar) was the first seabird reported to entangle macroplastic materials in its stomach (van Franeker et al., 2022). Polyethylene and polypropylene compounds account for a significant portion of marine landfills (Canopoli et al., 2020).

2.8.1.3. Atmospheric pollution by the plastic dumps

Plastic waste dumps are a growing source of atmospheric pollution, primarily caused by the release of hazardous chemicals during the degradation process of plastics. As plastics break down under sunlight and fluctuating temperatures, they emit volatile organic compounds (VOCs), microplastic particles, and toxic gases, including methane, carbon monoxide, and dioxins (Kida et al., 2023). The open burning of plastic waste, a common practice in some regions, further intensifies air pollution, releasing harmful pollutants that pose risks to human health and contribute to climate change. It drastically pollutes our atmosphere by releasing toxic gases, including dioxins, VOCs, CH₄, and greenhouse gases, as well as hazardous heavy metals such as As, Pb, Cd, and Hg, and lethal halogens like PCBs (Verma et al., 2016). These airborne emissions degrade air quality, leading to long-term ecological consequences.

2.8.1.4 Other plastic pollutions

Population growth and on-the-go plastic disposals of human beings are the main reasons behind plastic piles and their toxic releases (micro-plastics, nano-plastics, toxic additives, lethal gases, halogens, heavy metals, etc.) to the environment. The important anthropogenic activities that cause hassles to ecological sustainability are the following,

- a. Unscientific disposal of Industrial effluents containing plastic products pollutes water bodies (groundwater, freshwater, and marine). It affects soil microbial interactions, urban drainage systems, and other hydraulic infrastructures (Kichu & Devi, 2021).
- b. Excessive use of mulching films in agriculture—Although polyethylene film mulching has enormous advantages, its continuous use and disposal lead to toxic additives and microplastics leaching into agricultural fields. It modifies the microclimatic conditions of farm fields indirectly and, in turn, severely affects all types of soil interactions (Hasan & Tarannum, 2025).
- c. Negligent plastic disposal in various ecosystem types—unconditional plastic removal from mangroves, glaciers, water bodies (including freshwater such as lakes, ponds, streams, and rivers), estuaries, marine environments, groundwater (including their shorelines), and the atmosphere—has catastrophic effects on the ecosystem's flora and fauna. It also alters climatic conditions and ecological balances (Pandey et al., 2023).

2.8.2 Environmental impact of plastic debris

Plastic heaps have numerous deleterious effects, including suffocation, killing or rafting of organisms, dispersal (through floating or wind), and the introduction of invasive species, which affect biodiversity and trophic systems (Thushari & Senevirathna, 2020). The following six major categories of negative impacts were reported due to the excessive trashing of plastics.

They were

- a) Ingestion of plastics
- b) Entanglement of plastics
- c) Microbial interactions in various environments
- d) Groundwater contamination
- e) Greenhouse Effect and climate change
- f) Human livelihood

a. Ingestion of plastics

Plastic ingestion in animals causes numerous health hazards, including reduced fitness, altered behaviour due to endocrine imbalances, a false sense of satiation, gut obstructions, and impaired development and reproduction (Zolotova et al., 2022). Moreover, the toxic leachates of plastic can move up the food chain and cause biomagnified effects (Macali et al., 2018). According to a comprehensive review published by (Gall & Thompson, 2015), nearly all organisms in the marine environment are affected by plastic ingestion. Besides macroplastic ingestion, many scientists have recently reported microplastic ingestion in aquatic organisms (Marmara et al., 2023; Meaza et al., 2021).

Marine species, such as turtles, crustaceans, and seabirds, were reported to be most significantly affected by it (Thiel et al., 2018). Many recent studies have demonstrated the detrimental impact of ingestion on coral health, directly affecting photosynthetic activity and animal growth (Berry et al., 2019; Reichert et al., 2019). Additionally, several studies have demonstrated plastic ingestion in the fauna of freshwater bodies (Andrade et al., 2019; Faure et al., 2015; Windsor et al., 2019) and terrestrial animals, including carnivores, rodents, and ruminants (Thrift et al., 2022).

b. Entanglement of plastics

Plastic entanglements are most often found in the marine environment. It lethally kills organisms such as turtles, birds, sharks, and other mammals (Gregory, 2009). It is due to the excessive disposal of fishing materials (ghost gears), LDPE bags, PET bottles, plastic ropes, etc., into marine habitats (Afonso & Fidelis, 2023). Recently, scientists have reported a massive hike in rates of fatal ingestions and entanglements compared to past decades (Compa et al., 2022). The incidence of entanglement in freshwater biota is also increasing daily (Caruso et al., 2024).

c. Microbial interactions in innate environments

Toxic releases, such as additives, fillers, microplastics, and persistent organic pollutants, from various types of plastic material accumulations or LDPE-based agricultural mulching films can severely interfere with positive microbial interactions (Angnunavuri et al., 2020; Bandopadhyay et al., 2018). Moreover, it significantly

disrupts soil fertility by altering biogeochemical cycles, the health of the atmosphere, and ecological balances in the aquatic environment (Gunaalan et al., 2020; Sajjad et al., 2022). Thus, recently, several scientists have studied this alarming scenario and demonstrated the ability of a few microbes to adhere to the polymer and utilise it as an energy source (Lear et al., 2021).

d. Groundwater contamination

Harmful leachates and microplastics transported to groundwater sources through wind, rain, or direct plastic deposits may significantly impact the ecosystem. Additionally, water usage from such sources for irrigation and drinking affects the biomes of other ecosystems. LDPE bags and PET bottles are common plastic pollutants in similar aquifers (Sangkham et al., 2023).

e. Greenhouse Effect and climate change

Emissions of greenhouse gases and subsequent global warming are the catastrophic effects of plastic production, landfilling, recycling, and incineration. According to a recent study, global greenhouse gas emissions rebounded strongly after the pandemic period in 2020, increasing by 23% (Sikarwar et al., 2021). Between 2015 and 2050, the growth of the plastics industry, which contributes 10% to global warming, is predicted to release more than 56 billion metric tons of CO₂e (Carbon dioxide equivalent), accounting for 10-13% of the world's carbon footprint (S. Sharma et al., 2023). Earth's residual carbon resources have been depleted due to global greenhouse gas emissions and their continued presence in the environment, creating a dangerous feedback loop (S. Bolan et al., 2024). Moreover, it causes a

rise in global temperature, increases CO₂ concentration in the atmosphere, disrupts the water balance in marine and freshwater bodies, and accelerates the melting of glaciers. As proven by scientists, another significant consequence of global warming on the marine environment is the skewed sex ratio of turtles and coral bleaching, which leads to mortality and altered nutrient cycling (Hughes et al., 2018).

f. Human livelihood

Plastic trash impacts human livelihoods by increasing the risk of flooding in metropolitan areas and causing socio-economic losses in the tourism, real estate, and transport (including fisheries and shipping) sectors (Mihai et al., 2021). Plastic clogs in urban drainage systems have been the primary cause of the rise in water levels, rather than organic debris in drains (Lebreton & Andrady, 2019). Health hazards and biodiversity loss associated with plastic pollution also have a severe impact on human life.

2.8.3 Health Hazards of Plastic Accumulation

The pervasiveness of plastics in human life is colossal. However, extensive studies to prove health hazards caused by polymers are scarce; a few studies have demonstrated health problems, such as cardiovascular diseases, neuronal disorders, respiratory diseases, hormonal imbalances, reproductive issues, gut disturbances, skin diseases, oxidative stress, and cancers in the human body (Morrison et al., 2022). Recently, scientists have proven the presence of PVC-based nanoplastics (Mahadevan & Valiyaveetil, 2021) and airborne microplastic particles (Amato-Lourenço et al., 2021) in lung extracts.

Additionally, researchers have identified the presence of polyethylenes, polypropylenes, and polystyrenes containing microplastics in blood smears (Leslie et al., 2022). Although the toxic effects of plastic additives, such as phthalates and dioxins, on endocrine disturbances and respiratory diseases have been reported, the precise impacts of these additives and harmful fillers remain unclear.

2.9 Conventional degradation of Polyethylenes

The most common methods for degrading LDPE polymers are physical, chemical, mechanical, pyrolysis, and landfilling.

2.9.1 Physical Degradation Methods

Physical degradation of LDPE films can occur through exposure to sunlight, UV rays, weathering, changes in moisture, heat, and mechanical stress (Ghatge et al., 2020). It may release toxic volatile compounds, microplastics, and poisonous additives into the surrounding environment. It may perturb our environment (Wojnowska-Baryła et al., 2022). Several researchers have employed physical methods, including heat, steam, UV, and sunlight, as pre-treatment methods to enhance bio-attachment and promote further degradation (Raut et al., 2015). Recently, scientists have tried to utilise photocatalysis coupled with nanotubes to maximise the photo-oxidative degradation of LDPE films (Klein et al., 2018; Tofa et al., 2019). The fundamental structure of the polymer is altered by physical degradation, which causes minor chain cracking, changes in ductility, and splintering (Ceretti et al., 2023).

2.9.2 Chemical Degradation Methods

The use of chemical pro-oxidants, dye-sensitised nanotubes, and toxic chemicals such as TiO₂ and ZnO is a chemical method employed for the biodegradation of LDPE films (S. S. Ali et al., 2016; da Luz et al., 2014). These methods are not cost-effective and have harmful effects on our biosystem. The chemical degradation can oxidise long linear hydrocarbon chains and may cause the breaking (N. S. Ali et al., 2022).

2.9.3 Mechanical methods

Shredding LDPE materials into small pieces using mechanical force and further processing with high heat and pressure (pyrolysis) is another alternative to the degradation of the compound (Czajczyńska et al., 2017). This process is not a cost-effective method.

2.9.4 Drawbacks of other existing plastic waste management practices

Adopting a suitable management process for ending the life of plastics is a crucial issue. Currently, worldwide, various conventional and modern plastic waste management techniques are employed to mitigate the adverse effects of plastic waste (Maitlo et al., 2022) (Fig. 12). Nevertheless, practically all traditional methods have significant flaws. Financial impracticability, space requirements, energy requirements, volatile organic pollutant releases, and health hazards have prompted researchers to seek more sustainable and environmentally friendly solutions to address environmental concerns (Anand et al., 2023). LDPE with starch blends is another alternative method to LDPE

material use (Thakore et al., 2001). However, its stability and durability are inconsistent with those of usual LDPE films. Bioplastics are another alternative used in the market. However, its stability, durability, moisture-holding capacity, cost, and mechanical strength still need to be determined (Abe et al., 2021). Most household plastic items were conventionally removed by public burning in a vacant space. It releases CO, CO₂, and other toxic gases. It harms our health and the ecosystem. Thus, scientists sought a sustainable, robust, and biotechnological solution to mitigate the hardship of plastic dumps in our ecosystem. Apart from conventional methods, plasma treatment on LDPE is a novel pre-treatment technology introduced to biodegrade the polymer effectively. However, the cost and need for scientific expertise limit its popularity (Scally et al., 2018).

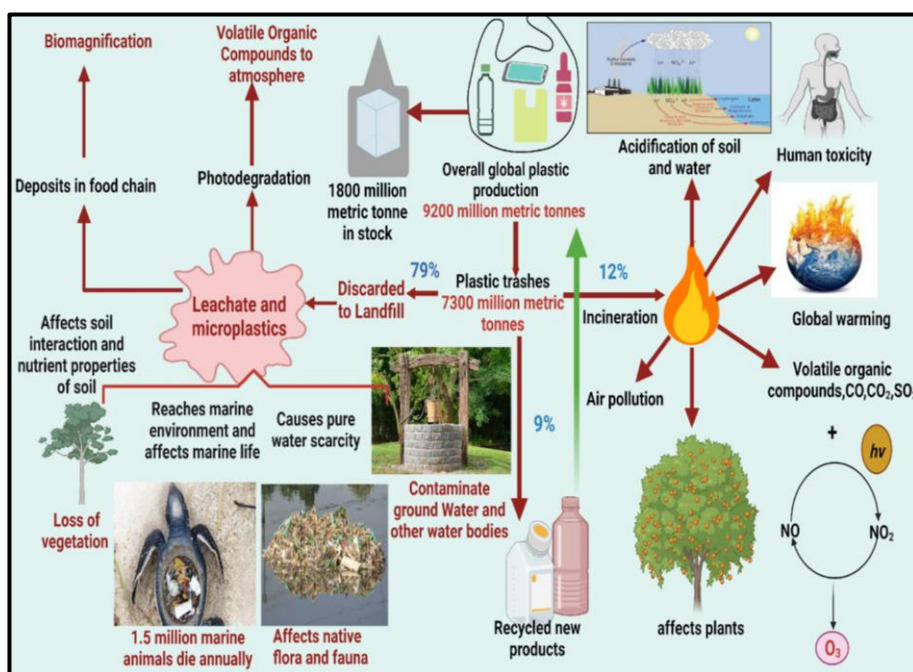


Fig 2.15 Drawbacks of standard conventional plastic waste management mechanisms

2.10 Factors controlling microbial biodegradation of polyethylenes

An eco-friendly and sustainable step to mitigate the environmental impact of polyethene accumulation has become a demand of modern society. To achieve this, the researchers have diverted their focus to microbial biodegradation. Nonetheless, it is a multifaceted phenomenon influenced by various biotic and abiotic elements. Abiotic factors, including sunlight, UV light, and temperature, were involved in the initial biodegradation process (Kong et al., 2025). The primary biotic factors encompass the quantity and viability of microbial cells (individual strains or effective consortia) attached to refractory surfaces, as well as their extracellular and endocellular enzyme activity on the polymer. The main factors influencing the microbial degradation of LDPE materials are modifications to the compound's surface form, size, crystallinity, elemental composition, molecular weight, types of functional groups, and additives (Cai et al., 2023). Besides these, several scientists have reported the pivotal role of biosurfactant production, specific chemoattractants, and membrane transporters in bio-attachments and degradation (Soni et al., 2025). However, various environmental factors, including temperature, pH, humidity, and nutritional conditions, affect the microbial strains attached to the polymer (Ahmed et al., 2018). Many natural or synthetic individual strains or consortia have been identified for bioremediation (Crystal Thew et al., 2024).

Due to the influence of physical factors on biodegradation, several scientists used steam, UV, sunlight, and compounds to enhance the attachment of microbial strains (Kundungal et al., 2021). Although

these pre-treatment methods improved the attachment of microbial strains, many scientists used untreated LDPE films to test the efficacy of natural strains in degrading long hydrocarbon chains (Dey et al., 2020; Harrat et al., 2024).

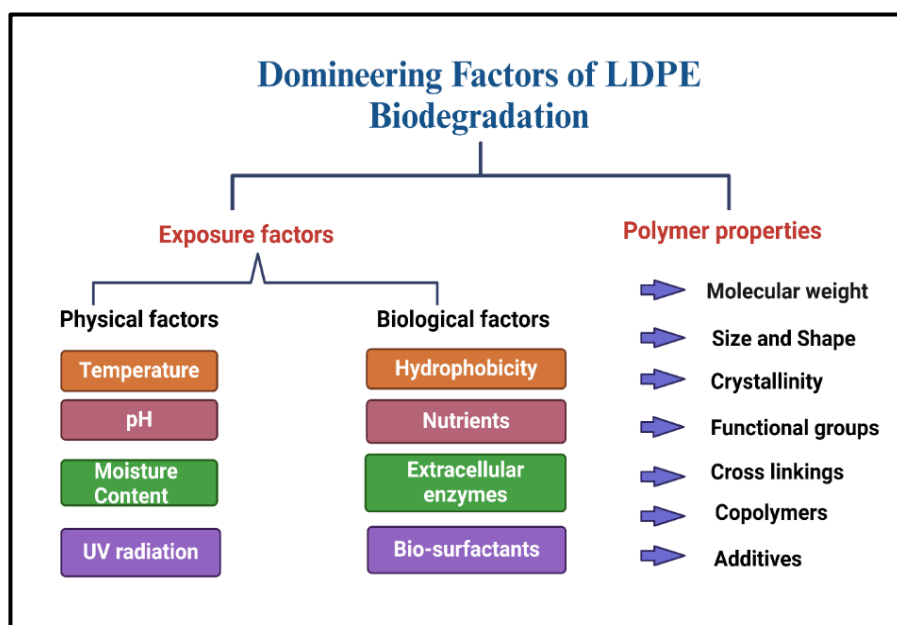


Fig 2.16 Factors controlling the biodegradation mechanism (Kijchavengkul & Auras, 2008).

2.11 Microbial Species in LDPE Biodegradation

2.11.1 Bacterial strains

Several bacterial genera have been reported to effectively deteriorate the surface of LDPE films, including *Pseudomonas* (L. Hou et al., 2022), *Bacillus* (Yao et al., 2022), *Staphylococcus* (Samanta et al., 2022), *Streptococcus*, and *Micrococcus* (Joshi et al., 2022). These potential bacterial species were mainly isolated from long-term waste dumps (details of the isolated cultures are presented in Table 2.3).

S. No	Bacterial pure cultures	Source of isolation	Weight loss	Degradation time	References
1	<i>Bacillus siamensis</i>	Plastic waste dump	4.7%	45 days	(Tarafdar et al., 2021)
2	<i>Bacillus cereus</i>	Dumps near coastal areas	4.1±0.81	42 days	(Jebashalomi et al., 2024)
3	<i>Pseudomonas aeruginosa</i> WD4	Plastic dumpsite	9.2%	100 days	(Shilpa et al., 2022)
4	<i>Serratia</i> sp.	Solid waste dump	40%	150 days	(Nadeem et al., 2021; Tiago et al., 2023)
5	<i>Bacillus</i> sp. PE3	Plastic Landfill	6.68%	30 days	(Kavitha & Bhuvaneshwari, 2021)

Table 2.3: Recently isolated highly potent bacterial strains from various plastic dumps

2.11.2 Bacterial consortia

The limitations of metabolic activities in individual strains for the biodegradation of recalcitrant compounds, such as LDPE, diverted the attention of several scientists to developing a practical consortium for the mechanism (Satlewal et al., 2008). Establishing any effective consortium generally adheres to two fundamental principles: the “top-down” and “bottom-up” approaches (Cao et al., 2022; Y. Zhang et al.,

2023). The top-down strategy utilises environmental factors to induce an existing microbiome, whether naturally present or synthetically created, to undergo ecological selection, thereby optimising its metabolic activities. However, a bottom-up approach requires a clear understanding of microbial strains' genomic and metabolic activities (Lawson et al., 2019). Unfortunately, comprehensive knowledge regarding microbial strains' genomic and metabolic activities for the biodegradation of LDPE materials has not been elucidated (Boschi et al., 2024). Our understanding of microbial diversity, composition, topologies, functional traits, and metabolic activities in natural microbial communities has recently improved due to advancements in molecular methods (Rüthi et al., 2023). A few bacterial consortia can effectively biodegrade other plastic materials in addition to LDPE compounds (Gao & Sun, 2021). Several early researchers have elucidated both natural (Wafaa et al., 2025) and artificially formulated bacterial consortia (Putchá & Kitagawa, 2024) for LDPE biodegradation.

Addressing the dynamics and stability of each bacterial species in consortia may help optimise biodegradation processes within a limited time frame for bioprocess designs aimed at the large-scale breakdown of polymeric materials (Muangchinda & Pinyakong, 2024). Microbes in consortia or co-cultures can produce several extracellular enzymes to degrade it effectively (Lokesh et al., 2023). The most crucial advantage of consortia in complex degradation is the division of labour among individual members (Shen et al., 2020).

In addition, several recent investigations have demonstrated the effectiveness of bacterial consortia for degrading LDPE, as well as breaking down various types of plastic materials (Edwards et al., 2022; Salinas et al., 2024).

S. No	Name of the Co-culture	Source of Isolation	Major Outcome	Reference
1	<i>Enterobacter</i> sp and <i>Pantoea</i> sp	Soil samples from plastic trash sites in Bangalore	81% weight loss for LDPE strips, 38% for LDPE over 140 days	(Skariyachan et al., 2018)
2	<i>Enterobacter</i> sp, <i>Enterobacter cloacae</i> and <i>Pseudomonas aeruginosa</i>	Soil samples from a landfill with cow dung	64–66% weight loss after 160 days of incubation	(Skariyachan et al., 2021)
3	Three species of <i>Vibrio</i> , three species of <i>Oceanimonas</i> , two species of <i>Paenibacillus</i> , one species of <i>Shewanella</i> , <i>Rheinheimera</i> , and <i>Bacillus</i>	Plastic debris in the coastal environment near the Andaman Islands	47–54% weight decrease after 120 days of incubation	(Gajendiran et al., 2016)
4	<i>Bacillus</i> sp and <i>Paenibacillus</i> sp	Sediment samples from landfills	14.7% weight loss within 60 days	(Park & Kim, 2019)

Table 2.4 Novel bacterial consortia isolated from various sources for LDPE biodegradation.

2.11.3 Fungal species

Fungi are better suited for degrading LDPE than bacteria because they grow in the soil more quickly and can spread their mycelial growth to

reach new areas (Okal et al., 2023). Most fungal species for LDPE biodegradation release enzymes such as hydrolases, peroxidases, oxidases, and oxidoreductases (Paço et al., 2017).

S. No	Name of the Fungal Culture	Source of Isolation	Key Findings	Reference
1	<i>Cladosporium sphaerospermum</i>	Plastisphere from the marshland plastic dump	15.23% weight loss in 7 days of incubation	(Sathiyabama et al., 2024)
2	<i>Aspergillus oryzae</i>	Landfill	36 ± 4% weight loss after 16 weeks at 37°C	(Ndahebwa Muhonja et al., 2018)
3	<i>Penicillium citrinum</i>	Landfill soil sample	38.82 ± 1.08% weight loss without pre-treatment	(Khan et al., 2023)
4	<i>Aspergillus terreus</i>	Rhizosphere soil of <i>Avicennia marina</i>	58.51 ± 8.14% weight reduction after 60 days at pH 7	(Sangale et al., 2019)

Table.2.5 Earlier investigations of fungal species in LDPE biodegradation *Aspergillus niger* (Volke-Sepulveda et al., 2002), *Aspergillus versicolor* (Pramila, 2011), *Aspergillus terreus*, *Aspergillus fumigatus* and *Fusarium solani* (B. Sharma & Jain, 2020; Zahra et al., 2010) and *Phanerochaete* (Bautista-Zamudio et al., 2023) were a few other previously reported fungal species for LDPE biodegradation. Several fungal species have been found to degrade LDPE and many other

petroleum and polymer compounds. For instance, marine fungi like *Alternaria alternata* have been reported to produce laccase and peroxidase enzymes that aid in degrading LDPE films, crude oil, and other petroleum products (Romero-Hernández et al., 2021). Fungal species, such as *Cladosporium halotolerans*, have been demonstrated to degrade LDPE, HDPE, and polyurethane compounds (Di Napoli et al., 2023). In the presence of intra- and extracellular enzymes, these long polymers were degraded into dimers or monomers, and through further metabolic processes, these compounds were converted into CH₄, H₂O, and CO₂ (J. Wang et al., 2019).

2.11.4 Fungal consortia

The elevated metabolic rate, strong biofilm formation, and enhanced enzyme activities of fungal consortia can become an effective and environmentally friendly bioprocess for the degradation of LDPE. Fungal colonisation on the polymer surface is facilitated by surface hydrophobins (an amphiphilic protein), which help aggregate the membranes of individual strains and introduce hydrophilicity to the polymer surface for biodegradation (Stanzione et al., 2022). They can make numerous surface modifications, such as pits and fissures, which make the fungal strains more accessible for degrading the recalcitrant surface of LDPE films. A few fungi can produce reactive oxygen species to degrade the homology of the LDPE basic structure (Gong et al., 2023).

S. No	Name of the Co-culture	Source of Isolation	Main Outcomes	Reference
1	<i>Aspergillus oryzae</i> , <i>Aspergillus flavus</i> and <i>Aspergillus niger</i>	Culture Collection Centre	26.15% mass reduction over 55 days of interaction	(DSouza et al., 2021)
2	<i>Penicillium simplicissimum</i> , <i>Alternaria alternata</i> , <i>Curvularia lunata</i> , and <i>Fusarium</i> sp.	Plastic dumps	27% weight loss within 3 months of incubation	(Sowmya, Bellibatlu, et al., 2015)
3	<i>Aspergillus fumigatus</i> and <i>Aspergillus carbonarius</i>	Mixed culture from plastic landfill	High weight loss percentage after pre-treatment of LDPE films compared to single isolates within 16 weeks	(El-Sayed et al., 2021)
4	<i>Sterigmatomyces halophiles</i> , <i>Meyerozyma guilliermondii</i> , <i>Meyerozyma Caribbean</i>	Yeast tri-culture from the gut of wood-feeding termites	63.4% reduction in tensile strength and 33.2% mass reduction of UV-sterilised LDPE after 45 days	(Elsamahy et al., 2023)

Table 2.6 A few investigations of the fungal/yeast consortium for LDPE biodegradation.

2.11.5 Actinomycetes

Actinomycetes are versatile organisms isolated from different ecozones that generate hydrolytic enzymes to biodegrade recalcitrant substances, such as LDPE, into simpler compounds (Amobonye et al., 2021). *Rhodococcus* and *Streptomyces* are the two main genera identified for the degradation of recalcitrant compounds.

S. No	Names of the Actinomycetes Cultures	Source of Isolation	Key Observations	Reference
1	<i>Rhodococcus rubber</i>	Plastic waste mixed with agricultural field soil	Degraded up to 8% within one month	((Orr) et al., 2004)
2	<i>Alcanivorax borkumensis</i>	Plastic waste from the Mediterranean Sea	Demonstrated catalytic enzyme (alkane hydroxylase) activity for LDPE degradation over 80 days	(Delacuvellerie et al., 2019)
3	<i>Streptomyces gougerotti</i> and <i>Micromonospora matsumotoense</i>	Marine environment	Effectiveness of UV-pretreated LDPE and PS films shown by the introduction of carbonyl groups	(Oliveira et al., 2022)

Table 2.7 Recent findings of the capabilities of actinomycetes strains in LDPE biodegradation.

2.11.6 Algal species

Microalgae are photoautotrophic organisms that can facilitate the biodegradation of LDPE materials. Microalgae are more suitable than other microbial species, as they do not produce endotoxins and can flourish without substantial organic carbon sources in photoautotrophic environments (Yan et al., 2016). Lignolytic and exopolysaccharide enzymes play a crucial role in the biodegradation process of LDPE.

S. No	Name of the Algal Species	Source of Isolation	Major Results	Reference
1	<i>Picochlorum maculatum</i>	Marine environment	Maximum weight reduction of 20% within 45 days of incubation	(Gowthami et al., 2023)
2	<i>Uronema africanum</i>	Domestic waste dumping site in a freshwater lake	Biodegradation is established within 30 days.	(Sanniyasi et al., 2021)
3	<i>Anabaena spiroides</i>	Landfills in ponds, pools, and ditches	Weight reduction up to 8.8%	(Vimal Kumar et al., 2017)

Table 2.8: Displaying the potentialities of different algal species in LDPE biodegradation.

2.11.7 Microbes in the insect gut for the degradation of LDPE film

The symbiotic microbial strains in the guts of various invertebrates, such as mealworms, waxworms, super worms, and snails, have demonstrated their outstanding capabilities to degrade various plastic polymers (S. S. Ali et al., 2021). A few gut microbes in the insect's gut are depicted below.

S. No	Name of the Insect	Microbes in the Gut	Potential Effects	Reference
1	Indian meal moth	<i>Enterobacter asburiae</i> YT1, <i>Bacillus</i> sp. YP1	<i>Bacillus</i> sp. degrades 11%, and <i>Enterobacter</i> sp. degrades 6% of polyethylene within 60 days.	(J. Yang et al., 2014)
2	Darkling beetle larvae (Larvae of <i>Zophobas atratus</i>)	<i>Citrobacter</i> sp.	56 mg LDPE/100 larvae/day within 28 days of incubation	(B.-Y. Peng et al., 2022)
3	Greater wax moth	Gut microbiota	5 mg LDPE degraded within 72 hours; LDPE in frass was observed	(Réjasse et al., 2022)
4	Waxworm (Larvae of <i>Plodia interpunctella</i>)	<i>Meyerozyma guilliermondii</i> , <i>Serratia marcescens</i>	15.87% weight reduction with <i>M. guilliermondii</i> , 3.57% with <i>S. marcescens</i>	(Lou et al., 2022)

Table 2.9 Recent evidence for the effectiveness of gut microbiota in LDPE biodegradation.

2.11.8 Cross-Kingdom consortia in LDPE biodegradation

Recently, several investigations have identified the potential of cross-kingdom co-cultures isolated from natural conditions for the biodegradation of recalcitrant compounds (Chigwada et al., 2023).

Continuous evolutionary modifications in the biosystem may be behind such consortia (Escalante et al., 2015). It alters the metabolic and intercellular communications of organisms (Duncker et al., 2021). It creates a novel microcosm with enhanced activities for the bioremediation of compounds (Esmaeili et al., 2013).

S. No	Name of the Cross-Kingdom Co-cultures	Source of Isolation	Major Results	Reference
1	<i>Clostridium</i> sp. and <i>Thiobacillus</i> sp. (bacterial strains) and <i>Dekkera</i> sp. (Yeast strain)	Culture collected from the culture collection centre	Bioengineering with bacterial-fungal consortia (BFC) enhanced external mass transfer (EMT), a limiting factor in PE degradation	(Fachrul et al., 2024)
2	<i>Bacillus</i> sp., <i>Trichoderma</i> sp., <i>Rhizopus</i> sp., and <i>Aspergillus</i> sp.	Isolated from LDPE compost	Marked weight loss in pure LDPE films, LDPE blended with TPS and SES	(Kučić Grgić et al., 2023)

Table 2.10 Novel bioengineering concepts to improve LDPE biodegradation using cross-kingdom co-cultures.

2.12 Strain improvement methods for biodegradation of LDPE compounds

Recently, scientists have attempted various methods to improve the capabilities of natural strains isolated from their native environments. The standard strain improvement methods include physical and chemical processes. In physical techniques, UV (250-290 nm) and gamma rays induce mutations in the strain, enhancing the biodegradation rate (Rostami et al., 2024). Ethyl methyl sulphonate

(EMS) is the most prevalent chemical alkylating mutagen that can produce CG to AT transitions in strains, enhancing biofilm formation (Talkad, 2018). An early study demonstrated that both UV and EMS applications on strains could improve the production of lipase-like enzymes in potential strains for LDPE biodegradation (Ritu et al., 2022).

2.13 Basic steps in the microbial degradation of LDPE films

Under environmental conditions, LDPE films mainly biodegrade in response to mechanical, chemical, and physical stress. However, the microbial biodegradation process includes five significant steps: biofilm formation, Biodeterioration by releasing extracellular enzymes or biosurfactants from microbial strains, biofragmentation, Assimilation, and Mineralisation.

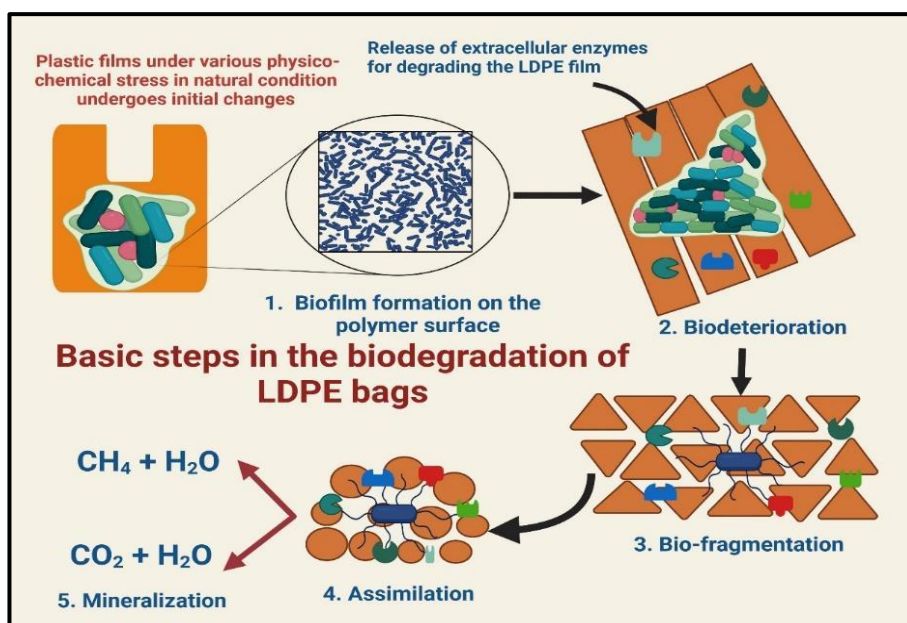


Fig. 2.17 Schematic representation of microbial degradation of the LDPE carrier bags.

2.13.1 Biofilm formation- Biofilms are bacterial communities that are densely packed and that form coordinated, functional microbial communities by encircling themselves with a matrix of extracellular polymeric substances (EPS) while growing on the surface of an inert material (Yin et al., 2019). As microorganisms infiltrate the LDPE film, the pores expand, causing fractures that weaken the polymer's structure by releasing acids into the pores, altering their pH, and accelerating the deterioration process.

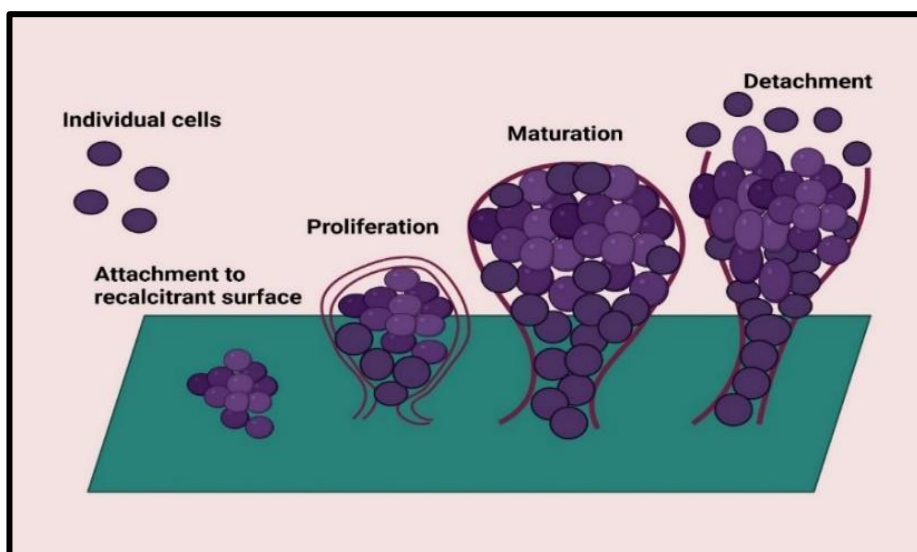


Fig. 2.18 Major steps in biofilm formation on LDPE film (Petrova & Sauer, 2016).

2.13.2 Biodeterioration: A physicochemical change occurs in the recalcitrant compounds by releasing extracellular enzymes excreted by the biofilm-forming microbial strains. A thicker sample will prolong the enzymatic degradation process, as the enzymes must penetrate further into the material to access the interior (Safdar et al., 2024;

Singh Jadaun et al., 2022). Their hydrophobic surfaces facilitate the enhanced adherence of bacteria to unaltered plastic polymers (Wilkes & Aristilde, 2017). The initial biofilm formation helps to add new functional groups to the polymer chain.

2.13.3. Bio fragmentation: The active release of free radicals by the microbial community may lead to polymer fragmentation into smaller carbon units called bio fragmentation. The macromolecular long C-C structures were broken into oligomers with 10-15 carbon atoms in the presence of potent microbial strains or consortia and further metabolised into smaller fragments. This metabolism is facilitated by enzymes, such as alkane monooxygenases (Yoon & Jang, 2021), laccases, esterases, and peroxidases (Suresh et al., 2023).

2.13.4. Assimilation: Oligomers and monomers produced during fragmentation are transported into the cytoplasm of microbes through transmembrane transporters and metabolised through the catabolic pathway to produce acids, alcohols, phenol, esters, alkenes, and alkane derivatives to meet the microbes' energy requirements (Kale et al., 2015).

2.13.5. Mineralisation: The complete degradation of the polymeric subunit converts the polymeric material to CO₂, N₂, CH₄, and H₂O, known as mineralisation (Pathak & Navneet, 2017). Initially, microbes oxidise LDPE materials by hydro-oxidation or oxo-biodegradation methods. The exact catabolic pathway that leads to complete mineralisation is still under study.

2.14 Role of key enzymes in LDPE biodegradation

Enzymes play a significant role in the microbial breakdown of LDPE materials. It non-toxically converts the complex polymer compound

into simpler molecules, such as CO₂ and H₂O, and subsequently produces high-value new biomass (Grima et al., 2000). The backbone of LDPE chains is strong and usually resistant to microbial attack. However, the hydrolytic enzyme can cleave the long hydrocarbon chain either by endo-cleavage (fragmentation along the chain) or exo-cleavage (at the terminus). It occurs in the presence of intra- or extracellular enzymes (Tokiwa & Calabia, 2004). Although a knowledge gap exists in deciphering the exact mechanism of enzymes for LDPE biodegradation, numerous reviews suggest different enzymes and their respective efficacies. Alkane hydroxylase, proteases, laccases, peroxidases, lipases, esterases, and dehydrogenases are the common enzymes identified for LDPE biodegradation.

2.14.1 Alkane hydroxylases

It is one of the pivotal microbial enzymes identified for LDPE biodegradation, with three coding regions: alkane monooxygenases (alkB), rubredoxin (alkG), and rubredoxin reductases (alkT) (Nie et al., 2014). Rubredoxin (an iron-sulfur cluster) and rubredoxin reductases are electron carriers that activate the enzyme's catalytic site, regulated by the alkB gene (van Beilen et al., 2002). The alkB gene clusters have six membrane-spanning helices and a conserved amino acid motif rich in histidine, an active site bridged by a glutamate residue, that can catalyse hydrolysis reactions such as hydroxylation, demethylation, and epoxidation. The reactions ultimately yield alcohol, aldehyde, carboxylic acid, fatty acids, and epoxides (Y. Ji et al., 2013). This enzyme belongs to the oxidoreductase enzyme category, which degrades alkanes by introducing oxygen atoms to the termini of the alkane chain. Furthermore, it leads to β -oxidation and mineralisation of long-chain hydrocarbons (H. J. Jeon & Kim, 2015). Different bacterial

species produce various types of alkane hydroxylases to degrade alkanes (Heris, 2024). The first *alkB* gene for the alkane hydroxylase enzyme was identified in *Pseudomonas putida* (S. C. Williams & Austin, 2022). A few strains demonstrated the presence of OCT operons in their plasmids, which regulate the *alkB* genes (Smits et al., 2003).

An early investigation has demonstrated that microorganisms expressing the alkane monooxygenase enzyme can fragment LDPE films through the terminal or sub-terminal oxidation pathway (Restrepo-Florez et al., 2014).

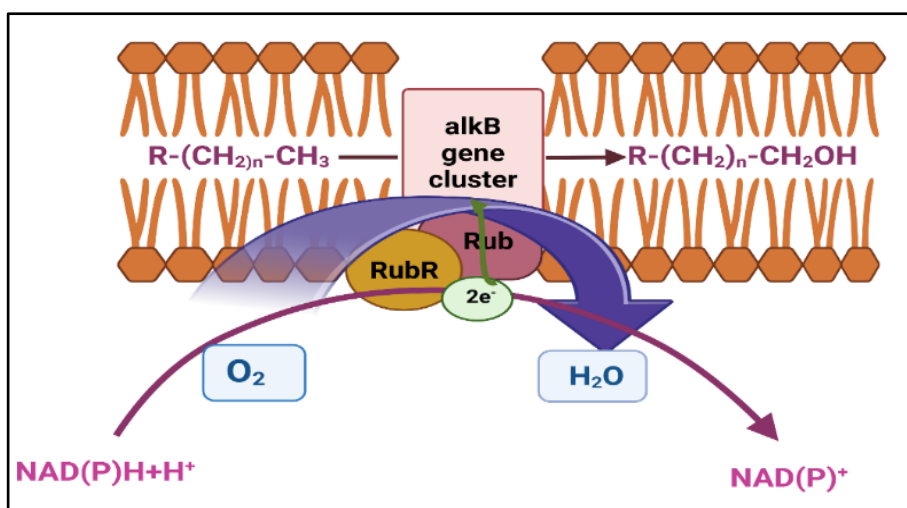


Fig 2.19 Architecture of aerobic *alkB* family alkane hydroxylase enzyme (AH) of *Pseudomonas putida* GPo1, the model organism, consists of *alkB* encoding the catalytic component, Rub, rubredoxin encoded by *alkG* & RubR, rubredoxin reductase encoded by *alkT* & its electron transfer system.

Another study demonstrated that the *alkB* gene is the active locus for the expression of the alkane hydroxylase enzyme responsible for LDPE biodegradation, as evidenced by the cloning of the *alkB* gene from *Pseudomonas* sp. E4 into *Escherichia coli* BL21, a strain not

involved in LDPE degradation (Gyung Yoon et al., 2012). The enzyme's presence can be identified by primer targeting (Nedi et al., 2024) or by performing an alkane hydroxylase enzyme assay (Yusoff et al., 2020). A few scientists have recently attempted to engineer plasmids with alkane hydroxylase genes in *Yarrowia lipolytica*, a yeast strain used for the biodegradation of LDPE (F. Liu et al., 2025).

S. No	Microorganism	Gene/ gene cluster identified for coding enzyme	References
1	<i>Pseudomonas aeruginosa</i> E7	The BCARB gene cluster codes for alkane monooxygenase, rubredoxin, and rubredoxin reductase.	(J.-M. Jeon et al., 2021)
2	<i>B. subtilis</i> , <i>B. wiedmannii</i> , <i>B. cereus</i> and <i>B. siamensis</i>	The alk B gene for the alkane hydroxylase enzyme	(Maroof et al., 2021)
3	<i>Paenibacillus</i> sp	Alk B	(Bardaji et al., 2019)

Table 2.11 A few studies have identified the presence of alkane hydroxylase enzymes and the gene or gene cluster responsible for enzyme expression in potential strains for LDPE biodegradation through primer targeting.

2.14.2 Laccases

Laccases are extracellular enzymes that facilitate the oxidative cleavage of LDPE film's amorphous area, which produces readily accessible carbonyl groups and significantly reduces the weight of LDPE film (Santo et al., 2013). The enzyme consists of dimeric or tetrameric glycoproteins; each monomer is linked to a copper centre. They participate in reducing molecular oxygen to water and oxidising phenolic and non-phenolic substances using oxygen as an electron

acceptor (Demarche et al., 2012). The enzyme's coordination centres (catalytic centres) consist of four copper atoms, categorised into three types of copper centres: type 1, type 2, and type 3 (Dwivedi et al., 2011). Many bacterial (Temporiti et al., 2022), fungal (Sánchez, 2020), and actinomycete (Zampolli et al., 2023) species have been reported to produce laccases for the biodegradation of LDPE. The presence of laccases in strains can be identified using a model substrate, ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), spectroscopically at 420 nm (More et al., 2011; Re et al., 1999).

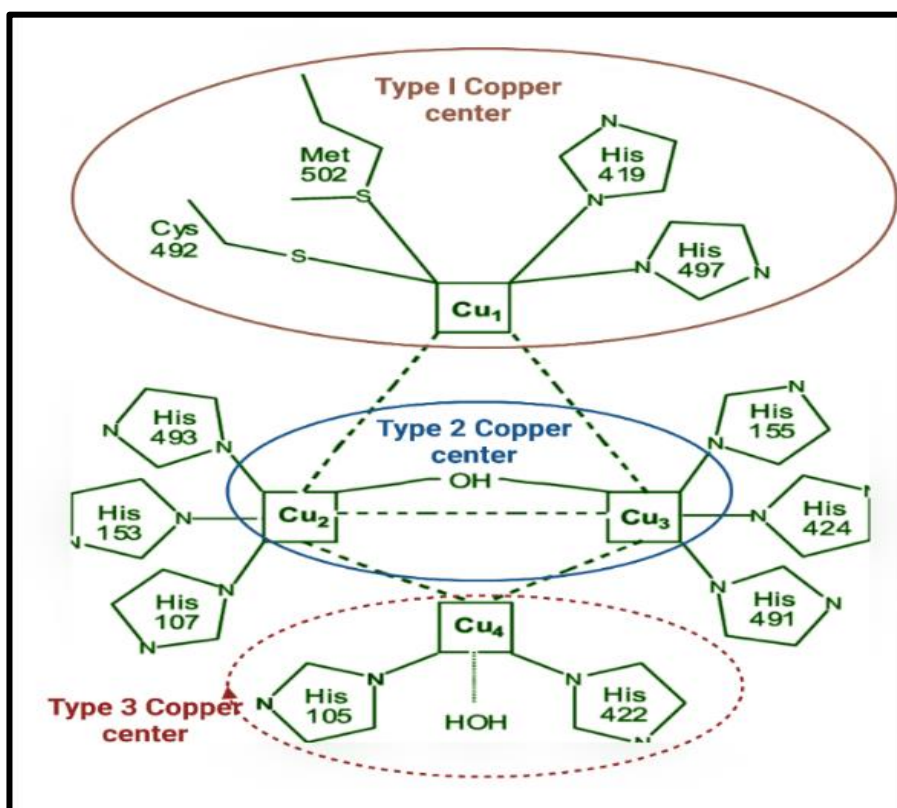


Fig. 2.20 Basic structure of Laccase enzyme with three copper centres

S. No	Organism	Reference
1	<i>Brevibacillus borstelensis</i> , <i>Brevibacillus parabrevis</i> , and <i>Pseudomonas putida</i>	(Muhonja et al., 2018)
2	<i>Pleurotus ostreatus</i>	(Gomez-Mendez et al., 2018)
3	<i>Trichoderma viride</i>	(Dorairaj et al., 2021)

Table 2.12 A few investigations prove the presence of laccase enzyme in microbial strains for LDPE biodegradation.

2.14.3 Peroxidases

They are extracellular oxidoreductase enzymes with a haem group capable of breaking the polymer backbone using H_2O_2 as an electron acceptor (Ameen et al., 2015). They can degrade high-molecular-weight polyethylenes (Kang et al., 2019). Different types of peroxidases are involved in LDPE biodegradation, including manganese peroxidases (Sowmya, Ramalingappa, et al., 2015), lignin peroxidases (Iiyoshi et al., 1998), and versatile peroxidases.

2.14.4 Manganese peroxidases (MnP)

The catalytic site of the enzyme features a propionate heme group (PHG) comprising two propionate molecules, amino acids such as aspartic acid (Asp 179) and glutamic acid (Glu 35 and Glu 39), Ca^{2+} for stability, and two water molecules (Y. Hou et al., 2001). One propionate group in PHG is bound with water molecules, and the other propionate is bound to Mn^{2+} . The catalytic function of heme MnP (manganese peroxidase) is initiated by free radicals in acidic conditions, with the highest level of activity observed at a specific pH

range of approximately 4.5-5.5 (Chandra et al., 2017). The addition of hydrogen peroxide to the enzyme assay in the culture media results in its cleavage, producing two hydroxyl free radicals. One of the radicals is then bound to the ferric MnP part ($\text{Fe}^{3+}\text{-MnP}$) and converted into Fe^{4+} oxo-porphyrin radical complex (compound I).

Meanwhile, the Mn^{2+} donates one electron to the porphyrin radical complex and forms Mn^{3+} by adding another radical to the propionate group of MnP. This process transforms compound I to II. Furthermore, this activates the enzyme's catalytic site for the biodegradation of polyethylenes into alkanes, alcohols, acids, and other compounds (Jin et al., 2023).

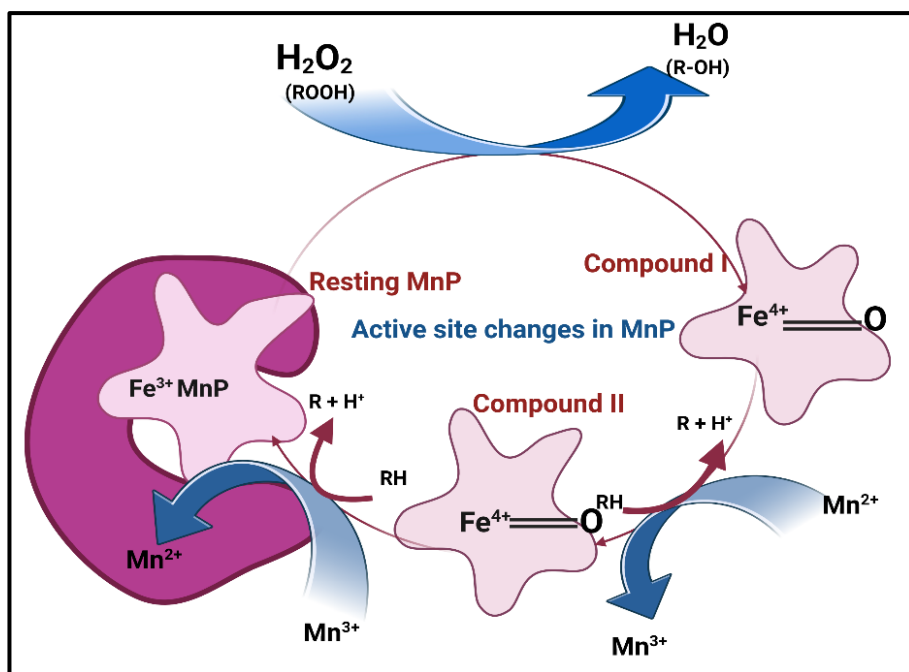


Fig. 2.21 Schematic representation of the manganese peroxidase enzyme active site for the catalysis of LDPE materials.

S. No	Organism producing MnP	References
1	<i>Phlebiopsis flavidoalba</i>	(Perera et al., 2023)
2	<i>Phanerochaete chrysosporium</i>	(Emami et al., 2020)
4	<i>Bacillus</i> sp. strain	(Nayanashree & Thippeswamy, 2015)
5	<i>Bacillus subtilis</i>	(Prabhawathi et al., 2014)

Table 2.13 Manganese peroxidase-containing microbes for LDPE biodegradation.

2.14.5 Lignin peroxidases (LiP)

It is one of the extracellular oxidoreductase enzymes with an exceptional capacity to oxidise non-phenolic aromatic compounds and pollutants (Catto et al., 2014). This oxidation process entails an oxidation-reduction reaction in which hydrogen peroxide acts as an electron donor, facilitating the removal of an electron from the pollutant and creating unstable free radicals. Afterwards, using non-enzymatic processes, another substrate molecule functions as the reducing agent, concluding the enzyme's catalytic cycle and liberating the substrate's original molecules. The discovery and purification of LiP and MnP first occurred in a culture of *Phanerochaete chrysosporium* in 1983 (Tien & Kirk, 1983). Similarly, the presence of such highly reactive radicals produced by this enzyme was reported in a *Bacillus* sp. inoculated in a mineral salt broth mixed with LDPE films for 120 days (S. Li et al., 2024). A recent study using *Pleurotus ostreatus* for LDPE degradation reported that the initial reactions were performed by the laccase (Lac) enzyme, lignin peroxidase (LiP),

manganese peroxidase (MnP), and unspecific peroxygenase (UP) enzymes (Maisto et al., 2024).

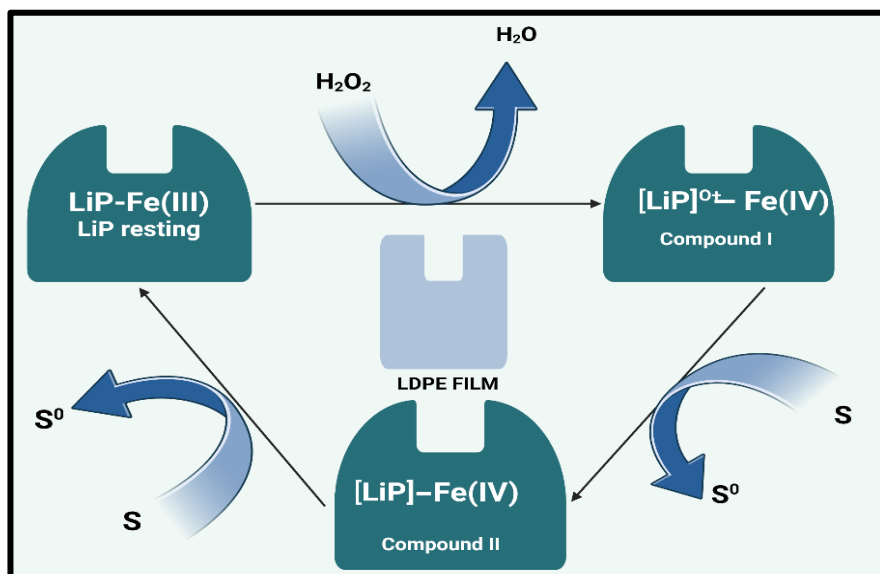


Fig.2.22 Conformation changes in the heme-catalytic site of lignin peroxidase enzyme for effectively degrading LDPE films (Redrawn image of (Falade et al., 2016)).

S. No	Organisms producing LiP	References
1	Bacterial consortium containing <i>Bacillus subtilis</i> , <i>Lysinibacillus sphaericus</i> , <i>Lysinibacillus fusiformis</i> , <i>Alcaligenes faecalis</i> , and <i>Kocuria rosea</i>	(Mohammadi et al., 2022)
2	Fungal consortia- <i>Diaporthe italiana</i> , <i>Thyrostroma jaczewskii</i> , <i>Collectotrichum fructicola</i> , and <i>Stagonosporopsis citrulline</i>	(Khruengsai et al., 2021)

Table 2.14 Studies showing the potentialities of strains to degrade LDPE compounds in the presence of the LiP enzyme are presented among them.

2.14.6 Versatile peroxidases (VP)

They are a hybrid form of manganese peroxidase and lignin peroxidase enzymes, displaying functionalities of both enzymes (Biko et al., 2020). In the presence of H₂O₂ oxidants, they can oxidise both low- and high-redox-potential pollutants. They can bind non-specifically to aromatic or non-aromatic compounds (Rodriguez, 2004). These notable attributes of VP have generated substantial interest in biotechnology. The enzyme was first reported in the fungal strain *Pleurotus eryngii* (Martínez et al., 1996). Several scientists have diverted their attention toward using VP-enzymes for the biodegradation of LDPE compounds (Twala et al., 2020).

2.14.7 Lipases

Lipases are enzymes that have specific targets against water-insoluble triglycerides composed of long fatty acids (Chahinian & Sarda, 2009). They exhibit a diverse spectrum of molecular sizes, substrate specificities, and catalytic activities. The X-ray crystallographic studies proved that the lipase enzyme has a lid-like structure (composed of fifteen amino acids), which is responsible for exposing the hydrophobic regions of the active site for substrate-enzyme activity (Derewenda et al., 1992). The enzyme can convert the insoluble triglycerides to free fatty acids. Several investigations have proven that bacterial (Khandare et al., 2022) and fungal strains (Srikanth et al., 2022) can degrade LDPE in the presence of lipase enzymes.

2.14.8 Esterases

Esterases are hydrolase enzymes that catalyse the hydrolysis of esters, breaking them down into alcohols and acids by adding water molecules (Fojan et al., 2000). Recently, a *Bacillus* species isolated from a landfill in Thailand was found to biodegrade LDPE (Khampratueng et

al., 2024). The presence of esterase enzyme in the biodegradation pathway proves the capabilities of the bacterial strains in LDPE biodegradation (P. Singh et al., 2024).

2.14.9 Proteases

It is also one of the hydrolase enzymes that modifies the LDPE film's surface. Introducing biofilm on the surface of LDPE film may be the reason behind the production of protease enzymes (Maroof et al., 2022). Many earlier studies have reported the involvement of protease enzymes and the introduction of nitrogen groups into the chemical structure of LDPE film (Taxeidis et al., 2023; Watanabe et al., 2009).

Other enzymes responsible for LDPE biodegradation

S. No	Microbial Strain	Enzyme	Mode of Action	References
1	<i>Rhodococcus</i> sp. enriched from seawater.	Glutathione peroxidase (GPx)	An active site with superoxide anion radicals assists in LDPE biodegradation.	(Rong et al., 2024)
2	<i>Alternaria alternata</i>	Glutathione peroxidase (GPx)	95% of samples achieved $\geq 5\%$ weight loss within 120 days due to high redox potential at the GPx active site	(Gao et al., 2022)
3	<i>Acinetobacter</i> sp	Cutinase	Hydroxylates the LDPE chain into alkanoic acid or alkanols	(G. H. Lee et al., 2023)
4	<i>Bacillus pacificus</i> and <i>Pseudomonas mucidolens</i>	Cellulase	Oxidises carbon backbone to CO ₂ and H ₂ O	(Krishnaswamy et al., 2022)

Table 2.15: Recently identified other types of enzymes in LDPE biodegradation.

2.15 Distinct mechanisms exhibited by the microbial system to mineralise the LDPE compound

2.15.1 Chemo-attractions

Chemotaxis is a conspicuous mechanism that brings the microbial strains closer to the recalcitrant compounds. It occurs through conformational changes in the multi-protein complex in the cytoplasm, which activates a signal cascade pathway to regulate the movement of flagella toward the recalcitrant compound (Ahmed et al., 2023). Recently, high-throughput transcriptome analysis of *Alcanivorax* sp. isolated from the plastisphere of a marine environment demonstrated the strain's chemoattraction mechanism towards the LDPE compound (Zadjelovic et al., 2022).

2.15.2 Membrane transporters

Most of the strains involved in hydrocarbon biodegradation possess various types of membrane transporters in their outer membrane, which transport compounds into the organism's cytoplasm for further degradation.

S. No	Name of the strain	Name of the membrane transporter	References
1	<i>Bacillus cereus</i>	ATP-binding cassette transporters	(Tao et al., 2023)
2	<i>Rhodococcus opacus R7</i>	ATP/GDP-binding integral membrane protein and ABC transporters	(Mishra et al., 2024; Zampolli et al., 2021)
3	<i>Rhodococcus ruber</i>	ABC transporters	(Gravouil et al., 2017)

Table 2.16 The presence of membrane transporters proved by transcriptome profiling in the membrane system of LDPE biodegrading strains.

2.15.3. Extracellular polysaccharides production during biofilm formation

It is an initial step in all types of hydrocarbon biodegradation. The non-availability of carbon or nitrogen resources contributes to the increased cell surface hydrophobicity among the LDPE film-attaching strains. It may elevate the biofilm formation on the polymer surface. A complex matrix encases microbial communities (Czaczyk et al., 2008). The main constituents of EPS include polysaccharides, proteins, and lipids (Stoodley et al., 2002). The potential strains' basic molecular mechanisms (genes and enzymes), cell secretions, and signalling molecules control biofilm formation (S. H. Ji et al., 2024). For instance, extracellular polysaccharide substances (EPS) secreted by microbes under stringent conditions can enhance cell communication, structural integrity, and mechanical stability of biofilms, thereby facilitating improved biodegradation (Payne & Boles, 2016). Organisms like *Pseudomonas aeruginosa* can release polysaccharide compounds, such as alginate, Pel, and Psl, which serve as a framework for the biofilm architecture (P. Gupta et al., 2016).

2.15.4 Biosurfactant formation

Biosurfactants are another microbial component that helps attach to hydrophobic hydrocarbon products. The biofilm attached to the polymers is responsible for the release of biosurfactants. These compounds, which are potential N-alkane and LDPE biodegraders, share a similar nature. It is mainly composed of glycolipid or lipoprotein in nature (Ron & Rosenberg, 2002). Rhamnolipids (a glycolipid biosurfactant that consists of rhamnose units linked with the

β -hydroxy fatty acids) are the common biosurfactants in the potential strains for LDPE biodegradation (Taghavi et al., 2022). *Bacillus* and *Pseudomonas* are usual producers of lipoprotein biosurfactants. A few researchers have demonstrated the ability of *Bacillus subtilis* to produce lipoprotein biosurfactants and its capability to degrade medium-chain alkanes of up to C19 length (Kim, 2003) and LDPE films (Vimala & Mathew, 2016). The biosurfactants in organisms' outer membranes can increase the substrate's hydrocarbon-water interface and net charge. It enhances the bioavailability of recalcitrant compounds. The primary biosurfactant detection methods include hemolysis, lipase test, BATH (Bacterial adhesion to hydrocarbon), Penetration assay, and emulsification assay (M. J. Singh & Sedhuraman, 2015).

2.16 Genes identified for LDPE biodegradation by using modern molecular techniques

Specific genes or clusters responsible for LDPE mineralisation have yet to be elucidated. A few scientists have reported the presence of genes such as *alkB*, which are responsible for the expression of the alkane hydroxylase enzyme, as well as the *LIP 1* and *LIP 2* genes for lipase enzymes, in potential strains responsible for LDPE biodegradation (Bowman & Deming, 2014; Mohanan et al., 2022). A recent study employed a combination of Oxford nanopore and Illumina technologies to investigate the chromosomal and plasmid genes of *Rhodococcus opacus* responsible for LDPE degradation (Firrincieli et al., 2022). In addition to the *alkB* gene, the *CYP153* gene, which codes for cytochrome P450 CYP153 alkane hydrolase, was demonstrated in

Alcanivorax borkumensis for LDPE biodegradation (Pinto et al., 2022). The genes responsible for long-chain alkane degradation, such as *AlmA* and *LadA*, were proven in *Alcanivorax dieseloei* (P. Wang et al., 2023) and *Geobacillus thermoleovorans* (Boonmak et al., 2014).

Numerous gene profiling studies using transcriptome analysis have demonstrated the activation of genes associated with ABC transporters, carbon metabolism, fatty acid oxidation, the TCA cycle, and stress response pathways (Fu et al., 2024; E. Yang et al., 2022).

2.17 Proposed pathway of LDPE biodegradation

Researchers in this arena have recently proposed a biodegradation pathway using high-throughput analysis. However, the exact mechanisms or pathways for biodegradation are still unclear. Several scientists have documented the presence of intermediates in metabolic pathways using analytical techniques such as FTIR and GC-MS (Alamer et al., 2023; Sutkar et al., 2025). Moreover, a recent transcriptome profile of LDPE-attached biofilms revealed upregulation in carbon and fatty acid metabolism (T. Zhang et al., 2025).

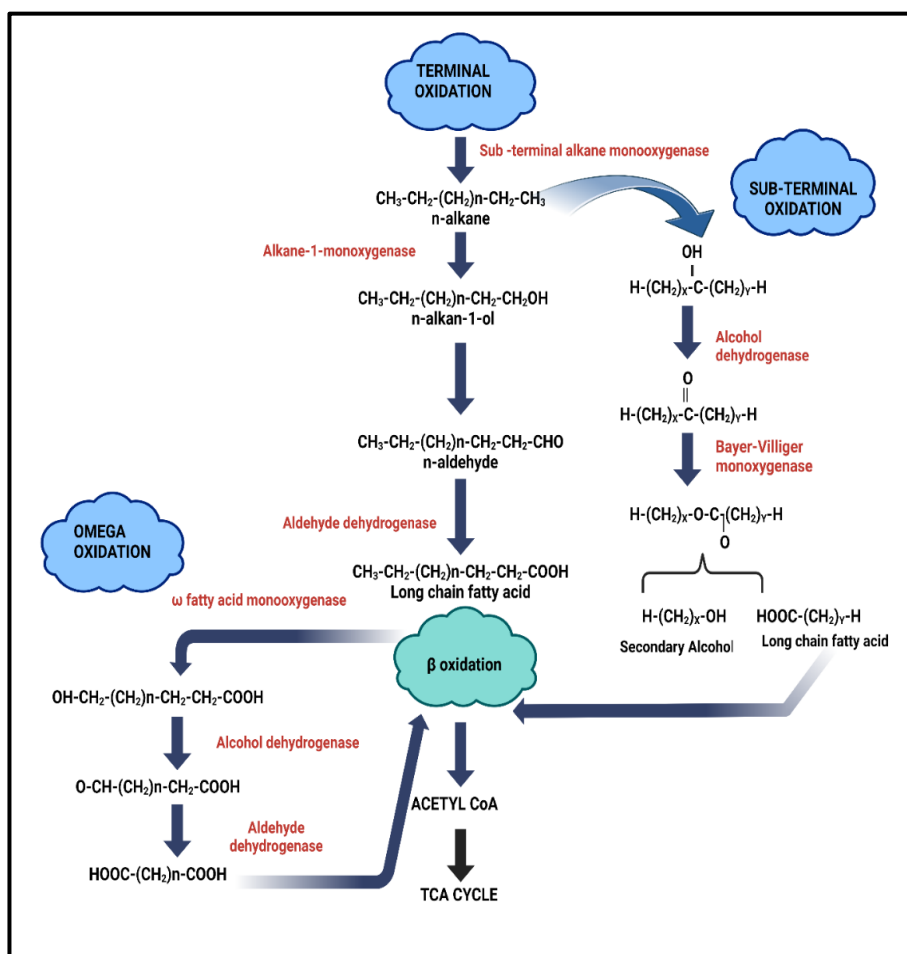


Fig. 2.23 Proposed pathways in LDPE biodegradation.

Still, the exact metabolic pathway by which the fate of LDPE material is demonstrated inside the bacterial cell is unknown. Thus, Scientists in our decade are constantly searching for a microbial system to overcome this daunting problem in our century.

CHAPTER 3

STUDY ON THE EFFICACIES OF BACTERIAL STRAINS SCREENED FROM PLASTIC LANDFILLS FOR ROBUST BIOFILM FORMATION ON LDPE FILMS

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3.1 Introduction

Plastic landfilling is a common practice worldwide. It serves as a primary disposal site for plastic waste from homes, industries, and commercial spaces (Dube & Okuthe, 2023). However, the per capita generation of plastic waste is substantially larger in developed countries than in developing and third-world countries, due to the correlation between the waste creation rate and a nation's economic and social prosperity (OECD, 2022; Velis et al., 2023). Almost all landfills require significant space and resources to maintain (Ozbay et al., 2021). A lack of knowledge about the safe and environmentally friendly removal of polymer compounds from our surroundings contributes to the daily increase in landfill size. In such depots, most polymeric compounds undergo substantial alterations in their structure and stability (Kumar Sen & Raut, 2015).

The natural fragmentation of long-chain polymers primarily happens in landfills through microbial activities. Therefore, it is regarded as a viable disposal option. Nonetheless, its prolonged breakdown processes and the production of harmful leachates have a significant impact on soil, water, and air quality. Furthermore, they may cause potential health risks, including cancer, hormone disruptions, and neurological impairments (Kabir et al., 2023).

Nowadays, plastic material landfills are common in terrestrial and aquatic environments. They cause the migration of macroplastic leachate and the dispersal of microplastics. Recently, plastic particles have been detected in blood samples, alveoli, intestinal tissues, and placental tissues (S. Liu et al., 2024; Saha & Saha, 2024; V. L.

Leonard et al., 2024). Recently, scientists from the University of New Mexico reported the presence of microplastics in brain tissues as well (Campen et al., 2024).

3.1.1 Existing terrestrial landfilling practices of plastic wastes around the globe

Landfills are often categorised into open dumps, semi-controlled, and sanitary landfills (Roy et al., 2022). Open dump landfills are the most common type of landfill. They are more prevalent in developing nations, where plastic waste is often indiscriminately discarded in vulnerable areas with low elevations. Due to inadequate management, deep depots create a unique habitat for birds, mosquitoes, fleas, bugs, worms, rats, and pathogenic microbes (Nuripuh et al., 2022). Recently, regulatory laws have outlawed open dumping in many industrialised regions of rich countries. It led to the transportation of plastic waste to developing nations, where it ultimately ends up in open debris sites (Ferronato & Torretta, 2019).

A few countries have implemented semi-regulated and sanitary dumping protocols to dispose of plastic waste materials (Agamuthu & Babel, 2023). Semi-controlled landfills are managed in approved dumpsites, where materials are sorted, shredded, and compacted on-site before disposal. To prevent plastic pile nuisances, the plastic trash is pulverised and levelled using bulldozers or crawlers, and subsequently, a topsoil layer is added to it. Although semi-regulated landfills have soil covers, they are not primarily engineered to limit toxic gas emissions or effluent discharges from plastic piles (Narayana, 2009).

Furthermore, sanitary landfills might be classified as sophisticated types of semi-controlled landfills. Apart from on-site activities such as solid waste sorting and soil coverings, these piles have specifically designed facilities to collect effluents and harmful gas emissions. Such dumps are prevalent in industrialised nations equipped with the capabilities to capture and treat leachate (Siddiqua et al., 2022). Sanitary landfills are scheduled to undergo landscape expansion by excavating new dumpsites after sealing the current ones to prevent saturation. The high cost, need for technical assistance, and high disposal rates cause plastic materials to end their lives in open landfills (Evode et al., 2021).

3.1.2 Peculiarities of long-run open plastic dumps

The primary factors contributing to the expansion of plastic waste are urbanisation, economic development, and population growth (Nayanathara Thathsarani Pilapitiya & Ratnayake, 2024). As of 2015, approximately 79% of landfills comprise plastic waste. The significant share comprises polyethylene materials (Geyer et al., 2017). Unlike conventional plastic waste, landfill plastic waste exhibited a markedly reduced concentration of carbon and sulfur, while displaying increased levels of oxygen, silicon, and aluminium (Wojnowska-Baryła et al., 2022). Disposing of such recalcitrant pollutants diverted scientists to study landfill degradation through physical or chemical conditions or by the action of biological agents. Moreover, several studies have demonstrated the release of numerous hazardous gases, leachates, and microplastics into the surrounding environment, thereby affecting our

environmental harmony (Campanale et al., 2020; R. Kumar et al., 2021; Skariyachan et al., 2022).

3.1.3 Long-run landfill soil- a microcosm for biodegradation of LDPE films

In recent years, numerous researchers have emphasised the significance of microbial processes in landfill soil for the secure biodegradation of long-chain polymers. The introduction of rDNA sequencing, metagenomics analysis, and transcriptome studies has enabled scientists to gain valuable knowledge about the capabilities of strains in long-term maintained landfills. Microbial biodegradation in landfills primarily involves bacteria and fungi, but the rates are slow due to the recalcitrance of LDPE (Tiwari et al., 2024). The continual exposure of plastic garbage to external factors converts it into a unique ecosystem inhabited by various microbes. A range of biotic factors—such as the inoculum's size and density, viable cell count, enzyme secretion and activity, use of microbial co-cultures, cell surface hydrophobicity, and surfactant production—and abiotic factors—including ultraviolet radiation, temperature fluctuations from climate change, chemical contaminants in landfills, moisture levels, and nutrient availability—contribute to the breakdown of polymeric substances into monomers, facilitating their integration with soil samples in landfill environments (Miloloža et al., 2022). Therefore, many scientists have utilised landfill soil to study biofilm formation and biotransformation processes occurring in natural settings and laboratory conditions, aiming to achieve a breakthrough. Nevertheless, a notable lack of studies exists on developing a biotechnological

method that utilises a natural strain or consortia to degrade LDPE films within a limited time frame efficiently.

3.2 Aim and Objectives

1. Sample collection from open plastic landfills in different parts of Kerala.
2. Study the physical and thermal properties of LDPE films purchased from the market and surface-sterilise the polymer strips for further studies.
3. Enrichment and screening of the potential strains from the soil samples collected from various landfills.
4. Preliminary investigations to identify the most effective bacterial strains from the enriched soil samples of each terrestrial plastic depot.
5. Perform morphological, biochemical, and culture characteristic studies and molecular and MALDI-TOF analyses to identify the most suitable strains from soil samples.
6. Study the initial growth pattern of the most competent bacterial strains isolated from each landfill soil.
7. Examine the efficacies of each isolated bacterial strain in forming strong biofilms on LDPE films in a carbon-free mineral salt broth.
8. Quantitative and qualitative analysis of enzymes produced by the biofilm formed on the surface of LDPE films.
9. Identify, isolate, and characterise biosurfactants from potent bacterial strains using oil displacement, emulsification, and FTIR analyses.

3.3 Review of Literature

Polyethylenes exhibit a semi-crystalline structure characterised by linear chains of carbon atoms bonded with hydrogen atoms, making them highly resistant to degradation. Several researchers have harnessed the biodegradation and bioconversion mechanisms of plastic waste released into the environment through the activities of bacterial, fungal, actinomycete, and photosynthetic algal species (Barone et al., 2024). Among the bacterial species, *Bacillus* and *Pseudomonas* are the most extensively researched bacterial genera for the breakdown of polyethylene (PE) (Roberts et al., 2020). Plastic biodegradation by bacterial strains is accompanied by biofilm formation, which enhances catalytic activities through close contact with the polymer (Katnic & Gupta, 2025). Numerous scientific studies have demonstrated that biosurfactants produced by specific bacterial strains can enhance biodegradation (Tiago et al., 2023). Fungal species, such as *Aspergillus* and *Penicillium*, also contribute to LDPE biodegradation, complementing bacterial activity (Sutkar et al., 2025).

3.3.1 *Bacillus* species as a potent bacterial strain isolated from landfills

Many recent reports have revealed the significance of *Bacillus* species as a promising innate strain isolated from long-term landfills for LDPE biodegradation. Upon thriving on synthetic mineral medium, an early study isolated *Bacillus* sp. and *Paenibacillus* sp. from landfill sediments, demonstrating a 14.7% biodegradation rate after 60 days of incubation (Park & Kim, 2019). Another study using the *Bacillus cereus* strain reported an almost 5% weight reduction and a 3.27%

reduction in crystallinity within 42 days of incubation in a carbon-free medium (Jebashalomi et al., 2024). The *Bacillus cereus* isolated from garbage sites was used to demonstrate substantial colonisation abilities and catalytic functions on LDPE films, establishing a distinct ecological niche (McFall et al., 2024).

3.3.2 *Pseudomonas* species as a competent strain for the biodegradation of LDPE films

Pseudomonas species are another common strain in open landfills (Lv et al., 2024). Recently, a scientific investigation by (Mouafo Tamnou et al., 2021) researchers reported the influence of temperature and the medium's pH on LDPE biodegradation using the *Pseudomonas* strain. Another inquiry with *Pseudomonas aeruginosa* isolated from a garbage site showed a weight loss of LDPE (9.2%) (Shilpa et al., 2022). Many scientists have demonstrated that the presence of enzymes such as laccases, manganese peroxidases, and lignin peroxidases in *Pseudomonas* strains is responsible for the chemical changes on the surface of LDPE (Cholewinski et al., 2022; Temporiti et al., 2022).

3.3.3 *Micrococcus* species as a potential strain for the biodegradation of LDPE films

A few researchers have reported the efficacy of *Micrococcus* as a potential strain for LDPE biodegradation (Nowak et al., 2011). An early study demonstrated that laccases, esterases, and alkane hydroxylases in *Micrococcus luteus* were the key catalytic agents

responsible for the degradation of LDPE films (Montazer et al., 2019; Muhonja et al., 2018).

3.3.4 Initial studies for establishing the degradation abilities of strains for biodegradation

Many researchers have performed weight reduction experiments, biomass assays, BATH tests, FDA tests, CO₂ evolution tests, protein analyses, and EPS analyses to demonstrate strong biofilm formation on LDPE films treated with bacterial strains (Maroof et al., 2021; Tarafdar et al., 2021). Apart from biofilm formation, several scholars have demonstrated the catalytic activities of potential strains and the structural peculiarities that lead to the biodegradation of hazardous polymers (Mohan et al., 2020).

3.4 Methodology

3.4.1 Sample collection and surface sterilisation of LDPE films

3.4.1.1 Soil sample collection from long-run plastic depot

The soil samples (approximately ten grams) mixed with small pieces of plastic film were collected in triplicate from three different locations of long-run plastic dumps in various parts of Kerala, India. Soil samples were collected from the top 10 cm of landfill surfaces to capture microbial activity. The garbage sites for enumerating bacterial strains were located at Alathur (10°37'49.8 "N, 76°32'10.2 "E), Laloore (10°51'26.8 "N, 76°18'25.3 "E), and Bhramapuram (10°00'40.6 "N, 76°36'35.1 "E). All the samples were scooped into gamma-sterilised plastic ziplock bags (15 cm in length) and placed in the chilled ice boxes. All samples were processed within a day of collection in our

laboratory. In addition, the physical parameters of each soil sample, such as pH (measured using a Soil Testing pH Meter from VCK, India) and temperature (measured with a Digital Soil Thermometer from Rapitest Lustre Leaf), were recorded at the sample collection sites and used in further studies.

3.4.1.2 Physical and thermal analysis of LDPE film

This current investigation utilised transparent LDPE films of 50-60 μ m thickness. We purchased plain, colourless LDPE bags (15cm $\times$ 15 cm) from Surya Polymers and Plastic Industry in Pudussery Central, Kanjicode, Palakkad. The thickness of all polyethylene bags was confirmed using a Yuzuki Electronic micrometre with 0.001mm resolution (Lingaraj et al., 2011). Moreover, the physical parameters, such as density, were calculated using the protocol of ISO 1183-1:2004 (Part 1 methodology), and specific gravity was determined using the procedure outlined in ASTM D792-08 (Xu et al., 2022). The weight changes due to time and temperature were measured using Thermo Gravimetric Analysis (TGA-DTA, HITACHI STA7300) (Dubdub & Al-Yaari, 2020).

3.4.1.3 Protocol of surface sterilisation of polymer film

Before each experiment, LDPE-based polymer films were surface-sterilised following the methodology described by (Kyaw et al., 2012). The LDPE films were primarily treated with 50 mL of 2% SDS (sodium dodecyl sulfate) for 10 minutes, followed by treatment with 50 mL of 70% ethanol for 30 minutes. Furthermore, they were washed with distilled water, and their moisture content was removed and dried.

All the LDPE films were at 55°C overnight in a hot air oven. Sterility was confirmed by incubating the treated LDPE films on nutrient agar for 48 hours at 37 °C.

3.4.2 Screening of potential strains from the soil samples collected from plastic dumps using the culture enrichment technique

A mineral salt broth without a carbon source, Carbon free liquid mineral salt medium (CFLMSM) was prepared to identify potential strains by adding chemical ingredients such as K₂HPO₄-7g, KH₂PO₄-2g, NH₄NO₃-1.2g, MgSO₄.7H₂O-0.8g, (NH₄)₂SO₄-0.1g, NaCl-0.05g, KNO₃-0.004g, FeSO₄.7H₂O-0.003g, 0.002g each of KCl, CuSO₄.5H₂O, MnSO₄.5H₂O, ZnSO₄.7H₂O and 0.001g of CaCl₂.2H₂O, per 1000 mL of distilled water at pH - 7.1 ± 0.2 (Pramila, 2011). During the initial phase of culture enrichment, positive control was established by adding one gram of soil samples collected from each spot of the plastic depot to 100 mL of freshly prepared CFLMSM and incubating at 25°C for 2-5 days until the final optical density at 610 nm (OD₆₁₀) reached a range of 0.6-0.9. Additionally, controls without a soil sample medium were maintained to corroborate the experimental results. We took 20 mL of the original enrichment liquid media and spun it at 10,000 rpm for 10 minutes in a benchtop cooling microfuge (Remi CM-12 Plus). The pellets were collected, and the supernatants were removed from the Eppendorf tubes. Each pellet in an Eppendorf tube was washed repeatedly with CFLMSM to remove even small traces of supernatant and mixed in 1.5 mL of carbon-free mineral medium. The aliquots were transferred to the conical flask containing 100 mL of sterilised CFLMSM. Eight LDPE films were placed in a conical flask for the

second enrichment stage. Control conical flasks without culture aliquots were maintained for each set of samples. The LDPE films from all the conical flasks were collected and placed in a saline solution (0.85% w/v), then shaken for 240 minutes at 180 rpm. Then, these were spread on Reasoner's (R2A) medium and incubated for two days at 37°C (Saleh et al., 2023). The total biomass in each sample was calculated in CFU/g (Reasoner & Geldreich, 1985), and the results were compared with control plates spread with the saline solution added without LDPE films from the enriched samples. The strains producing hydrolytic enzymes were further screened using the staining and destaining of the plates with Coomassie brilliant blue (CBB) (Howard & Hilliard, 1999). Finally, the bacterial strains were screened by repeated quadrant streaking.

3.4.3 Initial investigations to find out vital bacterial strains

3.4.3.1 Weight Loss Experiment

5 mL of 0.5 McFarland standard solution of each purified culture was centrifuged (Remi Centrifuge, RM1216) at 7500 g for 6 minutes to collect the pellets. The pellets were mixed with 80 mL of freshly prepared and sterilised CFLMSM broth. After adding the bacterial aliquots, the total volume in the conical flask was adjusted to 100 mL by adding CFLMSM. Furthermore, we have added sterilised LDPE films, weighing approximately 0.24 g, to the conical flask. Precautions were taken to add the 55-micron-sized, 0.03-g LDPE films to both the sample and control conical flasks. After that, the samples were kept at 30°C for 120 days. The weight reduction percentage of each film was calculated and recorded (Skariyachan et al., 2017). We kept CFLMSM

with sterilised LDPE films without bacterial addition at 30°C for 120 days to compare the results (control setup). Moreover, we have calculated the daily consumption rate of hydrocarbon constituents and their half-lives for LDPE films in both inoculated and uninoculated flasks (Auta et al., 2018).

$$\text{Daily uptake of polymer, K value} = -\frac{1}{t} \left(\ln \left(\frac{W}{W_0} \right) \right)$$

Where, t = Time of incubation in days

W = Residual weight of LDPE film after 120 days

W₀ = Initial weight

The half - life of LDPE film, $(t_{1/2}) = (\ln(2)) / K$

3.4.3.2 Fluorescein Diacetate Assay (FDA)

This assay measures the hydrolytic enzyme activities of microbial strains attached to the LDPE film (slightly modified protocol of (Green et al., 2006)). For this evaluation test, along with bacterial strains, eight LDPE strips were placed in 100 mL of CFLMSM and incubated for 30 days at 25°C (Jayan et al., 2023). Further, these LDPE films (within six hours after completing one month of incubation) were collected from CFLMSM and dipped in 60 mL of fluorescein diacetate sodium phosphate buffer (FDSPB) and incubated for 60 min, 120 min, 180 min, 240 min, 300 min and 360 min at 25°C, 180 rpm speed in an orbital shaker incubator (Kemi). After incubation, the culture-mixed buffer solution (2 mL) was transferred into an Eppendorf tube and centrifuged at 12000 rpm for 6 minutes using a CM-12 PLUS refrigerated centrifuge (REMI). The supernatants were carefully collected and transferred to a cuvette for taking spectrophotometric readings at 494

nm using a UV-VISIBLE spectrophotometer (Systronic AU,2701). Control setups were also maintained without treating bacterial colonies with LDPE films.

3.4.3.3 Bacterial Adhesion Test for Hydrophobicity (BATH) Assay

This test demonstrated strong biofilm formation on the long-chain hydrocarbon surface of LDPE film (Rosenberg et al., 1980). In this analysis, OD₆₀₀ bacterial cultures from CFLMSM mixed with LDPE films with an OD between 0.6 and 0.9 were collected in Eppendorf tubes (2 mL in each) and centrifuged at 7500g for 6-8 minutes (Caldas et al., 2005). After removing the supernatants, the pellets were washed three times with CFLMSM (R. K. Sarker et al., 2020). Finally, the pellets were mixed with 2 mL of CFLMSM and transferred to clean test tubes. Each tube was combined with an increasing volume of hexadecane (0, 0.05, 0.1, 0.15, and 0.2 mL), and all tubes were kept undisturbed for 2.5 hours. The aqueous solution in the test tube was carefully transferred into a cuvette, and spectrophotometric readings were taken at a wavelength of 600 nm. The CFLMSM without any bacterial inoculum served as a control. From the OD values, hydrophobicity percentages and reductions in turbidity were calculated (Hydrophobicity was calculated as the percentage decrease in OD₆₀₀ of the aqueous phase after hexadecane addition) (Tribedi et al., 2015), and the results were graphically represented.

3.4.5.4 CO₂ release test (Sturm test)

The gravimetric analysis of CO₂ evolution is considered strong evidence for bio-attachments to LDPE films (Bosco & Mollea, 2021). To perform this test, a self-made apparatus consisting of a conical flask with CFLMSM inoculated with or without bacterial-treated LDPE films (test and control), a receiver flask containing 100 mL of 1 M KOH solution, and clear connecting tubes was used. The leak-proof setups of the test and control were incubated for one month at room temperature to allow CO₂ to diffuse into the KOH solution. The trapped CO₂ in the receiver flask can be demonstrated by adding 0.1 M BaCl₂, which precipitates white crystals of barium carbonate (Ali Shah et al., 2013). The rate of CO₂ release to the KOH solution can be calculated by recording the difference in weights of barium carbonate precipitate crystals formed in the test and control flasks.

3.4.4 Identification of the most proficient bacterial species for the breakdown of LDPE films

Purified bacterial inocula from each enriched soil sample were identified using standard microbiological techniques, including morphological analysis, cultural character studies in selective media, staining methods, and biochemical tests (Koirala et al., 2021). Furthermore, they were identified by 16S rDNA sequencing, phylogenetic tree construction, and MALDI-TOF analysis.

3.4.4.1 Morphological and staining analysis of bacterial strains

All potential cultures from each soil sample were streaked in Nutrient media, and their morphological features were recorded (Demissie et

al., 2024). Gram staining, spore staining, capsule staining and motility tests were performed and documented. Colony morphology included size, shape, colour, and margin characteristics observed under a stereomicroscope.

3.4.4.2 Biochemical tests

The highly efficient strains were biochemically characterised by conducting tests such as IMViC (Indole, Methyl Red, Voges-Proskauer, and Citrate), catalase, oxidase, urease, triple sugar iron (TSI), and nitrate reduction (Lennox & Ackerman, 1984). Starch hydrolysis and gelatin hydrolysis were performed to identify the bacterial strains. The results were tabulated.

3.4.4.3 Biochemical Profiling using the Hi-Carbo Kit of Himedia

This study employed the Himedia kit (KB009) for carbohydrate test kits to determine the biochemical profiles of the isolated bacterial species that exhibited firm attachment to LDPE films for biodegradation (Alqasim et al., 2021). The kit comprises 35 carbohydrate fermentation tests and one control, which is arranged in three parts: Part A, Part B, and Part C. Part A has provisions for 12 tests; Part B comprises another 12 tests, and Part C contains 11 tests and one control well to check the complete biochemical activities of the organism. To conduct the test, peel off each part of the kit, aseptically inoculate each well with 50 μ L of each competent strain separately, except in the control well, and incubate the kits at 37 °C for 18-24 hours. Recorded the results.

3.4.4.4 Culture Characteristics Studies in Different Selective Media

For studying cultural characteristics, we have used Blood agar medium, Starch casein agar medium (Himedia M801-500G), Starch agar medium (Himedia M107S-500G) and Carboxy cellulose agar medium (Kasana et al., 2008). In addition, the cultural peculiarities of bacterial species were examined in various media, including HiChrome Bacillus agar (M1651), HiChrome Pseudomonas agar medium (M085), and Himedia mannitol salt agar medium (M118) (Alippi & Abrahamovich, 2019).

3.4.4.5 16S rDNA sequencing

Spin columns (purchased from BOGAR Biobee Stores Pvt Ltd, Coimbatore) were used to isolate DNA of the appropriate concentration from each of the most potent bacterial strains isolated from plastic-mixed landfill soils. The DNA concentration was assessed before sequencing using a Qubit 3.0 Fluorometer (Thermo Fisher) (Jahangiri et al., 2022). Then, the DNA molecule was amplified by a pre-designed PCR using 27F/1492R universal primers. Two denaturation phases are involved in the PCR reaction: the first occurs at 95 °C for five minutes, and the second is carried out at the same temperature for one minute. This cycle is repeated 25 times. Next, precise primers are introduced and bound at 50°C for 1 minute. Subsequently, the initial elongation occurs at 72 °C for 2 minutes. Then, an ultimate prolongation was executed at 70 °C for 14 minutes (Asghari Ozma et al., 2024). Following the completion of PCR reactions, the PCR products were washed with a Sigma-Aldrich clean-up kit to remove unincorporated nucleotides (Wróbel et al., 2023).

Sequencing was then performed using an ABI sequencer. Afterwards, the 16S rDNA sequences were uploaded into the NCBI BLAST similarity search tool for a BLAST analysis (Stalder et al., 2013). Subsequently, the most appropriate homologous sequences were extracted. The MEGA 11 software was used to conduct sequence alignment using the MUSCLE algorithm, construct a phylogenetic tree with the Neighbour-joining tree construction method, and identify outgroups based on the method described by (Li et al., 2025; Tamura et al., 2021). The resulting data were subsequently uploaded to GenBank.

3.4.4.6 MALDI-TOF-Mass spectroscopy analysis for bacterial species identification

For this analysis, 1 mL of a 16-hour suspension from efficient strains for the biodegradation of LDPE strips was taken in a clean and sterilised centrifuge tube using a micropipette. Then, 12 mL of 70% formic acid and the contents were mixed well by vortexing for 2-5 minutes. The tubes were incubated at 25°C for 45 minutes to achieve a homogeneous bacterial culture suspension. One millilitre of bacterial culture from each centrifuge tube was placed in the wells of separate MSP 96 steel plates for the MALDI-TOF-MS Microflex instrument (Bruker Daltonics) and air-dried for 5-10 minutes. Then, 2 mL of matrix solution from the Bruker company was added to the wells of the target plates, and each plate was loaded separately into the instrument (Zhu et al., 2015). Then, it compares the protein mass dendrogram of each sample with the control spectra in the database, and the Biotyper software of the instrument generates valid species-

specific profiling scores between 1.7 and 3 for bacterial strain identification (Asare et al., 2023).

3.4.5 Initial growth kinetics of individual bacterial strains on LDPE films

Examining the growth kinetics of individual strains under stressful conditions is advantageous for identifying the strain's capacity to attach to the complex geometry of the polymer. Each isolated bacterial species was inoculated into carbon-free broth with LDPE films and incubated in a static culture at room temperature. The cell growth of each strain was observed at 24-hour intervals for up to 5 days. For this periodic sampling, the LDPE films were collected at 24-hour intervals into a saline solution and shaken in a rotary shaker at 180 rpm for 5 hours (Salum et al., 2008). The absorbance of the culture-mixed saline solutions was measured at 610 nm using a spectrophotometer. Controls included CFLMSM without LDPE films to measure baseline bacterial growth.

3.4.6 Efficacy studies to prove strong biofilm formation on LDPE films by bacterial species such as OBA5, OLA2, and OBM3 isolated and enriched using landfill soils.

To demonstrate the effectiveness of each bacterial species isolated from various long-term dumps, we calculated the overall increase in cell population, determined the number of viable cells, measured protein content, examined pH fluctuations in the medium, and analysed EPS (extracellular polymeric substance) content.

Bacterial proliferation on the surface of LDPE polymer was quantified by placing films in a 0.85% solution of NaCl and mixing them by vortexing for 4-5 hours at 180 revolutions per minute. The bacterial suspensions were then serially diluted up to a dilution factor of 10^{-7} . Subsequently, 0.1 mL of the samples was spread onto Himedia's Luria-Bertani Agar M1151F medium and cultured for 48 hours at 37°C. Then, we calculated the total cell growth on the polymer film using the experimental data (Joseph et al., 2001).

$$\text{Total biomass count (CFU / cm}^2\text{ = } \\ \text{Log} \left[\left(\frac{\text{Average CFU}}{\text{Sample volume for spreading}} \right) \times \text{Dilution factor} \times \left(\frac{\text{Volume of the original suspension}}{\text{Surface area of LDPE film}} \right) \right]$$

The count of living organisms on LDPE films incubated for one and four months was calculated and graphically represented to prove the bacterial strain's competency to grow in a stressful environment.

In the FDA experiment, we assessed the viability of bacterial strains on LDPE films that were inoculated in CFLMSM for one month and four months of incubation at room temperature. The bacterial strains from LDPE biofilms were collected and placed in an FDPB (Fluorescein diacetate phosphate buffer) solution. The LDPE films collected during the assay were taken on the 5th, 10th, 15th, 20th, 25th, and 30th days of incubation, and then every month for four months, on the 30th, 60th, 90th, and 120th days of incubation.

Apart from the biomass count and FDA analysis, the protein content analysis by the Bradford assay can estimate the bioformation on LDPE films (Bradford, 1976). The bio-collected samples were gathered on the 30th, 60th, 90th, and 120th days of incubation and placed in a saline

solution for this experiment. The solution was spun at 12000 rpm for 6 minutes. The supernatant was collected, and 1 mL was transferred to the cuvette, where it was mixed well with 3 mL of the Bradford reagent. Subsequently, the supernatants were examined using a spectrophotometer (Visible Spectrophotometer—Aczet AV-5, India) at 595 nm. Typically, BSA is used as a benchmark for this assay, and a calibration graph is employed to determine the protein concentration (Brady & Macnaughtan, 2015).

pH variations in the carbon-free broth were carefully examined every 15 days using a pH test (pHTestr 3, Sigma-Aldrich, USA) for up to 120 days to determine bio-attachment. The test and controls, with or without bacterial treatment, on LDPE films were maintained to compare the results. Just before commencing the experiment, we adjusted the pH of the synthetic medium to 7.2 ± 0.2 using a 0.1 M potassium phosphate buffer. The results were presented in a graph.

The analysis of extracellular polysaccharide (EPS) content is strong evidence for detecting biofilm formation on long-chain hydrocarbon surfaces (Jiao et al., 2024). For this analysis, the bacterial suspensions on the films were detached by centrifugation at 7500g for 20 minutes. The resulting supernatants were dialysed overnight using a dialysis membrane disc (Spectra S/P11, 6000-8000 MWCO, 33mm diameter). The samples were taken to quantify the extracellular polysaccharides according to the protocol (DuBois et al., 1956).

3.4.7 Quantitative Enzyme Assays

3.4.7.1 Preparation of crude enzyme extract

Fifty millilitres of CFLMSM containing polyethylene films were inoculated with bacterial cultures at the exponential stage and incubated in a static culture mode at room temperature for 120 days. Control conical flasks without bacterial-treated LDPE films were kept in the same condition as the test samples to confirm the results. The specimens were collected at a frequency of every 15 days and spun for 10 minutes at 10000g. The supernatants were collected and subjected to enzyme assays.

3.4.7.1.1 Estimation of Laccase enzyme

The chemical component 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is essential for quantifying the presence of the laccase enzyme in biofilms that develop on the surface of polymers (Sheikhi et al., 2012). The experiment consisted of a 4 mL mixture containing 2 mL of buffer-sodium acetate (0.15 mM, pH 4.6), 1 mL of 0.2 mM L⁻¹ ABTS stock, and 1 mL of supernatant. The blank solution was maintained with all chemical reagents and a control conical flask containing a liquid suspension. All the solutions were incubated for 5 minutes using a controller-based UV-VISIBLE spectrophotometer (Systronics, model 117), and the absorbance of all samples (control and test) was analysed at 420 nm. The activity of a single enzyme unit is evaluated by quantifying the enzyme's capacity to oxidise one micromole of ABTS within one minute.

$$\text{Laccase enzyme activity (U / mL)} = \left(\frac{\Delta 420 \times \text{total volume of assay mixture} \times 10^6}{\epsilon_{\text{ABTS}} \times \text{sample volume}} \right)$$

$$\text{Rate of Absorbance, } \Delta 420 = \left(\frac{\text{Final absorbance after incubation} - \text{initial absorbance}}{\text{Difference of time for measuring final and Initial absorbance (min)}} \right)$$

$$\epsilon_{\text{ABTS}}_{420} = 360 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$$

3.4.7.1.2 Lipase Assay

UV-VISIBLE Spectrophotometry was used to monitor lipase activity in bacterial strains (pNPP) by measuring p-nitrophenyl palmitate (pNPP) (Pencreac'h & Baratti, 1996), which was conducted to detect enzyme production in the biofilms of LDPE films. The reaction solution comprises 80 μL of pNPP (0.8 mM) and 1.3 mL phosphate (potassium) buffer (55 mM) at pH 8.3, along with 120 μL of enzyme supernatant for the positive sample or 120 μL of mineral salt broth for the control sample. The mixture for the reaction was initially heated at 40°C for 10 minutes, and further the reaction was elevated to 45°C for 15-20 minutes. Then, the reaction was terminated by adding 1 mL of ethanol. A control experiment was conducted in parallel by combining all the reaction mixture components except the enzyme supernatant in the reaction mixture. The insoluble compounds were separated by spinning the reaction solution at 8,000 g for 5 minutes. The quantity of p-nitrophenol (NP) released, using its absorbance at 410 nm (Khan et al., 2023).

One unit of enzyme activity (U/mL) = 1 μmol release of p-NPP/minute (Zhao et al., 2021).

$$\text{Lipase enzyme activity (U / mL)} = \left(\frac{\Delta 410 \times \text{total volume assay mixture} \times 10^6}{\epsilon \text{ pNPP} \times \text{sample volume}} \right)$$

$$\text{Rate of absorbance, } \Delta 410 = \left(\frac{\text{Final absorbance after incubation} - \text{initial absorbance}}{\text{Difference of time for measuring final and initial absorbance (min)}} \right)$$
$$\epsilon \text{pNPP} = 18000 \text{ M}^{-1} \text{cm}^{-1}$$

3.4.7.1.3 Protease Assay

Protease enzyme activity was estimated by following the Sigma Aldrich protocol (Cupp-Enyard, 2008). For this assay, 2 mL of bacterial culture supernatants were spun at 4500g for 10 minutes and 1 mL of the culture supernatants of bacterial suspensions were mixed with 5 mL of the reaction mixture, which comprises 0.65% casein powder (6.5mg/mL) in a phosphate buffer (potassium) (50 mM) at pH 7.5 (Singh & Jha, 2015). Then, the mixture was homogenised by slow stirring and heated to 37°C for 10 minutes (temperature and stirring maintained using a shaking digital water bath). Cautions were taken to avoid boiling throughout the preparation, and the pH was corrected with HCl or NaOH. After incubation, the reactions in the mixture were stopped by adding 5 mL of 110 mM Trichloroacetic acid reagent. Blank and test solutions were prepared and incubated at 25°C for 45 minutes. The solution was vigorously centrifuged at 5000 revolutions per minute for 8 minutes to eliminate insoluble substrates. The supernatant of both blank and test (2 mL) was added to a Na₂CO₃ solution (5.5 mL, 500 mM) and phenol reagent-Folin Ciocalteu (0.5 mL), used for the estimation of protease enzyme in bacterial strains attached to LDPE film. The mixture was incubated for another 45 minutes in the dark until a blue hue appeared in the solution. The absorbance values were evaluated at 660 nm compared to a blank

solution. The tyrosine standard curve was built, and from its slope equation, μ mole of tyrosine equivalent release of test and blank were calculated, and further, the enzyme activity (U/mL) was computed as per the equation below,

$$\text{Protease enzyme activity (U/ mL)} = \left[\frac{\mu \text{ mole tyrosine equivalent release} \times \text{Total volume of assay}}{\text{Time of assay} \times \text{Volume of enzyme} \times \text{Volume used in colorimetric determination}} \right]$$

3.4.7.1.4 Estimation of Lignin Peroxidase Enzyme

Lignin peroxidase (LiP) activity was quantified using the following method (Fujii et al., 2013) with the essential compound Azure B. The sample solution, with a total volume of 2 mL (0.5 mL of each component), comprises sodium tartrate buffer at pH 4.2 (50 mM) along with azure B (35 μ M), H₂O₂ (100 mM), and culture supernatant. Control assay solutions were retained by adding 0.5 mL of synthetic broth instead of culture supernatant. The reaction commenced with the addition of 0.5 mL of hydrogen peroxide (H₂O₂). In the assay, Azure B's oxidation rate was quantified at a wavelength of 651 nm (Russell et al., 2011).

$$\text{Lignin peroxidase enzyme activity (U / mL)} = \left(\frac{\Delta 651 \times \text{total volume of assay mixture} \times 10^6}{\epsilon \text{ azure B} \times \text{sample volume}} \right)$$

$$\text{Rate of absorbance, } \Delta 651 = \left(\frac{\text{Final absorbance after incubation} - \text{initial absorbance}}{\text{Difference of time for measuring final and initial absorbance (min)}} \right)$$

$$\epsilon \text{ azure B} = 48,800 \text{ M}^{-1} \text{ cm}^{-1}$$

3.4.7.1.5 Manganese peroxidase assay

The presence of the enzyme manganese peroxidase was measured using guaiacol (2-methoxyphenol) via its oxidation to form a brown-coloured product (de Oliveira et al., 2009). The formation of coloured products can be determined at 465 nm using a UV-VIS spectrophotometer. The reaction solution consists of 0.4 mL of 0.1 M sodium lactate buffer, pH 4.5 (prepared by dissolving 11.2 g of sodium lactate powder in a 0.1 M lactic acid solution of 1000 mL), 0.1 mL of 1 mM guaiacol, 0.1 mL of 1 mM MnSO₄, and 0.4 mL of culture supernatant for the test sample. The reaction mixture of the control sample is maintained without the addition of enzyme supernatant—the reactions in both control groups and the initiated group were commenced by adding 0.1 mM H₂O₂ (30 µL). After five minutes of incubation, enzyme activity was measured at 465 nm using a UV-visible spectrophotometer (Naidu et al., 2017). At a temperature of 30°C, a single micromole of brown product produced per minute by an enzyme equals one unit of enzyme activity.

3.4.7.1.6 Esterase enzyme assay

This enzyme test utilised a slightly modified Sigma Aldrich protocol for the esterase assay (K. K. Bhardwaj et al., 2020). The reaction mixture (3 mL) comprises 2.87 mL of phosphate (potassium) buffer (55 mM), pH 8.0 at 25°C, 0.03 mL of O-nitrophenyl (butyrate), and 0.1 mL of enzyme solution (positive sample) or CFLMSM (control). Next, the reagents were incubated for five minutes each, and spectroscopic readings were taken at 420 nm.

$$\text{Esterase enzyme activity (U / mL)} = \left(\frac{\Delta 420 \times \text{total volume of assay mixture} \times 10^6}{\text{Millimolar extinction co-efficient of O-nitrophenol} \times \text{Sample volume}} \right)$$

$$\text{Rate of Absorbance, } \Delta 420 = \frac{(\text{Final absorbance after incubation} - \text{initial absorbance})}{(\text{Difference of time for measuring final and Initial absorbance (min)})}$$

$$\text{Millimolar extinction co-efficient of O-nitrophenol} = 4.6 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$$

3.4.7.2 Qualitative analysis of extracellular enzymes in various production media

Various production media were used to screen extracellular enzyme-producing strains in the biofilms on the LDPE surface. The laccase-producing strains were screened using a nutrient agar medium augmented with 0.5 mM guaiacol (Kameshwar & Qin, 2017). For lipases/esterases, tributyrin agar (Noormohamadi et al., 2013) and for proteases, skim milk agar (Shaikh et al., 2023) were utilised, in addition to nutrient-rich growth media supplemented with azure B, which were used to identify lignin and manganese peroxidase-producing strains (Rath et al., 2022).

3.4.8 Screening of bio-surfactants

Following a 15-day incubation period, the biofilms formed on LDPE films were collected and subjected to agitation in a saline solution for 4 hours at 180 revolutions per minute. The saline solutions with biofilms were centrifuged at 7500g for 25 minutes at 4°C (Deng et al., 2020), and the supernatants were collected in fresh Eppendorf tubes. The supernatants were subjected to bio-surfactant analysis using oil displacement tests and emulsification assays (Kiran et al., 2010).

3.4.8.1 Oil displacement analysis

This strategy detects the presence of microbial biosurfactants, which can alter the contact angle between oil and water. This simple and basic assay was devised by (Morikawa et al., 1993). In this test, to detect the surface pressure caused by biosurfactants, 10 μ L of each culture supernatant was added to the surface of 100 μ L of petrol placed over 25 mL of water. Within 30 seconds, oil displacement activity was observed due to the presence of biosurfactants. Control plates were maintained without adding culture supernatant to the crude oil film to validate the results.

3.4.8.2 Emulsification assays

The emulsification capacity of culture supernatants was assessed using a modified method (Cooper & Goldenberg, 1987). To measure this capacity, 2 mL of culture supernatant was mixed with 2 mL of crude oils, such as kerosene, petrol, and diesel, and swirled for 6 minutes. Checked its stable emulsification after 24 hours. Then, the emulsion rate percentage was calculated (Ibrahim et al., 2013) as follows,

$$\text{Emulsification rate in \%} = \left[\frac{\text{Height of emulsification layer}}{\text{Total height of aqueous layer}} \right] \times 100$$

3.4.8.3 Isolation and Characterisation of bio-surfactants

An acid and solvent extraction procedure was followed (Bijesh et al., 2015) to isolate the biosurfactant produced by the strain, with minor modifications. Bio-surfactant-containing bacterial strains were extracted from the mineral salt medium by centrifugation (15,000 rpm for 22 minutes at 4 °C). Then, each supernatant was collected and

mixed with 6 N HCl. Add HCl until the solution pH turns 2.0 and allow the extract to incubate in the refrigerator for 18 h to get a cloudy solution. The residues were extracted from the solution by centrifugation at 12000 rpm for 30 minutes at 4°C. They were extracted with equal volumes of chloroform-methanol solutions (2:1) and kept in a rotary shaker (Rotek shaker-RRS-04) at 250 rpm for six hours. The extracts were neutralised by adding 0.6 mL of ethanol to prevent the formation of methyl esters (Mandal et al., 2013). The biosurfactants from this solution were collected after pooling all the organic phases in each tube under vacuum, and the crude extracts were used for characterisation by FTIR analysis (SHIMADZU-IR Tracer-100).

3.4.9 Statistical analysis

All initial and conclusive investigations were conducted in triplicate ($n = 3$) to ensure reproducibility. All statistical computations were carried out in RStudio Cloud (v4.2.3), governed by the GNU Affero GPL v3 license. Results are reported as Mean $\pm$ SEM. Intergroup comparisons were analysed using Student's t-test in GraphPad (freely available software), with a threshold for significance set at $p < 0.05$ (95% confidence interval). Graphical representations of the data were generated using OriginPro 2018 (cloud-based).

3.5. Result

Study of the efficacies of bacterial strains screened from plastic landfills for the formation of resilient biofilms on LDPE films

3.5.1 Analysis of the physical characteristics of soil samples collected from Landfills

Soil samples (10 g) were taken in triplicate from three plastic landfills located in different areas of Kerala (Fig. 3.1), where LDPE films were collected in a pile after removing biological waste. The physical conditions of the soil samples, such as pH and temperature, were measured and used to formulate the media (Values depicted in Table 3.1). Our investigation utilised soil samples containing partially decomposed plastic fragments for bacterial isolation and subsequent studies.



Fig.3.1 Long-run plastic landfills selected for soil sample collection

S. No	Plastic Landfills	Physical parameters (Mean $\pm$ SEM) of the soil sample	
		pH	Temperature
1	Solid waste management unit of Alathur Thaluk at Vanoor, Palakkad.	7.29 $\pm$ 0.02	37.3 $\pm$ 0.841
2	Plastic waste unit of Thrissur Co-operation at Laloore, Aranattukara, Thrissur.	7.5 $\pm$ 0.06	38.033 $\pm$ 1.14
3	Solid waste management unit of Kochi Co-operation at Kakkanad, Kochi.	7.67 $\pm$ 0.064	38.77 $\pm$ 1.23

Table 3.1. Physical parameters of soil samples collected from various landfills associated with open plastic garbage landfills.

3.5.1.1 Inspection of physical parameters of LDPE films and their surface sterilisation

Before enrichment analysis, the physical characterisation (Table 3.2) of the purchased LDPE films was conducted. These LDPE films were sliced into pieces and treated with SDS and ethanol for the rest of the studies.

Material	Physical parameters	Result
LDPE Films	Thickness (in mm)	.0556 $\pm$ 0.002
	Density(g/cm ³)	0.85 $\pm$ 0.145
	Specific gravity(g/cm ³)	0.92 $\pm$ 0.115

Table 3.2 Physical Parameters of LDPE films collected from Surya Polymers and Plastic Industry in Pudukkottai Central, Kanjicod, Palakkad.

In addition to the physical characteristics of LDPE film, we conducted a thermal stability test using TGA-DSC analysis. From the result, we found that the maximum mass reduction occurs at 350 °C, and decomposition ends above 500 °C. According to (Chamas et al., 2020), the decomposition of a pure LDPE film starts at 390 °C and ends at 490 °C. It proves that the material we used for the study was LDPE. Still, the slight temperature change observed during decomposition in this analysis may be attributed to additives in the LDPE film (Zhu et al., 2017).

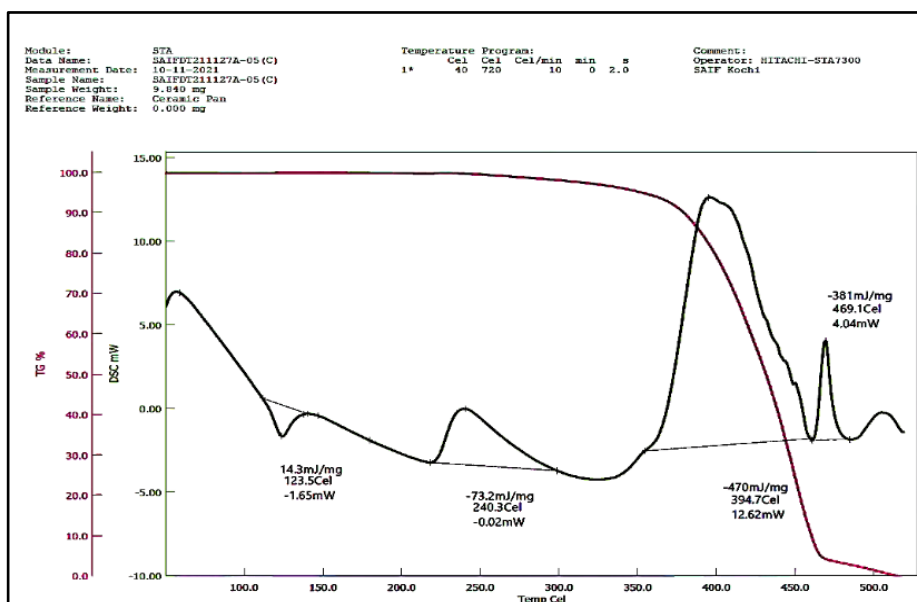
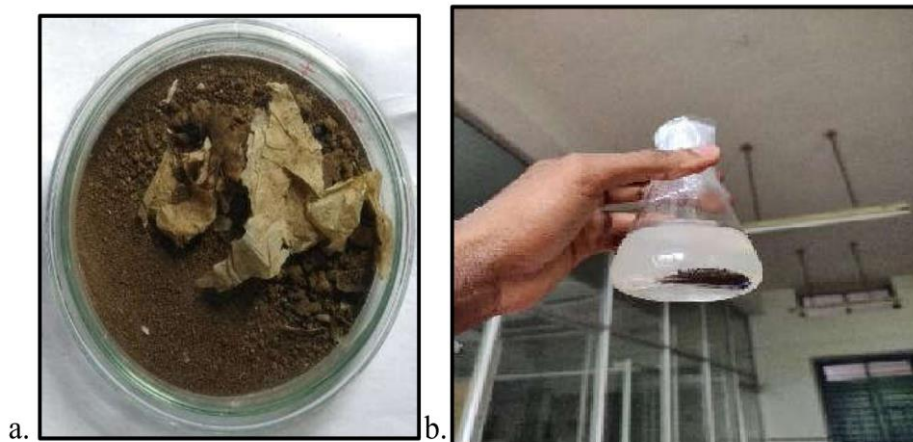


Fig.3.2 Thermo Gravimetric –Differential Thermal Analysis (TGA-DTA) to characterise the thermal property of the LDPE film collected.

3.5.2 Enrichment of bacterial species using soil samples collected from plastic garbage sites

During the initial enrichment period, all soil samples mixed with plastic pieces were collected from landfills and amended in CFLMSM

(Fig.3.3). Subsequently, the broth cultures were plated on R2A medium to determine the biomass count in CFU/g (See the results in Table 3.3). The biomass count results showed that plastic-mixed soil samples contained many potential strains, and their number was significantly increased by the enrichment of the medium's pressurised condition. Almost all the colonies isolated from the Vanoor dump were colourless and medium to large-sized compared to the other two garbage sites. However, we enumerated pigment-producing colonies, specifically those from large-sized colonies, in the Laloor and Bhramapuram dumps. Afterwards, the potential strains in each spread plate were screened using the Coomassie brilliant blue (CBB) staining and destaining method (see Fig. 3.4). The de-stained or transparent zone-formed colonies from each spot on the plastic plate were streaked repeatedly. The potential bacterial strains were purified (as shown in Fig. 3.4) and stored for further study.



c.



Fig.3.3 Enrichment of collected soil sample from a landfill. a. Soil sample collected from the plastic dump. b. soil sample mixed CFLMSM c. One month of enriched soil samples along with a control conical flask.

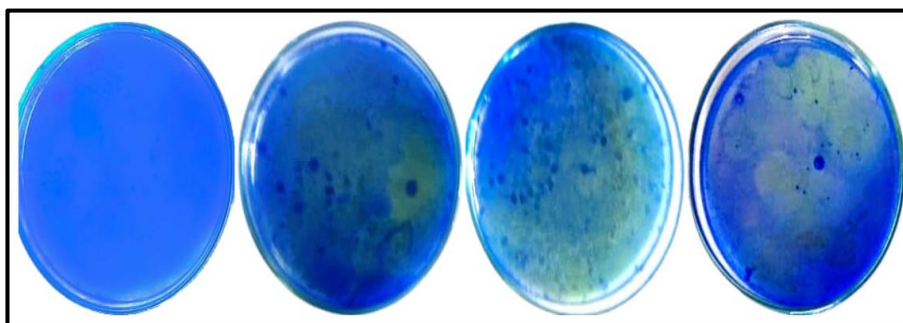


Fig.3.4 A few plates showing CBB staining and de-staining on R₂A media spread with soil samples and incubated for 20 minutes. A de-stained or zone-formed culture is used for further purification by repeated streaking. The results were compared with those of a control plate that did not contain soil samples.

Name of the Open Garbage Heap	Soil Sample Spot Code	Biomass (CFU/g on R2A Medium, Mean $\pm$ SEM)	Colonies with Destained Zones (CFU/g, Mean $\pm$ SEM)	Bacterial Colony Codes with Destaining Effect
Vanoor	VLS1	$3.27 \times 10^9 \pm 0.12$	$8 \times 10^6 \pm 0.176$	OBA5
	VLS2	$2.4 \times 10^9 \pm 0.09$	$5.9 \times 10^6 \pm 0.173$	OBA4
	VLS3	$4.83 \times 10^9 \pm 0.41$	$16.8 \times 10^6 \pm 0.208$	OBA3, OBA2 and OBA1
Laloor	LRS1	$3.1 \times 10^9 \pm 0.173$	$8.3 \times 10^5 \pm 1.453$	OLA1, OLA3
	LRS2	$3.4 \times 10^9 \pm 0.12$	$5.67 \times 10^5 \pm 0.88$	OLA2
	LRS3	$2.6 \times 10^9 \pm 0.145$	$3.33 \times 10^5 \pm 0.88$	OLA4, OLA5
Bhramapuram	BMS1	$3.2 \times 10^9 \pm 0.145$	$10 \times 10^4 \pm 1.16$	OBM3 , OBM1
	BMS2	$2.97 \times 10^9 \pm 0.18$	$6 \times 10^4 \pm 1.53$	OBM2, OBM4
	BMS3	$2.37 \times 10^9 \pm 0.145$	$3 \times 10^4 \pm 0.58$	OBM5

Table 3.3 Results of initial enrichment of capable bacterial strains from soil samples collected from various landfills to form effective biofilms on LDPE surfaces.

3.5.2.3 Purified strains after CBB staining



Fig. 3.5(a) Bacterial species were purified from soil samples collected from the Vanoor landfill, located at the solid waste processing unit in Alathur, Palakkad.



Fig. 3.5(b) Bacterial strains purified from soil samples from the Laloor landfill located at Thrissur corporation's plastic waste processing unit.



Fig.3.5(c) Bacterial species purified from soil samples from the Bhramapuram garbage site at the plastic waste management unit of Kochin Corporation.

3.5.3 Preliminary identification of the most potent bacterial strain for degradation from each soil sample after enrichment

3.5.3.1 Weight reduction of LDPE strips after four months of incubation

In this initial analysis, all the strains were incubated for 120 days in carbon-devoid mineral salt broth at room temperature. After the static culture incubation span, almost all the purified bacterial strains showed the ability to attach to the polymer. They contributed to weight

reductions (Results shown in Table 3.4(a), 3.4(b) and 3.4(c). Among the bacterial strains, the OBA5 isolated from the Vanoor dump has a maximum weight reduction percentage of 42.73 ± 1.105 . Following the OBA5 strain, OBM3, a bacterial species isolated from the Bhramapuram garbage heap, has exhibited $40.22 \pm 2.04\%$ weight depletion, and OLA2, isolated from the Laloer plastic pile, displayed $39.7 \pm 1.27\%$ weight depletion.

In addition to the weight reduction test, daily intake rates (K-rate) and half-life durations ($t_{1/2}$) of LDPE films from bacterial degradation were calculated. Significant results were observed with all OBA5, OBM3, and OLA2 strains in terms of both LDPE consumption rates and half-life reduction, demonstrating their effective degradation of non-pretreated LDPE films in a highly stressful environment.

3.5.3.1 Result of Weight Loss Experiments of LDPE films treated with purified bacterial strains isolated from various landfills in Kerala

S. No	Name of the Bacterial Species	Weight Drop Percentage (W%)	Daily Consumption Rate (g/day, K rate)	Half-Life (days, $t_{1/2}$)
1	CONTROL	1.63 ± 0.376	0.00014 ± 0.00003	5583.391 ± 1408.061
2	OBA1	20.67 ± 0.667	0.00174 ± 0.0002	412 ± 47
3	OBA2	14.996 ± 0.77	0.00136 ± 0.00008	514.7465 ± 49.8568
4	OBA3	11.65 ± 0.952	0.00103 ± 0.00091	680.4042 ± 60.3073
5	OBA4	8.09 ± 0.992	0.00086 ± 0.00011	834.2483 ± 109.7771
6	OBA5	42.73 ± 1.105	0.00452 ± 0.00029	154.803 ± 10.66253

Table 3.4(a) Weight loss of polyethylene films inoculated with bacterial suspensions isolated from the Vanoor Landfill.

S. No	Name of the Bacterial Species	Weight Drop Percentage (W%)	Daily Consumption Rate (g/day, K rate)	Half-Life (days, $t_{1/2}$)
1	CONTROL	1.32±0.194	0.00013±0.00003	6501.381±958.07
2	OLA1	26.1±2.01	0.001±0.0017	278.46±23.66
3	OLA2	39.7±1.27	0.00423±0.0002	163.82±7.25
4	OLA3	19.7±1.55	0.0016±0.0014	439.84±38.56
5	OLA4	15.8±2.04	0.0014±0.0002	516.93±42.3
6	OLA5	9.53±1.58	0.0026±0.00157	858.77±183.54

Table 3.4(b) Weight reduction of LDPE strips by adding isolated bacterial species from Laloor landfill.

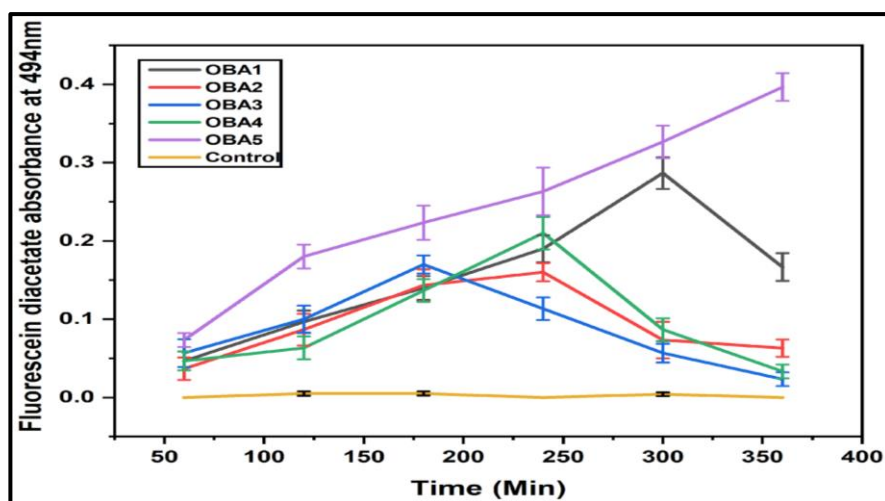
S. No	Name of the Bacterial Species	Weight Drop Percentage (W%)	Daily Consumption Rate (g/day, K rate)	Half-Life (days, $t_{1/2}$)
1	CONTROL	1.22±0.4897	0.0001±0.00003	8389±2386.042
2	OBM1	21.33±0.96	0.002±0.0001	350.31±17.82
3	OBM2	31.22±0.97	0.0031±0.0002	221.99±8.968
4	OBM3	40.22±2.04	0.0043±0.0002	162.0133±6.29
5	OBM4	29.3±1.15	0.0029±0.0001	240.45±11.34
6	OBM5	20.77±1.44	0.0019±0.0002	361.01±28.2

Table 3.4(c) Weight loss of LDPE strips treated with isolated bacterial colonies from the Bhramapuram landfill soil samples in CFLMSM.

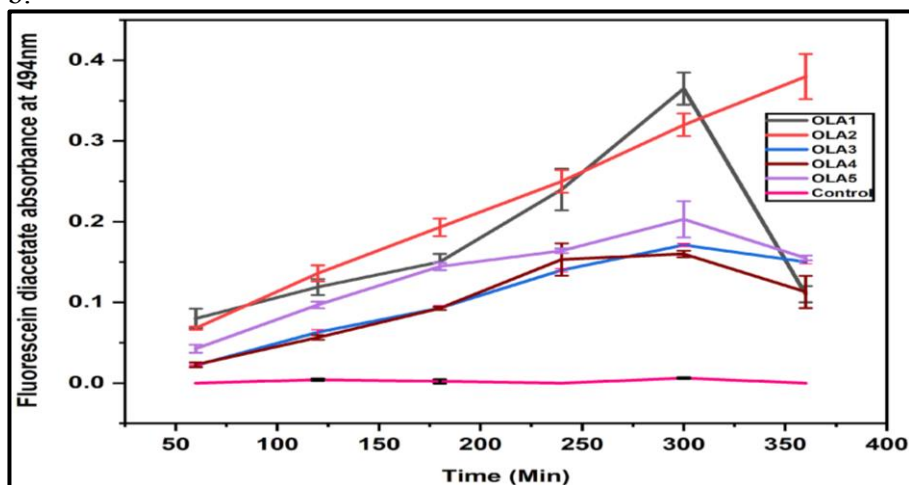
3.5.3.2 FDA Analysis

This test quantifies the prevalence of viable microbial strains that establish strong biofilm on the surface of the polymer((Orr) et al., 2004). The present study observed a progressive enhancement in the conversion of FDA (3'6' diacetyl fluorescein (FDA) within six hours on the 30th day of incubation in a carbon-devoid mineral salt medium. Hydrolysis of the FDA enables us to determine microbial metabolic activities (Schnürer & Rosswall, 1982). The coloured product fluorescein can be quantified using a spectrophotometer at 494 nm and represented as an origin graph (Tribedi & Sil, 2013).

a.



b.



c.

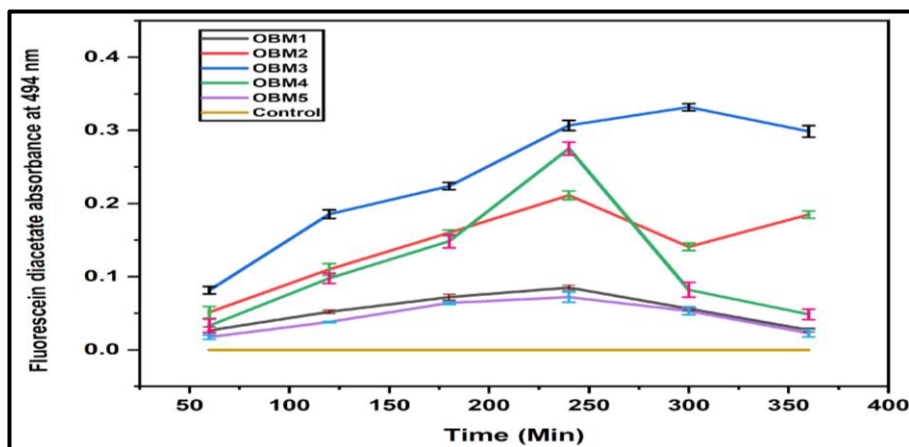


Fig.3.6 The outcomes of FDA assays for potential landfill-derived strains were evaluated against controls without adding corresponding bacterial isolates. a. Vanoor plastic dump b. Laloor dump c. Bhramapuram dump. After 30 days of incubation, during the initial six-hour period, the most potent bacterial species, such as OBA5, OLA2, and OBM5, exhibited rising absorbance.

3.5.3.3 BATH tests

BATH tests were conducted to assess the cell surface hydrophobicity (CSH) of purified strains from the soil samples of landfills. In the

current investigation, the pellets of purified strains from landfill soil samples displayed solid adhesion to the organic phase, consisting of increasing concentrations of hexadecane (0.05 mL, 0.1 mL, 0.15 mL, and 0.2 mL). Among the bacterial species we screened from various accumulation spots, the strains OBA1 and OBA5 of Vanoor, OLA1 and OLA2 of Laloore, and OBM1, OBM2, and OBM3 of Bhramapuram garbage sites exhibited a high percentage of turbidity reduction. Among all these inocula, the strain OBA5 showed the highest rate of turbidity reduction (around 50%), followed by OBM3 (47.23%) and OLA2 (45.35%) (all results depicted in Fig. 3.7(a), 3.7(b), and 3.7(c). Several recent researchers reported an increase in the turbidity reduction percentage in the aqueous phase and a rise in the hydrophobicity of cultures.

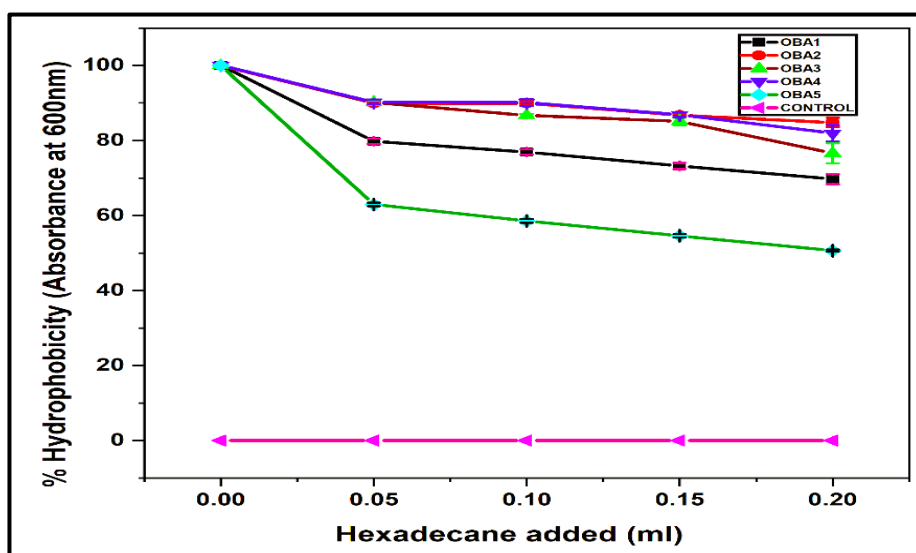


Fig.3.7(a) Result of BATH analysis of bacterial species isolated from Vanoor open plastic dump and compared with the results of the control setup without adding bacterial species. The strain OBA5 (green colour graph line) showed a maximum reduction in hydrophobicity in the aqueous phase of $49.372 \pm 0.27043\%$ after adding 0.2 mL of hexadecane.

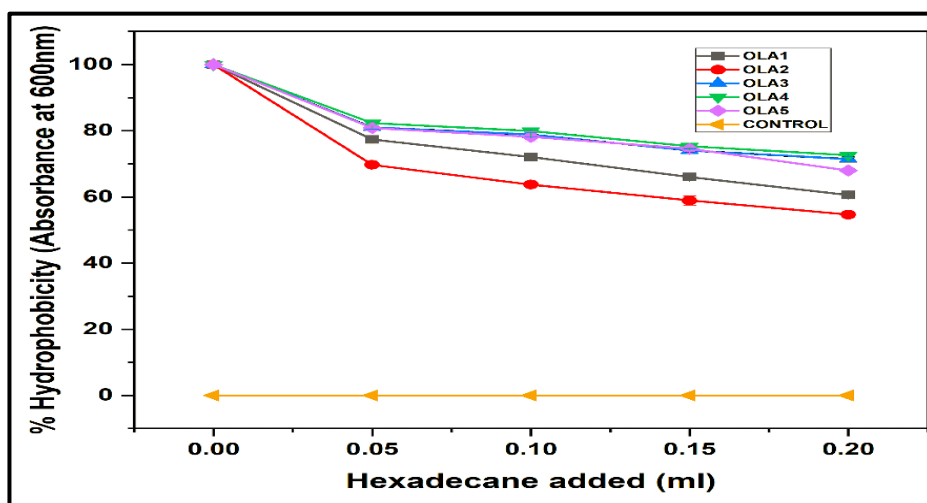


Fig.3.7(b) Result of BATH analysis of bacterial species isolated from Laloor plastic waste pile. The OLA2 strain (red graph line) exhibited an enhancement in turbidity reduction and a reduction in hydrophobicity in the aqueous phase, attributed to the migration of bacterial strains to the organic phase ($45.35 \pm 0.85\%$) in the aqueous phase of the BATH solution. The test is validated using a control setup without adding bacterial strains.

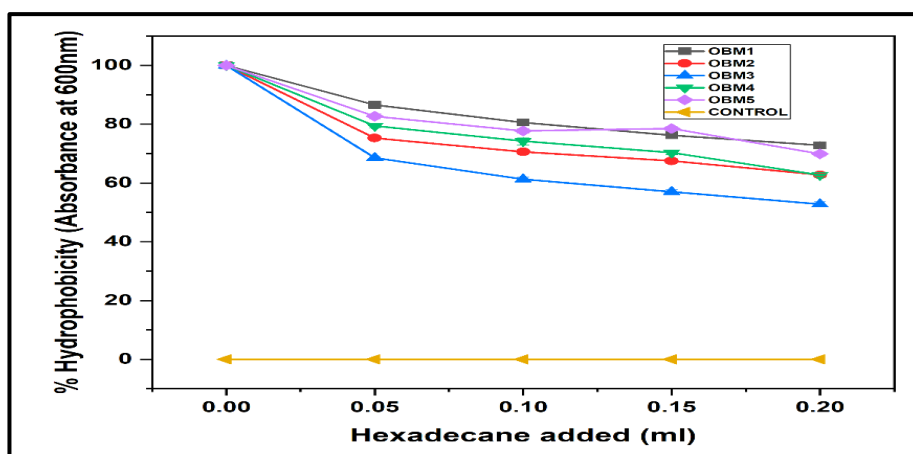
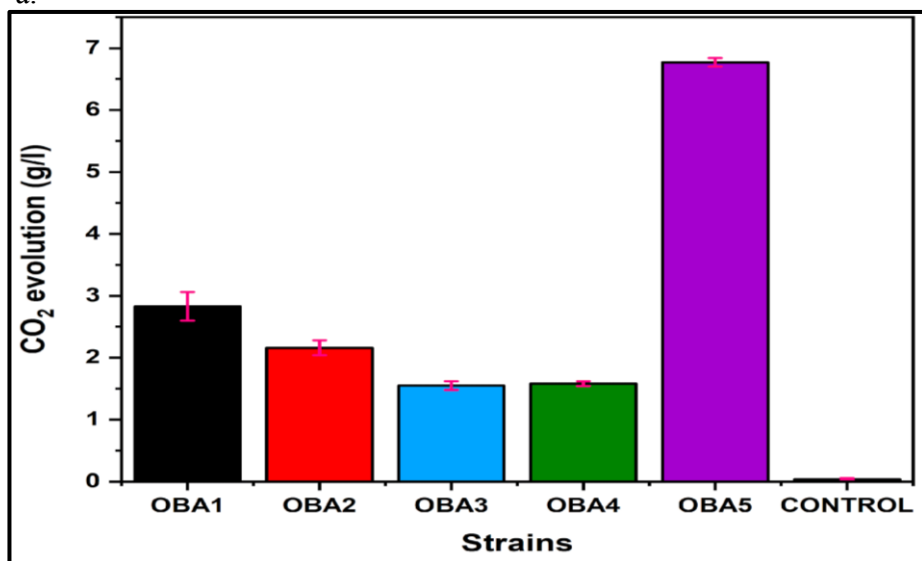


Fig.3.7(c) The BATH assay demonstrated the Hydrophobic interaction of microbial strains isolated from the soil samples of Bhramapuram landfill spots. In this analysis, the strain OBM3 showed elevated hydrophobic interaction (47.23%) with the organic solvent hexadecane. The results were compared to a baseline control without any bacterial introduction.

3.5.3.4 CO₂ estimation test (Gravimetric analysis of Sturm test)

This test indirectly confirms the capabilities of isolated strains to degrade LDPE films inoculated in a carbon-devoid broth for one month. This experiment was conducted in a self-assembled setup (see Fig.3.8(1)). Even though all the strains isolated from open plastic dumps showed CO₂ production, the strains OBA5 and OBA1 of Vanoor dump, OLA2, OLA1, and OLA5 of Laloor, and OBM3, OBM2, and OBM4 exhibited a high amount of CO₂ during 30 days of incubation (Rate of CO₂ production in g/L displayed as a graph in fig.3.8(2)). Among all the setups for the test, the bacterial species with the OBA5 added unit showed the highest CO₂ production rate (6.77 ± 0.07 g/L), followed by the OBM3 unit (6.3 ± 0.06 g/L) and the OLA2 unit (5.62 ± 0.26 g/L).

a.



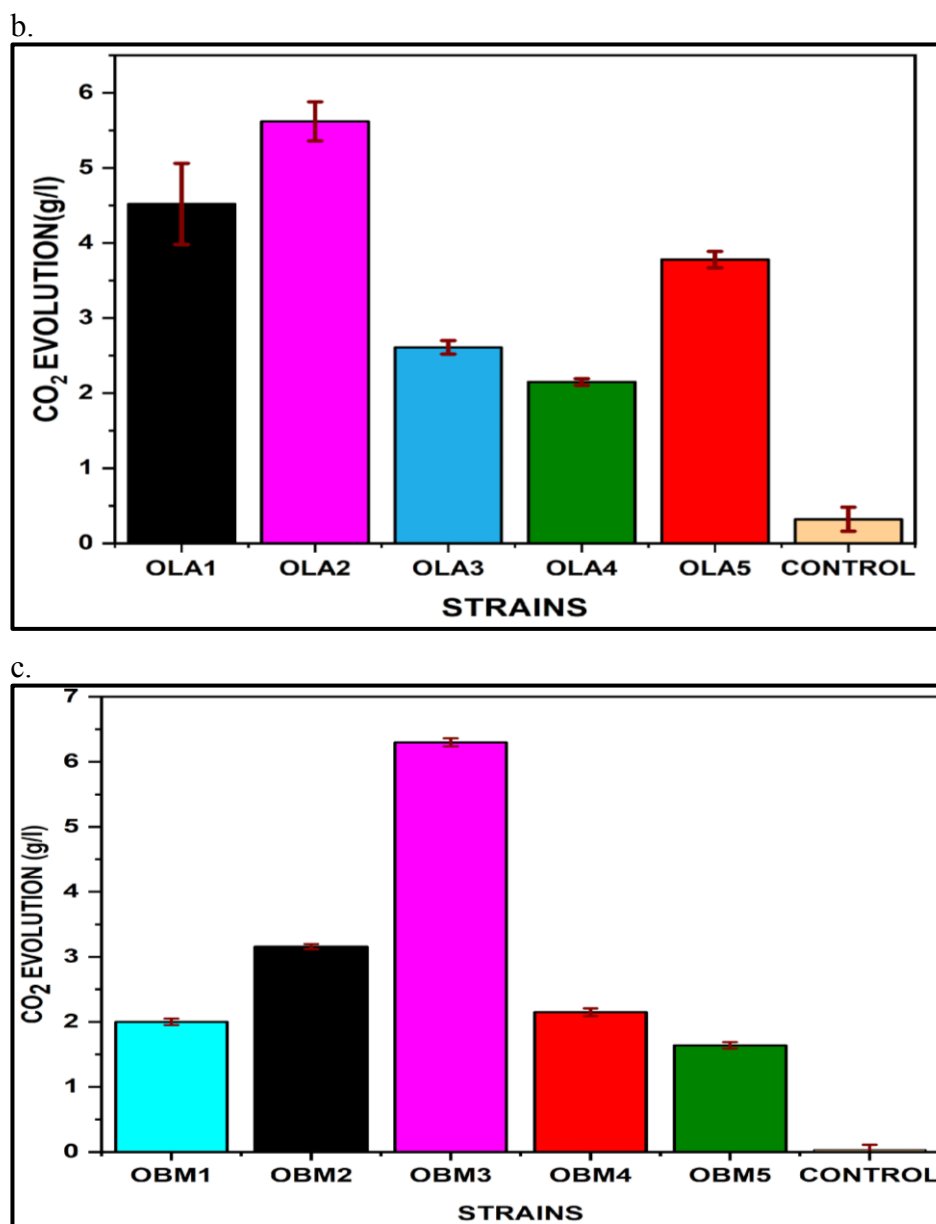


Fig.3.8 Results of CO₂ evolution tests of potential bacterial strains. a. Vanoor dump, b. Laloor dump c. Bhramapuram trash. All results were validated using an experimental setup that excluded the addition of bacterial strains to LDPE films.

3.5.4 Identification of the most potential bacterial strains OBA5, OLA2 and OBM3 from various landfills of Kerala for LDPE biodegradation

Upon initial investigations, we observed that the bacterial strain OBA5 from the Alathur trash, OLA2 from the Laloor plastic dump, and OBM3 from the Bhramapuram plastic dump exhibited the most significant and consistent affinity for LDPE films. We purified these organisms and used them to identify the strains.

3.5.4.1 Results of Morphological and Microscopic Analysis

Following Bergey's manual systematics for bacteriological identification, we recorded morphological staining analysis and displayed it in Table 3.5.

S. No	Name of the analysis	Features/Test	The result of bacterial strains		
			OBA5	OLA2	OBM3
1	Morphological Analysis in Basal Media	Colony Size and Shape	Significant, Round, slightly convex edge	Medium, mucoid, slightly convex edge, irregular spreading-edged	Medium, circular, convex/entire margin
		Colour of colony	Off White	Blue-green pigmented	Bright yellow pigmented
2	Staining	Gram Staining	G+ve Rod-shaped	G-ve, Rod-shaped	G +ve, Cocci-shaped
		Spore Staining	Positive, Ellipsoidal endospore	Negative	Negative
		Capsule	Negative	Positive	Positive
3	Motility	Wet mount	Positive	Positive	Negative
		Sulphide Indole Motility Medium	Positive (Spread in stab line)	Positive	Negative

Table 3.5 Morphological and staining features of capable strains such as OBA5, OLA2, and OBM3 isolated from various landfills

3.5.4.2 Biochemical tests of potential strains

We have conducted a series of biochemical tests, which are summarised (Table 3.6).

S. No	Name of the biochemical test	Biochemical test results of potential bacterial strains		
		OBA5	OLA2	OBM3
1	Indole	-	-	-
2	MR	-	-	-
3	VP	+	-	+
4	Citrate	+	+	-
5	Catalase	+	+	+
6	Oxidase	+	+	+
7	H ₂ S production	-	-	-
8	Urease	+	-	+
9	Gelatin hydrolysis	+	+	+
10	Starch hydrolysis	+	+	+
11	Casein hydrolysis	+	+	+
12	Hemolysis	Alpha	Beta	Non- haemolytic
13	Growth at 45°C	+	+	+
14	Carbohydrate fermentation			
	1. Glucose	+	+	+
	2. Fructose	+	-	+
	3. Sucrose	+	+	+
15	Nitrate reduction test	-	+	+
16	TSI test	K/A	K/K	A/K
17	Coagulase test	-	-	-
18	Esculin hydrolysis	+	+	+

Table 3.6 Result of biochemical test of competent bacterial species (OBA5, OLA2, and OBM3) isolated from the long-run plastic heap (- means negative and + means Positive).

3.5.4.3 Biochemical tests using Hi Carbo Kit of Himedia (KB009 kit)

Thirty-five carbohydrate tests were conducted for OBA5, OLA2, and OBM3 to assess adherence to LDPE films, and a control test was performed to confirm the bacterial species further.

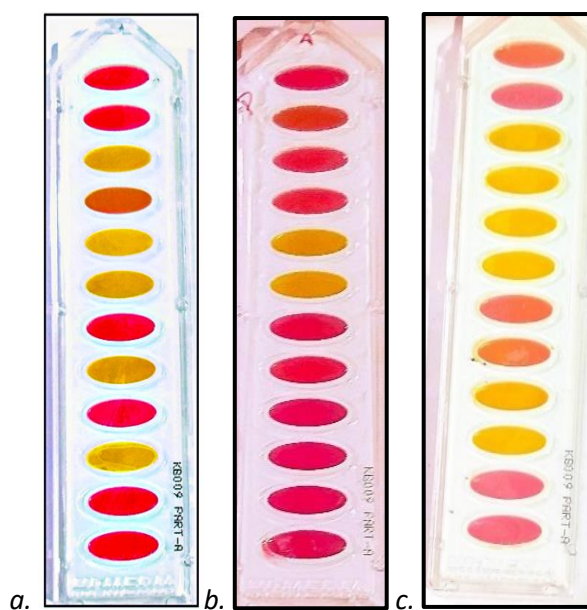


Fig.3.9(a) Result of biochemical tests- KB009-Part A of isolated strains from various landfills. a. OBA5 isolated from Vanoor dump b. OLA2 from Laloor dump c. OBM3 of the Bhramapuram dump sites.

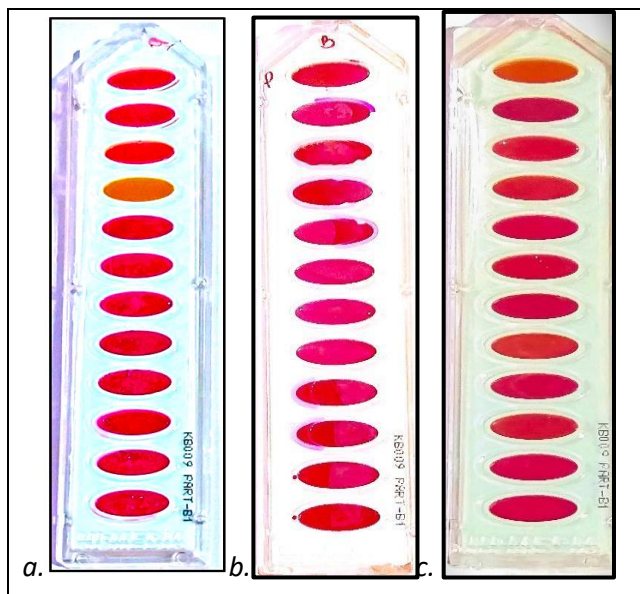


Fig.3.9(b) Result of biochemical tests- KB009-Part B of 12 carbohydrates. a. OBA5 from Vanoor dump b. OLA2 from Laloor dump c. OBM3 from the Bhramapuram dump.

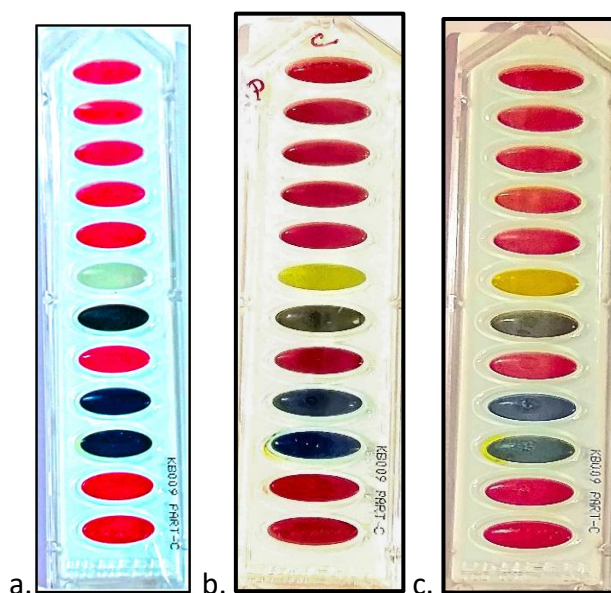


Fig.3.9(c) Result of biochemical tests- KB009-Part C of 11 carbohydrates and one control. a. OBA5 from Vanoor dump. b. OLA2 from Laloor dump c. OBM3 from the Bhramapuram dump.

Apart from red to yellow colour changes in regular carbohydrate analysis tests, a few tests exhibit distinct colour changes for positive results. They were

1. ONPG (Orthonitrophenol- β -D-galactopyranoside) Test: - This assay measures the activity of β -galactosidase. It showed a positive result with a yellow colour in the medium for the OLA2 and OBM3 strains, and a negative result with a colourless medium for the OBA5 bacterial species.
2. The esculin hydrolysis test quantifies the capacity of bacterial species to break down esculin into esculetin when exposed to β -glucosidase or esculinase enzymes. In this analysis, the black

colour in the media wells of all the bacterial strains indicates positive results.

3. Citrate test: This test detects the ability of microorganisms to utilise citrate as a carbon source. All the strains used for the test displayed positive results by converting the green colour to blue.
4. Malonate utilisation test: In this analysis, we assess the capacity of microorganisms to use sodium malonate as their exclusive carbon source. The strains OBA5 and OLA2 introduced into the media wells showed colonies of blue colour. However, the strain OBM3 showed negative results, with a green colour in the media after 24 hours of incubation.

Based on the analyses, we have assumed that OBA5 belongs to the *Bacillus* species, OLA2 belongs to the *Pseudomonas* species, and OBM3 belongs to the *Micrococcus* species.

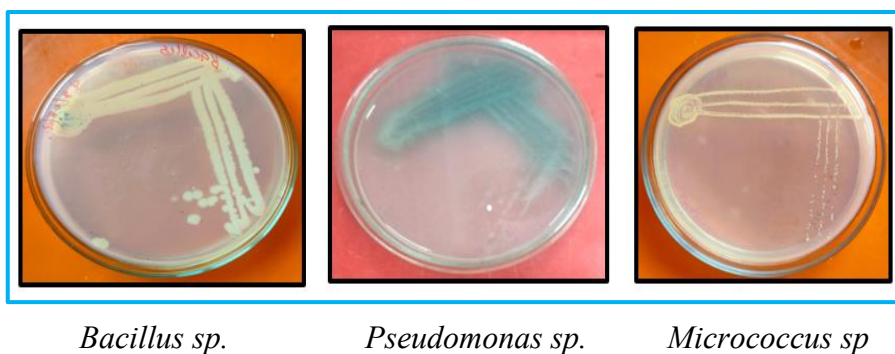


Fig.3.10 Pure cultures of potential strains in nutrient agar were used for further 16S rDNA sequencing and MALDI-TOF analysis to identify the genus and probable species.

3.5.4.4 Result of the culture characteristics of the most potent strains isolated from long-run plastic heaps in selective media

All the isolated strains were streaked on various selective media, and the bacterial species were identified after biochemical tests confirmed their identities.

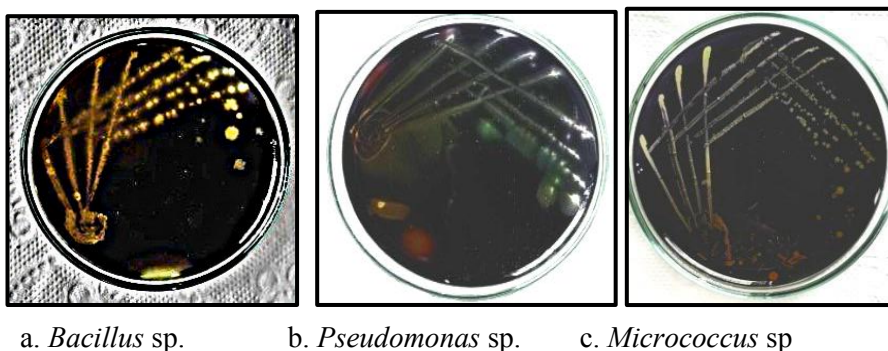


Fig.3.11(1) Demonstration of caseinase activity using starch casein agar.

The appearance of zone clearance in each plate after adding an iodine solution gave us a positive result.

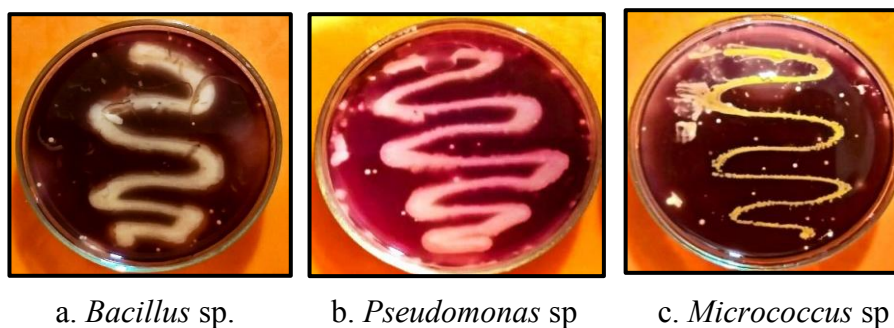


Fig.3.11(2) Starch hydrolysis activity using starch agar. We obtained a positive result for all three bacterial species by zone clearance in all plates after flooding with 2 mL of iodine solution.

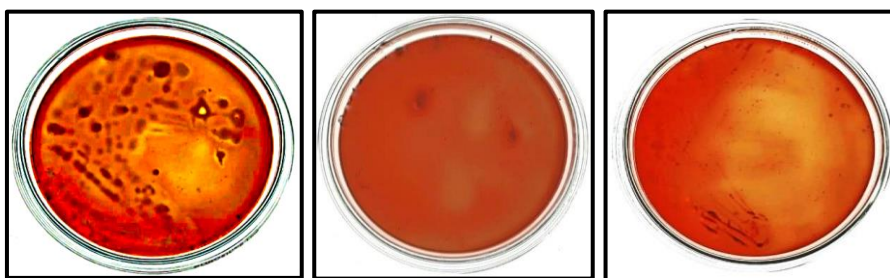
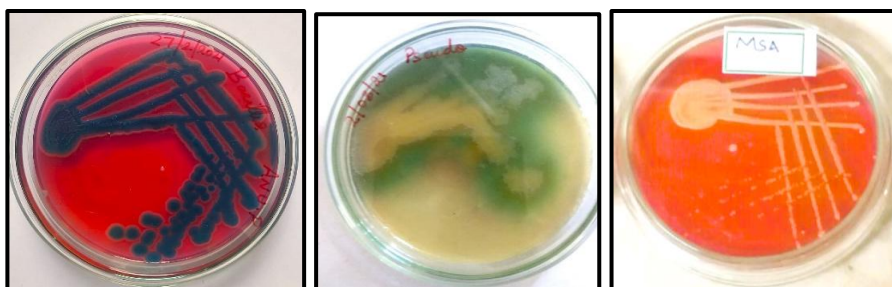
a. *Bacillus* sp.b. *Pseudomonas* sp.c. *Micrococcus* sp.

Fig.3.11(3) Cellulase activity using carboxy cellulose agar. The light orange colouration of 1% Congo red added to CMC (Carboxymethyl Cellulose) agar indicates the presence of cellulase activity, which is more evident in *Bacillus* species than in the other two species.

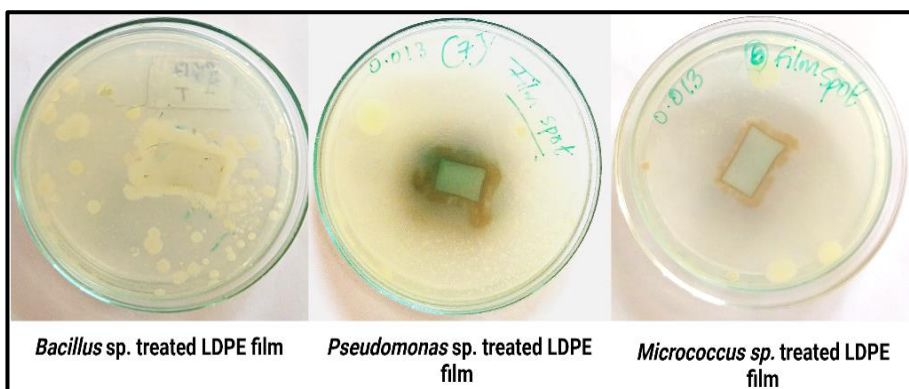


a

b

c

3.11(4) Bacterial species on the specific media. a. *Bacillus* sp on Hi-Media Bacillus agar b. *Pseudomonas* sp on hi-media pseudomonas agar c. *Micrococcus* sp on mannitol salt agar.

*Bacillus* sp. treated LDPE film*Pseudomonas* sp. treated LDPE film*Micrococcus* sp. treated LDPE film

3.11(5) LDPE films inoculated in CFLMSM for 120 days and placed on the R2A media.

3.5.4.5 Result of 16s r DNA sequencing

Furthermore, isolated strains were confirmed by 16S rDNA sequencing in an ABI sequencer. The obtained sequences were then used to search for the most homologous sequences using the NCBI-BLAST tool. Then, the novelty of each strain was proved by phylogenetic tree construction. Each Dendrogram depicting an outgroup (see fig.3.5.4.5(1),3.5.4.5(2), and 3.5.4.5(3)) confirmed the uniqueness of the isolated bacterial strains, and all were deposited in GenBank with accession numbers OLA823002.1 as *Bacillus cereus* NJD1, OL823063 as *Pseudomonas aeruginosa* NJD2 and OL823065 as *Micrococcus luteus*.

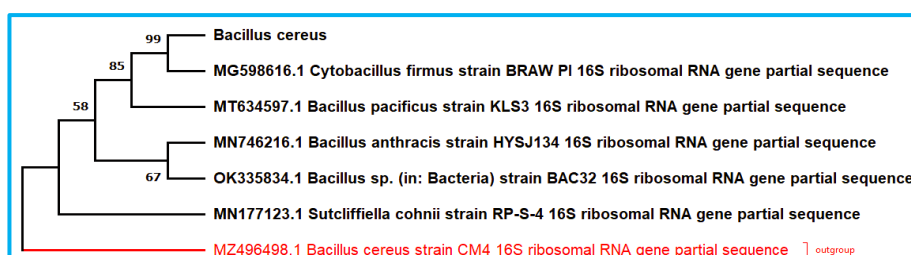


Fig.3.12(1) Phylogenetic tree depicting the isolated bacterial strain, *Bacillus cereus*. The outgroup determines the evolutionary root of the organism.

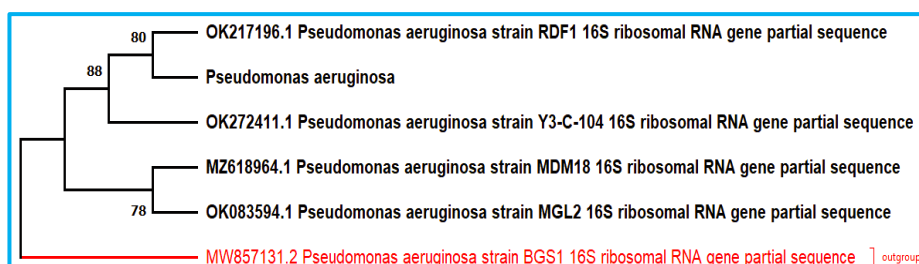


Fig.3.12(2) Phylogenetic tree constructed using homologous sequences retrieved by NCBI-BLAST analysis to depict *Pseudomonas aeruginosa*.

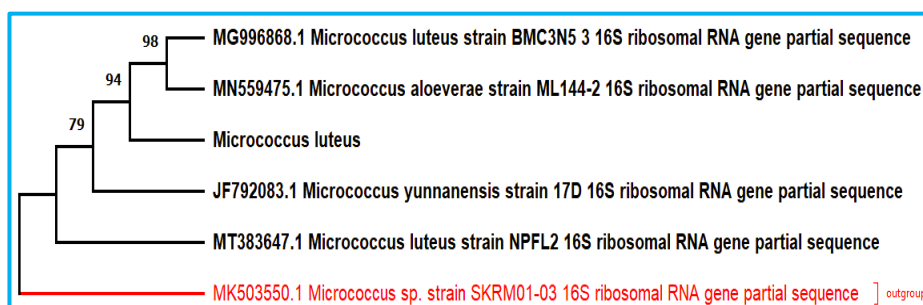


Fig.3.12(3) Phylogenetic tree constructed using 16S rDNA sequence of *Micrococcus luteus*. It shows the homologous sequences and outgroup of the isolated strain.

3.5.4.6 MALDI-TOF analysis of the potential strains isolated from various landfills

3.5.4.6(a) MALDI-TOF analysis of *Bacillus cereus*

It is one of the most sophisticated and straightforward methods for identifying the genus and species levels of unknown organisms (Sommerer, Centeno, and Rossignol 2007). The strain-specific protein mass fingerprints (PMFs) generated by Bruker Daltonics' Microflex LT-PC-based MALDI-TOF device for each strain were compared with the reference data stored in the instrument's database (Wang et al., 2012). Harnessing the strains' PMF data, the instrument software calculated an arbitrary score value to identify the strain (Szmigiel et al., 2021).

S. No	Strain	Organisms (best match)	Score values	Descriptions
1	OBA5	<i>Bacillus cereus</i>	2.208	fixed genus and expected species identification (J. Tao et al., 2009).
2	OLA2	<i>Pseudomonas aeruginosa</i>	2.612	Highly probable species identification (Desoky et al., 2022).
3	OBM3	<i>Micrococcus luteus</i>	2.257	Secure genus identification and probable species identification.

Table 3.7 Results of the best matches from the biotype software for each strain using the score values of each organism.

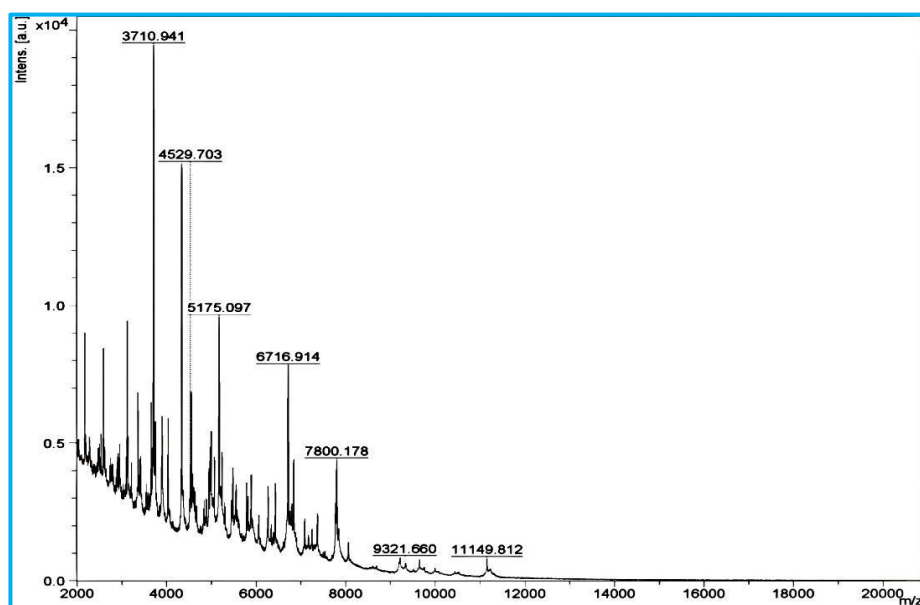


Fig.3.13(1) Protein mass fingerprint of *Bacillus cereus*

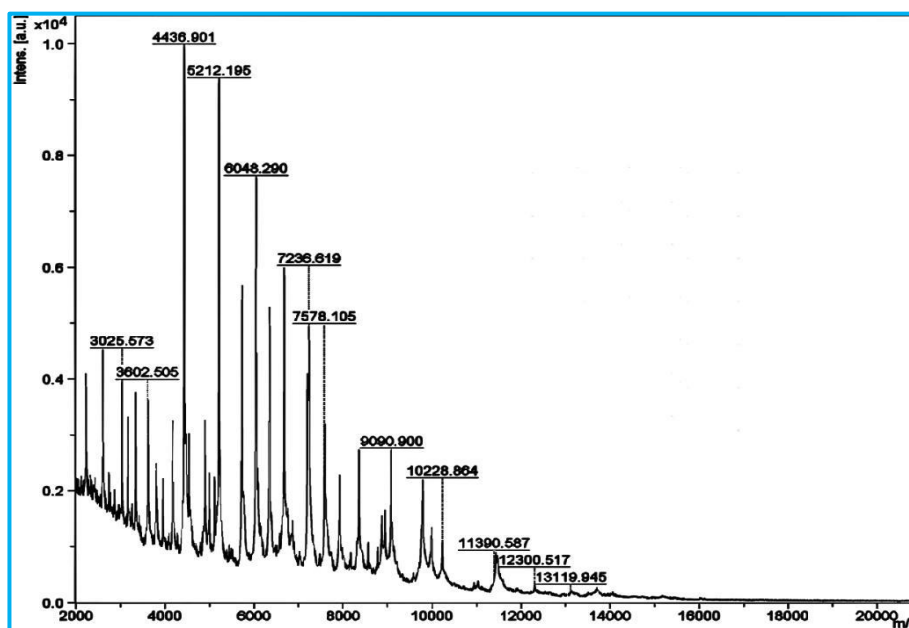


Fig.3.13(2) Protein mass fingerprint of *Pseudomonas aeruginosa*

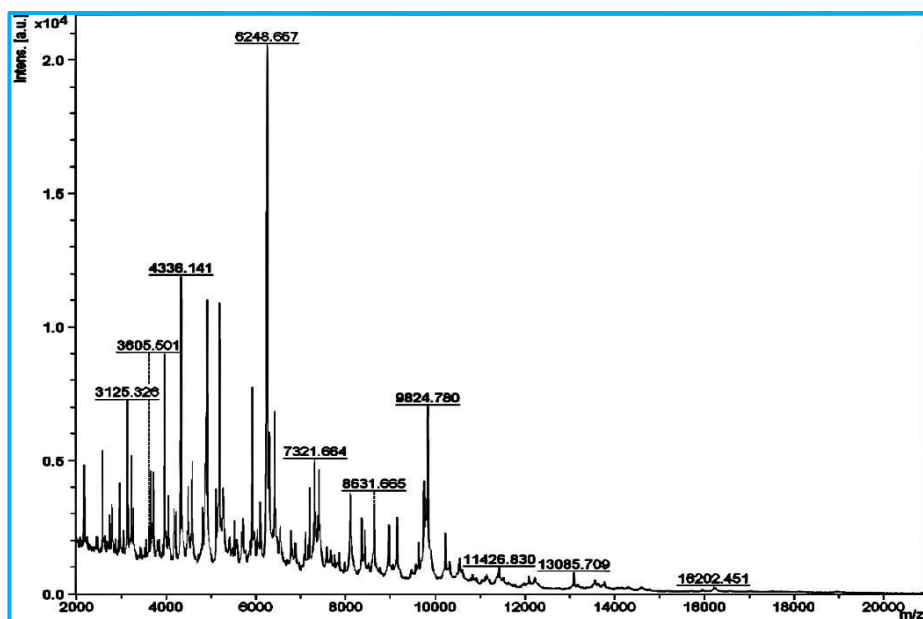


Fig.3.13(3) Protein mass fingerprint of *Micrococcus luteus*

3.5.5 Quantification of biofilms on LDPE surface

Biofilm development on LDPE is a complex assembly of microorganisms in a specified habitat. Altered structural and physiological circumstances facilitate bacterial adaptation to the hostile surface of polymers. Microbes' surface roughness is a crucial factor that creates a conducive environment for bacterial attachment. It represents the initial stage in the biodegradation of LDPE films.

To demonstrate the initial biofilm, we observe the presence of organisms on LDPE films inoculated with potent strains in a carbon-free medium.

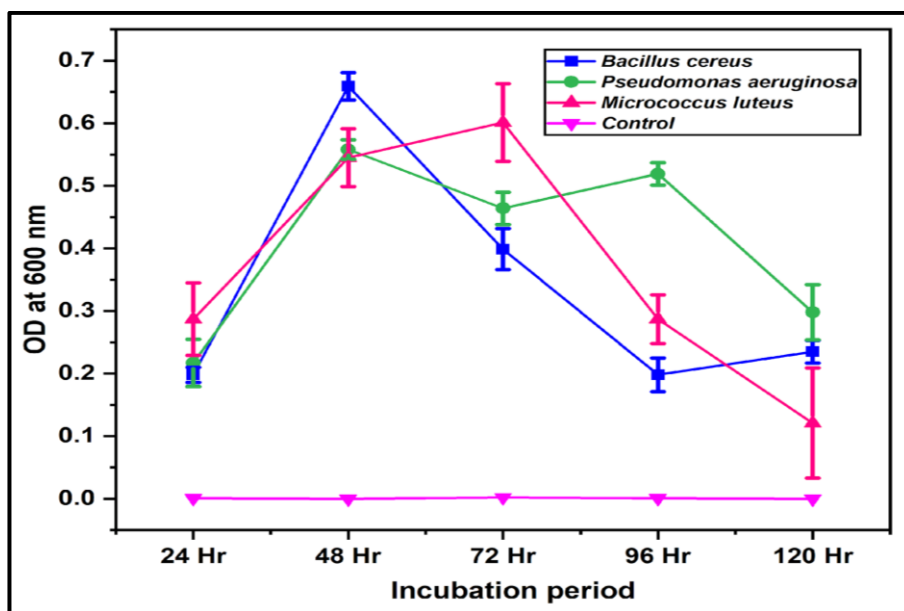


Fig.3.14 Biomass count on LDPE films in CFLMSM incubated by *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Micrococcus luteus* for the first five days. This result indicates the efficacy of the bacterial strains isolated from landfills in adhering to LDPE films. Biomass counts of bacterial strains incubated with LDPE films were compared to control setups without bacterial inoculation.

The maximum biomass in the first five days of *Bacillus cereus* and *Pseudomonas aeruginosa* was 48 hours. However, the maximum biomass of *Micrococcus luteus* was reached within five days of incubation. We conducted this study for further analysis.

3.5.6 Empirical investigations to demonstrate robust biofilm development on LDPE films by the competent bacterial species isolated from landfill soil.

To determine the efficacy of the isolated strains in forming biofilms on LDPE films inoculated in a carbon-free liquid medium, we calculated the total cell count on the films, strain viability, estimated total protein content, pH change of the media, and EPS (extracellular polymeric substances) count.

3.5.6.1 Determination of total cell growth count (Total biomass count)

All species were counted for this test regardless of their metabolic stage: logarithmic, stationary, or death. It measures the bioactivities of potential strains adhered to the complex surface of LDPE films (S. H. Ji et al., 2024). This adherence may lead to colonisation and the introduction of metabolites to the hydrophobic surface of LDPE films (Sooriyakumar et al., 2022). Each experiment involving three separate competent organisms was conducted at room temperature using a static culture method in highly stressed media.

3.5.6.1.1 Result of total biomass count of *Bacillus cereus*

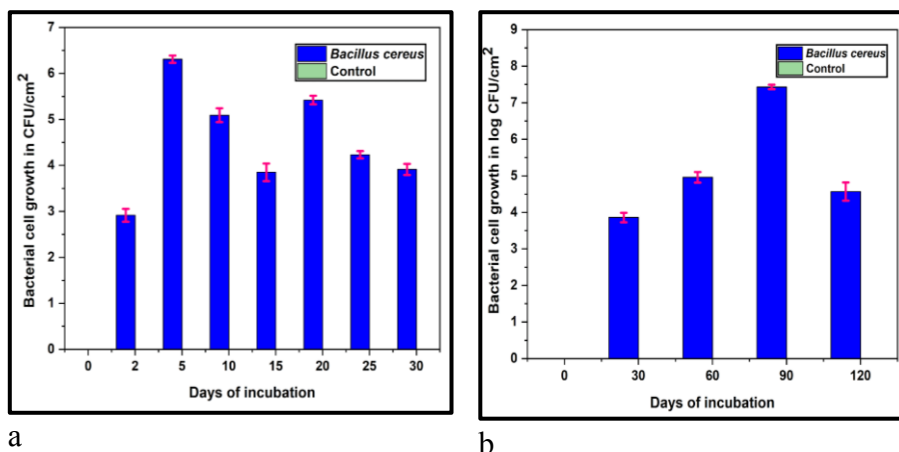


Fig.3.15(1) Biomass count of *Bacillus cereus* in CFLMSM at 30°C a. for 30 days b. for 120 days.

In this analysis, we studied the total *Bacillus cereus* cell count for 0, 2, 5, 10, 15, 20, 25, 30, 60, 90, and 120 days. The strain exhibited a maximum cell count on the 5th day of the 30-day incubation analysis and the 90th day of the 120-day incubation (See Fig. 3.15(1). The results demonstrate potent bioactivity during incubation on LDPE films.

3.5.6.1.2 Result of biomass count of *Pseudomonas aeruginosa*

In this test, we calculated the cell count of *Pseudomonas aeruginosa* for 30-day (0, 3, 5, 10, 15, 20, 25, and 30 days) and 120-day (0, 30, 60, 90, and 120 days) incubation periods. In the initial one-month incubation study, the maximum biomass count appeared on the 25th day of incubation. During the 120-day incubation period, the 60th-day films exhibited the highest cell count (results shown in Fig. 3.15(2). All four months of incubation demonstrated the strain's adherence to the hydrophobic LDPE film surface, confirming the organism's potency.

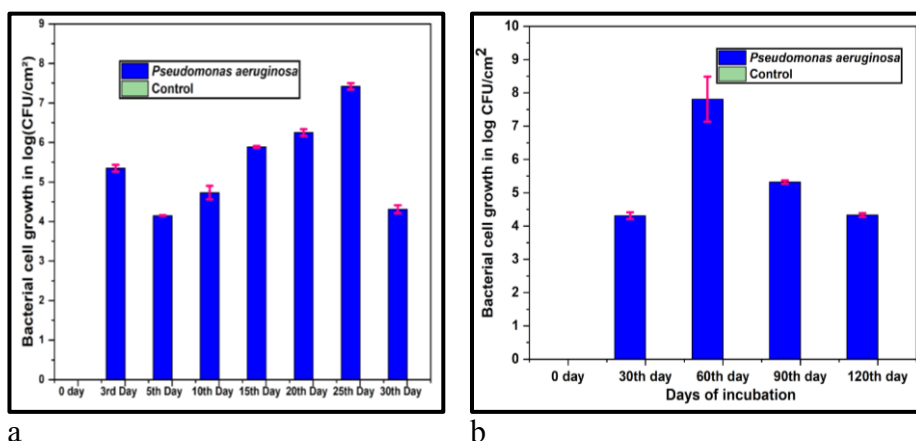


Fig.3.15(2) Biomass count of *Pseudomonas aeruginosa* in CFLMSM: a. after 30 days of incubation, b. after 120 days of incubation (all flasks incubated at 30°C under static culture conditions).

3.5.6.1.3 Result of biomass count of *Micrococcus luteus*

In this test, we calculated the cell count of *Micrococcus luteus* for 30-day (0, 2, 5, 10, 15, 20, 25, and 30 days) and 120-day (0, 30, 60, 90, and 120 days) incubation periods (see results in 3.15(3)).

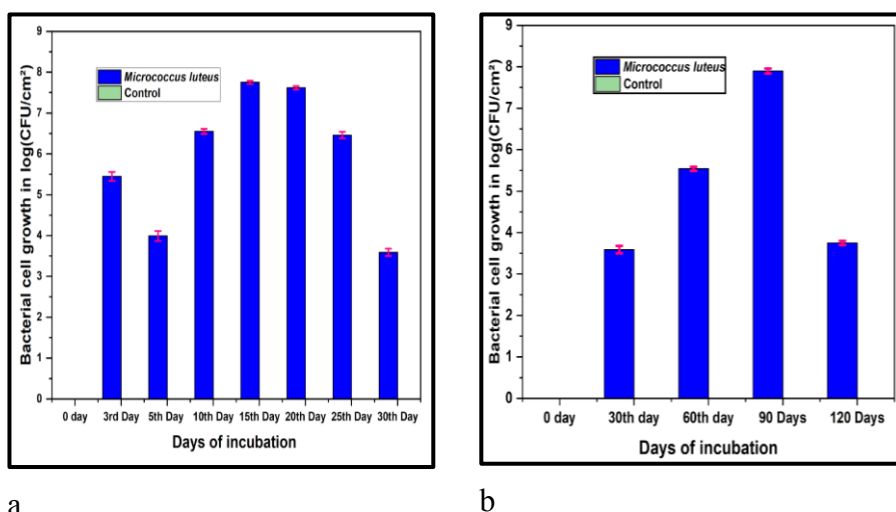


Fig.3.15(3) Biomass count of *Micrococcus luteus* in CFLMSM at room temperature (static culture): a. for the initial 30 days, b. for 120 days of incubation.

The maximum biomass count was observed on the 15th day of the initial 30-day incubation period. After 120 days of incubation, the 90-day LDPE films showed the highest biomass count. These results also confirm the successful adherence of *Micrococcus* strains to the chemical structure of the polymers.

3.5.6.2 FDA test for 30 and 120 days of incubation

FDA testing determines the viability of isolated bacterial strains on LDPE films (Shilpa et al., 2023). This test only accounts for the organisms in the logarithmic phase, and its results (shown in Figs. 3.16(1), 3.16(2), and 3.16(3)) demonstrate the strains' capabilities to form strong biofilms on the surface of LDPE film.

3.5.6.2.1 FDA analysis of *Bacillus cereus*

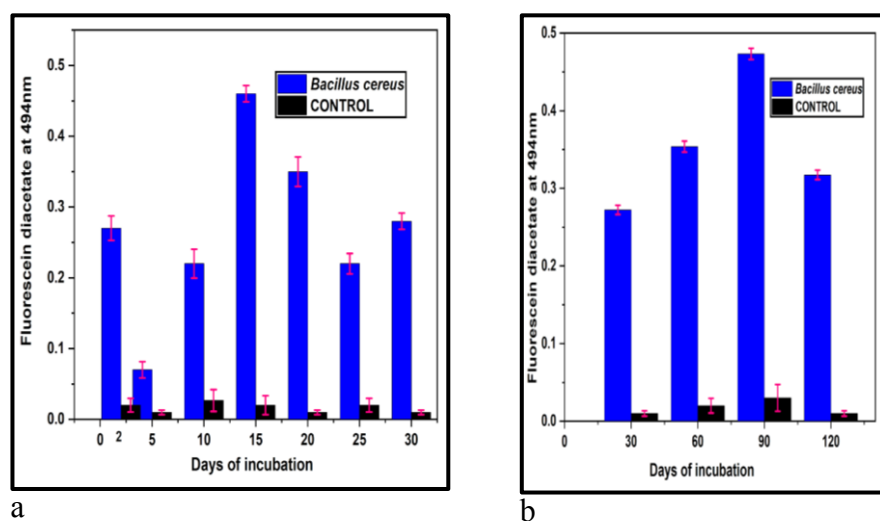


Fig.3.16(1) Result of the FDA test of *Bacillus cereus* incubated in a carbon-devoid medium with LDPE strips. The LDPE films were collected from incubation flasks at 2,5,10,15,20,25, and 30 days (a) and 30, 60, 90, and 120 days (b) following the growth pattern of *Bacillus cereus*.

In this test, we observed a high rate of metabolically active *Bacillus cereus* cells in the conical flask of carbon-free broth with LDPE films. Within the group of conical flasks maintained for 30 days, the LDPE strips incubated for 15 days showed the highest cell viability rate. Similarly, among the four-month conical flasks incubated, the LDPE strips at 90 days displayed the highest rate of living cells.

3.5.6.2.2. FDA test of *Pseudomonas aeruginosa*

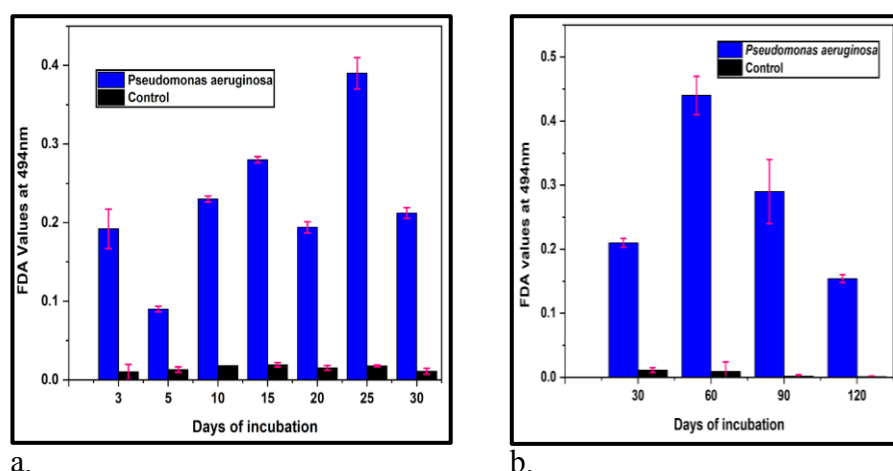


Fig.3.16(2) Outcome of the FDA analysis of *Pseudomonas aeruginosa*: a. for 3,5,10,15,20,25, and 30 days of incubation, and b. FDA results within thirty days of incubation in CFLMSM.

The presence of metabolically viable *Pseudomonas* sp was detected on LDPE, and the results were summarised in 3.5.6.2.2. Among the conical flasks, the maximum FDA value was recorded on the 25th day of incubation. For 120 days, the highest number of living cells was observed on the 60th day of incubation. It demonstrated the ability of *Pseudomonas* species to attach to polymer pieces inoculated in a carbon-free liquid mineral salt medium.

3.5.6.2.3 FDA Analysis of *Micrococcus luteus*

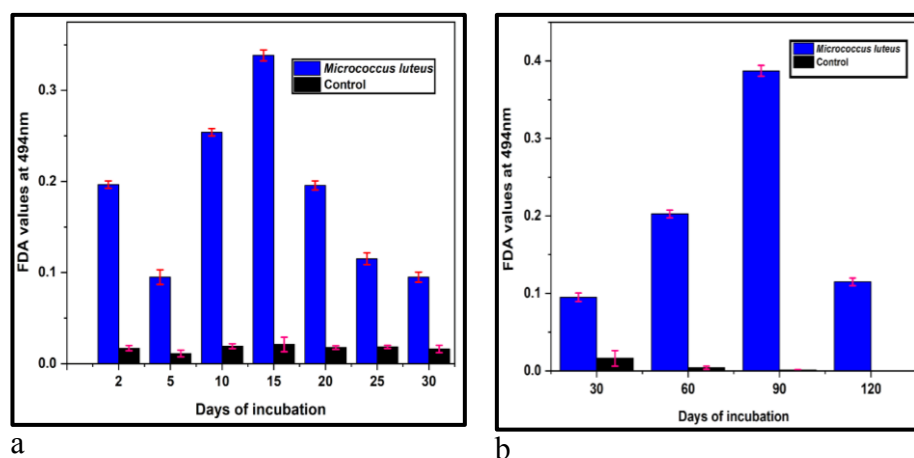


Fig 3.16(3) Biochemical activities of *Micrococcus luteus* on LDPE films in a pressurised condition of carbon-free mineral broth. a. for 2,5,10,15,20,25, and 30 days of incubation. b. for 30,60,90, and 120 days of incubation.

The FDA analysis of *Micrococcus luteus* on LDPE films revealed the presence of living organisms. Considering the test result, among the 30 30-day incubation flasks, the 15-day-old LDPE films showed the highest FDA value. In a 120-day experiment, its value peaked on the 90th day of incubation.

The FDA test results of the three competent strains demonstrated their efficacy in forming biofilms on hydrocarbon surfaces, utilising the LDPE films as their energy source, and producing hydrolytic enzymes (Schnürer & Rosswall, 1982). The difference in the metabolic rate may be due to the growth curves of microbial strains.

3.5.6.3 Protein content estimation from the biofilm attached to the LDPE films

Enhanced bacterial biomass on the LDPE film may increase protein concentration (S. H. Ji et al., 2023). This test gives precise information regarding the catalytic activities of the potential strains we used in the study. It was conducted by collecting bio-attached LDPE films from a conical flask incubated for 30, 60, 90, and 120 days with *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Micrococcus luteus* (the results are summarised in Fig. 3.17).

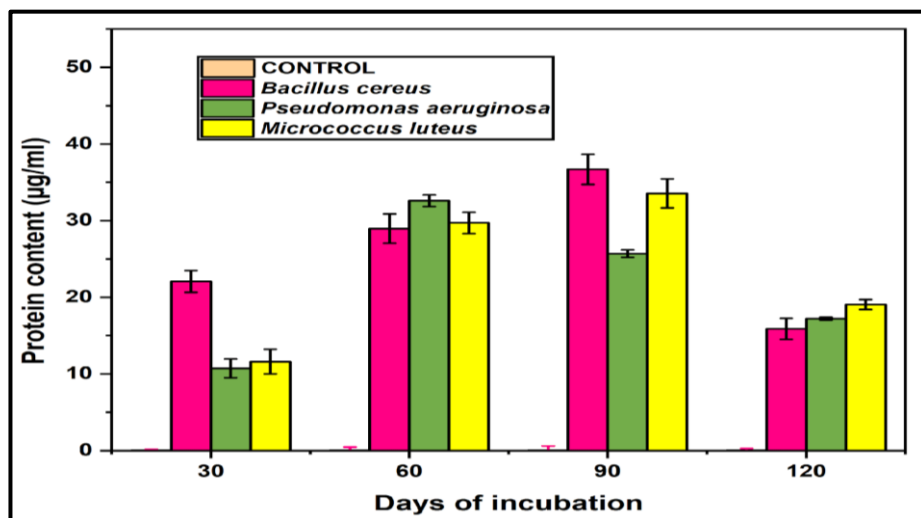


Fig.3.17. Results of protein estimation of all three potential strains on the polymer strips. Each result was compared with that of the LDPE films without bacterial inoculum.

We observed a high protein content on the 90th day of incubation with *Bacillus* species (36.7 ± 1.97 µg/mL). The maximum protein content of *Pseudomonas aeruginosa*-treated LDPE films was reported at 60 days of incubation (32.59 ± 0.755 µg/mL). It could be because the organism reaches its fast-proliferating stage after 60 days of incubation. Similar

to the *Bacillus* species-treated LDPE film, the *Micrococcus luteus* strain biofilm on the polymer also exhibited a significant protein content at 90 days of incubation ($33.54 \pm 1.89 \mu\text{g/mL}$). It shows the solid adherence of the organisms on the polymer surface.

3.5.6.4 Influence of pH change in media due to the growth of microbial strains inoculated to LDPE films.

In this analysis, the *Bacillus cereus* inoculated conical flask exhibited a pH change from 7.28 ± 0.04 to 4.64 ± 0.09 . The *Pseudomonas aeruginosa*-inoculated medium showed a pH change from 7.3 ± 0.04 to 4.97 ± 0.059 , and the *Micrococcus luteus*-inoculated medium displayed a decline in pH from 7.29 ± 0.059 . The data were compared by recording the pH of CFLMSM without bacterial inoculation (control).

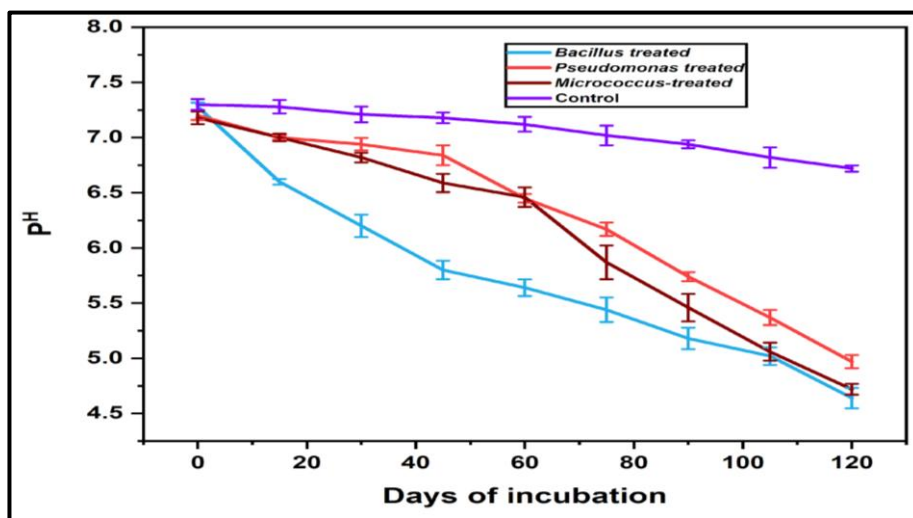


Fig.3.18 pH change in the carbon-free medium inoculated with the isolated bacterial strains. The results were compared with the control and represented in the graph.

These significant changes indicate the organisms' production of acidic metabolites and their ability to assimilate the intrinsic compound as their energy source (Mallisetty et al., 2023).

3.5.6.5 Extracellular polymeric substance (EPS) production in the potential strains

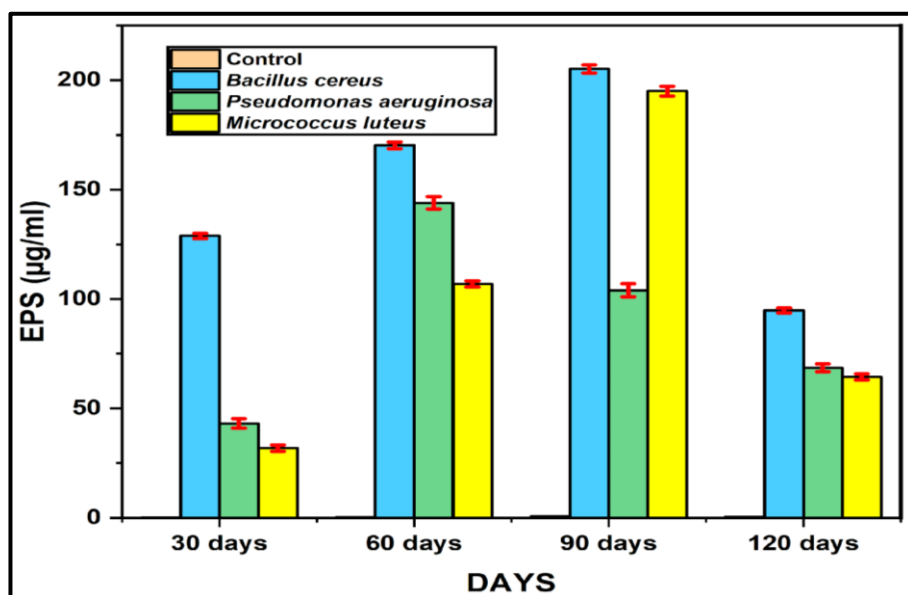


Fig.3.19 Estimation of EPS production by potential strains on the LDPE film. All results were compared with control setups, excluding bacterial cultures to ensure accuracy.

Among the strains, *Bacillus cereus* exhibited significant EPS generation over a 90-day incubation period, with measurements of 128.83 ± 1.22 µg/mL at 30 days, 170.21 ± 1.5 µg/mL at 60 days, and 205.21 ± 1.82 µg/mL at 90 days. At 120 days, they exhibited a sharp decline in EPS production (94.79 ± 1.1 µg/mL). Similarly, EPS production was observed in the other two potential strains, *Pseudomonas aeruginosa* and *Micrococcus luteus*. The highest rate of EPS production for *Pseudomonas* sp was observed on the 60th day of

incubation ($143.96 \pm 2.92 \mu\text{g/mL}$). The highest rate of EPS production by *Micrococcus* sp. was recorded on the 90th day of incubation ($195 \pm 2.2 \mu\text{g/mL}$) (Results depicted in Fig.3.19). The comparatively elevated EPS formation throughout the incubation period for all three strains signifies their capacity to adhere to the resistant surfaces of LDPE films.

Enzyme analysis

Each potential strain isolated produced a few extracellular enzymes on the LDPE films during its catalytic activities. The culture supernatants were collected and subjected to quantitative and qualitative analysis.

3.5.7.1 Quantitative Assays of Enzymes

The analysis was conducted over fifteen-day intervals.

3.5.7.1.1 Laccase assay

Laccases are the primary extracellular enzymes involved in LDPE biodegradation, with oxygen serving as the primary electron acceptor (Suresh et al., 2025). All three strains exhibited the production of this enzyme, as shown in Fig.3.20.

All three microbial strains exhibited a notable rate of laccase production throughout their four-month incubation period. *Bacillus cereus* attained peak laccase activity in carbon-free synthetic media at 90 days of incubation ($51.9 \pm 1.142 \text{ U/mL}$). At 60 days of incubation, *Pseudomonas aeruginosa* demonstrated its maximum laccase enzyme activity ($61.8 \pm 1.3 \text{ U/mL}$). Furthermore, *Micrococcus luteus*,

identified as a promising strain, yielded the highest laccase enzyme production at 90 days of incubation (65.75 ± 1.59 U/mL).

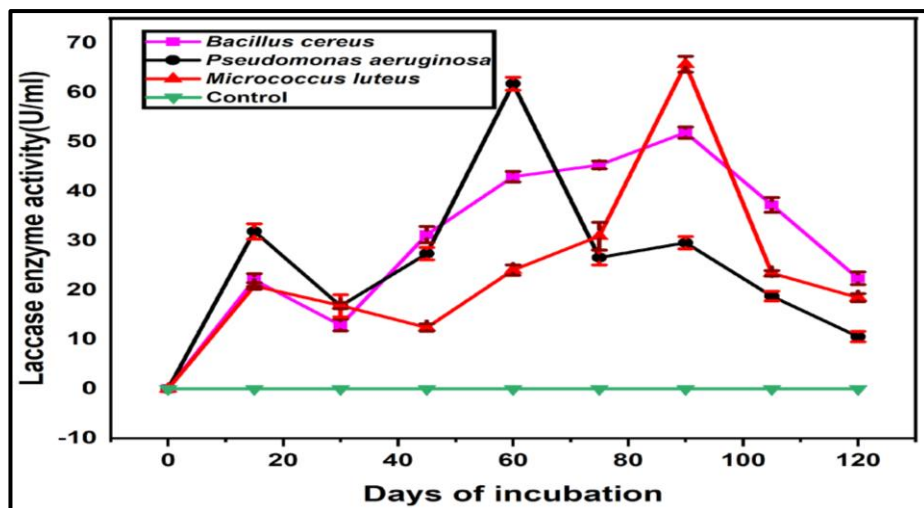


Fig.3.20 Graphical representation of laccase enzyme activity of *Bacillus cereus* (violet line in the graph), *Pseudomonas aeruginosa* (black-lined), and *Micrococcus luteus* (red-lined) during 120 days of incubation, along with control (without enzyme suspension-green lined).

3.5.7.1.2 Lipase Assay

The effectiveness of isolated candidates in generating lipase enzymes was measured to illustrate the hydrolysis of ester linkages in LDPE films inoculated in CFLMSM for four months (Kim et al., 2024). In the isolated strains, *Bacillus cereus* has shown high enzyme activity among the bacterial strains at 15 days (80.67 ± 1.21 U/mL) and 90 days (67.58 ± 0.997 U/mL) of incubation. The *Pseudomonas aeruginosa* strain isolated from the Laloor dump showed the highest production at 60 days of incubation (78.52 ± 1.59 U/mL). Another potential candidate isolated from the Bhramapuram open landfill has demonstrated the highest enzyme rate at 90 days (80.11 ± 1.88 U/mL).

All three strains exhibited significant lipase enzyme activity during the incubation period.

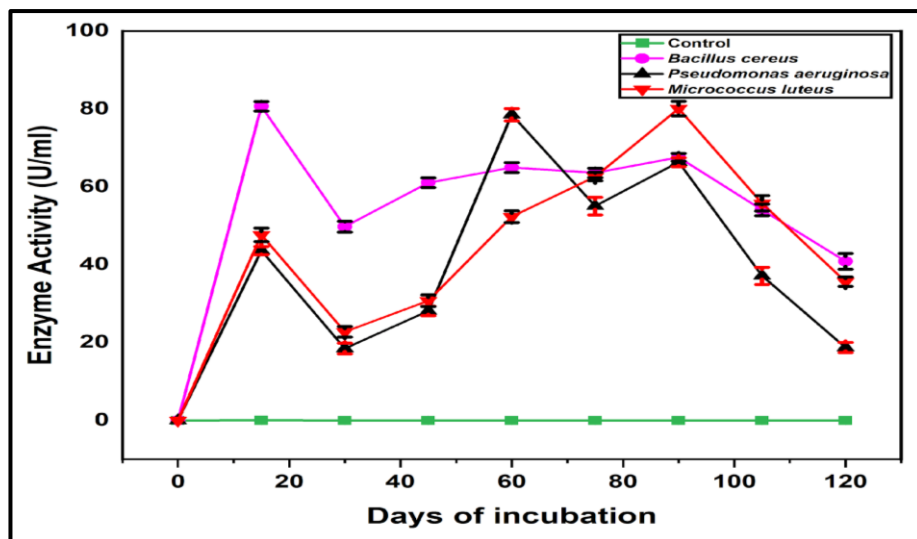


Fig.3.21 Lipase enzyme activity of *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2 and *Micrococcus luteus* NJD3 during 120 days of incubation. Results validated using a control setup devoid of enzyme suspension.

The maximum lipase production of *Bacillus* sp occurs at 15 days of incubation ($80.67 \pm 1.21 \text{ U/mL}$), *Pseudomonas* species at 60 days of incubation and *Micrococcus luteus* strain at 90 days of incubation.

3.5.7.1.3 Protease assay

Proteases are another group of extracellular enzymes that can introduce amide linkages to the recalcitrant surface of LDPE, facilitating biodegradation (H. Bhardwaj et al., 2013; Roohi et al., 2017).

In our analysis, we found that all three strains produced significant levels of protease enzymes (see Fig. 3.22). Among the potential strains, *Bacillus cereus* NJD1 exhibited the highest protease production rate at 90 days of incubation ($137.67 \pm 1.45 \text{ U/mL}$). The other two competent

strains, *Pseudomonas aeruginosa* (maximum protease production occurred at 60 days, 80.74 ± 1.96 U/mL) and *Micrococcus luteus* (enzyme peak observed at 90 days, 90.99 ± 1.06 U/mL), exhibited notable protease activities.

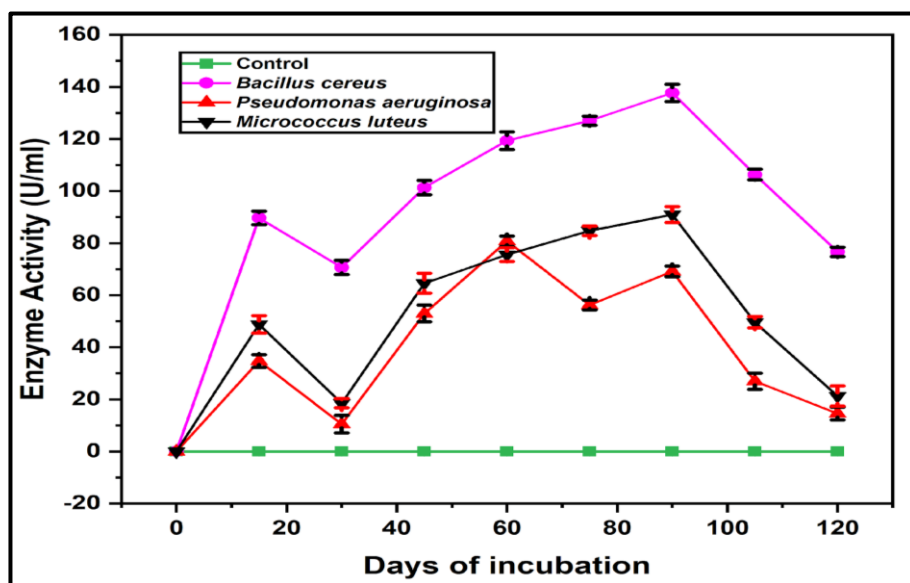


Fig.3.22 Protease enzyme activity of *Bacillus cereus*, *Pseudomonas aeruginosa* and *Micrococcus luteus* during 120 days of incubation. The experimental outcomes were validated using a control.

3.5.7.1.4 Manganese peroxidase and Lignin peroxidase enzyme activities for LDPE biodegradation

Several recent investigations have demonstrated the specificity and stability of manganese peroxidases (MnPs) and lignin peroxidases (LiPs) in the biodegradation of LDPE (Sowmya et al., 2015). In the present study, only *Pseudomonas aeruginosa* exhibited a notable rate of these enzymes (see figures 3.23(a) and 3.23(b)).

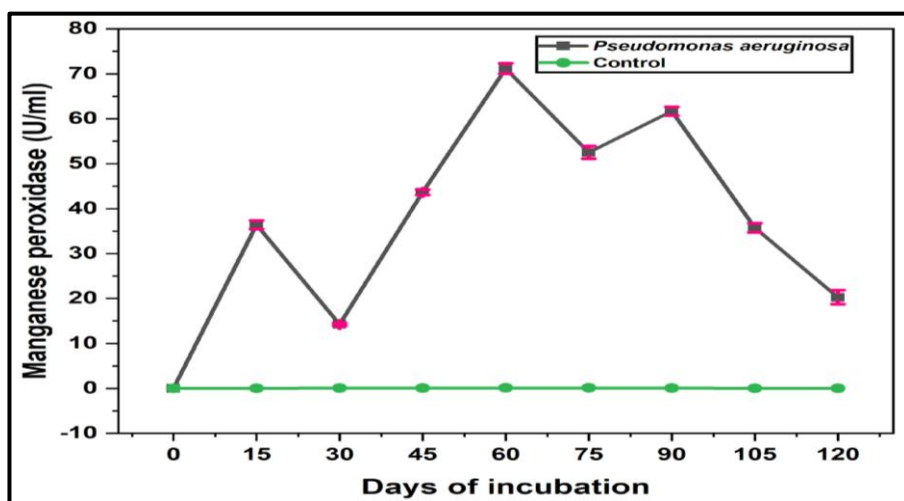


Fig.3.23(a) Manganese peroxidase enzyme activity of *Pseudomonas aeruginosa* during 120 days of incubation. Each experiment was benchmarked against controls.

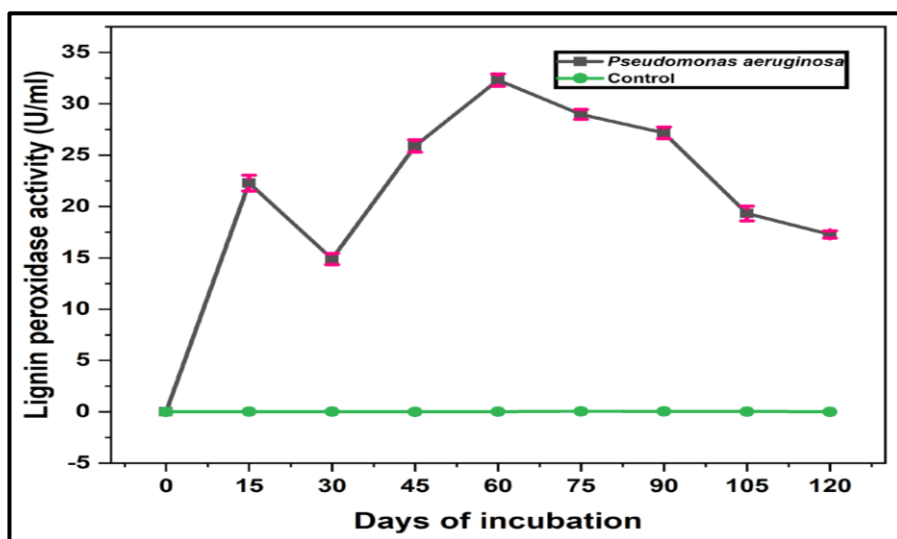


Fig.3.23(b) Lignin peroxidase enzyme activity of *Pseudomonas aeruginosa* during 120 days of incubation. Controls, devoid of bacterial inoculation and enzyme supernatants, were used for comparative assessment of all results.

3.5.7.1.5 Esterase Assay

These extracellular enzymes catalyse the conversion of the water-soluble simple esters produced after the initial biodegradation of LDPE films for a few weeks into simpler molecules and among the competent strains isolated from landfills for LDPE biodegradation, *Micrococcus luteus* NJD3 only exhibited a moderate rate of esterase production. The organism exhibited the maximum production rate in biofilms attached at three months of incubation (results displayed in Fig. 3.24).

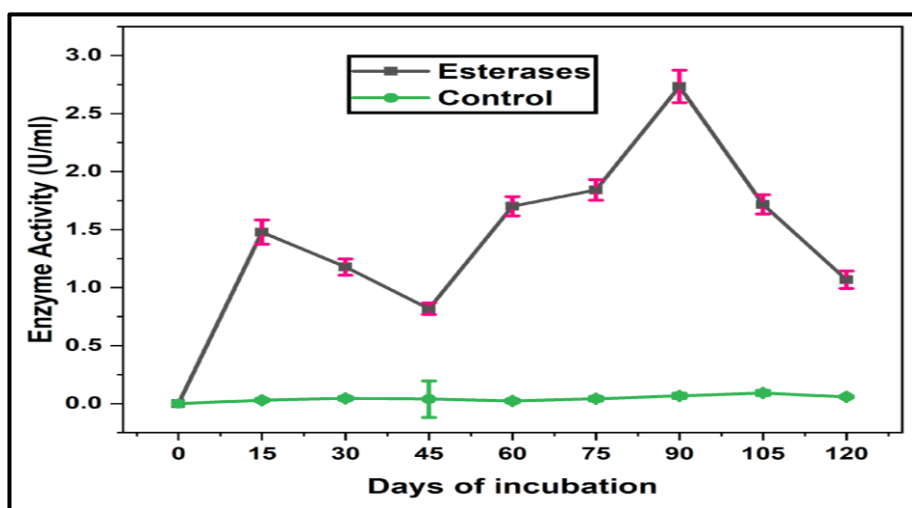


Fig.3.24 Result of esterase assay of *Micrococcus luteus* attached to LDPE films incubated for 120 days in CFLMSM. The outcome of each experiment was validated using a control setup.

3.5.7.2 Qualitative assays of enzymes

All three capable strains, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Micrococcus luteus*, showed positive results for laccase, lipase, and protease enzymes. Additionally, *Pseudomonas aeruginosa* exhibited positive results only for the manganese peroxidase and lignin peroxidase enzymes.

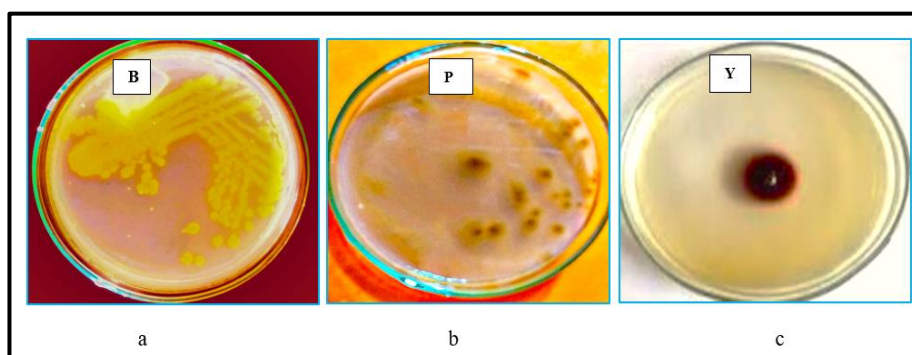


Fig.3.25 Laccase assay. The guaiacol-supplemented media plates formed yellow- or brown-coloured colonies after ten days at 37°C, indicating the presence of the laccase enzyme (N. S. Ali et al., 2022).

The guaiacol in the medium is oxidised in the presence of the laccase enzyme in the isolated strains to produce yellow to brownish zones around the colonies (Siroosi et al., 2016). The laccase enzyme catalyses oxidation reactions and forms water molecules (Senthivelan et al., 2019). Thus, laccases can oxidise long-chain hydrocarbons, such as LDPE films, and accelerate their biodegradation (Santo et al., 2013).

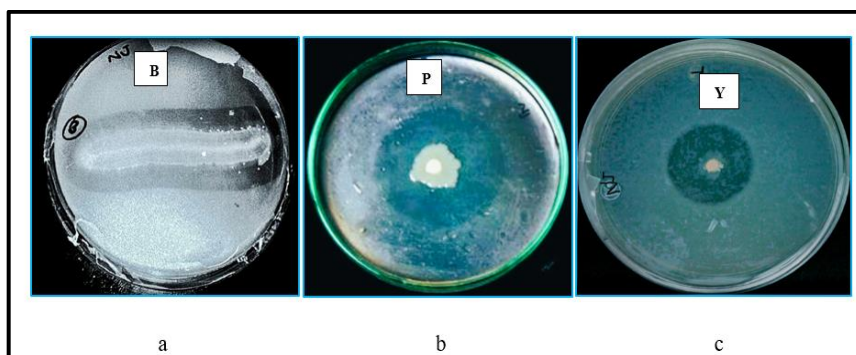


Fig.3.26 Lipase assay of potential in tributyrin agar medium. The halo zones produced around the colonies designate the lipase production by the strains. a. *Bacillus cereus* b. *Pseudomonas aeruginosa* c. *Micrococcus luteus*

Tributylin is the main component of the tributyrin agar medium (used to detect protease enzymes) and is insoluble in water. The bacterial lipases produced by the microbial strains can hydrolyse the compound into water-soluble butyric acid (S. S. Ali et al., 2023). It demonstrates the efficacy of lipase enzymes in hydrolysing water-insoluble triglycerides in LDPE into simple compounds (Fernandez-Lopez et al., 2023).

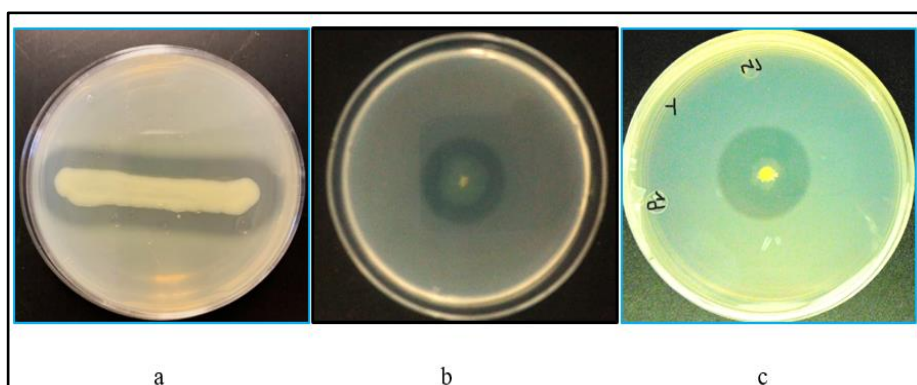


Fig.3.27 Protease assay in skim milk agar medium. The halo zones around the colonies indicate potential strains' protease enzyme production, such as *Bacillus cereus*. b. *Pseudomonas aeruginosa* c. *Micrococcus luteus*

The halo-zones produced around the colonies in skim milk agar proved the production of protease enzymes. All three strains inoculated into a carbon-free medium exhibited distinct zones around the colonies (Jones et al., 2007). Several investigations have demonstrated the ability of various *Bacillus* and *Pseudomonas* species to produce protease enzymes for the biodegradation of LDPE-based compounds (Mohanani et al., 2020; Najam et al., 2025).

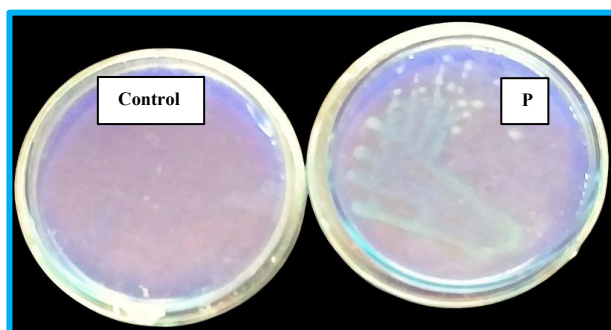


Fig.3.28 Azure B assay for detecting the presence of manganese peroxidase and lignin peroxidase production by *Pseudomonas aeruginosa* on the LDPE films.

The microbes that secrete these enzymes can decolourise or degrade azure B dye (Kavitha & Bhuvaneswari, 2021). Several scientists used this assay to demonstrate the presence of manganese peroxidase and lignin peroxidase production on LDPE films inoculated with efficient strains isolated from plastic dumps for polymer degradation (Iiyoshi et al., 1998; A. Kumar & Arora, 2022).

3.5.8 Bio-surfactants in the isolated strains for LDPE biodegradation

3.5.8.1 Result of oil displacement test

This indirect test identifies the presence of biosurfactants in the isolated strains. They are amphiphilic substances that can aggregate across aqueous phases. They can be secreted extracellularly or formed on the surfaces of microorganisms (Nayarisseri et al., 2018). Our analysis used petrol without bacterial inoculum as the control and petrol with bacterial adhesion as the test (see the results in Figs. 3.29(a), 3.29(b), and 3.29(c)).

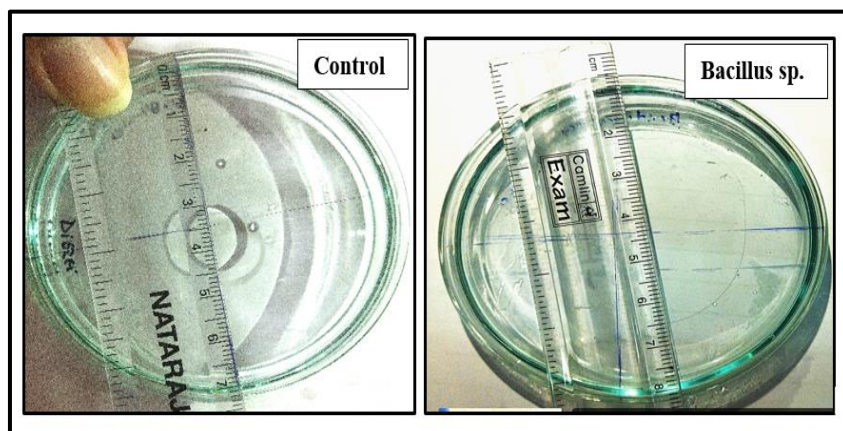


Fig.3.29(a) Oil displacement test using *Bacillus cereus* as a bacterial inoculum in the positive control.

In the above analysis, the oil drop diameter increased from 15 ± 1.25 mm (in the control plates) to 45 ± 4.08 mm (in the *Bacillus*-added test plates), indicating the presence of biosurfactants in the bacterial strain. Several studies have demonstrated the presence of such compounds using this simple protocol (Buhori et al., 2024).

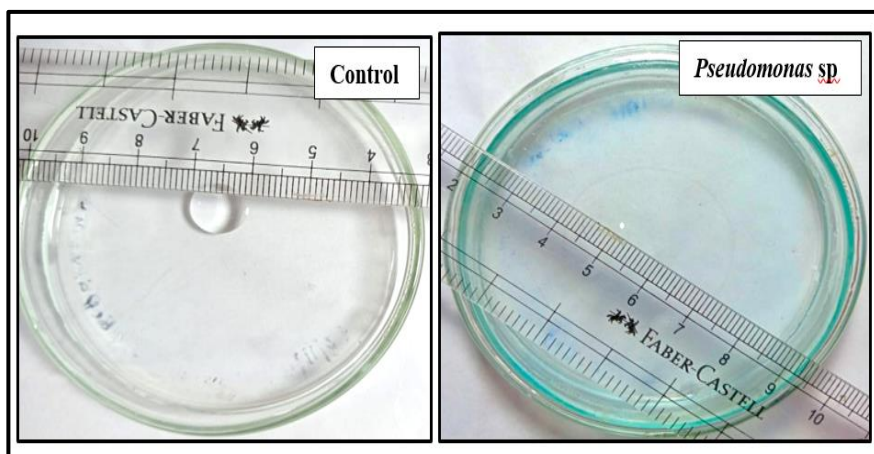


Fig.3.29(b) Oil displacement test using *Pseudomonas aeruginosa* as a bacterial inoculum in positive control.

The oil displacement test using the strain proved the presence of biosurfactant by increasing the drop diameter from $10 \text{ mm} \pm 1.5 \text{ mm}$ (Control) to $30 \text{ mm} \pm 5.15 \text{ mm}$ (*Pseudomonas* added test plate). Similar studies using *Pseudomonas* sp. have demonstrated the test's efficacy in identifying the presence of biosurfactants (Ghatge et al., 2020; Taghavi et al., 2022).

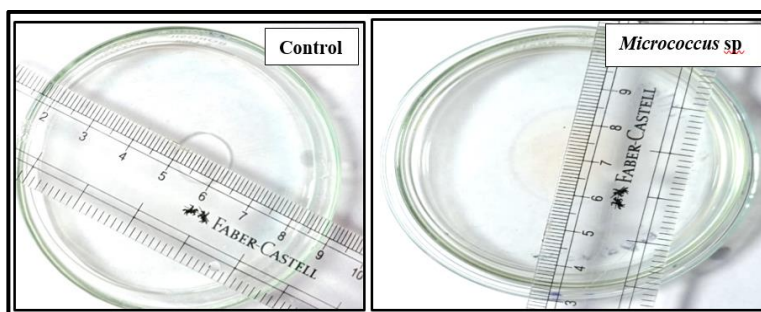


Fig.3.29(c) Oil displacement test using *Micrococcus luteus* as a bacterial inoculum in positive control.

Using *Micrococcus luteus* as a bacterial inoculum, we obtained an oil drop diameter of up to $28.5 \pm 5.69 \text{ mm}$, compared to the control without bacterial culture addition (diameter of $12 \pm 1.14 \text{ mm}$). The results showed that the strain also produces biosurfactants. Few reports have been published on the production of biosurfactants by the *Micrococcus* strain for degrading polymer films (Rani et al., 2020; Yilmaz et al., 2009).

3.5.8.2 Result of the emulsification capacity of potential strains using various petroleum products

In this experiment, we analysed the strains' emulsifying ability across multiple petroleum compounds like kerosene, petrol, and diesel. Such evidence suggests that the isolated strain produces emulsifying agents for the biodegradation of petroleum-based synthetic polymers.

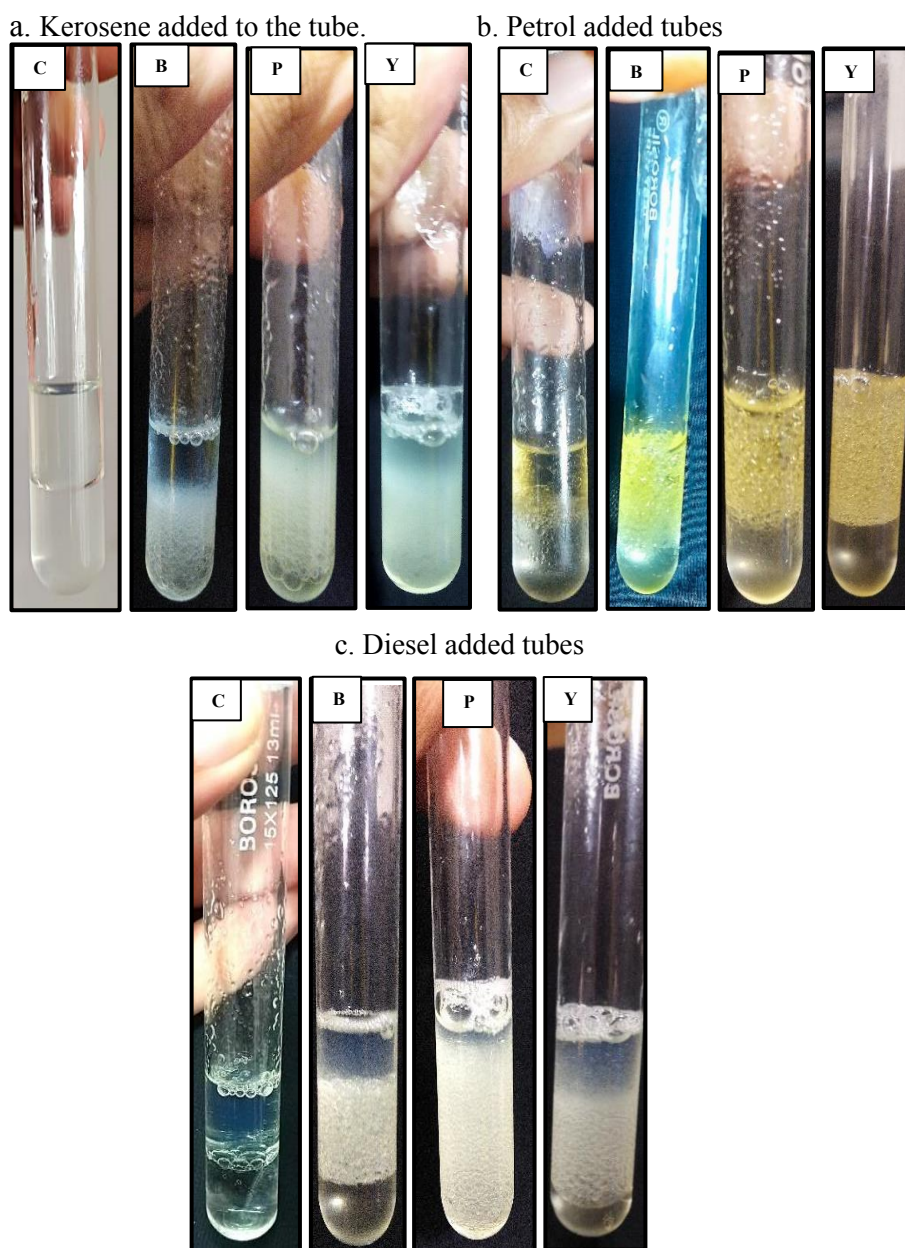


Fig.3.30 Stable emulsification layers after 24 hours of culture supernatants with petroleum compounds (Kerosene, Petrol, and Diesel) with culture supernatant of *Bacillus cereus* (tube B), *Pseudomonas aeruginosa* (tube P), and *Micrococcus luteus* (tube Y) and Control (without culture supernatant).

Stable foam formations were observed even after 24 hours in all test tubes with kerosene, petrol, and diesel, incubated with all three potential strains isolated from various plastic dumps. *Bacillus* sp. exhibited an emulsification rate of 62.5% with kerosene, 72.5% with petrol, and 75% with diesel. *Pseudomonas* sp. displayed 75% emulsification with kerosene, 75% with petrol, and 100% with diesel. Similarly, 80% emulsification was observed in the petrol tubes, and 75% in diesel for *Micrococcus* sp. added to the emulsification tubes. However, *Micrococcus* sp exhibited only 37.5% emulsification with kerosene.

3.5.8.3 Characterisation of biosurfactants isolated from potential strains by FTIR analysis

Biosurfactants are surface-active extracellular molecules that facilitate the formation of biofilms on complex, thin-film surfaces. They encourage a strong interaction between the polymer's hydrophobic ends and the biofilm's hydrophilic compounds (Katemai et al., 2008). It reduces the interfacial tension and solubilises the toxic pollutants (E et al., 2013). The FTIR analyses of biosurfactants isolated from each strain proved their nature and the presence of functional groups.

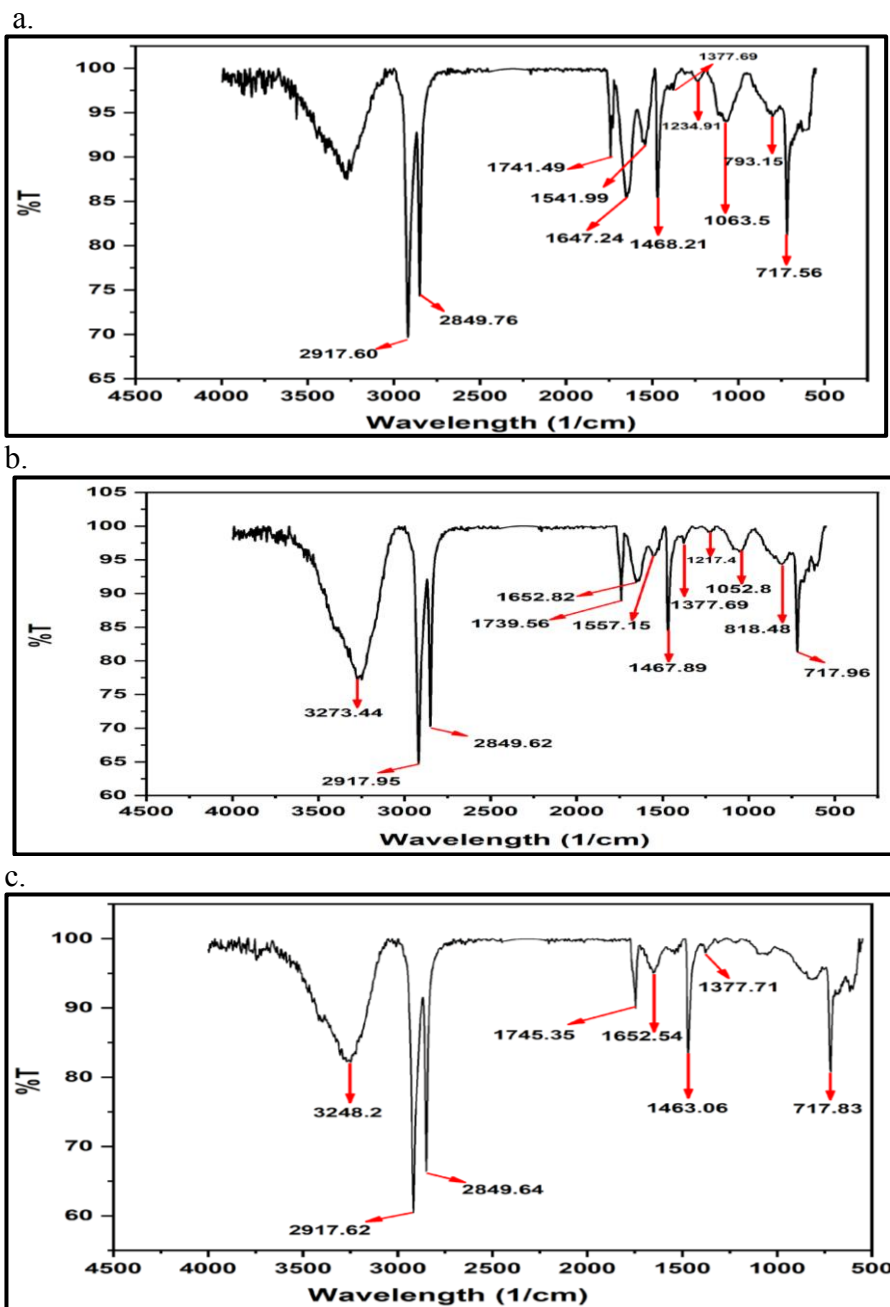


Fig.3.31 FTIR-ATR result of bio-surfactants of *Bacillus cereus* NJD1 (a), *Pseudomonas aeruginosa* NJD2 (b), and *Micrococcus luteus* NJD3 proving the presence of nitro-compound functional groups and ester functional groups.

Interestingly, all the strains' FTIR data showcased several very close peaks of amino and carbonyl ester groups (despite each strain showing slight variations in their transmission rates). The results were depicted in Table 3.8

S. No	Name of the absorption peak with its frequency range	Absorption peak in the FTIR spectrograph treated with bacterial strains			Functional group
		<i>Bacillus</i> sp	<i>Pseudomonas</i> sp	<i>Micrococcus</i> sp.	
1	C-H stretchings (2820-3000 cm^{-1})	2917.60 and 2849.76 cm^{-1}	2917.95 and 2849.62 cm^{-1}	2917.62 and 2849.64 cm^{-1}	alkanes
2	C=O stretching (1730-1750 cm^{-1})	1741.49 cm^{-1}	1739.56 cm^{-1}	1745.35 cm^{-1}	esters
3	N-H bendings (1580-1655 cm^{-1})	1647.24 cm^{-1}	1652.82 cm^{-1}	1652.54 cm^{-1}	amines
4	N-O stretching (1500-1550 cm^{-1})	1541.99 cm^{-1}	1557.15 cm^{-1}	-	nitro-compound
5	C-H bending (1455-1470 cm^{-1})	1468.21 cm^{-1}	1467.89 cm^{-1}	1463.06 cm^{-1}	alkanes
6	C-H bending (1375-1390 cm^{-1})	1377.69 cm^{-1}	1377.69 cm^{-1}	1377.71 cm^{-1}	aldehydes
7	C-N stretching (1018-1250 cm^{-1})	1234.91 cm^{-1}	1217.4 cm^{-1}	-	amines
8	C-O stretching (1050-1085 cm^{-1})	1063.5 cm^{-1}	1052.8 cm^{-1}	-	primary alcohol
9	C-H bending (700-830 cm^{-1})	793.15 and 717.56 cm^{-1}	818.48 and 717.96 cm^{-1}	717.83 cm^{-1}	alkanes

Table 3.8 Similarity in FTIR bands of extracted biosurfactants from isolated strains, with potential for biofilm formation on LDPE films.

The extremely identical fingerprints found in the isolated strains demonstrated the effectiveness of the strains attaching to the polyethylene surface. Moreover, reported molecular vibrations of all identified bacterial strains indicated the presence of amines, other nitrogen-containing groups, and carboxy ester groups, confirming the bio-surfactant's lipopeptide properties. These findings were supported by several earlier investigations (Thavasi et al., 2011; Varadavenkatesan & Murty, 2013).

3.6 Discussion

This study examined possible bacterial strains in soil samples contaminated with LDPE pieces collected from long-term landfills in Kerala. Samples were gathered from various Alathur, Laloore, and Bhramapuram plastic depots. Identifying site-specific physical factors enabled us to develop the inoculation media for initial enrichment and subsequent analysis. Numerous earlier scientific pilot studies have facilitated the identification of microbial adhesion on polymers steeped in a long-term heap due to continuous changes in climatic conditions (Harrison et al., 2014; Lear et al., 2021). The initial screening of potent bacterial species was performed by CBB flooding. De-staining around the colonies may occur due to the release of extracellular hydrolytic enzymes from the effective strains (Skariyachan et al., 2018).

In the initial analyses, the bacterial species OBA5, OBM3, and OLA2 treated-LDPE films showed a significant weight reduction in the polymer film. Additionally, we calculated the increased rate of daily polymer consumption and the reduction rate of the half-life span. Indeed, earlier researchers reported similar results, demonstrating the

capacity of bacterial species-enriched carbon-free medium to degrade LDPE films (Biki et al., 2021; Fang et al., 2024). However, our recent study presents compelling evidence that indigenous microbes in open landfills can spontaneously degrade non-pre-treated LDPE films at room temperature.

FDA tests on LDPE films in carbon-free conditions proved the presence of viable cells. One possible explanation for the FDA's breakdown of fluorescein into a coloured end-product may be the existence of extracellular enzymes such as proteases, lipases, and esterases (K. Tao et al., 2021).

The BATH test was conducted to demonstrate the increase in cell surface hydrophobicity resulting from prolonged interaction with highly hydrophobic compounds (R. Sarker et al., 2021). Due to the rise in cell surface hydrophobicity, the migration of strains to the organic phase of hexadecane (akin to LDPE used in this assay) demonstrated in this test resulted in a reduction in turbidity in the aqueous phase of the BATH solution. Our analysis revealed a 50% reduction in turbidity for the OBA5 bacterial species, followed by 47% and 45% reductions in turbidity for OBM3 and OLA2, respectively. It indicates the potential roles of isolated bacterial strains in biofilm formation on polymer surfaces. Several earlier investigators have demonstrated that the BATH test suggests the possible role of microbes in biofilm formation on polymers (Gupta et al., 2016).

The fundamental premise of the CO₂ release test is the biomineralisation of elongated linear hydrocarbons, resulting in the formation of chemicals such as carbon dioxide and water (Tantawi et

al., 2025). In our analysis over one month, the OBA5-treated LDPE films showed the highest CO₂ production rate (6.77 ± 0.07 g/L), followed by the OBM3 unit (6.3 ± 0.06 g/L) and the OLA2 unit (5.62 ± 0.26 g/L). In line with these results, a previous study documented a significant level of carbon dioxide (CO₂) generation via the treatment of *Bacillus subtilis* at 8.1 g/L and *Pseudomonas aeruginosa* at 8.86 g/L on low-density polyethylene (LDPE) materials following a four-month incubation period (Pathak & Navneet, 2017). Several prior studies have demonstrated that the generation of carbon dioxide (CO₂) indirectly indicates the microbial breakdown of low-density polyethylene (LDPE) molecules (Chamas et al., 2020; Tareen et al., 2022; Zhang et al., 2022).

After preliminary efficacy studies, the potential strains were identified through morphological analysis, culture characteristic studies, biochemical tests, 16S rDNA sequencing, and PMF analysis using the MALDI-TOF instrument. Finally, the strains were deposited to GenBank as *Bacillus cereus* NJD1 (access no. OLA823002.1), *Pseudomonas aeruginosa* NJD2 (access no. OL823063) and *Micrococcus luteus* (access no. OL823065).

To validate the biofilm formation, we conducted a total biomass count, a viable test, estimation of protein content, pH variation in media analysis, and extracellular polymeric substance analysis.

Initially, a biomass count of five days was conducted to prove the strain's ability to adapt to stressful conditions. Furthermore, the significance of biofilm formation for one month and four months by all three potential strains manifested their ability to attach to the

hydrocarbon chain. Many previous works have employed the biomass count method to demonstrate biofilm formation on the polymer under pressurised conditions in a mineral salt medium (Esmaeili et al., 2013; Maroof et al., 2022).

The biomass count quantifies the overall number of living and non-living microorganisms. In contrast, the viable count exclusively assesses the catalytically active strains (Davey, 2011) over an extended incubation period of 30 to 120 days. In the current analysis, the growth pattern of the isolated bacterial strains showed that all three strains exhibited a significant viability rate on the LDPE films. It indicates the strain's capabilities to adhere to polymer surfaces.

Quantifying proteins in surface-adherent biomass effectively establishes biofilm development (Haney et al., 2018). The protein estimation of all the potential strains demonstrated their adaptability to pressurised conditions and their ability to utilise hydrocarbons in the polymer as an energy source (Harshvardhan & Jha, 2013). Numerous previous studies have demonstrated this analysis as a key test for detecting solid biofilm formation on the recalcitrant surfaces of long-chain hydrocarbon compounds (Alaidaroos, 2023; Zhai et al., 2023).

Another validation study of the change in the pH of potential strain-provided media indicates the introduction of metabolites and exoenzymes into the LDPE films, which directs polymer degradation (Rong et al., 2024). The medium's pH drop or rise may be due to the nature of the inoculum added to LDPE films and the metabolites released to CFLMSM during incubation (Dey et al., 2020; Gong et al., 2023; Samanta et al., 2020). In our analysis, all three bacterial species

significantly reduced the pH of the medium during their incubation period. A recent study reported that *P. aeruginosa* can degrade polyethylene at pH 7 and temperatures between 30–35 °C (Mouafo Tamnou et al., 2021). Moreover, the acidic pH below 4.5 at 37°C-44°C may adversely affect the strain's ability to degrade LDPE compared to the alkaline conditions of the medium (Ma et al., 2023). Our experiment also showed reduced microbial activity on the LDPE films at pH levels below 4.5. All three strains have shown notable changes in the medium's pH, which validates the strains' metabolic activities in a carbon-free synthetic medium.

The EPSs mainly consist of polysaccharides, proteins, and DNA molecules (J. Ji et al., 2020). The production of such components on the LDPE film is strong evidence for the biofilm formation of bacterial strains on the polymer's surface (Soni et al., 2025). Moreover, EPS formation can protect microbial strain biofilms from environmental pressures (Saharan et al., 2024). Although all three strains in our study exhibit a significant amount of EPS production, *Bacillus* species produce a higher amount of EPS. A few earlier scientists demonstrated the EPS production for forming the microbial matrix, which provides stability and integrity for biofilm formation (Zineba et al., 2015).

The catalytic activities of the strains on LDPE films are a crucial investigation that proves the strong adherence of the isolated strains to the polymer surfaces. Even though the potential strains in our investigation were isolated from three different plastic dumps in various parts of Kerala, we could demonstrate the production of polyethylene-targeted enzymes, such as laccases (Jin et al., 2023),

lipases (Fecker et al., 2018; Timucin et al., 2016; Wilkes & Aristilde, 2017) and proteases (De Jesus & Alkendi, 2023), in all the potential bacterial strains qualitatively and quantitatively. Numerous scientists have indicated that the optimal pH for enzyme synthesis ranges from 4-9 (For laccases, it ranges from 4.5-6.5 (Akram et al., 2022; Shraddha et al., 2011); for lipases, it is between 4.5 and 7 (Ritu et al., 2022); and for proteases, it ranges from 4-9 (Abusham et al., 2009)). Our analysis revealed that the pH medium during incubation ranged from 4 to 7.6. However, the exact mechanism of all three extracellular enzymes produced by microbial strains, as well as their activities on LDPE films, remains unclear. Previous research studies have indicated that laccase enzymes decompose LDPE films by oxidising hydrocarbon chains, generating free radicals via electron transfer, and adding carbonyl groups, which weaken or scission the chain (Yao et al., 2022). Our validation studies demonstrated the presence of carbonyl groups by FTIR and micro-ATR-FPA imaging.

In the case of lipases, the enzyme has a lid-like structure, and the opening of this lid reveals hydrophobic regions in the active site, which facilitate catalysis at the lipid-water interface (Derewenda et al., 1992). Although the exact mechanism of the enzyme and gene responsible for its production is not precisely understood, the two genes responsible for lipase enzymes (LIP1 and LIP2) were identified in *Pseudomonas chlororaphis* using the primer targeting method (Mohan et al., 2022). In a recent study using the lipase-producing *Aspergillus niger*, it was demonstrated that a hydrophobic pocket near its active site can accommodate a polymer for biodegradation (Safdar et al., 2024). Our

study showed significant amounts of lipase enzyme production in the biofilm inoculated with *Bacillus* sp., *Pseudomonas* sp., and *Micrococcus* sp., given as the inoculum. Ester bond stretching and its metabolic products were demonstrated in our validation experiments.

The direct role of protease enzymes remains unclear. A few scientists have demonstrated its indirect role in facilitating bacterial colonisation on recalcitrant surfaces and introducing nitrogen-containing functional groups (Cabiglieria et al., 2025). The FTIR result in the validation chapter of our investigation proved this mechanism of the protease enzyme.

Moreover, our research demonstrated the presence of lignin peroxidase (LiP) and manganese peroxidase (MnP) enzymes in *Pseudomonas* sp. (Gkoutselis et al., 2021; Maisto et al., 2024). A small amount of esterase enzyme production is found in *Micrococcus* sp. An earlier study reported that both LiP and MnP enhance the activity of laccase enzymes (multi-copper oxidases), thereby accelerating the oxidation of synthetic polymers (A. Kumar & Chandra, 2020). Esterases hydrolyse simple esters, such as triglycerides (X. Liu et al., 2021). It may be an indication of the mineralisation of the LDPE backbone.

Many have reported the importance of the presence of biosurfactants in potential microbes forming robust biofilms on recalcitrant compounds. According to (Eras-Muñoz et al., 2022), bi-surfactants in polymeric compound biofilms are surface-active agents that can lower surface tension and contribute to the emulsification and biodegradation of hydrophobic substances. They have hydrophilic and hydrophobic moieties that demix at liquid-liquid, liquid-gas, or liquid-solid

interfaces (Santos et al., 2016). Our current study screened bio-surfactant molecules from all competent strains identified for LDPE biodegradation. We performed an oil displacement test with petrol and emulsification assays using petrol, diesel, and engine oil to determine the presence of biosurfactants. Additionally, we isolated and characterised the nature of biosurfactants using FTIR analysis and demonstrated their lipoprotein nature.

3.7 Conclusion

The current study highlights the presence of highly potent bacterial species in the samples from long-term garbage sites that can form robust biofilms on the polymer. The preliminary tests, including weight reduction, FDA testing, BATH analysis, and CO₂ evolution test, provided reliable evidence for robust biofilm formation on polymer strips. The current study reported a high rate of weight reduction in non-pretreated LDPE films treated with isolated strains under a stressed condition of CFLMSM at room temperature in a static environment. Among the bacterial strains screened from landfill soils, OBA5 from the Alathur plastic dump showed the maximum weight reduction at $42.75 \pm 1.105\%$, followed by OBM3 ($40.22 \pm 2.04\%$) isolated from the Bhramapuram garbage heap and OLA2 ($39.7 \pm 1.27\%$). All confirmatory studies of capable organisms were conducted using the static culture method in carbon-free media.

Furthermore, the extensive biofilm formation on LDPE films was assessed by conducting total cell counts, FDA tests, protein estimations, pH changes in the medium, and EPS production tests. The presence of bacterial enzymes in all three strains (Laccases, lipases,

proteases, manganese peroxidases, lignin peroxidases, and esterases) was demonstrated both qualitatively and quantitatively to identify the metabolic activities of the bacterial strains in degrading the compound. Moreover, we analysed the presence of bio-surfactants in all the enriched bacterial cultures using oil displacement and emulsification tests. Thereafter, the isolated bio-surfactants were characterised by FTIR analysis, and the FTIR data proved the nature of bio-surfactants as lipoproteins in all strains.

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CHAPTER 4

VALIDATION OF LDPE BIODEGRADATION USING COMPETENT STRAINS SCREENED AFTER PRELIMINARY SCIENTIFIC INVESTIGATION

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4.1 Introduction

The environmentally friendly and secure biodegradation of LDPE film is a primary objective of the contemporary scientific community. The consumption and entanglement of harmful plastic pollutants by marine creatures, including dolphins, seabirds, turtles, and fish, lead to significant ecological degradation. Consequently, global researchers are actively engaged in this topic to enhance microbial activity for the bioremediation of persistent contaminants. Recent systematic reviews have highlighted the scientific methods for demonstrating LDPE biodegradation (Kumar Sen & Raut, 2015; Matjašič et al., 2021). Most validation studies focus on deviations in the mechanical, structural, thermal, and chemical properties of LDPE films before and after microbial degradation.

4.1.1 Mechanical deviations of LDPE films due to microbial attack

Several biodegradation studies have focused on reducing the thin film's mechanical properties to demonstrate the microbes' ability to assimilate the compound (Khruengsai et al., 2023). Numerous biodegradation studies have employed the Instron Universal Mechanical Testing System (UMTS) to assess the mechanical properties of polymer samples.

4.1.2 Instruments to Detect Structural Stability Analysis of LDPE Films

Techniques such as XRD analysis can be used to determine structural changes, specifically changes in crystallinity, in LDPE films (Depan et al., 2021). This technique can manifest the difference in

crystallinity in the LDPE strips due to the action of microbes. As the crystallinity decreases, the surface becomes increasingly amorphous, which enhances the degradation capabilities of the strain (Adithama et al., 2023). A polymer's crystallinity significantly impacts its physical properties, including tensile strength, modulus, hardness, stiffness, melting point, and resistance to cracking (Lin et al., 2020).

Another technique is using a TGA-DTA instrument for thermogravimetric analysis of polyethylene films. This instrument determines the thermal stability of LDPE films and displays a degradation curve between 450-480°C in non-degraded films (Bockhorn et al., 1999). The deviation in thermal stability indicates a difference in the polymer's structure (Kojnoková et al., 2021).

4.1.3 Surface topography analyses

The colonisation of microbial strains on the surface of the polymer modifies its topography. It is primarily because of the enzymes and other metabolites produced and released by the microbes. Fluorescent microscopy (Morales Ramos et al., 2024; Tribedi et al., 2015), SEM with EDAX analysis (Skariyachan et al., 2022), and AFM (Muangchinda & Pinyakong, 2024) were employed to analyse the grooves, pits/cracks, and other modifications induced on the surface due to biofilm formation. For SEM analysis, films of LDPE were coated with an electrically conducting material (gold, silver, or platinum) via sputtering. The coatings also enabled the low-conductivity material surface to emit secondary electrons, which could be reflected to generate high-resolution images (Kwiecińska et al., 2019). Similar topography analysis techniques, such as AFM, provide

high-resolution nanometric imaging and quantitative measurements of surface changes due to microbial adhesion (Beaussart et al., 2014).

4.1.4 Validation methods to demonstrate chemical changes in the polymer due to the adherence of microbes

FTIR (Fourier Transform Infrared Spectroscopy) analysis is the most crucial validation method for demonstrating chemical changes in microbial-treated LDPE films. This non-destructive approach generates vibrations using infrared energy across the polymer's molecular bonds. FTIR analysis data can be utilised to compute carbonyl and double bond indices, as well as polymer crystallinity. Numerous earlier studies have shown that the absorbance at 4000 cm^{-1} reflects the formation of carbonyl bonds, whereas alterations in the C–H alkane group are observed around $2750\text{--}3000\text{ cm}^{-1}$ (D. Das & Dutta, 2022; Fattouh et al., 2019). Furthermore, degraded fractions with -OH groups were suggested at 1000 and 1700 cm^{-1} ; the development of ketone or aldehyde C=O groups was depicted at $1710\text{--}1750\text{ cm}^{-1}$, and the presence of CH_2 at 1465 cm^{-1} further illustrated the impact of bacteria on polymers.

Plastic polymer degradation produces gaseous end products, which can be analysed using GC-MS (Mazaheri & Nazeri, 2023). The technique can also verify the polymer's chemical homogeneity and determine the chemical structure, molar mass, and weight distribution of the end groups. Several previous studies have demonstrated the production of acids, aldehydes, fatty acids, alkenes, and alkanes as end products (M. Sangale et al., 2019).

A few studies have demonstrated the chemical changes associated with biofilm formation using XPS (X-ray photoelectron spectroscopy), ESI-MS, and NMR analysis.

4.2 Aim and Objectives

1. Validate the topographic changes observed on LDPE films resulting from the interactions of potential bacterial strains screened from landfill soil samples using SEM, SEM-EDAX, AFM analysis, and water contact angle measurements. Assess mechanical strength reductions in LDPE films using tensile testing to confirm bacterial degradation.
2. Characterise the chemical changes in the LDPE films (both sample and control) using FTIR and FTIR-ATR-FPA imaging analysis and compare the results with those of the control films to assess the strain's efficacy in biodegrading the recalcitrant compound within 120 days in a carbon-devoid mineral salt medium.
3. Identify the catalytic end products of bacterial metabolism using GC-MS chromatography analysis.

4.3 Review of Literature

The bacterial adhesions on the LDPE films indirectly reveal the potential of microbial strains to utilise recalcitrant compounds like LDPE as an energy source. Based on several recent studies, validation of LDPE degradation involves scanning electron microscopy, FTIR, and GC-MS analysis. In many early studies, tensile testing, FTIR, XRD, and SEM analysis of the polymer films demonstrated the

degradation of polymers (Kumar Sen & Raut, 2015; Pathak & Navneet, 2017). Several previous studies have employed different analytical techniques to demonstrate biofilm formation and its efficacy in biodegradation.

S. No	Techniques used	Changes in the LDPE films	References
1	Water contact angle	Contact angle reduction	(Awasthi et al., 2017)
2	Tensile strength	Mechanical strength reduction, modification of tear strength, and elongation	(Nwuzor et al., 2023)
3	XPS	Surface chemical changes by oxidation and carbonyl group formation	(Di Giulio et al., 2024; Dorey et al., 2018)
4	CHNS analysis	Thickness difference in the LDPE film	(R. Moharir & Kumar, 2021)
5	SEM	Surface damages- pits, holes, and erosions	(Khan et al., 2023)
6	FE-SEM	Surface erosions, cavity size, and more changes in surface topography	(Kim et al., 2024)
7	SEM-EDAX	Significant changes in elemental compositions	(Febria et al., 2024; Jebashalomi et al., 2024)
8	AFM	Surface roughness parameters	(Li et al., 2020)
9	FTIR	Structural changes in the surface by calculating carbonyl (ester and keto) and internal double bond indices	(R. V. Moharir & Kumar, 2019; Pramila, 2011)
10	FTIR-ATR	High spatial resolution is needed to detect chemical and molecular changes in the surface.	(Campanale et al., 2020; Hedir et al., 2022)

11	FTIR IMAGING	Chemical structure and molecular modification in the LDPE films	(J. Yang et al., 2014)
12	GPC-HT	The molecular weight of LDPE films	(Nguyen et al., 2024)
13	GC-MS	Degradation products were Analysed	(M. K. Sangale et al., 2019)
14	GC-FID	End products of polymers	(Shahnawaz et al., 2016; Skariyachan et al., 2016)
15	NMR	Changes in physicochemical properties	(Menzel et al., 2022)
16	ESI-MS	Chemical compounds formed by biofilm formation on the LDPE films	(Ghatge et al., 2020)

Table 4.1 Previous studies illustrate techniques for demonstrating LDPE biodegradation through microbial attachment.

Several scientists utilised various physical pre-treatments (heat, UV treatments, gamma radiation, steaming, and plasma treatments (Sanchis et al., 2006; Tribedi & Dey, 2017) and chemical treatments (ethyl methyl sulfonate, nitric acid treatment and nitro-methyl guanidine) (Chaudhary et al., 2021; Rani et al., 2021) to achieve better results in the validation study of LDPE biodegradation. However, several previous validation studies have demonstrated that pre-treatments can introduce different functional groups (such as carbonyl and nitro groups) and alter internal bonds in the inert carbon backbone, often resulting in reduced hydrophobicity (Albertsson et al., 1998; (Orr) et al., 2004). Therefore, many recent scientists have used non-pre-treated LDPE films for validation studies (Dey et al., 2020).

4.4 Methodology

4.4.1 FE-SEM analysis

The bacterial-treated LDPE strips, incubated for 120 days, were collected and surface-sterilised with a 2% sodium dodecyl sulphate solution in 150 mL conical flasks. Then, they were shaken continuously (REMI RS-12R Mini Rotary Shaker) for five hours. Afterwards, the LDPE films were collected from the SDS solution, placed in a beaker containing distilled water, and shaken for an additional four hours to remove the biofilms attached to the polymer films. It was then washed three times with 70% ethanol, and subsequently, these films were repeatedly washed in distilled water and kept in a digital hot air oven (Relitech, 42L, 350×350×350 mm) for one day in sterilised Petri dishes at 50°C. Gold nanoparticles (thin layer) were sputter-coated onto the strips in a vacuum after they were sliced into 1 × 1 cm pieces. To visualise the surface erosions, the samples (both controls and test samples) were placed on the sampling port of the ZEISS-FESEM (Field Emission Scanning Electron Microscope), and images of the polymer films were taken for comparison.

4.4.2 SEM-EDAX analysis

In our current investigation, EDAX analyses were conducted with SEM analyses using an energy-dispersive spectrometer (SEM-EDAX Jeol 6390LA/OXFORD XMX N). For this, bacterial-inoculated and bacterial-free LDPE films were utilised. This analysis used high-energy X-ray beams to bombard LDPE films, obtaining chemical

fingerprints. Furthermore, the AztecLive 6.0 EDS Software aids in determining the weight and atomic element percentages of nitrogen and carbon.

4.4.3 Atomic force microscope imaging

Surface damages on the exterior layer of LDPE films were assessed using Raman-AFM imaging with a Zeiss Axioscope and a WITEC alpha 300 RA (tapping mode) (WITEC GmbH, Ulm, Germany). After 120 days, all the LDPE films from the carbon-devoid liquid broth were withdrawn and surface sterilised with a 2% SDS solution, a 70% ethanol solution, and distilled water. Then, dried films at 60 °C were used for the 2D and 3D analyses. Furthermore, surface roughness parameters were calculated using WITEC software.

4.4.4 Measurement of contact angle

A droplet of water was applied to LDPE strips obtained from either control or bacterial-treated conical flasks and subjected to surface sterilisation to evaluate surface modifications. The water contact angle was measured using a video-based optical instrument, the OCA 15 plus model from GMBH, with SCA20 software. To assess the change in surface hydrophobicity on both control and treated LDPE films (Chen et al., 2025), water contact angle images were obtained within 5–10 seconds.

4.4.5 Tensile strength testing to detect the change in mechanical strength of the polymer film

The sample and control of 6.5 x 1.3 cm LDPE sheets were collected separately from CFLMSM after 120 days. The surface was sterilised

with SDS, ethanol, and distilled water and then dried for 16 hours for this analysis. ASTM D882-18 prepared the specimens. The experiment utilised a Tinius Olsen UTM (Universal Testing Machine) - H50KL, with a 50 kN capacity, to test the mechanical strength of LDPE films. Individual LDPE films were placed on the sample holders and stretched at a speed of 3 mm per minute. The Horizon software of the tensile instrument was used to calculate mechanical strength parameters, including ultimate tensile strength, percentage of elongation at break, and shear strength of LDPE films.

4.4.6 Infrared Fourier-transform spectroscopy (FTIR)

4.4.6.1. Determination of spectral data and computation of indices

The LDPE films were aseptically removed from CFLMSM after four months of incubation with (test) or without bacterial inoculation (control), and then surface-sterilised following the protocol (Kyaw et al., 2012). Then, these films were spectrally studied using an ATR-FTIR Shimadzu. Subsequently, each polymer film was sectioned into 1 × 1 cm fragments and scanned across the wavelength range of 4000–500 cm⁻¹ (Cavallo et al., 2020). The results were presented independently for each set of samples and control using the OriginPro 2018 (64-bit) program, showing the transmittance percentage versus wavelength in cm⁻¹. Using FTIR spectral data, carbonyl (Keto and ester carbonyl) and internal bond indices (Vinyl or double bond) were calculated, and the results are illustrated in the Origin graph.

4.4.6.2 Micro-ATR-FPA analysis

We examined the spectrochemical imaging of LDPE films using a micro-ATR/FTIR imaging device (Bruker Focal Plane Microscope, Hyperion 3000, with a micro-ATR/FTIR system). We captured and compared pictures of the focal plane array (FPA) of both test and control films.

4.4.7 Gas chromatography-mass spectrometer analysis of LDPE films

LDPE films (2.5×2.5 cm) (ten films with an average weight of 0.3 g) were taken from a mineral salt medium and cut into small pieces after removing the biofilm. Place all the ingredients in a beaker and stir with 12 mL of chloroform. The extracts, which had evaporated at room temperature, were then ultrasonically agitated, and the mixture was kept at 60°C for three hours. Next, combine two millilitres of chloroform with each extract and filter it with a PTFE filter (0.45 μ m). The detection of degraded products was performed on a hybrid GC-MS instrument (Shimadzu model QP2010S) using a Rxi-5Sil MS column with helium as the carrier gas. Spectral lines were obtained by using GC-MS Solutions software.

4.5 Results and Discussions

We confirmed the efficacy of bacterial strains isolated from open plastic dumps in degrading polymers using various validation experiments. Statistical significance was assessed using one-way ANOVA with $p < 0.05$ for all experiments using GraphPad Prism software.

4.5.1 FE-SEM analyses

In the current investigation, the FE-SEM micrograph of all three potential organisms exhibited biofilm formation after 90 days of incubation. Furthermore, up to 120 days of incubation revealed several surface erosions, grooves, fissures, and pits in the film. The changes in bacterial-inoculated LDPE films kept in mineral salt broth (MSB) for 90 and 120 days were compared with those in control films without bacterial inoculation. Our research found no noticeable structural alterations in the control films incubated in the carbon-devoid medium for 90 and 120 days.

4.5.1.1 FE-SEM imaging using *Bacillus cereus* strain-treated LDPE film

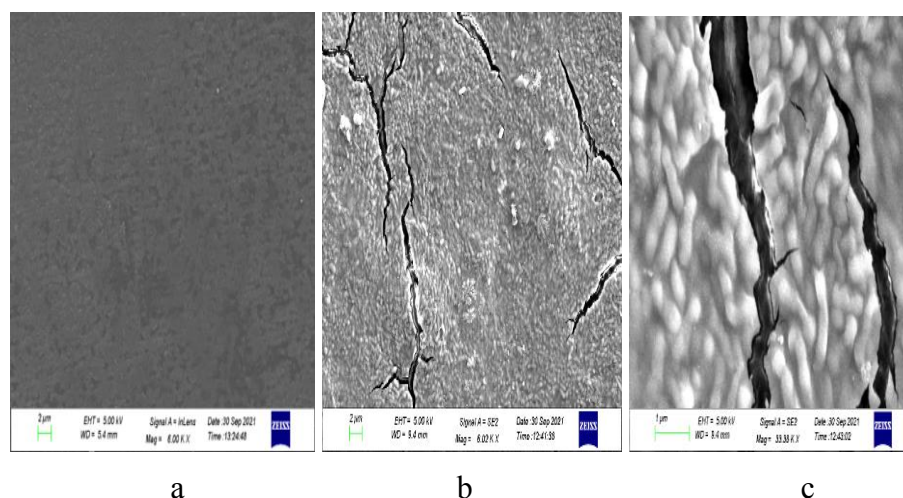


Fig. 4.1(a) FE-SEM images show *Bacillus cereus* NJD1-induced deep fissures, pits, and biofilms on LDPE films inoculated in a synthetic carbon-free medium after 120 days (images b and c), along with a control film without any bacterial adhesion (image a).

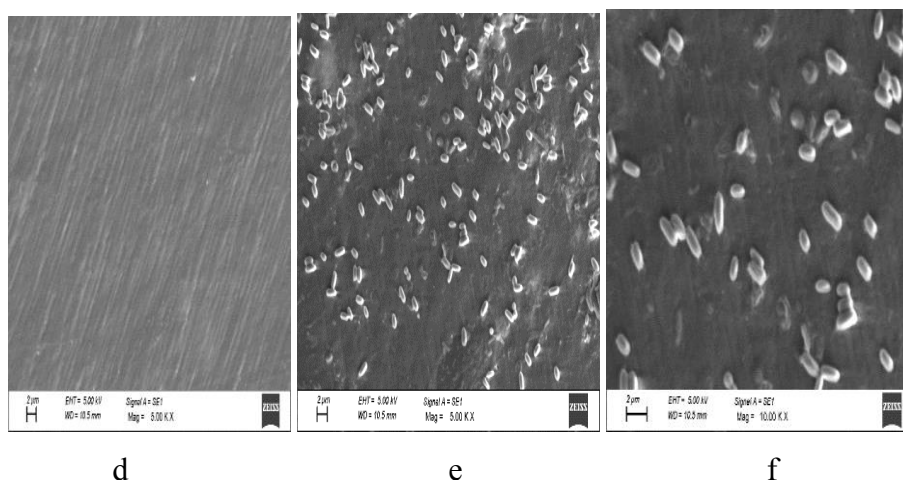


Fig.4.1(b) The micrograph (FE-SEM) of rod-shaped *Bacillus cereus* NJD1 demonstrates bacterial attachments after 90 days of incubation. Image a. Control images b and c. Different focuses of LDPE films with biomass attachment.

4.5.1.2 FE-SEM micrographs of polyethylene strips inoculated with *Pseudomonas* sp.

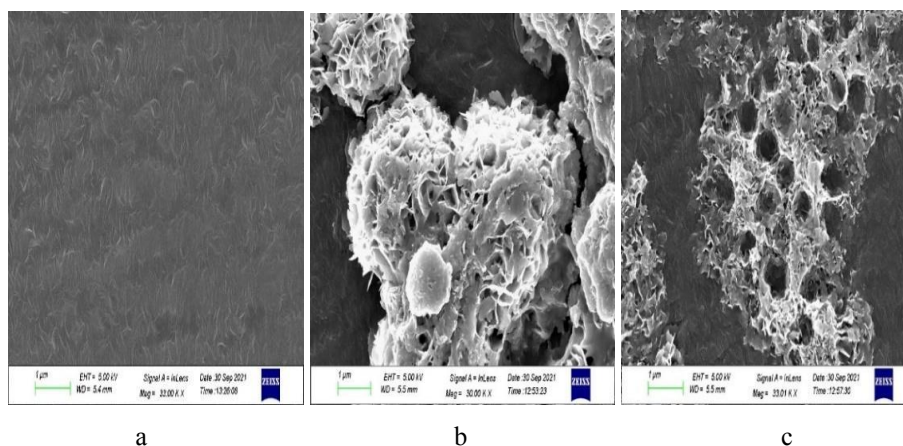


Fig.4.2(a) FE-SEM micrograph showing structural changes on the LDPE film due to the catalytic activity of *Pseudomonas aeruginosa* NJD2 after 120 days of incubation at higher magnification (30,000 x and 33,000 x). Images b and c show magnified bacterial-treated LDPE films, which show structural modifications such as flake-flower-like formations, pits, erosions, grooves, and fissures. Image A depicts the control film without bacterial inoculation.

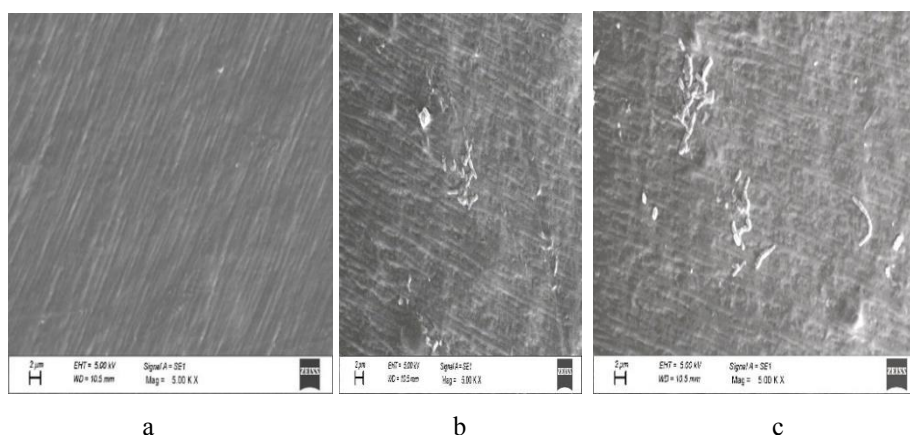


Fig.4.2(b) The scanning electron microscopy image displays surface colonisation and penetration of rod-shaped *Pseudomonas aeruginosa* NJD2 after 90 days of incubation at 5,000x magnification. a. A control film without any bacterial attachment. b. SEM micrograph of focus 1 of LDPE film with bacterial colonies. Image of focus 2 of LDPE film attached with *Pseudomonas* sp

4.5.1.3 FE-SEM experiments with *Micrococcus luteus*

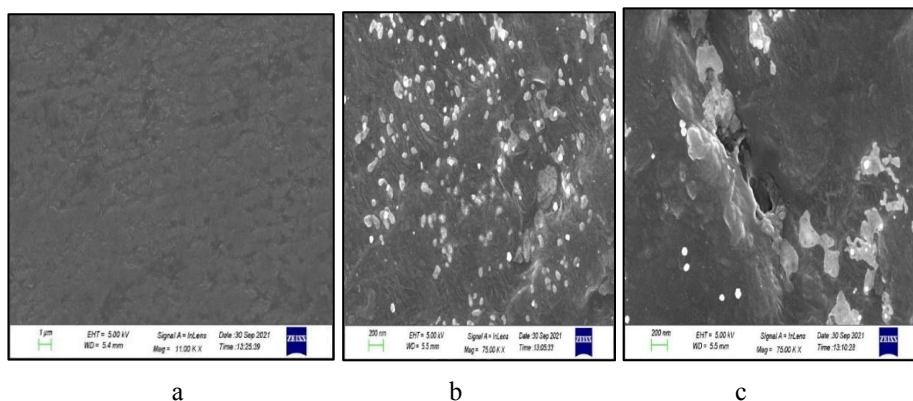


Fig 4.3(a) The FE-SEM micrograph of *Micrococcus luteus* NJD3-treated LDPE films after 120 days. Image A shows the control film, and images B and C indicate surface irregularities, including deep fissures and inner-layer colonisation due to biofilm formation by *Micrococcus luteus* NJD3.

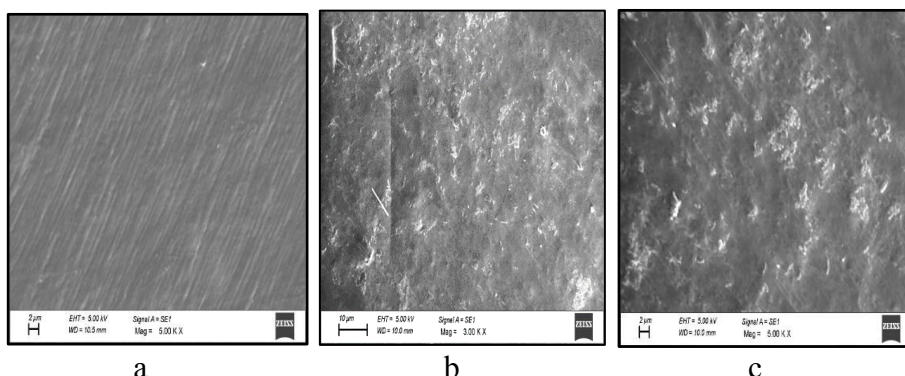


Fig.4.3(b) FE-SEM micrograph showing bacterial attachments on the LDPE films inoculated in CFLMSM for three months (90 days). a. Control LDPE film devoid of bacterial attachment. b. shows the firm attachment of the coccoid-shaped *Micrococcus* colonies. A 5000x magnified SEM image of biofilm at 90 days of incubation.

4.5.2 SEM-EDAX analysis

Our study utilised surface-sterilised LDPE films obtained after 120 days of incubation in enrichment broth for SEM—EDAX analyses to detect elemental changes (primarily carbon and nitrogen) and deterioration. LDPE films treated with all three organisms showed significant changes in their carbon and nitrogen elemental compositions, as measured in weight per cent (wt%) and atomic weight per cent (atomic wt%).

The EDS table calculated using Aztec Live 6.0-EDS software clearly showed the elemental changes of C and N elements in the case of *Bacillus* sp-treated films compared to the control film. In the control LDPE films, the carbon element's weight and atomic weight percentages were 100% and 0 % for the nitrogen element. However, the *Bacillus*-treated LDPE films displayed a weight percentage of 61.15% carbon and 38.85% nitrogen, respectively. It confirms the strong adherence of isolated *Bacillus cereus* NJD1 to the long-run depot on LDPE films (See Fig. 4.4(a)).

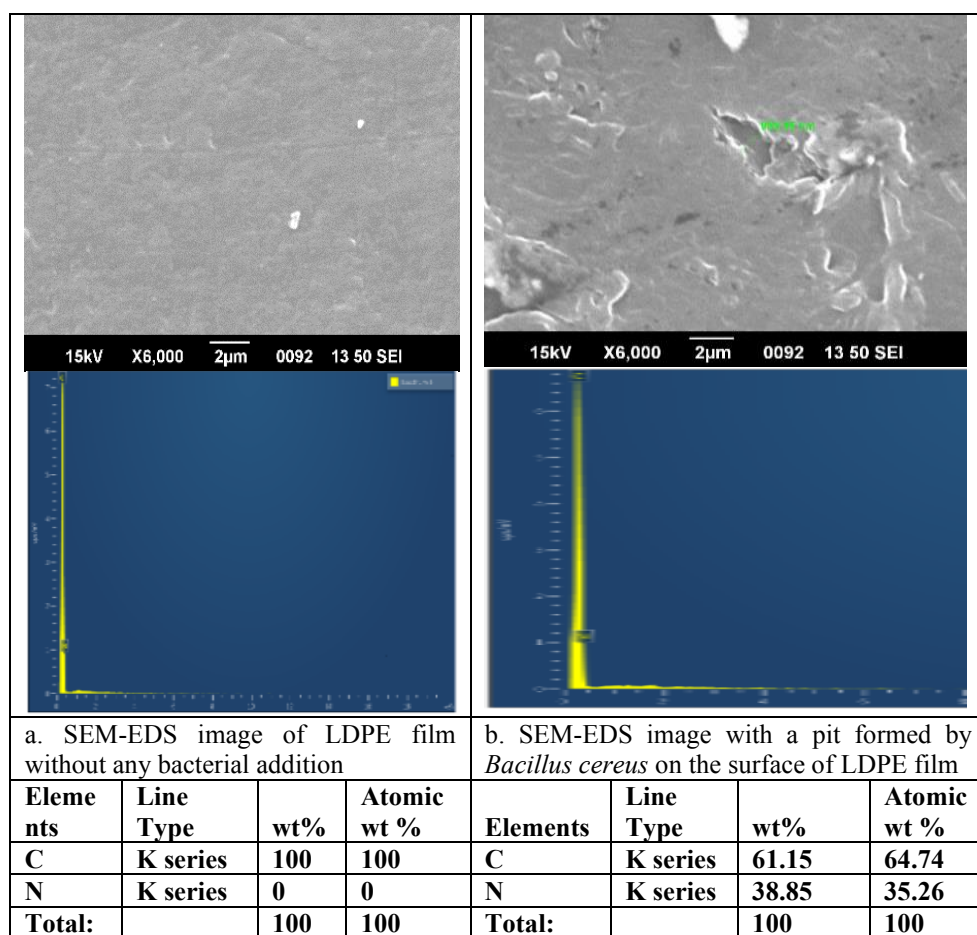


Fig.4.4(a) SEM-EDS analysis of LDPE film treated with *Bacillus cereus* NJD1. The SEM images revealed structural details, and the EDS analysis indicated elemental changes (see tables) (Jasmin Vijitha et al., 2023) on the surface of the LDPE films after four months of incubation (Mohammadi et al., 2022).

In the investigation of *Pseudomonas*-treated LDPE films, carbon elements' weight percentage (wt%) and atomic weight percentage (atomic wt%) changed from 100% to 60.66% and 64.26%, respectively. Similarly, the wt% and atomic wt% of nitrogen elements increased from 0% to 39.34% and 35.7%, respectively. In addition, during SEM analysis, we observed several structural aberrations,

including pits, fissures, and cracks, on the LDPE films incubated for four months in the carbon-free medium. These changes firmly support the capabilities of *Pseudomonas aeruginosa* to degrade polymer strips.

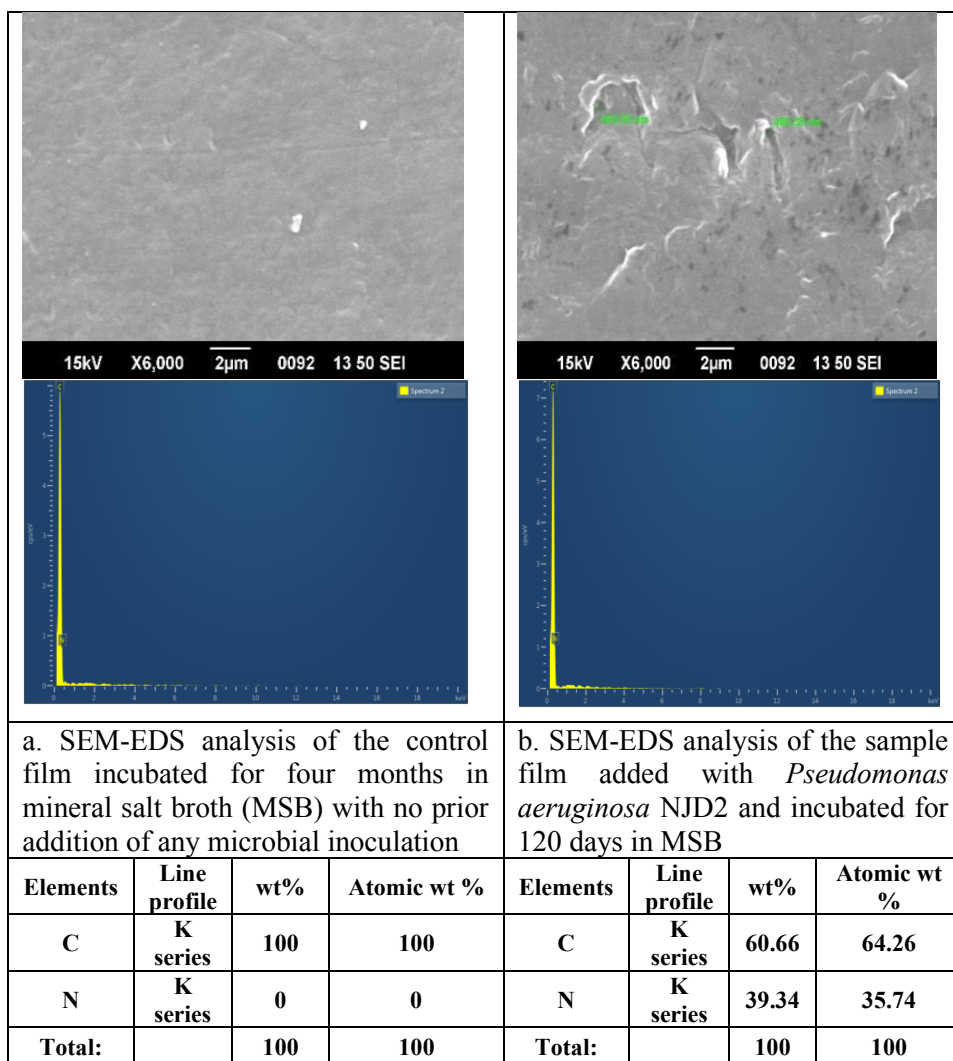


Fig.4.4(b) SEM-EDS imaging of LDPE film pieces inoculated with *Pseudomonas aeruginosa* NJD2. The SEM images revealed surface structural abnormalities, including pits up to 453.2, compared to the control. Additionally, EDS analyses showed elemental changes in carbon and nitrogen.

Like the other analysis, SEM-EDAX analyses of *Micrococcus luteus* NJD3-treated LDPE strips have also shown distinct structural and elemental changes in nitrogen and carbon elements compared to the control film's SEM-EDAX test.

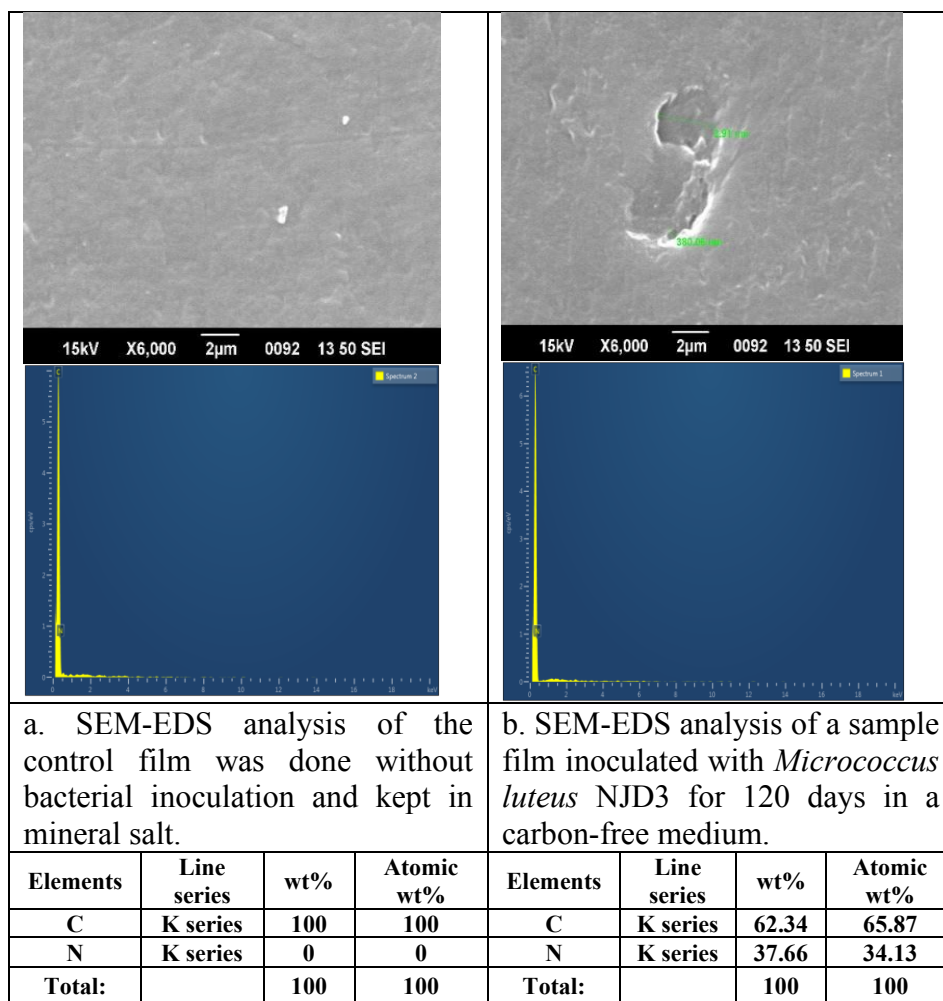


Fig.4.4(c) SEM-EDS micrograph of LDPE films treated with *Micrococcus luteus* NJD3 and control LDPE films without bacterial inoculation. The elemental changes in carbon and nitrogen were calculated using EDS software and are depicted in the tables.

In this analysis, the carbon element weight percentage (wt%) decreased from 100% to 62.34%, and the atomic weight percentage (atomic wt%) decreased to 65.87%. Meanwhile, the nitrogen element wt% increased from zero to 37.66%, and the atomic wt% % (atomic weight percentage) increased from zero to 34.13%.

Carbon reduction in all three bacterial strains demonstrates their ability to cleave carbon chains in polymers. At the same time, the increase in nitrogen may result from the development of a functional biofilm on the polymer or the adhesion of metabolites to its hydrocarbon backbone during biodegradation (Skariyachan et al., 2021). In contrast to previous records (Eriksen et al., 2013; Skariyachan et al., 2018), our study found elevated elemental changes in the EDS spectra. It highly recommends the potentialities of isolated strains for the biodegradation of LDPE films.

4.5.3 AFM analysis

AFM is a precise technique for visualising and quantifying topographic changes and surface unevenness (Dufrêne, 2002; Sridharan et al., 2021). In our current investigations, all three potential bacterial species-treated LDPE films showed escalated roughness variables (surface roughness mean data (S_a), maximum surface height (S_z), roughness in root mean square (S_q), maximum height of peak (S_p), and maximum depth of pit (S_v)) compared with the control samples. Control LDPE films showed minimal roughness changes, confirming bacterial-induced alterations.

4.5.3.1 AFM analysis of LDPE film inoculated with *Bacillus cereus*

NJD1

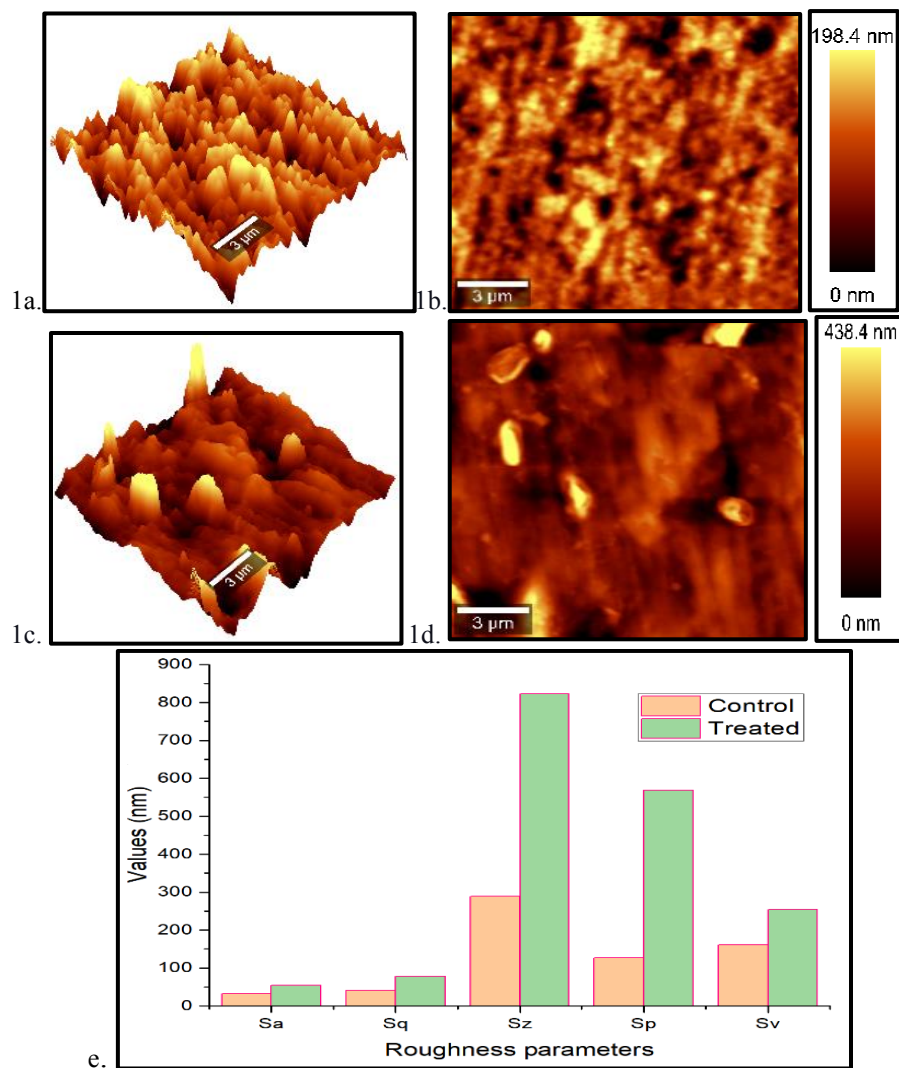
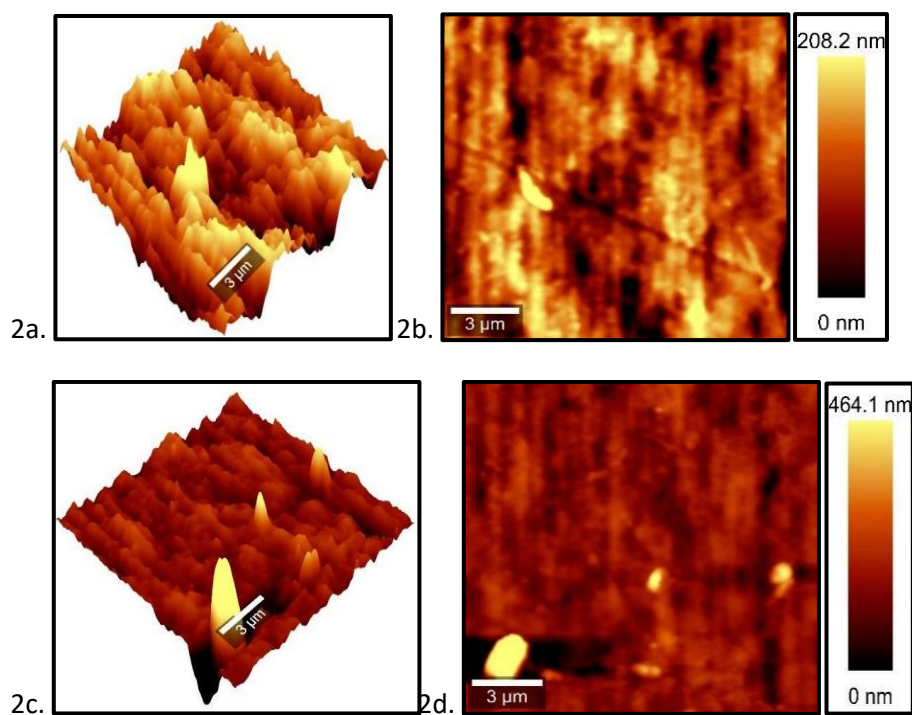


Fig.4.5(a) AFM data of *Bacillus cereus* NJD1-inoculated LDPE film after 120 days of incubation. AFM-3D and AFM-2D images of the control were shown in images 1a and 1b. Images 1c and 1d displayed AFM-3D and AFM-2D of the sample. Image 1e shows a graph of the roughness parameters from the WITEC software of the AFM instrument.

The physical alterations caused by *Bacillus* biofilms were evident in the AFM micrographs of LDPE films (see Fig.4.5(a)). All computed roughness parameters were higher in the samples treated with the *Bacillus* inoculum compared to the non-treated samples with the *Bacillus* strain. It indicates the formation of several surface depressions on the polymer due to the firm attachment of *Bacillus cereus* NJD1.

4.5.3.2 Atomic force microscopy images of *Pseudomonas aeruginosa* inoculated LDPE films

Compared to control LDPE films, the high roughness parameters of *Pseudomonas*-treated LDPE films revealed pits, fissures, and corrosion formation on the LDPE film surface.



e.

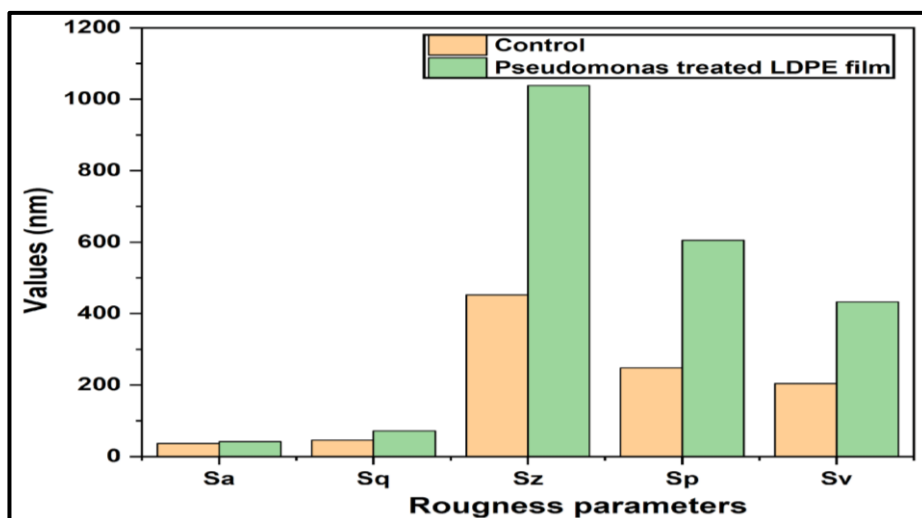
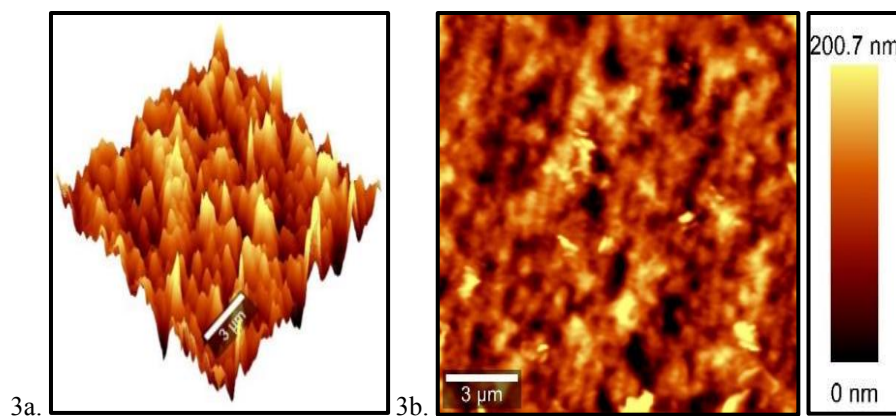


Fig.4.5(b) AFM micrograph reveals topographic changes on LDPE strips resulting from treatment with isolated *Pseudomonas aeruginosa* NJD2 over 120 days in CFLMSM. Images 2a and 2b are AFM-3D and AFM-2D of LDPE film without bacterial inoculation, and Images 2c and 2d depict AFM-3D and AFM-2D of *Pseudomonas*-treated LDPE film. Image 2e (Origin graph) represents the roughness variables of control and sample LDPE films, indicating the effective biofilm formation on the polymer.

4.5.3.3 AFM micrograph of *Micrococcus luteus* treated LDPE film



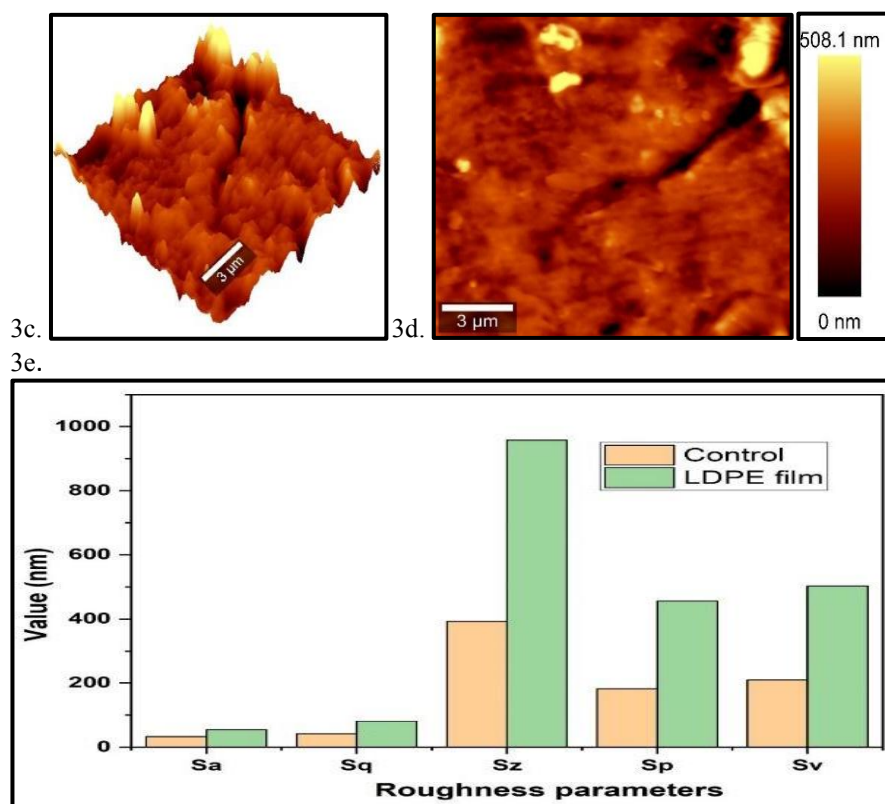


Fig.4.5(c) AFM micrograph and increase in surface roughness by adding *Micrococcus luteus* after 120 days of incubation in CFLMSM. The 3D and 2D AFM micrographs illustrate a comparison of LDPE film without bacterial strain addition (control) (Images 3a and 3b) and LDPE film with the addition of *Micrococcus luteus* NJD3 strain (Images 3c and 3d). Image 3e illustrates the unevenness of the LDPE film's surface.

The observed variations in surface topography between the 3D and 2D AFM images of LDPE films with *Micrococcus luteus* NJD3 and without bacterial inoculation validated the strain's effectiveness in the biodegradation process. Furthermore, we have identified an increase in the sample's surface roughness variables in the bacterial-treated LDPE using statistical data from the AFM software (WITEC).

4.5.4 Water Contact Angle (WCA) Assessments of LDPE films treated with potential strains



Fig.4.6(a) WCA measurements of *Bacillus cereus* NJD1. a. Video captured images (five in number) of LDPE films without bacterial addition, inoculated in the synthetic, carbon-free medium for 120 days. b. WCA images of *Bacillus* strains inoculated on LDPE films.

In our test, the contact angle for the control films of the first set of experiments, both with and without inoculation of *Bacillus cereus* NJD1, was $91.06 \pm 1.13^\circ$. For the isolated treated films, it was $67.63 \pm 0.82^\circ$. However, our study with *Bacillus cereus* NJD1 showed a decreased contact angle without pre-treatments on the LDPE films.

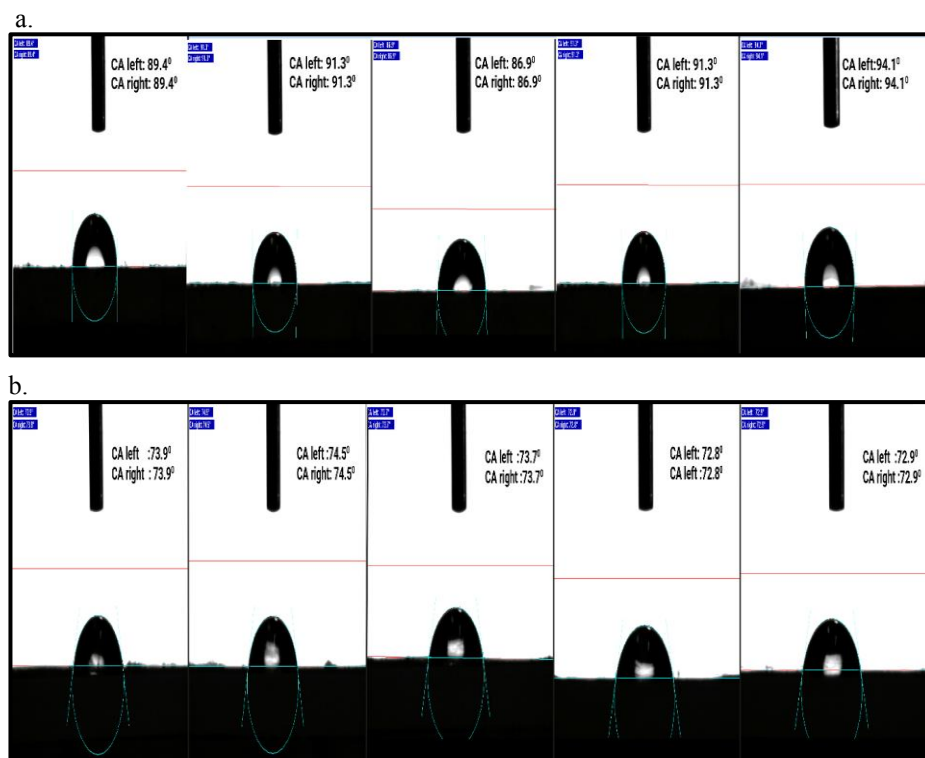


Fig.4.6(b) Demonstration of LDPE biodegradation by measuring the water contact angle after inoculating with LDPE strips with *Pseudomonas aeruginosa* for 120 days. a. WCA observations of control films without bacterial attachment. WCA images of *Pseudomonas aeruginosa* NJD2 treated LDPE films.

The WCA result of the *Pseudomonas*-treated LDPE film after 120 days was $73.56 \pm 0.32^\circ$, and the control film was $90.54 \pm 2.64^\circ$. The reduction of the water contact angle by *Pseudomonas aeruginosa* colonisation provides strong evidence for biofilm formation. Furthermore, these attachments reduced hydrophobicity and introduced wettability, facilitating subsequent colonisation and degradation of polymers.

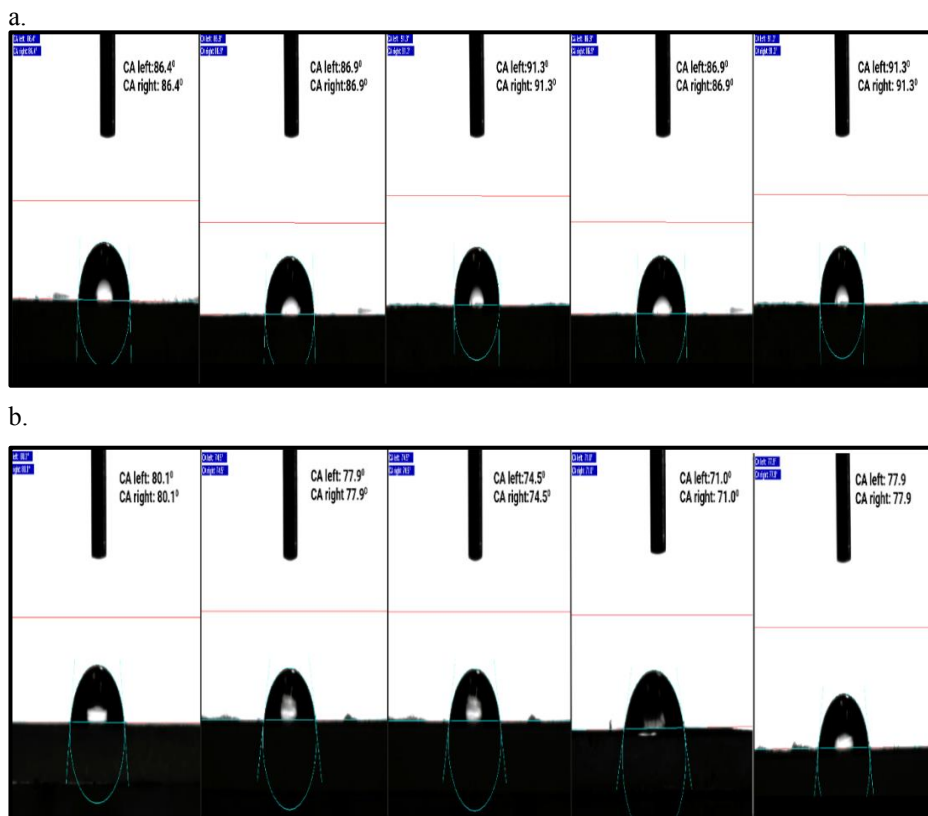


Fig.4.6(c) WCA measurements captured by a video-based WCA instrument to illustrate the degradation of LDPE film pieces inoculated with *Micrococcus luteus* for a prolonged period of four months in mineral salt broth deprived of a carbon source. a. WCA data of Control LDPE film without bacterial inoculation. b. Water contact angle observations of *Micrococcus luteus* NJD3-treated LDPE film.

In the third set of experiments, the control films exhibited a water contact angle of $90.68 \pm 0.94^\circ$, indicating the polymer's hydrophobic nature and its ability to withstand an aqueous environment. However, in our sample, LDPE film-treated *Micrococcus luteus* NJD3 exhibited a reduced contact angle of $76.28 \pm 1.6^\circ$, indicating the isolated strain's ability to utilise the hydrocarbon chain as its energy source.

S. No	Name of the bacterial strain	Contact angle of control LDPE films	The contact angle of bacterial-treated LDPE films
1	<i>Bacillus cereus</i> NJDI	91.06±1.13°	67.6±0.82°
2	<i>Pseudomonas aeruginosa</i> NJD2	90.54 ± 2.64°	73.56 ± 0.32°
3	<i>Micrococcus luteus</i> NJD3	90.68±0.94°	76.28±1.6°

Table 4.2 Consolidated results of contact angle measurements of isolated bacterial strains

The significant changes in the hydrophobicity of LDPE films, without any pre-treatment, confirmed the efficacy of all three strains in degrading the compound. Among the isolated strains, *Bacillus cereus* exhibited the highest reduction in contact angle, nearly 26%, compared to the control LDPE film, followed by *Pseudomonas aeruginosa*, with a decrease of almost 19%, and *Micrococcus luteus*, with a reduction of 16%.

4.5.5 Measurement of mechanical strength of isolated bacterial strain inoculated LDPE strips by tensile strength estimation

In addition to topographic changes in LDPE films due to bacterial addition, we observed notable alterations in the polymer's mechanical strength when our isolates were added to LDPE films compared to LDPE films devoid of bacterial addition. We extracted dump-bell-shaped LDPE strips from the control and sample flasks to measure the width, thickness, force, and extension length (Refer to Figs. 4.7(a), 4.7(c), and 4.7(e)). Additionally, we used these data to calculate the elongation percentage at break, ultimate tensile strength, and tear strength of LDPE films to determine how bacterial inoculations affected surface homogeneity.

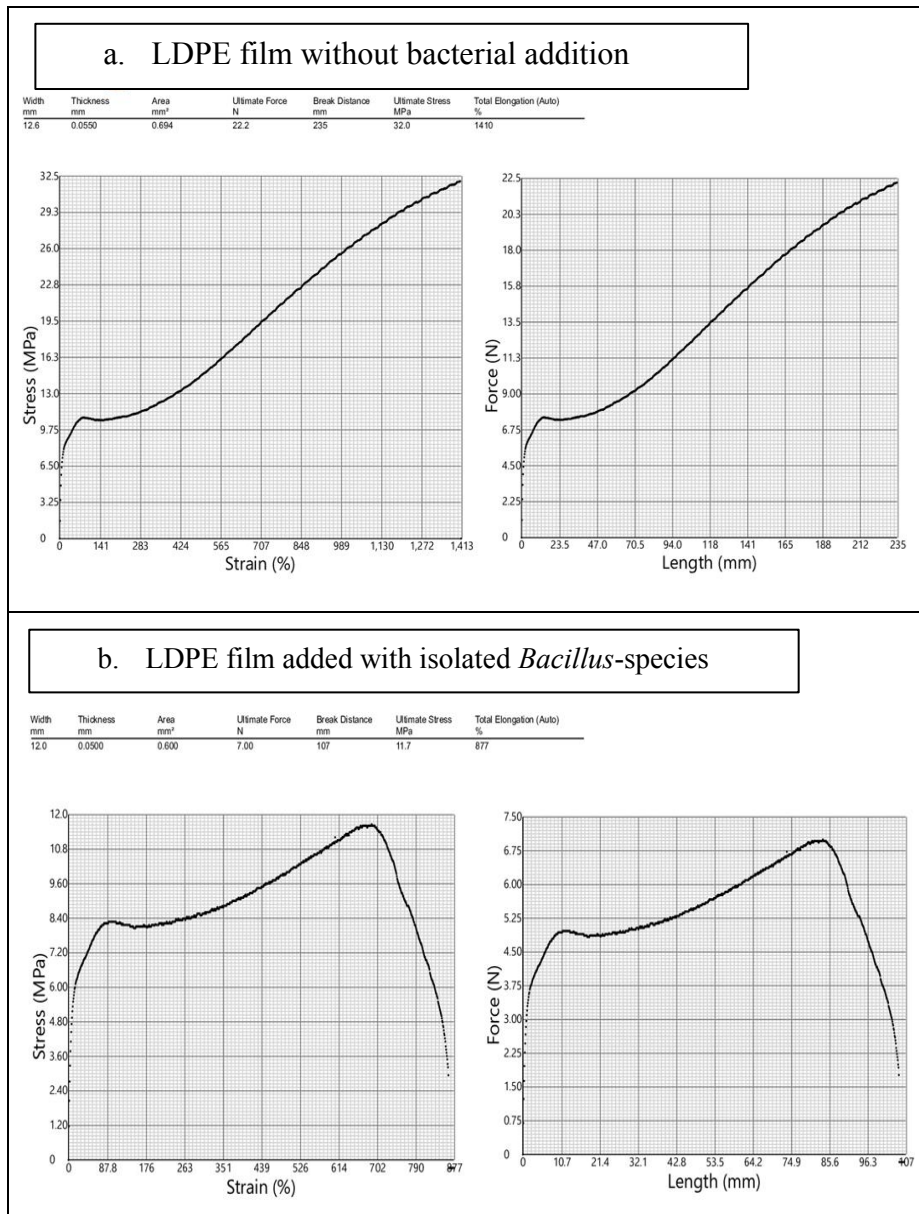


Fig.4.7(a) Tensile data of LDPE film without (control) or with inoculation of *Bacillus cereus* strain(sample). The parameters measured during tensile testing help us determine the change in stiffness of LDPE film before and after bacterial inoculation.

Compared to the control, we observed a significant decrease in tear strength (N/m) (65.6%), ultimate tensile strength (MPa) (66.6%), and total elongation or strain percentage (41.8%) in the *Bacillus*-inoculated LDPE films. It may be due to the robust biofilm formation of the *Bacillus* strain.

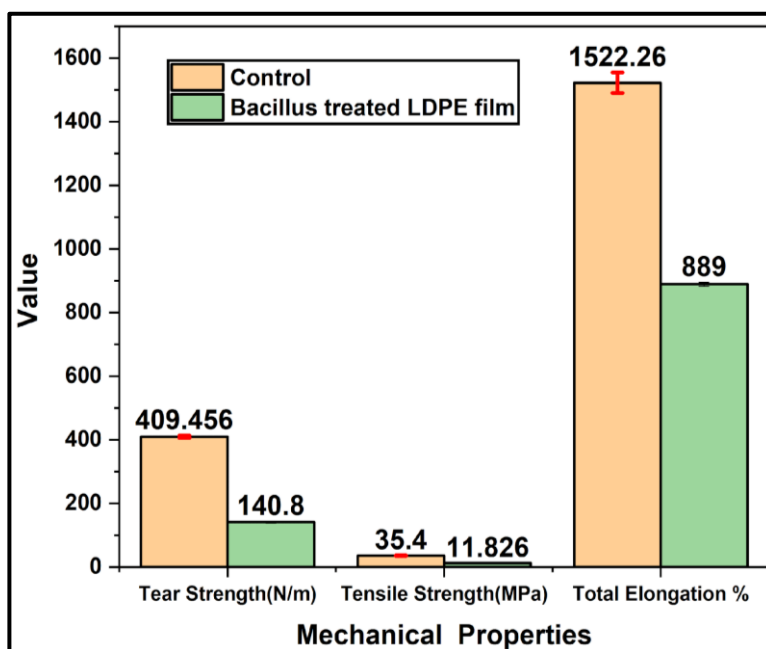


Fig.4.7(b) Graph showing the change of tensile properties of *Bacillus cereus* on LDPE films

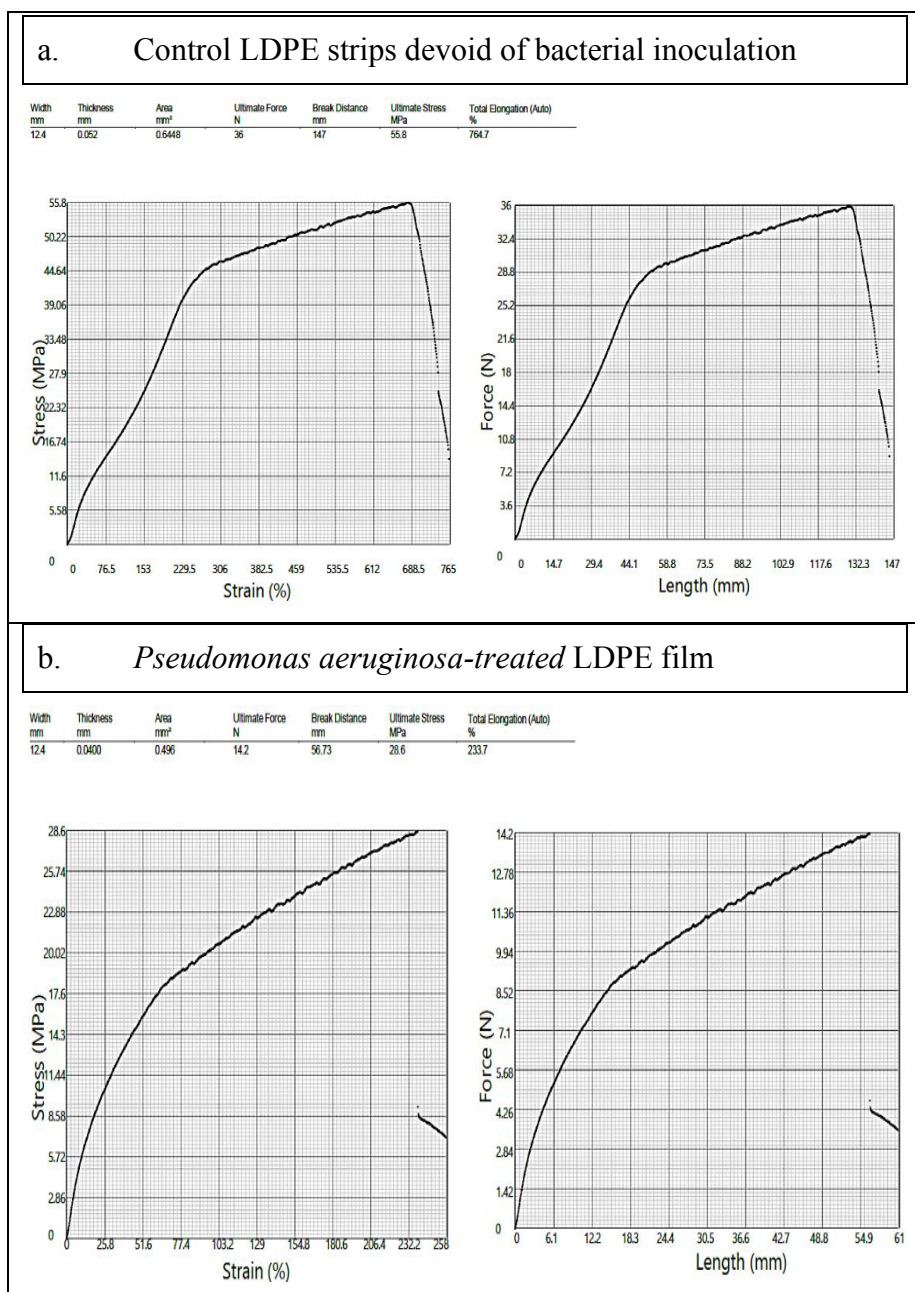


Fig.4.7(c) Tensile strength analysis of control LDPE strips devoid of bacterial inoculation and *Pseudomonas aeruginosa*-treated LDPE film.

After 120 days of incubation, the tear strength (N/m) of LDPE films reduced to 56.78% and tensile strength (MPa) to 56.17%. The total elongation percentage diminished up to 71.8% by the inoculation of *Pseudomonas* strains to the LDPE films, compared with the polymer film without bacterial addition (View the results in Fig.4.7(d)).

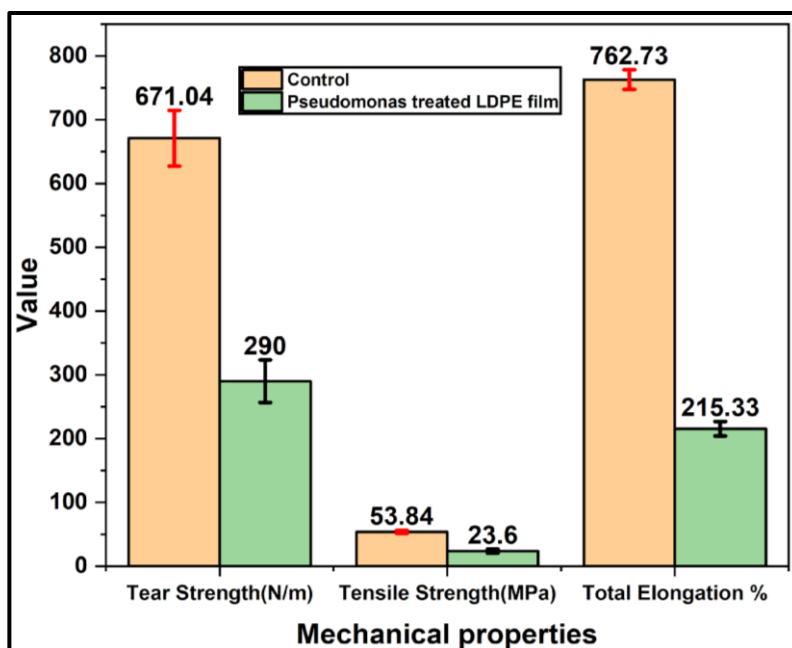
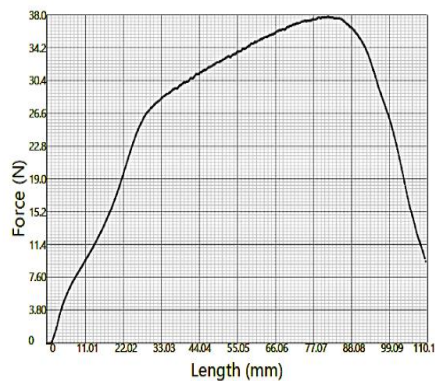
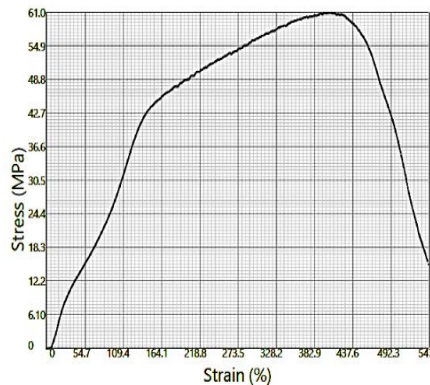


Fig.4.7(d) Graph showing the mechanical strength reduction of LDPE strips added with *Pseudomonas aeruginosa* strain.

a. LDPE films without bacterial inoculation

Width mm	Thickness mm	Area mm ²	Ultimate Force N	Break Distance mm	Ultimate Stress MPa	Total Elongation (Auto) %
12.4	0.0500	0.620	37.8	107	60.9	547

b. LDPE films with the addition of *Micrococcus luteus*

Width mm	Thickness mm	Area mm ²	Ultimate Force N	Break Distance mm	Ultimate Stress MPa	Total Elongation (Auto) %
12.3	0.04	0.4816	12	53.0	24.91	211.76

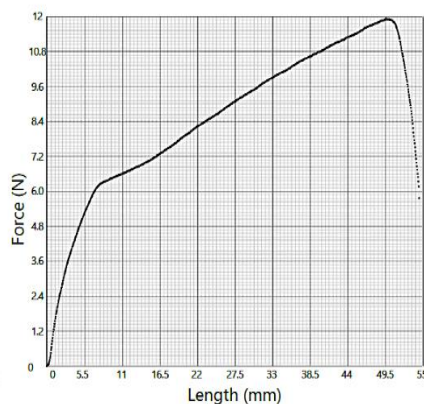
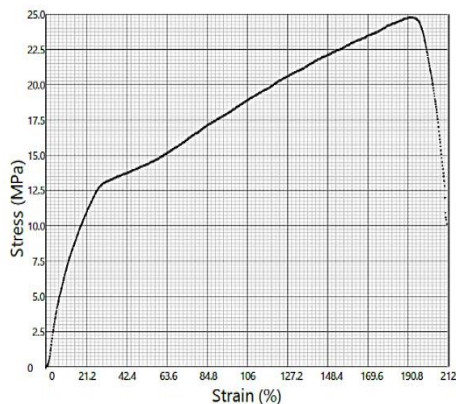


Fig.4.7(e) Tensile reduction of LDPE strips without or with the addition of *Micrococcus luteus* NJD3 for 120 days.

In contrast with the tensile testing data of the control LDPE films, *Micrococcus* sp. added films exhibited a depletion in tear strength (N/m) almost 60% reduction), tensile strength (MPa) (nearly 59.3%

depletion) and elongation percentage deviation (close to 60.33%) (Results displayed in Fig.4.7(f)). Such changes have heterogeneously appeared leading to notable differences in surface stiffness within the LDPE films exposed to such an environment for a quarter of a year.

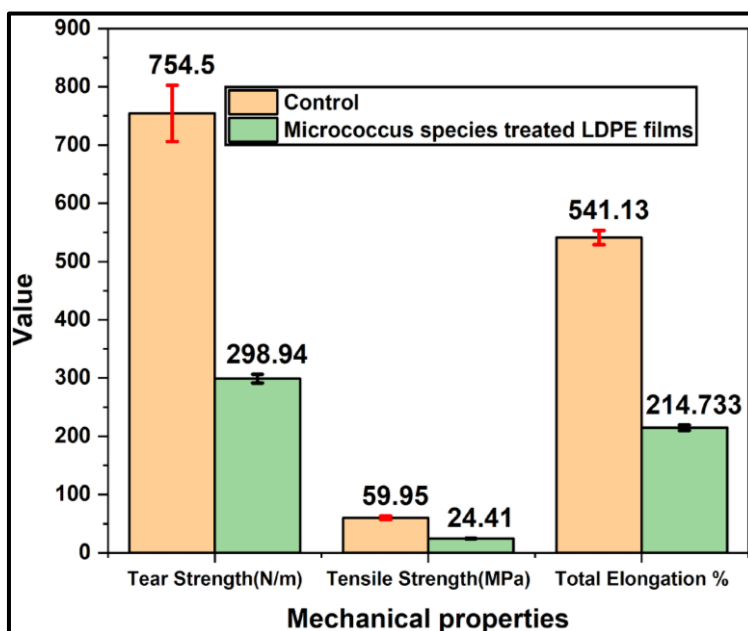


Fig.4.7(f) Graph showing reduction in mechanical properties of LDPE strips after inoculation with *Micrococcus* strains for 120 days

4.5.6 FTIR analysis of LDPE strips in a stressed condition of mineral salt broth

LDPE films were subjected to FTIR analysis after the bacterial strains isolated from long-run dumps were added to the polymer's basic structure to confirm the chemical changes that occurred. The results were compared with control films that did not contain microbial strains and were incubated in a carbon-devoid medium for 120 days. The main

components of these assessments specifically focus on spectral analyses and index computations.

4.5.6.1 FTIR assessments of LDPE films inoculated with *Bacillus cereus* NJD1 strain

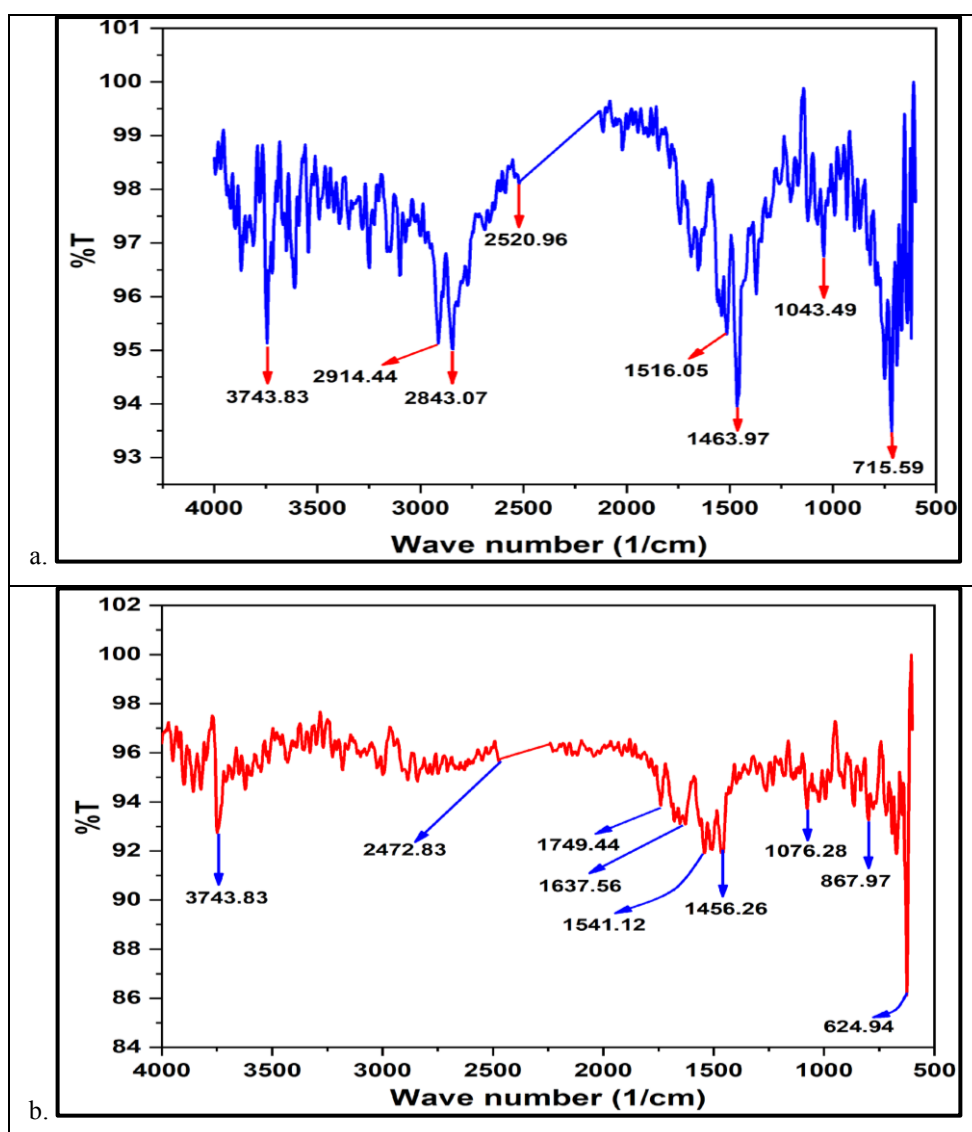


Fig.4.8(a) FTIR analysis of *Bacillus cereus* NJD1-inoculated LDPE film (image b) and its control without bacterial inoculation (image a).

S. No	Wave number cm^{-1}	Peak nature	Interpretations
1	3743.83	O-H Stretching	Possible oxidation or moisture absorption due to prolonged incubation in liquid media.
2	2914.44	C-H Stretchings	Symmetric or asymmetric stretching of $-\text{CH}_2$ groups in polyethylenes.
3	2843.07	C-H Stretchings	Symmetric or asymmetric stretching of $-\text{CH}_2$ groups in polyethylenes.
4	2520.96	O-H Stretching	Minor oxidations in pure LDPE
5	1516.05	C-H bendings	Bending in $-\text{CH}_2$ or $-\text{CH}_3$ groups, common in polyethylenes.
6	1043.49	Either C-O Stretching or C-C Stretching	It may be due to slight oxidation.
7	715.59	Rocking vibrations of CH_2	Crystalline structure of polyethylenes.

Table 4.3(a) FTIR peak interpretations of control LDPE film without *Bacillus* sp. inoculation. It shows no specific peak, indicating no degradation.

The FTIR spectra of the control LDPE film showed that the compound remained chemically stable over the 120-day incubation period. Its spectrum showed only the standard stretching in LDPE film kept under moisture conditions, such as minor oxidation by O-H bond addition due to prolonged incubation in liquid media and C-H stretching or bending.

S. No.	Wave number cm^{-1}	Peak nature	Interpretation
1	3743.83	O-H Stretching	Possible oxidation or moisture absorption
2	2472.83	O-H Stretching	The presence of carboxyl groups in the degradation
3	1749.44	C=O Stretching	Indicates oxidation leading to aldehydes, ketones or carboxylic acids
4	1637.56	C=C Stretching	Suggests the formation of vinyl or unsaturated bonds due to degradation
5	1541.12	N-O Stretching	Presence of proteins or peptide residues from bacterial activities.
6	1456.26	CH ₂ Bending (Alkanes)	Common polyethylene backbone bendings.
7	1076.28	C-O Stretching (Esters, alcohols)	It could be due to the oxidation of microbial metabolic activity products.
8	867.97	C-H Bending (Alkenes groups)	Possible degradation products
9	624.94	Halogenated compounds	Likely from plasticisers in LDPE

Table 4.3(b) FTIR peak illustration of LDPE films treated with *Bacillus cereus* NJD1 (*FTIR Functional Group Database Table*, accessed via *InstaNANO*,2021). It shows several peaks manifesting the degradation of LDPE films.

4.5.6.2 FTIR fingerprints of LDPE films treated with *Pseudomonas aeruginosa* NJD2 strain

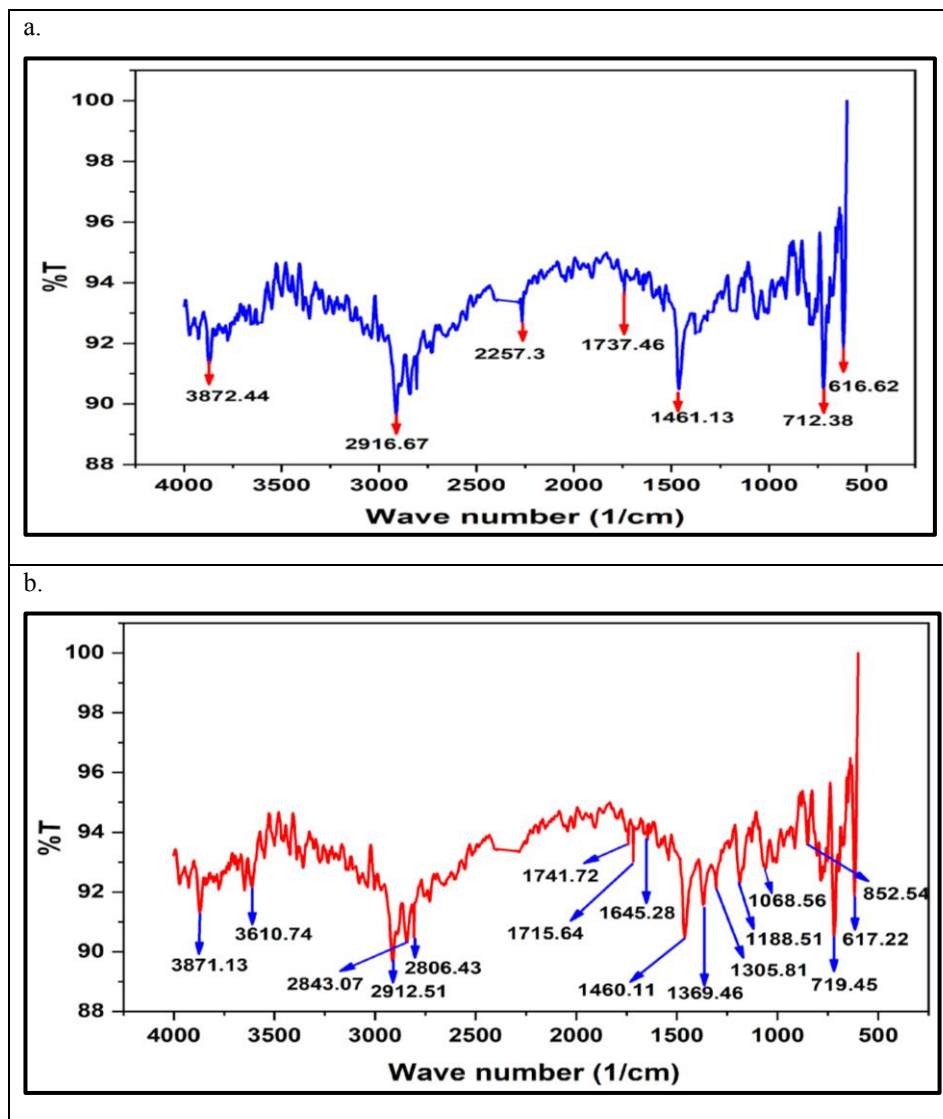


Fig.4.8(b) FTIR spectrograph of LDPE film without (image a) or with (image b) treatments by *Pseudomonas aeruginosa* NJD2. Table 4.5.6.2a. FTIR peaks of the control LDPE film without *Pseudomonas* sp. addition and their explanations.

S. No	Wave number cm ⁻¹	Peak nature	Interpretation
1	3872.44	O-H stretching	Possible oxidation or moisture absorption due to prolonged incubation in liquid media.
2	2916.67	C-H stretchings	Symmetric or asymmetric stretching of -CH ₂ groups in polyethylenes.
3	2257.3	C≡N stretchings	Minor impurities in polymer films.
4	1737.46	C=O stretchings	Mild oxidations
5	1461.13	C-H bendings	Bending in -CH ₂ or -CH ₃ groups, common in polyethylenes.
6	712.38	Either C-O stretching or C-C stretching	It indicates the crystalline nature of the polymer.
7	616.62	Halogenated compound	Maybe from plasticisers in the LDPE film.

Table 4.4(a) FTIR peaks of the control LDPE film without *Pseudomonas* sp. addition and their explanations.

Like previous control spectra, the LDPE film, used to compare the results of *Pseudomonas*-treated LDPE film, also exhibited C-H stretching and bending peaks of hydroxyl groups. C≡N impurities may be due to environmental stress or oxidation.

S. No	Wave number cm^{-1}	Peak nature	Interpretation
1	3871.13 3610.74	O-H stretching	Possible oxidation of LDPE films due to microbial action
2	2912.51 2843.07 2806.43	C-H stretchings	The slight shift in peaks suggests chain scission or molecular changes.
3	1741.72 1715.64	C=O stretching	Novel peaks of C=O bonds due to oxidative degradation of LDPE film
4	1645.28	C=C stretching	Indicate the presence of unsaturated bonds due to the breakdown of the polymer chain.
5	1460.11 719.45	C-H bendings	Modification in the polymer backbone
6	1369.46	O-H bendings	It indicates the presence of phenol/alcohol
7	1188.51 1068.58	C-O-C stretching	Suggests the formation of esters, ethers or alcohols, indicating microbial attacks (Daniel et al., 2018)
8	1305.81	C-N stretching	Indicates bacterial proteins or amine functional groups
9	852.54	C=C stretching	Indicates degradations in the LDPE films
10	617.22	Halogenated compound	Presence of plasticisers

Table 4.4(b) FTIR peak interpretations of *Pseudomonas aeruginosa* NJD2-treated LDPE films.

4.5.6.3 Chemical modification of LDPE films by the treatment of *Micrococcus luteus* NJD3 by FTIR data analysis

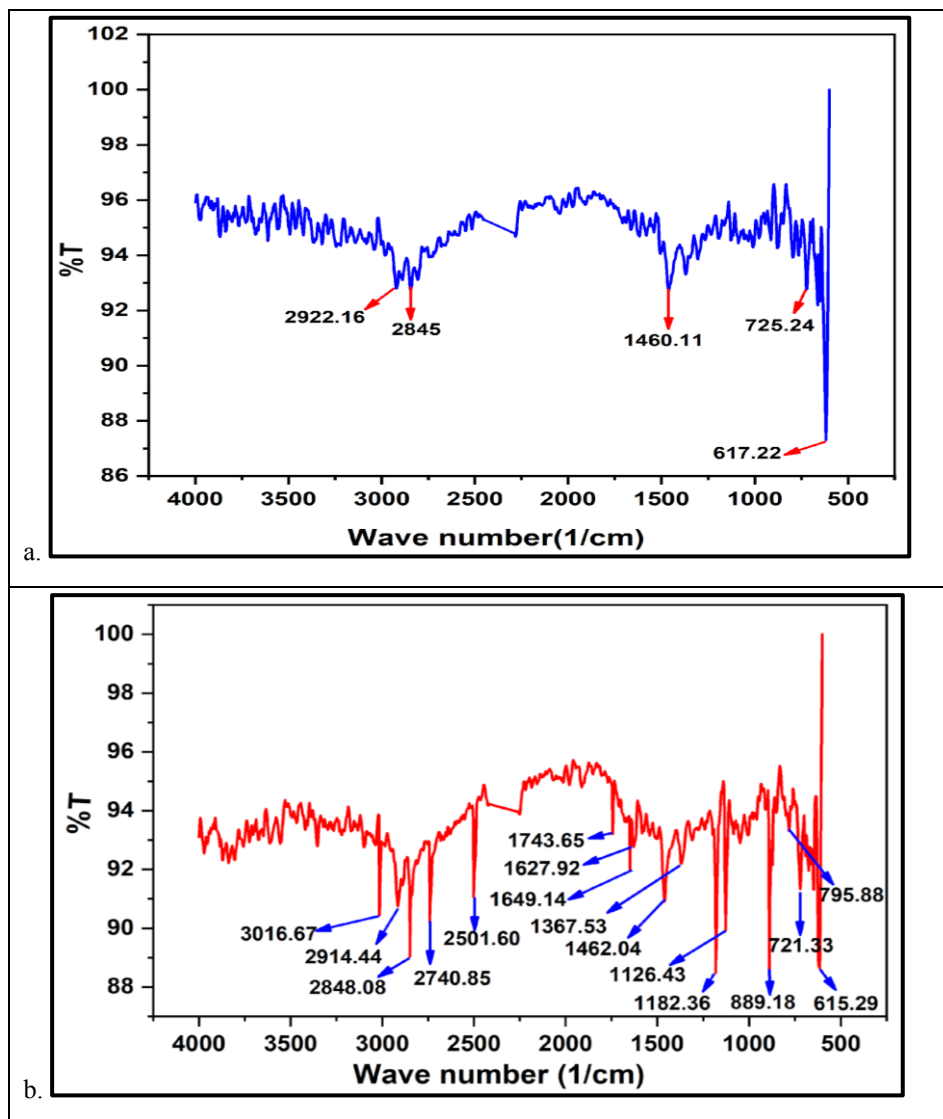


Fig.4.8(c) FTIR absorption peaks of LDPE film in a mineral salt medium without bacterial addition (control, image a) and polymer films with *Micrococcus luteus* addition (image b).

S. No	Wave number cm^{-1}	Peak nature	Interpretations
1	2922.16 2845	C-H stretching	The alkane functional group indicates the essential nature of LDPE.
3	1460.11	CH_2 bending	Minor impurities in polymer films.
4	725.24	$\text{C}=\text{O}$ stretchings	Mild oxidations
5	617.22	C-H bendings	Bending in $-\text{CH}_2$ or $-\text{CH}_3$ groups, common in polyethylenes

Table 4.5(a) FTIR peak illustrations of control LDPE films (without adding *Micrococcus luteus* NJD3).

The control spectra showed only the fundamental component stretchings of LDPE films, such as alkane stretchings and bending peaks. The mild oxidations and minor impurities may be due to the prolonged incubation of LDPE strips in mineral salt broth.

S.No	Wave number cm^{-1}	Functional group	Interpretation
1	3016.67 2914.44 2848.68	$-\text{CH}_2$ symmetric and asymmetric stretching	Polymer backbone alteration in alkane or alkene groups
2	2740.85	C-H stretching	Suggests the presence of a novel aldehyde group, indicating bacterial degradation
3	2501.60	O-H stretching	Indicates the presence of carboxylic acid
4	1743.65	$\text{C}=\text{O}$ stretching	Proves the presence of an aldehyde group
5	1649.14	$\text{C}=\text{C}$ stretching	Indicate the presence of a vinyl group (an unsaturated group)

			due to the breakdown of the polymer chain.
6	1627.92	N-H bending	Suggest the presence of protein or catalytic compounds on the polymer
7	1462.04 721.33	C-H bending	Molecular rearrangement in the polymer
8	1367.53	O-H bending	It indicates the presence of phenol
9	1188.36 1126.43	C-O-C stretching	Suggests the formation of ethers or alcohols, indicating microbial attacks
10	889.18 795.88	C=C bending	Oxidation of polymer
11	615.29	Halogenated compound	Presence of plasticisers

Table 4.5(b) FTIR absorption peaks of *Micrococcus*-treated LDPE film.

The specific absorbance bands denote chemical modification on the LDPE strips incubated with *Micrococcus luteus*.

4.5.6.4 FTIR indices analysis of potential strains using FTIR data

Based on the FTIR data, we calculated the carbonyl (keto (KI), ester (ESI), and vinyl (VBI)) and internal bond indices (Sudhakar et al., 2008), which are graphically presented in Fig. 4.9.

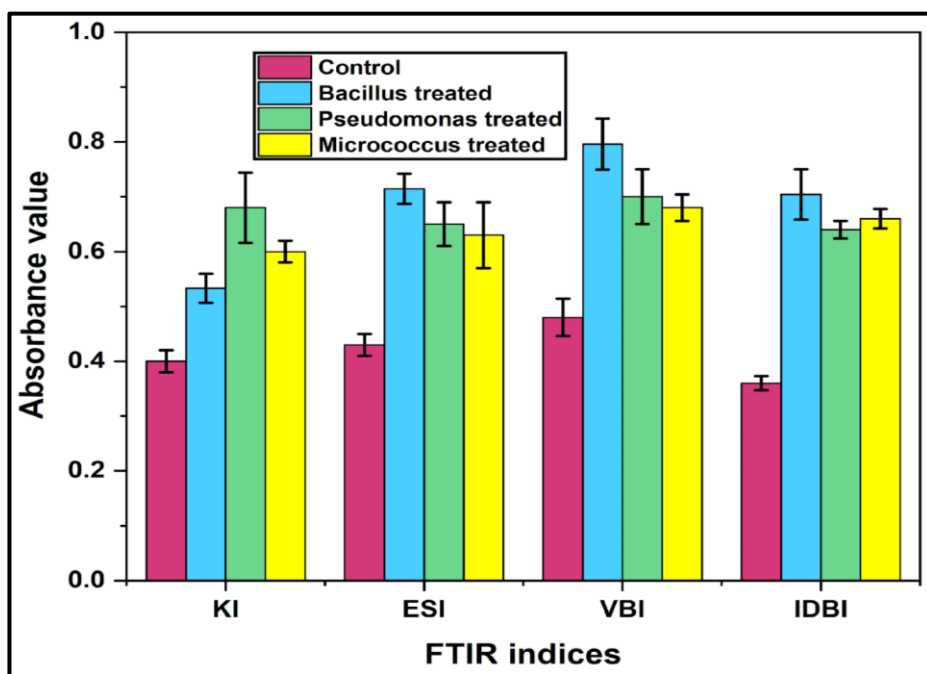


Fig.4.9 Image of FTIR indices of all potential bacterial strains. It manifests the hike of the carbonyl group in the LDPE film for mineralisation.

4.5.6.5 FTIR-ATR-FPA imaging

The micro-ATR (micro attenuated total reflectance) analysis further confirmed the fingerprints illustrated in the FTIR spectrum.

4.5.6.5a. FTIR-ATR-FPA images of *Bacillus* sp.

Bacillus species-treated LDPE film exhibited stretching/bending of carbonyl, alkyl, alkene and nitro functional groups in a 1000-2000 cm^{-1} bandwidth.

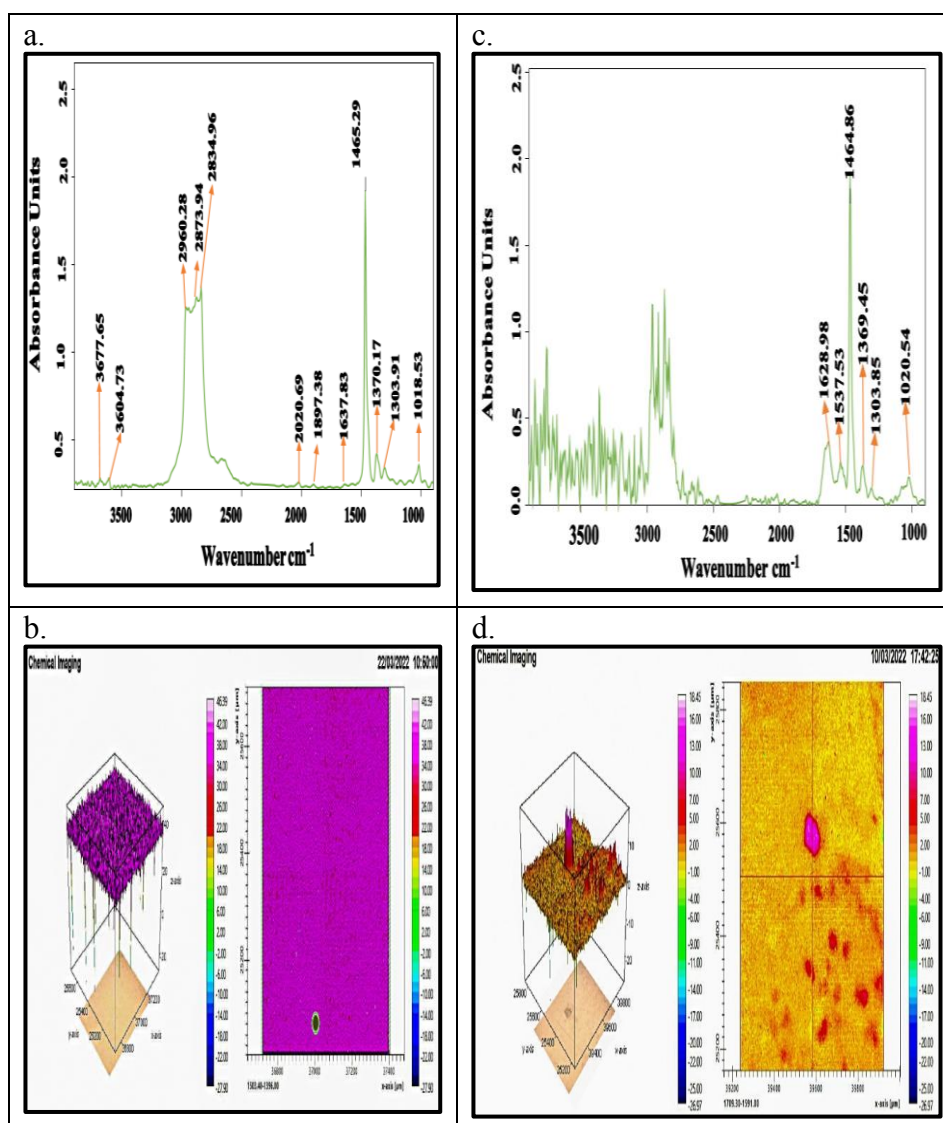


Fig.4.10(a) Attenuated total reflectance Fourier transform spectroscopy coupled with focal plane array imaging (ATR-FTIR-FPA imaging) (Ferreira et al., 2015; A. K. Sharma et al., 2015) using a Hyperion Micro-ATR (Micro-attenuated total reflectance) microscope to validate the chemical alterations in the LDPE pieces with (image c and d) or without incubation (image a and b) of *Bacillus cereus*-NJD1 for four months (H. Sharma & Neelam, 2023).

4.5.6.5b Visualisation of FTIR-ATR-FPA images of *Pseudomonas* sp. treated LDPE films.

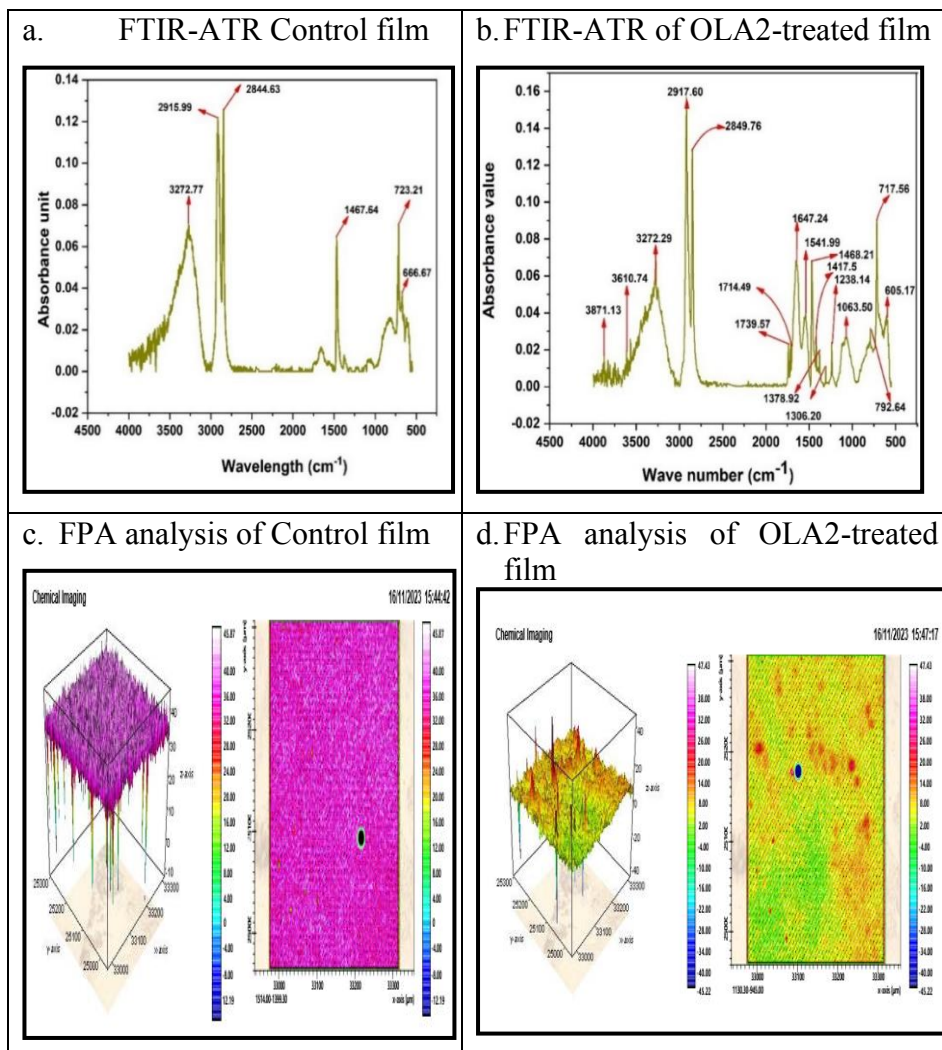


Fig.4.10(b) FTIR-ATR-FPA of LDPE film analysis using *Pseudomonas aeruginosa* NJD2. Images a and b are the results of micro-ATR analysis of control and *Pseudomonas* sp. treated LDPE films. Images c and d are the FPA images of LDPE strips (with or without *Pseudomonas* sp. inoculation). Compared to the control LDPE film (bright violet image), the FPA image of bacterial-treated polymer strips displayed a green-yellow-red colour code in each pixel, and its spectrum shows chemical alterations in the range of 1000-2000 cm⁻¹.

4.5.6.5c Demonstration of topographic changes in *Micrococcus* species treated LDPE film by Micro-ATR coupled FPA imaging

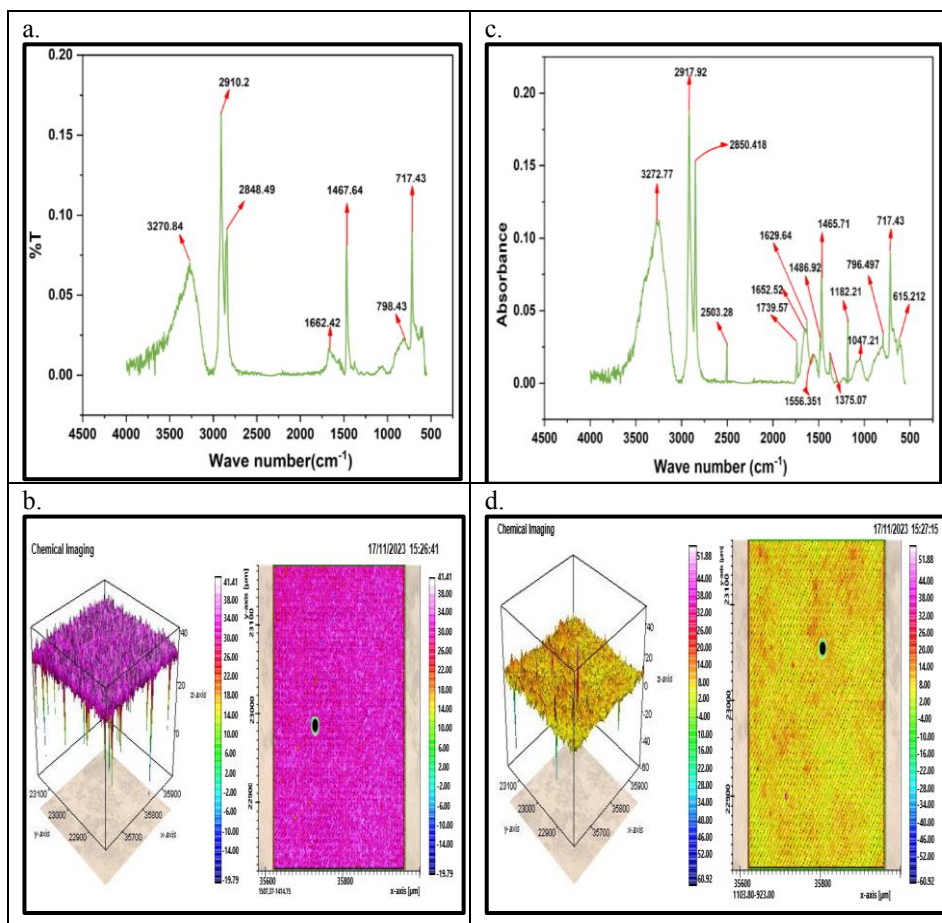


Fig.4.10(c) The images show micro-ATR of LDPE film without bacterial adhesion (control) and with *Micrococcus luteus* NJD3 adhesion (Images a and c) and FTIR microscopic imaging (FPA analysis) (Images b and d). The colour changes in FPA analysis prove the change in molecular vibrations on the LDPE film.

The FTIR-ATR spectrum of bacterial-treated LDPE films confirmed the presence of stretching peaks, including carbonyl, alkene, nitro,

hydroxyl, alkane and amine groups (Tripathi & Melo, 2017), as well as bending bands associated with C-H and C=C bonds. The results are similar to those of this study's IR spectrum, indicating the formation of aldehydes, carboxylic acids, and esters due to the enzymatic activities of the bacterial inoculum, which suggests similar catalytic activities by these bacterial strains.

In the FPA imaging, the strain exhibited a yellow-red colour code in the bacterial-treated image, in contrast to the light violet colour in the control. It may be due to chemical changes (bending of the C-H group, stretching of the C=O group, stretching of the C-C group, and stretching of the alkene) within the 1000-2000 cm^{-1} bandwidth.

4.5.7 GC-MS analysis of LDPE strips inoculated in synthetic mineral broth

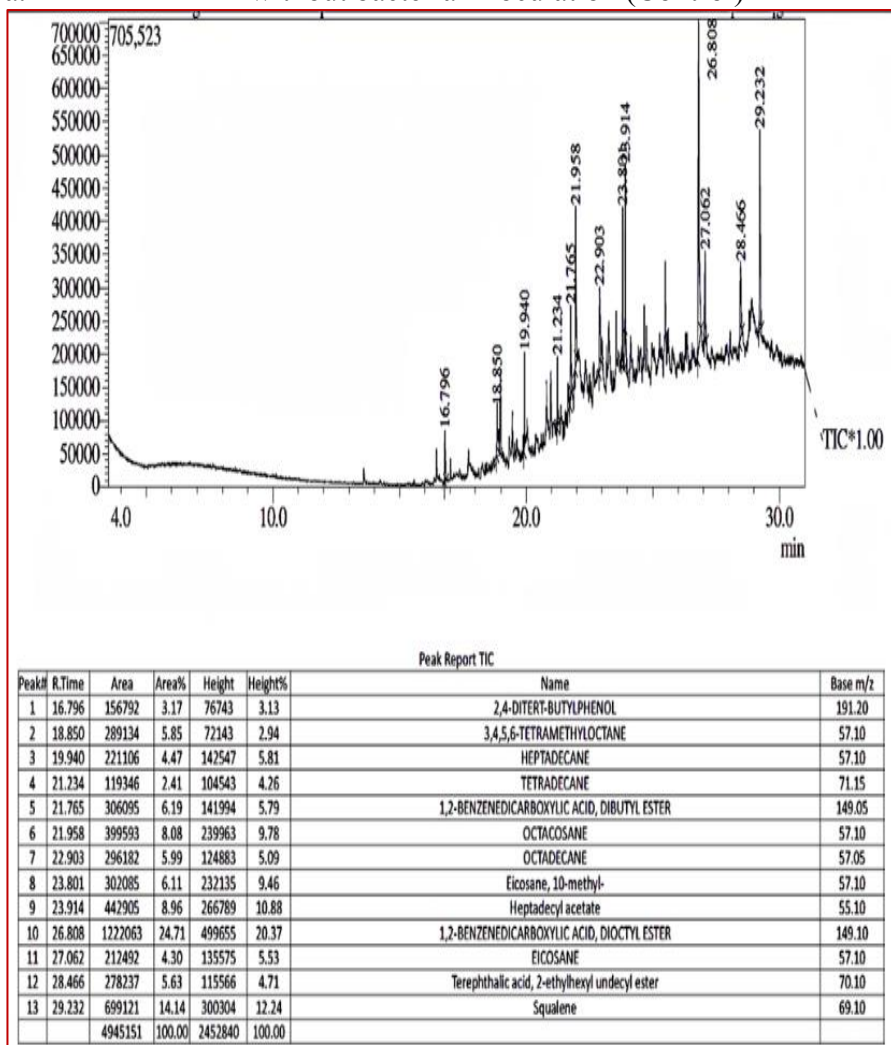
By employing the GC-MS tool in our current investigation, we found that the spectral lines of all isolated bacterial species exhibited either biodegradation intermediates or simple biodegradable compounds, compared to the control films, which did not contain any bacterial suspensions.

4.5.7.1 Comparison of GC-MS chromatogram with or without adding *Bacillus* sp. to the LDPE films

The spectrograph of the control LDPE film only demonstrated native hydrocarbons, additives, and plasticisers. They were highly stable and chemically inert components. It showed no significant sign of bacterial degradation. Compared to the control LDPE film spectrum, the *Bacillus*-treated films displayed fewer spectral bands of phthalate esters, diesters and other complex compounds (Akash et al., 2025) (results displayed in Fig.4.11(a)). The exact reason behind this

reduction in peak lines remains unknown. The *Bacillus*-treated LDPE films exhibited branched mid-alkanes (Undecane), plasticiser degradation products (1,2-benzene dicarboxylic acid), and halogenated by-products (2-bromododecane). However, this may be due to the strain's catalytic activities, which mineralise a few compounds in the polymer.

a. LDPE film without bacterial inoculation (Control)



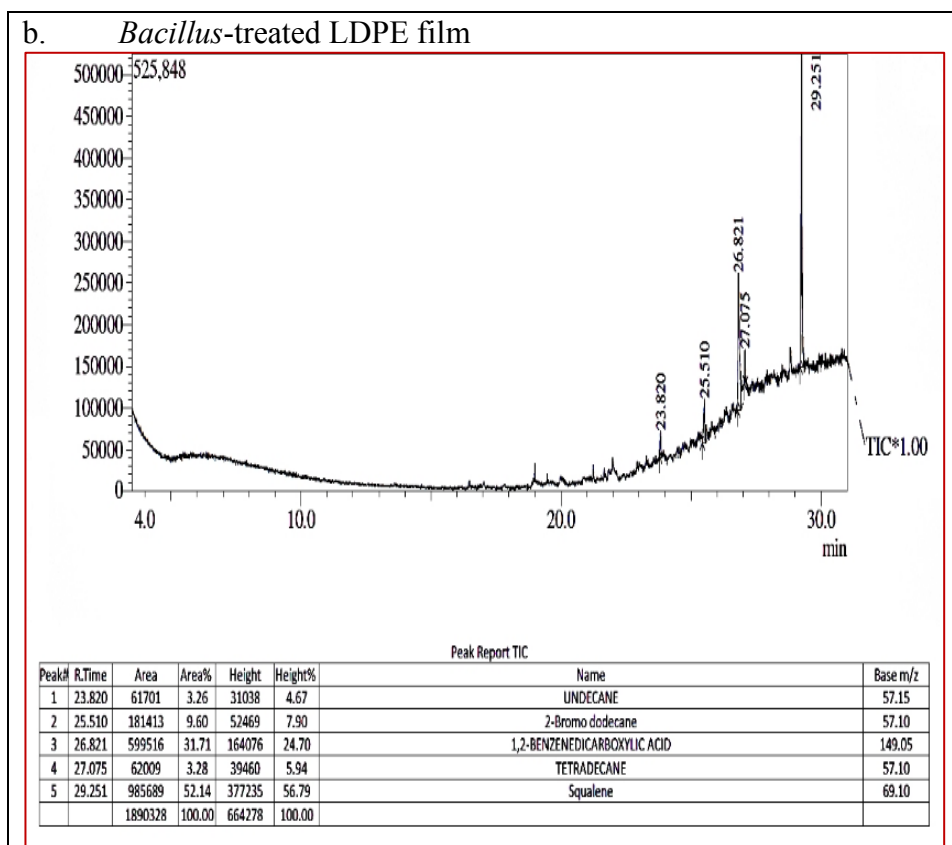


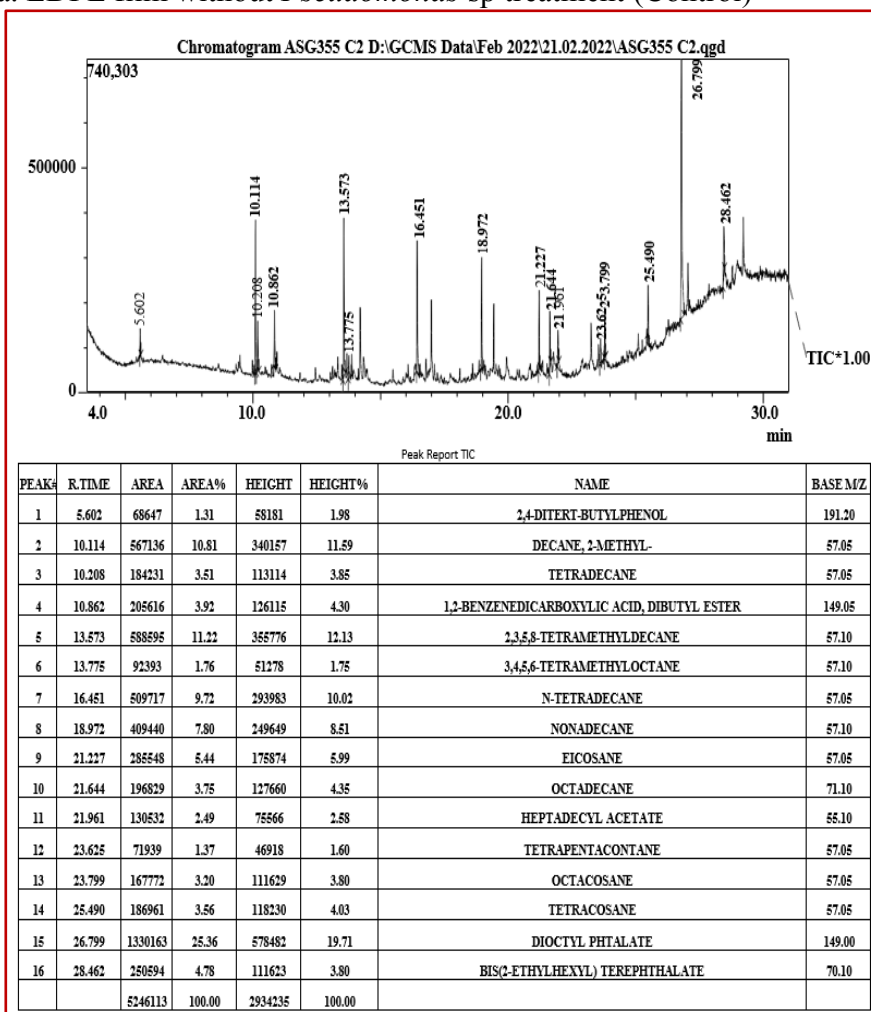
Fig.4.11(a) GC-MS chromatogram of LDPE films without (Image a) and with *Bacillus* suspensions (Image b).

4.5.7.2 GC-MS Analysis of LDPE strips added with *Pseudomonas* sp.

We demonstrated the existence of hydrocarbons, saturated fatty acids, phenols, esters, and other volatile intermediates crucial to biodegradation using GC-MS studies of LDPE strips added with *Pseudomonas aeruginosa* NJD2. In this analysis, the spectrograph of the control LDPE film also showed highly stable and chemically inert compounds. The bacterial-treated LDPE film showed peaks for alkanes, including decane, tetradecane, and heptadecane, indicating

popolyethylene cleavage. The branched alkanes (2,6,10,15-tetramethyl heptadecane), degraded plasticisers (1,2 Benzene dicarboxylic acid), phenolic antioxidants (phenol 3,5-Bis (1,1 dimethylethyl) and new metabolites (Tridecanoic acid methyl ester and squalene) peaks in the GC-MS image illustrated the catalytic activities of *Pseudomonas* strain attaches with LDPE films in minerals salt medium for 120 days.

a. LDPE film without *Pseudomonas* sp treatment (Control)



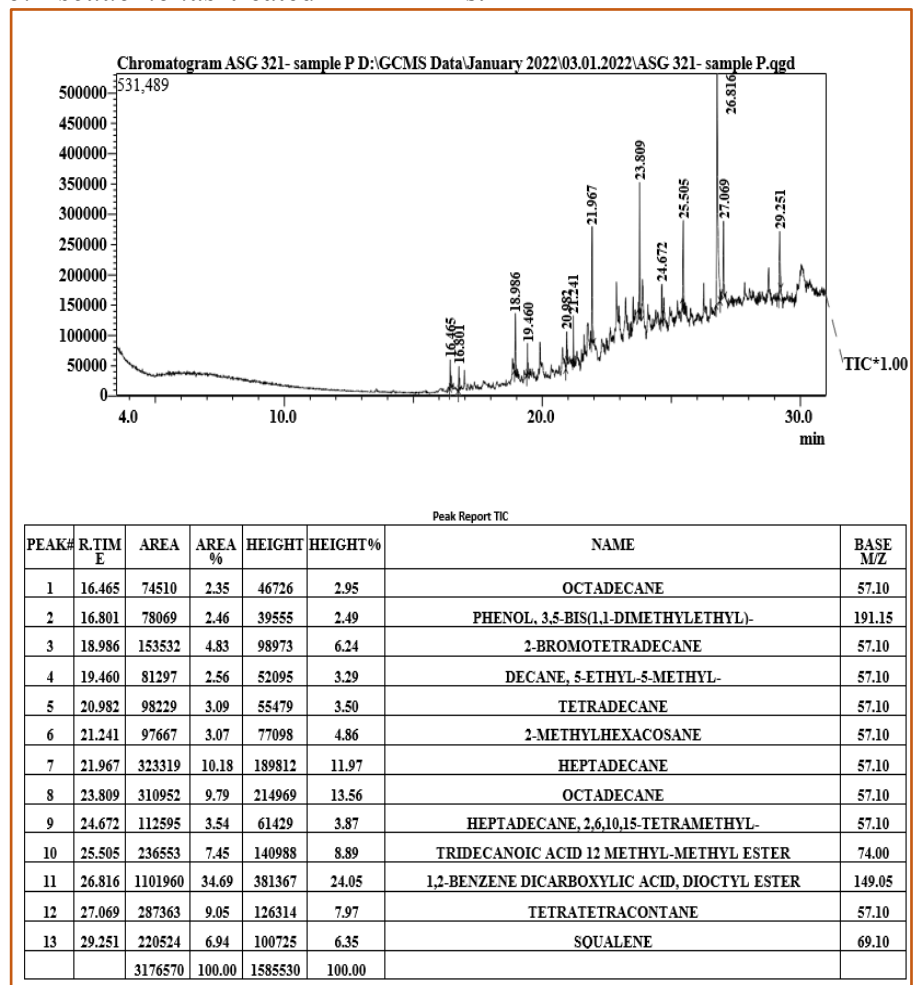
b. *Pseudomonas*-treated LDPE films.

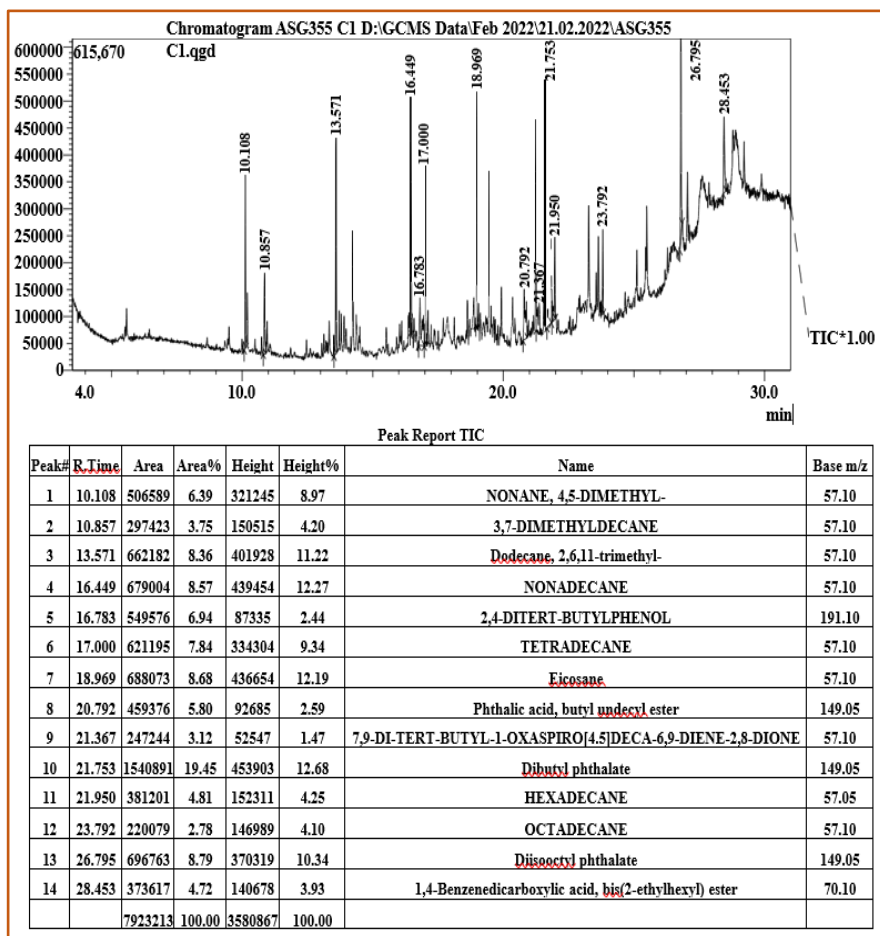
Fig.4.11(b) Image of GC-MS analysis. a. LDPE films without *Pseudomonas* sp addition(control) b. *Pseudomonas aeruginosa* treated LDPE film.

4.5.7.3 GC-MS Fingerprints of LDPE Films Treated with *Micrococcus* sp.

In contrast to the control LDPE film, the GC-MS images of *Micrococcus*-treated LDPE films showed several degradation products or transformations resulting from the strain's catalytic activities. The

GC-MS images of the control LDPE films showed only a hydrocarbon profile, with no plasticisers, stabilisers, or additives detected. All the contents were chemically stable. Evidence for polymer degradation: The detection of shorter alkanes, such as nonane, undecane, and tetradecane, suggests the cleavage of the polymer backbone.

1. Transformation of LDPE film: The presence of branched alkanes, such as heptadecane 2,6,10,15-tetramethyl and octadecane, indicates partial degradation of the long hydrocarbon chain in the LDPE film.
2. Plasticiser degradation: Phthalic acid and its derivatives (Phthalic acid, 4-cyanophenyl heptyl ester, and 1,2-benzene dicarboxylic acid) highlighted the plasticiser transformation due to microbial enzymes.
3. Oxidative products from LDPE biodegradation, such as fatty acid methyl esters like tetradecanoic acid 12-methyl ester, indicate the presence of an oxidative intermediate in LDPE biodegradation.
4. Microbial metabolite production: The *Micrococcus*-treated LDPE films exhibited a spectral fingerprint of squalene. It is a biosynthetic compound produced by a bacterial strain or released due to oxidative stress on polymer strips.

a. LDPE films non-treated with *Micrococcus* sp. (control)

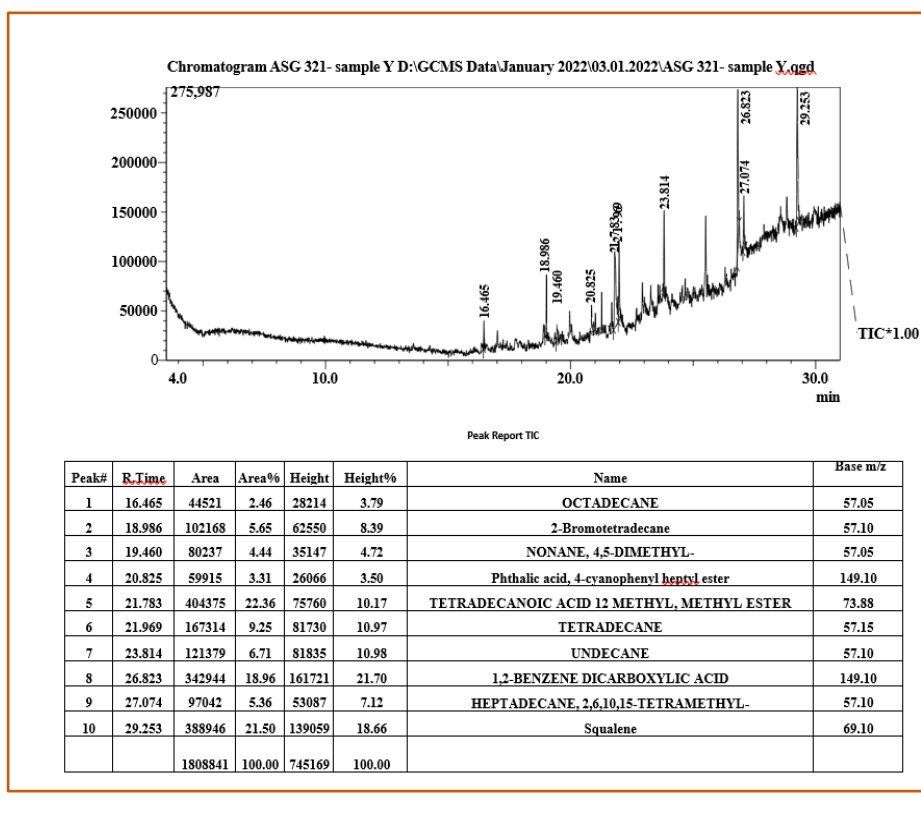
b. *Micrococcus*-treated LDPE film

Fig.4.11(c) Images of GC-MS analysis of LDPE film inoculated in carbon-free broth without bacterial biofilm (a) and *Micrococcus luteus*-treated LDPE film after 120 days (b).

Every data analysis utilising the three isolates verified their effectiveness in forming robust biofilms and their ability to show discernible changes in the mechanical properties of polyethylene films.

4.6. Discussion

This chapter discusses validation studies using sophisticated analytical instruments to demonstrate LDPE degradation by potential bacterial strains isolated from plastic landfills. It includes topographic alteration

analyses, mechanical strength deviations, studies illustrating chemical changes in LDPE films and metabolic product analysis. To investigate topographic alterations, we employed FE-SEM, SEM-EDAX, AFM, and video-based water contact angle analyses. Universal tensile testing machines demonstrated mechanical strength differences between bacterial-treated and non-treated LDPE films. FTIR and Micro-ATR-FPA imaging were used to manifest the chemical changes in the LDPE films. GC-MS analyses were used to demonstrate metabolic products of degradation.

The FE-SEM analyses were performed to illustrate the strong bio-attachments and surface modifications, such as pits, cracks, and flower-like fissures, on the surface-sterilised LDPE films incubated for three and four months in a carbon-source-devoid mineral broth (Khampratueng et al., 2024). All three strains exhibited significant alterations on the surface, and after prolonged incubation (120 days), bacterial colonisation appeared in the inner layers of the polymer. The modifications in the LDPE films could result from the activities of exopolysaccharides or enzymes released by biofilms attached to the polymer (Han et al., 2020; Whitfield et al., 2015). Multiple prior research endeavours have yielded identical findings in examining potential strains on polymer films (Dimassi et al., 2022; Ren et al., 2019). Several species of *Bacillus*, *Pseudomonas*, and *Micrococcus*, when treated on LDPE films, showed similar results (Anand et al., 2023; Maleki Rad et al., 2022; Nademo et al., 2023).

Another validation experiment for demonstrating surface alteration was SEM-EDAX analysis. SEM-EDAX images and data of all

bacterial-treated LDPE films proved significant holes, cracks and other surface alterations. Moreover, they used to demonstrate elemental changes, such as those involving carbon and nitrogen. Our investigation showed elevated weight and atomic weight percentage values for carbon and nitrogen elements compared to the control LDPE film without bacterial inoculation after 120 days of incubation in CFLMSM. These changes strongly demonstrated the strain's ability to utilise the structural elements of LDPE strips as its energy source (Oluwole et al., 2022; C. Yao et al., 2022)

AFM, used in our validation study, helped us prove nanoscale alterations on the surface of LDPE film (Sathiyabama et al., 2024). In our current analysis, the AFM data of all three bacterial strains showed significant differences in roughness values. It demonstrated the strain's ability to utilise LDPE films as its carbon source and to produce enzymes or metabolites that form physical irregularities on the hydrocarbon chains (Ji et al., 2024; Ren et al., 2019). Although many researchers have used this technique to demonstrate adhesion on LDPE film, a few have illustrated significant changes in surface roughness, similar to our analysis (Abo ElSouad, 2025).

This water contact angle measurement provides insight into the capabilities of the microbial strains to alter the surface hydrophobicity of polymers, which ultimately enables them to degrade. The contact angle reduction observed in the current study may be attributed to the unique catalytic properties of hydroxyl groups or their incorporation into the polymer (Jaiswal et al., 2022). A water contact angle below 90° indicates the solid surface's hydrophilicity, while an angle above

90° signifies its hydrophobicity (Samanta et al., 2020). In addition, the wettable environment created on the polymer surface by the strains may lead to subsequent colonisation and further improved biodegradation (Malachová et al., 2020). In the early days, many researchers performed similar validation experiments to demonstrate the ability of strains to colonise LDPE film (Ji et al., 2023; Y. Zhang et al., 2022).

Tensile testing of polymer films involves examining the difference in tensile properties between LDPE film with and without bacterial inoculation. The mechanical properties (Basaran et al., 2012) showed comparable reductions in bacterial species treated with LDPE films. Several previous researchers have verified similar findings, validating the efficiencies of bacterial strains in degrading LDPE films (Khruengsai et al., 2021; Szlachetka et al., 2021).

FTIR analysis revealed the hydroxylation and oxidation of the long-chain hydrocarbon chains in LDPE films treated with efficient bacterial strains for 120 days (Park & Kim, 2019). Compared to the FTIR data of the control LDPE film (un-inoculated with bacterial strains), all three bacterial strains treated with LDPE films displayed similar absorbance bands, including peaks associated with hydrocarbon chain oxidation, polymer backbone degradation, and the catalytic activities characteristic of the bacterial species. The establishment of oxidation and carbonyl groups on the treated films is strong evidence of bacterial adherence by a potential strain to recalcitrant surfaces (Syranidou et al., 2019). The C=O stretches between 1680 cm⁻¹ and 1790 cm⁻¹ have been shown in numerous investigations to degrade

polyethylene samples by producing ketone or aldehyde groups, which allows the chemical to enter the mineralisation route. (Gajendiran et al., 2016; Saha et al., 2021; Soleimani et al., 2021). In the presence of microbial enzymes, polymer films with carbonyl groups may further transform into simpler and degradable molecules that can progress to the final mineralisation phase (Jayan et al., 2023). Our GC-MS results further confirmed this result, which shows several metabolic intermediates. Peaks between 1750 and 1640 cm^{-1} in the current data show the existence of the vinyl group ($\text{C}=\text{C}$ stretching at 1645.28 cm^{-1}), the ester carbonyl group (1741.72 cm^{-1}), and the keto-carbonyl group (1716.64 cm^{-1}). The $\text{C}=\text{C}$ stretching in all the strain-treated LDPE films, in the $1635\text{-}1650\text{ cm}^{-1}$ range, indicates the formation of vinyl or unsaturated bonds. Introducing such bonds into polyethylene film may help mitigate its degradation (Mukherjee et al., 2018; Nademo et al., 2023). Alkenes can be produced either by the polyketide synthase route or by the decarboxylation of fatty acids; however, the mechanism by which bacteria induce vinylene in polyethylene remains unknown (Kang & Nielsen, 2017). The ester compounds formed by the biodegradation of the LDPE polymer can be converted to carboxylic acids and alcohols in the presence of esterase/lipase enzymes (Sumpavapol et al., 2010). Many precedential scientific studies have validated the presence of ester formation during the mineralisation of LDPE film (Harshvardhan & Jha, 2013; Peixoto et al., 2017). The $\text{C}=\text{C}$ bending mode, observed in the range of $790\text{-}895\text{ cm}^{-1}$ for all strains, indicates oxidation of the polymer (Harrat et al., 2024).

C O–O–C bonds in the FTIR data of LDPE film treated with bacterial strains indicate the formation of alcohols, ethers, or esters on the hydrocarbon chain in the 1050–1250 cm^{-1} area, which strongly supports microbial attack on the polymer (Khandare et al., 2021). Several recent investigations have suggested that the emergence of such peaks on polymer films may be attributed to the presence of laccase enzymes produced by microbial strains (S. Jiang et al., 2022; Navas et al., 2019). The stretches and bends between 1370 and 1050 cm^{-1} indicate the presence of alcohols, carboxylic acids, amines, esters, and ethers (Vimala & Mathew, 2016).

O–H bending in the range of 1310–1420 cm^{-1} indicates the formation of phenol or alcohol on the LDPE film. This study's GC–MS analysis further confirmed the presence of phenol compounds on LDPE film. The hydroxylation of the polymer chain is highly required for carbonyl group formation, which can be converted to either an ester in the presence of an esterase or lipase enzyme (Yoon & Jang, 2021) or an alcohol in the presence of an alkane hydroxylase enzyme (M. P. Das & Kumar, 2015; Parthipan et al., 2017). Carboxylic acid is present in the polyethylene film when O–H stretching occurs between 2450 and 3300 cm^{-1} in addition to O–H bending (Nirosha et al., 2015). The absorption band at 3550–3900 cm^{-1} suggests that alcohol or such oxidations may be due to prolonged incubation of LDPE films (W.-K. Yang et al., 2024). The C–H bending absorption peaks, located between 1400 cm^{-1} and 1465 cm^{-1} , as well as those between 700 cm^{-1} and 880 cm^{-1} , underscore the presence of long alkane chains in the plastic film. Several researchers have reported similar results, indicating that C–H

bending is a sign of biodegradation in potential bacterial biofilms introduced to LDPE films (Di Pippo et al., 2023). The altered transmittance of the treated polymer films throughout the 2847-2925 cm^{-1} range indicates a reduction in the C-H stretching attributed to the functional group. It may be attributable to microbial attack (Tarafdar et al., 2021). The CH_2 group scissoring (in-plane bending vibration, which reduces the bond angle and the alkene group) (Lin-Vien et al., 1991).

The N-O peak indicates microbial adherence and their catalytic activity on the films at 1541.12 cm^{-1} in the *Bacillus* sp. -treated LDPE film, the aromatic amine (C-N) peak at 1305.81 cm^{-1} in the *Pseudomonas* sp. -treated polymer film, and the amine group peak (N-H) at 1627.92 cm^{-1} in the *Micrococcus*-treated LDPE film. The EDAX and ATR-FPA analyses conducted in this study confirmed the presence of nitrogen element additions in the polymer. According to a recent study, *Bacillus*-produced biosurfactants or nitric oxide synthase enzymes may be responsible for adding the N-O group to the polymer structure (Z. Yao et al., 2022).

The absorption bands at 624.94 cm^{-1} , 617.22 cm^{-1} , and 615.29 cm^{-1} in the *Bacillus* sp., *Pseudomonas* sp., and *Micrococcus* sp. -treated LDPE film indicate the presence of a brominated plasticiser. It is further confirmed in our GC-MS analysis. Typically, the surface bromination of LDPE is the reason behind this chemical vibration, which can enhance its wettability and hydrophobicity (Kowalczyk et al., 2016). Many previous researchers have presented similar FTIR data to demonstrate the capabilities of innate organisms isolated from long-

term plastic piles (Gupta & Devi, 2020; Hou et al., 2022; Shilpa et al., 2023).

By examining the peaks in the FTIR spectra of LDPE strips added with each bacterial species, we found several closely spaced absorption bands (see Table 4.7), indicating similar catalytic activities despite the strains being isolated from different waste sites. Apart from similar peaks in spectra, each isolated strain showed distinct fingerprints (see Table 4.6). Several scientists have reported similar results in their investigations (Montazer et al., 2020; Nadeem et al., 2021).

S. No	Name of the Potential Bacterial-treated LDPE films	Unique peaks in cm^{-1}
1	<i>Bacillus cereus</i> NJD1-treated LDPE film FTIR spectra	N-O stretching (1541.12), C-H bendings at 867.97, and O-H stretching (2472.83) (Boukherroub & Szunerits, 2009).
2	<i>Pseudomonas aeruginosa</i> NJD2-treated LDPE film FTIR spectra	O-H stretching (3871.13 and 3610.13 cm^{-1}), C-N stretching (1305.81) (Lacsado et al., 2023).
3	<i>Micrococcus luteus</i> NJD3-treated LDPE film FTIR-spectral peak	C-H stretching (2740.85), O-H stretching (2501.60) and N-H bending (1627.92) (Bhing et al., 2025).

Table 4.6 Unique fingerprints (Stretching/bending peaks in the FTIR spectra of each isolated bacterial strain).

S. No	Name of the absorption peak with its frequency range	Absorption peak in the FTIR spectrograph treated with the bacterial strains in cm^{-1}			Functional group
		<i>Bacillus</i> sp	<i>Pseudomonas</i> sp	<i>Micrococcus</i> sp.	
1	C-H bendings (1400-1465 cm^{-1}) (700±20-880±20 cm^{-1})	1456.26	1460.11, 719.45	1462.04 and 721	Alkanes
2	C=O stretchings (1715-1750 cm^{-1})	1749.44	1741.72 1715.64	1743.65	Aldehydes
3	C=C stretchings (1635-1650 cm^{-1})	1637.56	1645.28	1649.14 1627.92	Vinyl group or unsaturated group
4	C-H stretchings (2800-3020 cm^{-1})	-	2912.51 2843.07 2806.43	3016.67 2914.44 2848.08	Alkanes
5	O-H bendings (1310-1420 cm^{-1})	-	1369.46	1367.53	Phenol/alcohol
7	C-O stretching (1050-1250 cm^{-1})	1076.28 cm^{-1}	1068.56 1118.51	1182.92 1126.43	Primary alcohols
8	C=C bendings (850-895 cm^{-1}) 790-830	867.97	852.54	889.18 795.88	Oxidation of Alkene
9	C-Br stretchings (400-690 cm^{-1})	624.94	617.22	615.29	Bromine-substituted compound

Table 4.7 List of similar peaks in all three capable strains for LDPE biodegradation.

The computation of FTIR indices using FTIR data reveals bond modifications and chemical conversions in hydrocarbons when bacterial species are inoculated onto LDPE films. The FTIR indices indicate changes in the film's internal structure, crystallinity, and hydrophobicity (Zerbi et al., 1989). The bacterial enzymes in the potential strains may oxidise the polymer and metabolise complex compounds to simple metabolites, such as primary alcohols, aldehydes,

ketones, and fatty acids. As a result, FTIR indices increase and further mineralise to H₂O and CO₂ via β -oxidation (Schober et al., 2023). The carbonyl indices identified in this study are significantly higher than those previously reported (Novotny *et al.*, 2018). Furthermore, a recent study demonstrated the efficiency of laccase enzymes, which add carbonyl groups to the polymer, and lipase enzymes for ester hydrolysis in the biodegradation of LDPE (N. Zhang et al., 2022). Besides the main path, it may transform into alkenes and, in turn, be catabolised to epoxides in alternative metabolic routes.

Micro-ATR coupled with FPA imaging is another validation method that manifests the biodegradation of LDPE films. Most of the peaks in the micro-ATR spectrum of each strain showed similarity to the FTIR data of that strain's spectrum, specifically in the range of 1000-2000 cm⁻¹. Moreover, this experiment's FPA imaging (FTIR imaging) helped us visualise the chemical modification in the LDPE film at a pixel level. Compared to the colour code of the control FPA, which ranges from light violet, the deep blue image appeared in the *Bacillus*-treated LDPE film. In contrast, the *Pseudomonas*-treated LDPE film exhibited a green-yellow-red colour, and the *Micrococcus*-treated LDPE film showed a yellow-red colour. It confirmed the depth of chemical shifts on the polymer surface. A few recent scientific investigations have demonstrated the efficiency of micro-ATR-FPA analyses in showing LDPE biodegradation (Mendoza et al., 2025; Tagg et al., 2015)

Ultimately, GC-MS analyses identified the metabolic intermediate products resulting from the LDPE degradation process. Upon examining the GC-MS fingerprints of LDPE films, the control films

(without bacterial addition) exhibited many innate compounds of polymer strips (fatty acid esters), which are intricate and resistant to degradation. However, the *Bacillus* species-treated films exhibited numerous hydrocarbon peaks, indicating the biodegradation of the polyethylene backbone. Several previous scientific reports have suggested the presence of alkanes in microbial-treated LDPE films as an indication of depolymerisation (Sanniyasi et al., 2021). Additionally, the current evaluation of *Bacillus*-treated polymer films revealed novel peaks in the sample spectrum for compounds such as undecane and halogenated hydrocarbons, including 2-bromododecane. The bromine molecules are added to the hydrocarbon chain in the inner layer stabilisers or the polymers' outer surface modification agents to improve the compound's stability and wettability (Desai & Singh, 2004; Z. Jiang et al., 2023). These compounds may become visible in the bacterial-treated GC spectrum due to surface layer cracks or deep pits formed by the bacterial strain on the polymer. Our current study's SEM analyses have demonstrated the adhesion of strains on the inner and outer layers of the LDPE film, as well as their ability to pit or fracture the polymer. Our study's FTIR spectrum also confirms the presence of bromine compounds. These metabolic products can also enhance the polymer's hydrophilicity. Most interestingly, all the end products illustrated in the *Bacillus cereus* NJD1-treated spectrum were non-toxic intermediates of the depolymerisation of LDPE films. It manifests the strain's efficacy in attaching to complex and harmful substances.

GC-MS analysis of polymer strips inoculated with *Pseudomonas aeruginosa* NJD2 manifested the presence of several hydrocarbons, saturated fatty acids, phenols, esters, and other volatile intermediates important in the biodegradation process. Numerous prior investigations have demonstrated the potential impact of *Pseudomonas aeruginosa* in the bioremediation of LDPE films (Medić et al., 2020). The long-chain alkanes such as 2-methylhexacosane ($C_{27}H_{56}$) and tetrateracotane ($C_{40}H_{82}$) were present in the *Pseudomonas*-treated LDPE films. Scientific work has reported similar peaks (Khandare et al., 2022). The ester compound in our chromatogram, namely 1,2-benzene dicarboxylic acid dioctyl ester, was recently identified as a volatile compound in the GC-MS analysis of LDPE film treated with *Cladosporium sphaerospermum*, a highly promising fungal species isolated from a plastic garbage site. Moreover, a distinct degraded compound, phenol 3,5-bis(1,1-dimethyl-ethyl) ($C_{14}H_{22}O$) (Sai Latha et al., 2020), was demonstrated in our chromatogram of the bacterial-treated film, which was also reported in an early GC-MS study (Abraham et al., 2017) of incubating *Aspergillus nomius* on LDPE films for 90 days. A fatty acid ester peak of tridecanoic acid, specifically the 12-methyl ester, elucidated in this study, may provide strong evidence for bacterial adherence to LDPE film (Lattuat et al., 2002). In another study, GC-MS analysis of polymer pieces treated with the *Pseudomonas aeruginosa* WD4 strain, isolated from a plastic dumpsite, revealed a peak corresponding to the alkane compound 5-ethyl-5-methyl decane (Shilpa et al., 2022). In our study, such identical peaks were also displayed. The alkane peak of heptadecane and the

methylated alkane, heptadecane,2,6,10,15-tetramethyl, may be intermediates of long-chain hydrocarbons (J. Yang et al., 2015). In our investigations, volatile alkanes, such as octadecane and tetradecane, were commonly found in control and bacterial-treated films. However, many long-chain alkanes, esters, aromatic compounds, and plasticisers were unique to the chromatogram of the control LDPE film. They were not detected in the GC-MS reports of *Pseudomonas aeruginosa*-inoculated LDPE films. It explains the strong biofilm formation and its catalytic effects on degrading polymer films.

The spectrum of the *Micrococcus luteus*-treated LDPE film revealed several new peaks for fatty acid esters, alkanes, bacterial metabolites, and plasticiser intermediates, in contrast to the GC-MS chromatogram of the LDPE film without bacterial biofilm (control film). They were tetradecanoic acid 12-methyl, methyl ester, phthalic acid,4-cyanophenyl heptyl ester, Undecane,1,2-benzene dicarboxylic acid, heptadecane 2,6,10,15-tetramethyl,2-bromotetradecane, and squalene. The volatile compound, tetradecanoic acid 12-methyl, methyl ester (a methylated fatty acid ester), may be formed due to the catalytic activity of the solid adherence of the bacterial strain on the polymer. Similar methylated fatty acid ester products were recently reported in the GC-MS analysis (Lee et al., 2023). A unique alkane compound, Undecane, and a methylated alkane compound, heptadecane 2,6,10,15-tetramethyl, reported in our study, demonstrate the ability of the isolated strain to utilise long-chain hydrocarbons as an energy source (Jeon et al., 2021). Furthermore, recent research has revealed that the esterase enzyme generated by certain bacterial strains can convert

complex phthalates—used as plasticisers in LDPE films—into less hazardous compounds.

Similarly, in the current analysis, enzyme analysis studies revealed the presence of esterase enzyme production by *Micrococcus luteus* NJD3 after incubation with LDPE films in a mineral salt medium. A few phthalates, often called phthalic acid esters (PAEs), were detected in the control LDPE film. These include 1,4-benzene dicarboxylic acid, bis(2-ethylhexyl) ester ($C_{24}H_{38}O_4$), phthalic acid butyl undecyl ester ($C_{23}H_{36}O_4$) (Y. Wang et al., 2025), dibutyl phthalate ($C_{16}H_{22}O_4$), and diisooctyl phthalate ($C_{24}H_{38}O_4$). These were applied to the LDPE films as plasticisers (Uzamurera et al., 2023). Additionally, the surface of control LDPE displayed 2,4-ditert-butyl phenol ($C_{14}H_{22}O$) and 7,9-ditert-butyl 1-oxaspiro (4,5) deca-6,9-diene-2,8-dione ($C_{17}H_{24}O_3$), which act as antioxidants (Vera et al., 2023). These compounds harm our surroundings and damage the endocrine and reproductive systems of humans. Surprisingly, most of these compounds disappeared or transformed into less toxic compounds or metabolic products in the GC-MS chromatogram of bacterial species inoculated on LDPE strips. In the bacterial-inoculated spectrum, we observed only 1,2-benzene dicarboxylic acid (Phthalic acid, PA) and a nitrogen-containing phthalic acid derivative, 4-cyanophenyl heptyl ester. A prior study by (P. Wang et al., 2021), which isolated a bacterial species of *Achromobacter* from activated sludge, demonstrated the metabolic pathway for the conversion of di-(2-ethylhexyl) phthalate ester (DEHPE, a PAE) into phthalic acid (PA). It revealed the strain's ability to biodegrade the toxic compound. Likewise, the peak of 1,2-

dicarboxylic acids in the present GC-MS investigation has also validated the transformation ability of PAE into PA in the presence of *Micrococcus luteus*. The bromination in the LDPE film is one of the vital routes for improving its surface properties. Thus, the presence of 2-bromotetradecane in the GC-MS spectrum of the bacterial-treated polymer film may be attributed to the interaction of bromine with hydrocarbons through cracks and indentations formed by biofilm adherence. Moreover, the squalene peak observed in the bacterial-treated fingerprint may be attributed to the strong attachment of the isolated strain to the LDPE film (Song et al., 2020).

All the validation studies demonstrated the efficacy of *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, and *Micrococcus luteus* NJD3.

4.7 Conclusion

This chapter examined the efficacy of isolated and enriched bacterial strains, specifically *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, and *Micrococcus luteus* NJD3, in degrading low-density polyethylene (LDPE) films. To demonstrate degradation, we employed several reliable and sophisticated analytical instruments. Biofilm formation and changes in surface morphologies, including the formation of pits, cracks, or fissures, were demonstrated by SEM analysis. In addition to the topographic deviations, the elemental differences in carbon and nitrogen on the polymer surfaces were demonstrated by SEM-EDAX analyses. The difference in surface roughness of LDPE strips with and without bacterial inoculation was demonstrated using AFM data. Another method used to illustrate surface alteration in our research was the measurement of contact

angles using a video-based optical instrument from GmbH. It validates the reduction in hydrophobicity resulting from robust biofilm formation and enzymatic reactions on LDPE films. The mechanical strength measurements, conducted using a universal tensile machine (Tinius Olsen), confirmed the reduction in tensile properties. Through analysing FTIR chromatograms (utilising Shimadzu ATR-FTIR apparatus) of both untreated and treated polymer strips, we discerned unique functional groups and chemical modifications on the surfaces of LDPE films injected with bacterial cultures. These alterations may result from the enzymatic activities of bacterial strains that generate biofilms. Moreover, we calculated keto-carbonyl, ester-carbonyl, vinyl-carbonyl, and internal double bond formation values and represented them accordingly. FPA images were captured using a Hyperion 3000 inverted microscope and an ATR-FTIR spectrometer to confirm the chemical changes in LDPE films. The degradation intermediates by the action of each bacterial species were recorded using a GC-MS spectroscope. All the validation experiment results demonstrated the efficacy of each isolated bacterial strain from the natural depot in degrading LDPE film.

CHAPTER 5

FORMULATION OF AN IN VITRO BACTERIAL CONSORTIUM FOR ACCELERATING THE BIODEGRADATION OF LDPE FILM

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5.1 Introduction

The alarming upswing in plastic waste has prompted scientists to develop a novel, safe, and cost-effective microbial co-culture process for biodegrading complex polymers. Many recent researchers have explored the synergistic effect of microbes to achieve better degradation (Cao et al., 2022). The coexistence effect could be attributed to microbes' stability, functional resilience, and ability to complete complex degradation tasks (Ojha & Skariyachan, 2024).

However, not all strains within the consortia may participate directly in the comprehensive decomposition of toxic pollutants; some may facilitate adhesion, while others may degrade or convert complex components into simple sugars. Meanwhile, a few may only mineralise them into simple sugars, fatty acids, and other end products. This systematic allocation of metabolic functions alleviates the responsibility of each cell to mineralise the harmful and resistant elements of contaminants (Ben Said & Or, 2017).

Microbial consortia or co-cultures can be isolated from the natural environment or formulated under artificial conditions. To design effective artificial consortia, scientists mixed pure cultures isolated from different natural environments to establish a multi-enzyme catalytic metabolism pathway for better bioremediation of polyethylene compounds (Putcha & Kitagawa, 2024). In addition, an artificial microbial consortia system has a more defined division of labour and elevated capabilities for metabolising various target products (Jones & Wang, 2018). A few bacterial strains in a consortium have proved their capabilities to form effective biofilms in

the presence of extracellular polymeric substances. High catalytic strains were selected to bio-remediate toxic compounds. Lower frequency of mutation, low toxicity, and high endurance are the standard features of consortia.

5.1.1 Strategies for Developing Microbial Consortia

The microbial consortia rely on two primary principles: top-down and bottom-up (Lyu et al., 2024; C. Zhang et al., 2023). The top-down technique utilises carefully selected environmental elements to stimulate a functioning microbiome, whether naturally occurring or intentionally created in vitro, to make ecological decisions that enhance the intended biological processes. A comprehensive understanding of physical, chemical, and biological elements that facilitate or hinder the individual or consortium is required to create a suitable consortium using a top-down method. It often overlooks the intricate mechanisms between the strains (Lawson et al., 2019).

Recent advancements in molecular biology tools, including high-throughput sequencing and multi-omics approaches, have empowered researchers to adopt bottom-up strategies to alter bacteria's genomic organisation and metabolic pathways (C. Zhang et al., 2023). To construct a bottom-up design, we require a more profound understanding of the genomic setup for catalytic activities and cell-cell communications (Liang et al., 2022). The mechanism is primarily based on the genomic and metabolic network of an individual strain, as well as its metabolic fluxes between and within the population, to form an effective consortium. Therefore, scientists typically favour top-down processes for elucidating the earliest interactions of microbial

populations with harmful polymer surfaces (Vargas-Suárez et al., 2019). However, a combination of top-down and bottom-up approaches is required to overcome the significant bottlenecks in developing effective consortia.

5.1.2 The effect of microbial consortium in LDPE biodegradation

Researchers have focused on formulating new microbial consortia for the effective and sustainable degradation of LDPE (Khampratueng & Anal, 2025). This approach offers significant benefits over pure isolates for the bioremediation of long-chain hydrocarbons (Sah et al., 2011). Microbial consortia are deemed optimal because they enhance the rate of safe detoxification via co-metabolism or inadvertent degradation (Nunes et al., 2024). The isolation and further enrichment of microbial consortia from their natural habitats offer extensive opportunities and applications in the remediation of toxic pollutants, such as those found in LDPE materials.

5.2 Aim and Objectives

1. Formulate an in vitro consortium using isolated potential strains.
2. Check the attachment of strains to LDPE film preliminarily by weight reduction experiment.
3. Verify the initial bio-attachment by BATH test, FDA test and CO₂ evaluation test.
4. Confirm the biofilm formation by estimating the EPS, protein content, medium pH change, and viability test.
5. Manifest the catalytic activities of the consortium inoculated on LDPE films by quantitative analyses of enzyme production.

6. Validate the consortium's biodegradation efficiencies by SEM, SEM-EDAX, AFM, contact angle measurements, tensile testing, FTIR, and GC-MS analysis.

5.3 Review of Literature

Natural or in vitro microbial formulations have recently gained acceptance in the bioremediation mechanism of LDPE films (Pinto et al., 2022; Salinas et al., 2024). Extended exposure of microbes to polyethylene compounds in the ecosystem may account for their evolutionary adaptations (Escalante et al., 2015).

S. No.	Microbial strains in the consortium	Nature of consortium	Effectiveness on LDPE films	References
1	<i>Enterobacter</i> sp. and <i>Pantoea</i> sp.	Isolated from plastic landfills and formulated as a consortium in in vitro conditions	High rate of weight loss (81%) within 120 days of incubation	(Skariyachan et al., 2016)
2	<i>Bacillus vallismortis</i> , <i>Pseudomonas protegen</i> , <i>Stenotrophomonas</i> sp. and <i>Paenibacillus</i> sp	Enumerated from a deep dump of plastics	75% weight loss in 120 days of incubation	(Skariyachan et al., 2017)
3	<i>Arthrobacter</i> sp. and <i>Streptomyces</i> sp.	Plastic dumps in the agricultural field	Biofilm formation within 90 days of incubation	(Han et al., 2020)

Table 5.1 Recent scientific investigations have demonstrated the efficacy of bacterial consortia for the remediation of LDPE films. All the Bacterial strains in the consortia were isolated from different plastic depots in terrestrial ecosystems and formulated as a consortium in the laboratory.

Recently, scientists have demonstrated that microbial consortia on LDPE films can also readily adapt to other recalcitrant pollutants, exhibiting accelerated enzyme production and metabolic activities (Bhatia et al., 2018). Most microbial consortia were isolated individually from natural ecosystems and then mixed in laboratory conditions (Gad et al., 2024; Patowary et al., 2016) to enhance biodegradation rates.

S. No	Consortia-bacterial species	Effectiveness	References
1	<i>Bacillus safensis</i> and <i>Bacillus amyloliquefaciens</i>	An 18% weight reduction in 30 days of incubation	(Waqas et al., 2021)
2	<i>Bacillus</i> and <i>Sphingobacterium</i> species	Isolated from a landfill. Extracellular enzymes, such as esterases, were produced to form cavities and rough surfaces.	(Khampratueng et al., 2024)
3	<i>Bacillus</i> sp and <i>Enterobacter asburiae</i>	Isolation from the gut of the larvae of <i>Ploida interpunctella</i> (Indian meal moth) proved the new carbonyl group formation on LDPE films.	(Y. Lou et al., 2020; Yang et al., 2014)

Table 5.2. A few *Bacillus* species contain a bacterial consortium capable of biodegrading LDPE.

Interestingly, the bacterial consortium extracted from the gut of various insect larvae has also proven effective for LDPE bio-degradation (H. Lou et al., 2022; Yin et al., 2020). It involves complex mechanisms, including physical (chewing), chemical (dissolution by acids in the alimentary canal), and biological (metabolic activities of gut symbionts) alterations of LDPE films (Cassone et al., 2020). In addition to these findings, the plastisphere, which is closely linked to

the marine environment, has been shown to display various bacterial co-cultures (Joshi et al., 2022; Syranidou et al., 2019).

Bacillus and *Pseudomonas* species were frequently identified as the predominant bacterial species in the formulated consortia for LDPE biodegradation (Pathak & Navneet, 2017). Consortia comprising *Bacillus subtilis* and *Bacillus licheniformis* exhibited cracks and effective colonisation on the polymer surface (Yao et al., 2022a).

Mixed cultures of *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Pseudomonas syringae* exhibited biofilm formation within 40 days of inoculation, and extended incubation up to 120 days demonstrated weight loss (Kyaw et al., 2012).

S. No	Consortia-bacterial species	Effectiveness	References
1	<i>Pseudomonas aeruginosa</i> and <i>Enterobacter</i> sp.	The cow dung formulated consortium showed high efficacy in degrading pre-treated LDPE ($64.25 \pm 2\%$) and polypropylene ($63 \pm 2\%$) strips under UV light and kept for incubation at 37°C for 160 days.	(Skariyachan et al., 2021)
2	Some <i>Pseudomonas</i> include <i>P. putida</i> , <i>P. fluorescens</i> , <i>P. aeruginosa</i> and <i>P. stutzeri</i> , <i>Vibrio alginolyticus</i> , <i>Flavobacterium</i> sp., and <i>Anabaena</i> sp.	Degrade LDPE film in the presence of various percentages of starch under a slightly alkaline P^{H} of 7.5	(Veethahavya et al., 2016)
3	<i>Pseudomonas aeruginosa</i> and <i>Brevibacterium</i> sp.	At multiple temperatures and P^{H} , the bacterial consortium degrades LDPE films.	(Dwicania et al., 2019)

Table 5.3 *Pseudomonas* sp. containing co-cultures/mixed cultures for LDPE biodegradation

One of the most recent studies employed a sequential and induced enrichment strategy to characterise plastic-degrading microbial consortia from intentionally polluted microcosms (Salinas et al., 2023). Such consortia have a broad spectrum of enzymes, including lipases, cutinases, and polyphenol oxidases. Several other researchers have constructed effective bacterial-fungal consortia using various polluted environmental samples for LDPE biodegradation (Fachrul et al., 2024; Kućić Grgić et al., 2023). Furthermore, based on GC-MS and FTIR findings, as well as enzyme studies, several delineated metabolic pathways for the degradation of LDPE materials have been identified (W. He et al., 2024). Recently, researchers have begun to utilise multi-omics technologies to gain deeper insights into the functional gene activities of potential strains for the biodegradation of recalcitrant polymers (Sahoo et al., 2021; Wright et al., 2021; Y. Zhang et al., 2023).

5.4 Materials and Methodology

5.4.1 Initial formulation of the consortium and its enrichment

To create an artificial consortium under in vitro conditions, pure cultures of *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, and *Micrococcus luteus* NJD3 were cultured in Reasoner's broth for 48 hours and removed from the incubator upon achieving optimum growth, indicated by an optical density (OD₆₁₀) range of 0.7-0.9. The samples were centrifuged for 12 minutes (at 12,000 rpm) using a Remi RM-1216 Microcentrifuge. After carefully discarding the supernatant, the pellets were washed with freshly prepared CFLMSM (carbon-free liquid mineral salt medium) at least three times. Finally, the pellets

were resuspended in 2 mL of CFLMSM broth solution. All aliquots were combined and resuspended in 100 mL of CFLMSM.

Additionally, we have incorporated surface-sterilised LDPE sheets, each weighing 0.30 g, resulting in a total of 0.24 g per 100 mL of carbon-free broth. Control flasks have been maintained without bacterial inoculation. Both treatments were initially incubated at room temperature for one month (Shilpa et al., 2023). A 20 mL CFLMSM broth culture with an optical density at 610 nm ranging from 0.7 to 0.9 was collected and spun at 15,000 rpm for 7 minutes using the Remi centrifuge (model RM1216). Pellets were harvested after removing the supernatants. Then, the pellets were washed with CFLMSM and resuspended in freshly prepared 2 mL of CFLMSM. All of the aliquots were then employed for the second stage of enrichment. For this, the conical flasks were kept statically at room temperature for 120 days. A control without respective culture aliquots was maintained for each set of bacterial enrichments. The LDPE films from all the conical flasks were collected and placed in a saline solution (0.85% NaOH) and agitated for 5 h at 180 rpm. For the subsequent biodegradation reactions, we purified the bacterial consortia cultures from biofilms of LDPE films.

5.4.2 Weight reduction experiment

5 mL of the consortium from the saline solutions was obtained for each experiment, confirming the OD₆₁₀ value of the bacterial culture between 0.7 and 0.9. The collected saline solutions were spun at 15,000 rpm for 7 minutes to isolate the consortia pellets. Using the static culture method, the pellets were resuspended in 100 mL of

freshly prepared CFLMSM, along with surface-sterilised LDPE films, and cultured for 120 days at room temperature. The total weight of each LDPE film before its introduction into the conical flask was standardised at 0.03 g. After incubation, all the polymer strips were collected and subjected to surface sterilisation to eliminate biofilm adherence before weighing (Shimadzu-ATX84R). The comparative weight variation of each film relative to the control films has been recorded and quantified as the percentage reduction in the weight of the polymer films (Awasthi et al., 2017). Furthermore, we assessed the consortium's daily consumption of LDPE films and determined the polymer's half-life (Auta et al., 2018).

5.4.3 Preliminary studies for establishing the growth of bacterial consortium on LDPE films

5.4.3.1 Estimation of hydrophobicity by the BATH test

The bacterial adhesion test for hydrophobicity (BATH) was conducted to demonstrate the hydrophobic nature of the formulated bacterial consortium, using a modified protocol illustrated by (Rosenberg et al., 1980). For each test set, 8 mL of consortium in saline solution with an OD₆₁₀ of 0.7-0.9 was aliquoted into four Eppendorf tubes and centrifuged at 15,000 rpm for 7 minutes using a Remi CM-12 PLUS cooling centrifuge (Ahmed et al., 2018). The collected pellets were suspended in 2 mL of CFLMSM and transferred to four test tubes. Add the different volumes of hexadecane (0.05, 0.1, 0.15, and 2 mL) to each test tube, mix well for 2 minutes, and allow it to stand for 3 hours to observe the phase transition. Then, the aqueous phase was carefully separated, and OD₆₀₀ values were measured. Control samples without

hexadecane were maintained at baseline OD₆₀₀ values. The recordings were used to compute the hydrophobicity percentage and depletion in turbidity to analyse the capabilities of the strain to attach to the hydrocarbon compound (Devi et al., 2023).

5.4.3.2 Fluorescein diacetate test (FDA test) to detect biofilm on LDPE film

This assessment evaluates the sustainability of biofilms on polyethylene film surfaces (Tribedi et al., 2015). The LDPE films, incubated in CFLMSM with the consortium for 5, 10, 15, 20, 25, and 30 days, were subsequently immersed in fluorescence diacetate phosphate buffer (FDPB). The samples were incubated at 25 °C and agitated at 180 rpm in a Kemi Orbital shaker for 1 hour. Two millilitres of the FDPB buffer were harvested and centrifuged at 12,000 rpm for 5-6 minutes. The culture supernatants were carefully taken to record absorbance at 494 nm using a Systronic UV-VISIBLE spectrophotometer. The FDPB buffer, devoid of inoculation, served as the control.

5.4.3.3 CO₂ evolution by the inoculation of bacterial strains for 120 days

The test followed the modified protocol of (Pramila, 2011) using a self-constructed leak-proof apparatus comprising a conical flask containing CFLMSM inoculated with donor flask either with bacterial consortium-treated LDPE films (positive control) or without them (negative control) and a receiver flask containing 100 mL of 1M KOH solution and transparent connecting tubes. The presence of trapped CO₂ in the receiver flask after 120 days of incubation was demonstrated by adding 0.1M BaCl₂, which precipitated white barium

carbonate crystals. The weight differences in the positive and negative controls were used to measure the CO₂ release.

5.4.4 Confirmatory tests to establish biofilm formation by the in vitro consortium

5.4.4.1 Estimation of extracellular polysaccharide (EPS) content

It is a confirmatory assay for biofilm formation. We extracted the biofilm into a saline solution via centrifugation (10,000 rpm for 25 minutes), and the resultant supernatant was dialysed overnight using a dialysis membrane disc (Spectra S/P11,6000-8000 MWCO,33 mm diameter). The dialysis used deionised water at 4°C. The EPS content was determined using the phenol-sulfuric acid method (Bohutskyi et al., 2015).

5.4.4.2 pH change in the carbon-free broth

Checked the pH change of CFLMSM estimated every 15 days, with intervals up to 120 days. Positive-negative control conical flasks were maintained with or without bacterial treatment. The initial pH of the medium was 7.2 ± 0.2 , using a 0.1 M potassium phosphate buffer. The results of the test were represented in a graph.

5.4.4.3 Estimation of protein content

The biofilm's protein content was estimated to confirm the strong adherence of consortia to LDPE films. The Bradford assay (Bradford, 1976) was used to estimate the protein content. The standard protein curve was plotted using the known protein concentration (BSA). The absorbance of the culture supernatant at 30, 60, 90, and 120 days was measured at 595 nm to calculate the protein concentration in µg/mL.

5.4.4.4 Estimation of the FDA test for 120 days

The FDA test was conducted on LDPE films administered with bacterial consortia collected from conical flasks at 30, 60, 90, and 120 days to assess the viability of the strains in the biofilms. The test was performed according to the protocol illustrated by (Green et al., 2006). The FDA test was also conducted on control LDPE films without bacterial addition to evaluate the impact of the strains on hydrolysis.

5.4.4.5 Enzyme activities by the strains

For the analysis, the specimens for crude enzyme extracts were collected from CFLMSM conical flasks (both from the bacteria consortia-treated (sample) and non-treated(control)) at an interval of 15 days and centrifuged (10,000 rpm for 12 minutes) (Muangchinda & Pinyakong, 2024). Then, the supernatants were collected and subjected to various enzyme assays, such as the Laccase assay (Re et al., 1999), lipase assay (Nawani et al., 2006), protease assay (Tsuchida et al., 1986), lignin peroxidase (Archibald, 1992), Manganese peroxidase (de Oliveira et al., 2009) and esterase activity (Kameshwar & Qin, 2017). Each enzyme assay of treated specimens was compared with the control samples (control CFLMSM without bacterial addition on LDPE films was used to assess the enzyme activities). All the results were plotted in Origin graphs.

5.4.5 Validation studies of the laboratory-mixed bacterial consortium for LDPE biodegradation

5.4.5.1 Scanning Electron Microscopy Analysis

After the treatment, the polymer strips were retrieved and surface-sterilised. Then, the moisture content was entirely removed by placing

it in the oven overnight at 55°C. Before micrographing, the polymer strips were treated with gold nanoparticles. Then, using the FE-SEM instrument of the ZEISS family, we analysed the microbial consortia and the topographic changes on the polymer film.

5.4.5.2 SEM-EDAX analysis

Scanning electron microscopy-energy dispersive X-ray spectroscopy analyses (Gonzalez-Gil et al., 2021) were conducted on LDPE films inoculated in CFLMSM with artificial bacterial consortium inoculation and without bacterial strain inoculation (control). This test was performed using the Jeol-SEM-EDAX-6390LA/ OXFORD XMX N instrument to detect surface changes and analyse chemical changes that occurred due to the attack of the bacterial consortium (using the instrument's EDS software).

5.4.5.3 Atomic force microscopy analysis

The WITEC 300—alpha AFM instrument was used to compare the surface roughness of artificially formulated consortia-added LDPE films with the polymer strips without microbial addition. For this analysis, the treated LDPE films with in-vitro consortia and the control films without inoculation were taken from the conical flasks after 120 days. They were dried by placing them in Petri dishes at 55°C overnight. Furthermore, these films were used to capture 2D and 3D micrographs for topological analysis. In addition, using WITEC software, the surface roughness parameters were calculated.

5.4.5.4 Measurement of contact angle using water droplet (WCA)

Aged LDPE films in CFLMSM medium for 120 days were taken and surface sterilised. Then, a drop of water molecule was placed on the

polymer film, and the contact angle measurements were recorded by a video-based GMBH contact angle instrument (Germany). This analysis compared the efficacy of a lab-mixed bacterial consortium against WCA of non-microbial-treated LDPE films (Y. Wang et al., 2024).

5.4.5.5 Ultimate tensile strength testing

This study examined the mechanical strength of LDPE films. In it, the LDPE strips of 6.5×1.3 cm were harvested from the medium for the analysis and surface sterilised (the samples were prepared according to the procedure established by ASTM D5882-18). Thickness, film area, force, tensile strength, break distance, and extension at failure were measured using a UTM (Universal Testing Machine) (Tinus Olsen; 50 kN) with a crosshead speed of 3 mm/min. The instrument's Horizon software helps us quantify the mechanical properties of LDPE film incubated with or without inoculation of bacterial strains (Control LDPE films maintained baseline tensile properties, confirming bacterial-induced reductions).

5.4.5.6 FTIR analysis of artificial consortia-treated LDPE films

5.4.5.6.1 FTIR-fingerprinting and indices calculation

The FTIR-ATR (Shimadzu) was performed on consortium-treated LDPE films and non-microbial-treated LDPE films after incubation in a carbon-free liquid medium under strenuous conditions. Then, the LDPE films (after removing biofilms from the treated samples) were sectioned into 1 cm × 1 cm pieces, and spectra were recorded (Q. He et al., 2018). Control LDPE films were incubated in CFLMSM without bacteria for 120 days to account for abiotic changes. The FTIR data generated by the instrument were plotted as a graph in Origin Pro software, and the FTIR indices (carbonyl and internal bond indices)

were calculated. The index values were plotted in a graph to analyse the differences between the treated LDPE and control polymer films.

5.4.5.6.2 FTIR imaging

To view the chemical changes on the LDPE film, we used a micro-attenuated total reflectance focal plane array (Micro-ATR-FPA) imaging instrument (Hyperion-3000 (Germany)) and a Vertex 80 FTIR microscope with a 2D-focal plane array (300×300 μm). Our study recorded micro-ATR-FPA images (1000-2000 cm^{-1}) of LDPE films with or without bacterial addition.

5.4.5.7 Gas chromatography-mass spectroscopy analysis

Nearly ten LDPE films (from both control and consortium-treated groups) were recovered from a mineral salt medium and subsequently fractured into tiny pieces following the removal of the biofilm. Integrate all constituents in a beaker, subject to ultrasonic agitation with 12 mL of chloroform. The extracts that had evaporated at room temperature were subjected to ultrasonic agitation and then held at 60 °C for three hours. Subsequently, combine 2 mL of chloroform with each extract and filter through a PTFE filter (0.45 μm). The filtered samples were also analysed using a hybrid GC-MS apparatus with GC-MS solution software (Jiang et al., 2019; Nakata et al., 2020). All the results of the control and treated samples were compared, and the degraded products were reported.

5.4.6 Statistical analysis

All the tests to prove the efficacy of the consortia-attached LDPE films were conducted in triplicate and recorded as mean $\pm$ SEM values using R-studio (R version 4.3.2). OriginPro software in 2018 was used to

draw graphs in the experiment. The significance level of all values was further tested by one-way ANOVA using GraphPad Prism (an open-source platform).

5.5 Result

5.5.1 Formulation of bacterial consortium in a carbon-free synthetic medium

The isolated species from natural plastic depots, *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, and *Micrococcus luteus* NJD3, were added to a carbon-free synthetic broth and sequentially enriched to form an artificial consortium stable at room temperature.



Fig.5.1 The enrichment of formulated bacterial consortium in a mineral salt medium for four months by adding bacterial cultures to a conical flask and comparing results with its control conical flask without bacterial inoculation.

All biodegradation experiments were conducted under static culture conditions. Continuous enrichment of the strains made them a stable inoculum to attach to the LDPE films. Our study used LDPE film without any pre-treatments apart from surface sterilisation. The optimum temperature for all the strains was observed to be between 28-33°C. The consortium containing a mineral salt medium showed

ample turbidity and visible biofilm formation with the LDPE film. It indicates the efficacy of bacterial strains that attach to the LDPE strips.

5.5.2 Enhanced weight loss due to adherence of bacterial consortium to LDPE film

The weight reduction attained by the formulated co-culture over 120 days evidenced the isolated strains' ability to engage with LDPE films in a controlled setting of static culture at 25-30°C incubation. This initial test determines how well the consortia adhere to and break down the complicated polyethylene component. Compared to the original weight of LDPE film, the consortia adhered to LDPE films exhibited a weight loss of $54.67 \pm 1.28\%$ upon adding the laboratory-prepared bacterial consortia. Furthermore, we have determined the formed consortium's daily consumption rate (K) to be $0.0066 \pm 0.0023\text{g}$, and the half-life of LDPE film treated with the consortium is 105 ± 3.7 days. This amplified effect may result from the consortium's beneficial influence on bacterial cultures on polyethylene films.

5.5.3 Results of initial investigations to prove the biofilm formation of consortia strains on the polymer surface

5.5.3.1 BATH test

BATH test showed a steady decrease in the hydrophobicity percentage with an increasing volume of hexadecane added to CFLMSM. It indicates the efficacy of bacterial strains to attach to hexadecane, an organic compound used in this analysis. The addition of 0.2 mL of hexadecane to the consortia pellets mixed in the carbon-free medium resulted in the highest hydrophobicity drop ($30.072 \pm 0.59\%$) in its

aqueous phase. However, the untreated hexadecane sample exhibited a higher hydrophobicity percentage in the aqueous phase compared to the aqueous phase of the organic compound-treated sample. It indicates the migration of highly hydrophobic bacterial colonies to the increased surface area of the highly hydrophobic compound (hexadecane). This study strongly validates the efficacy of bacterial strains in forming strong attachments to the polymer compounds.

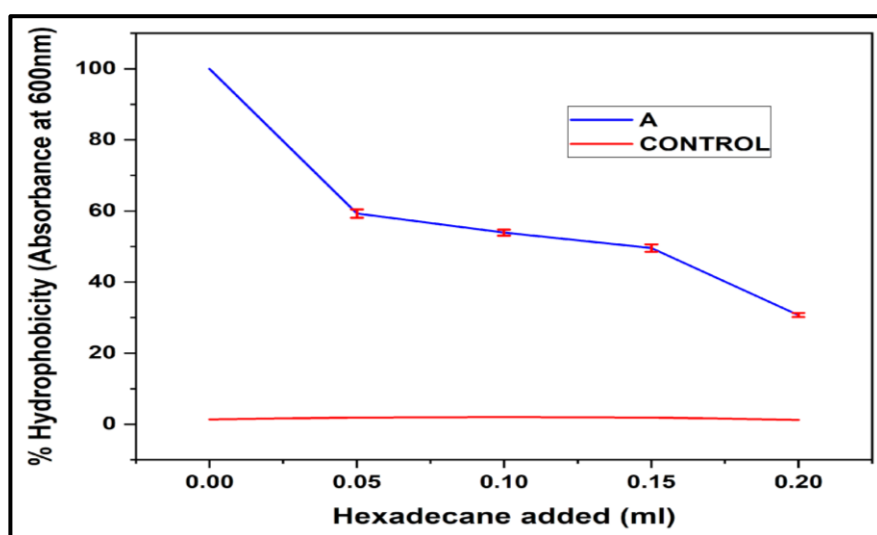


Fig.5.2 Results of the BATH test of biofilm of bacterial consortia on LDPE films. The blue line in the graph exhibits a maximum decrease in the hydrophobicity percentage of the strains in the aqueous phase when 0.2 mL of hexadecane is added to the consortia pellets mixed in the carbon-free medium ($30.072 \pm 0.59\%$).

5.5.3.2 FDA analysis of LDPE films incubated for 30 days along with the artificial co-culture in a carbon-free medium

In this FDA analysis, the viable cells on the LDPE films were tested at 5- to 30-day intervals of incubation to assess the consortium's efficacy in forming a biofilm on the polymer.

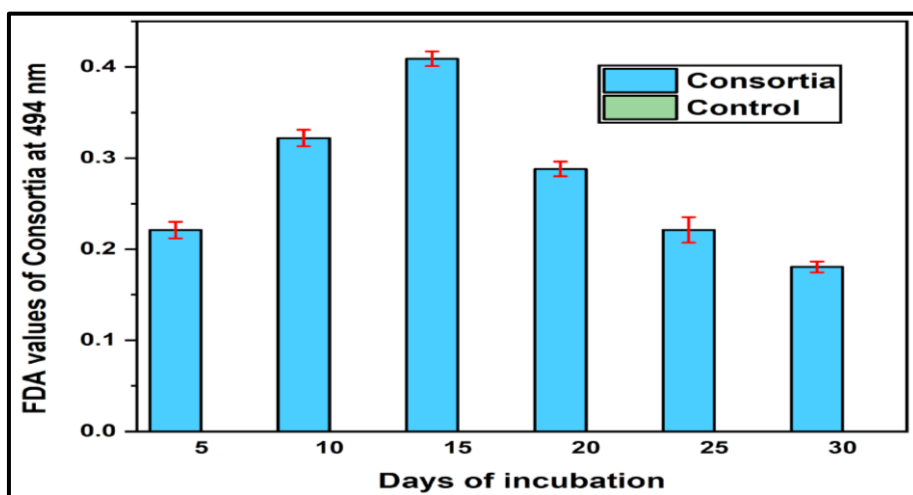


Fig.5.3 Results of the FDA test of LDPE strips attached with formulated consortia for one month of incubation. FDA test results show an increasing absorbance at 494 nm, indicating viable biofilm formation on LDPE films over 30 days.

5.5.3.3 CO₂ evolution test (Gravimetric Sturm test)

This experiment proves the catalytic activities of consortia on the LDPE films in a prolonged incubation period. A self-modified experimental setup was used to detect CO₂ production. In the current analysis, the rate of production of CO₂ within 30 days due to the treatment of bacterial consortia on LDPE films was reported as $10.3 \pm 0.61\%$ g/L.

5.5.4 Experimental evidence for biofilm formation on LDPE films in a carbon-devoid mineral medium for four months

5.5.4.1 EPS (Extracellular polymeric substances) Analysis

In this analysis, we found maximum EPS production (466.88 ± 3.8 µg/mL) at 90 days of incubation of LDPE films in the CFLMSM by bacterial consortia inoculation. As the biofilm formation escalates, the synthesis of extracellular polymeric substances (EPS) intensifies. In

our preliminary studies, the maximum growth of two organisms (*Bacillus cereus* and *Micrococcus luteus*) in the consortia was at 90 days of incubation. The overall results of our research, which included incubation periods of up to 120 days, demonstrated strong biofilm formation.

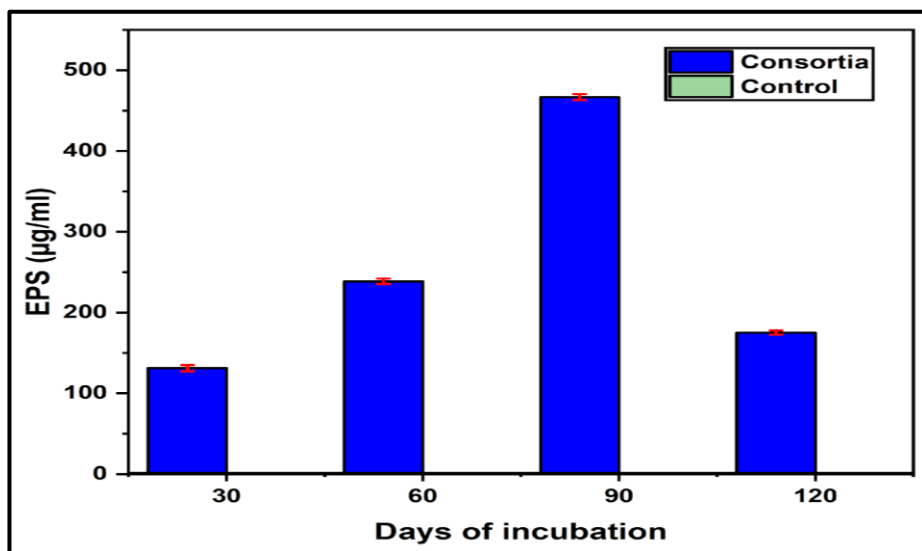


Fig 5.4 Graphical representation of extracellular polymeric substances (EPS) produced by the consortium on the LDPE films for stable biofilm formation (Abdul, 2020).

5.5.4.2 Estimation of pH changes in the inoculation medium

The pH changes in the CFLMSM medium inoculated with the consortia indirectly indicate biofilm formation on the LDPE films added to it and its catalytic activities. Compared to the medium's pH in the control flask (7.41 ± 0.084), the medium inoculated with the consortia (3.83 ± 0.12) exhibited a significant decline in the pH value (Results shown in Fig. 5.5).

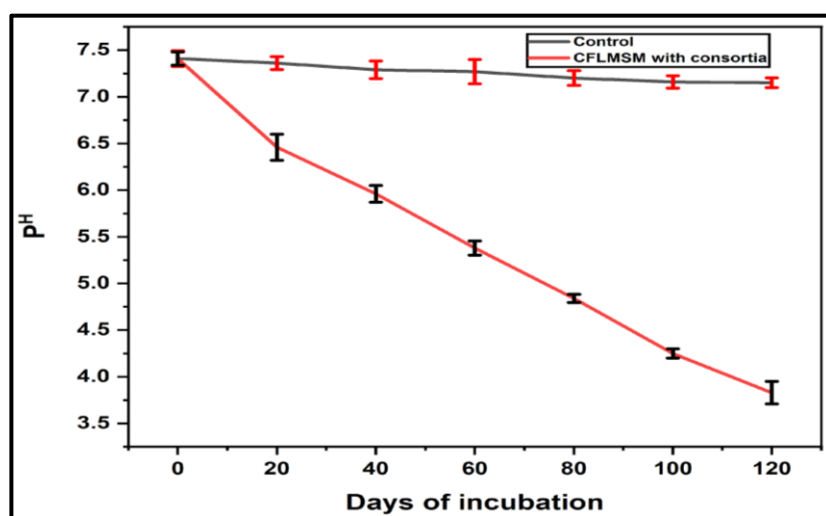


Fig.5.5 Graphical representation of change in pH of medium inoculated with consortia (red line) for an incubation period of 120 days compared with the control flask medium without the addition of formulated consortia.

5.5.4.3 Estimation of protein in the consortia-treated LDPE films

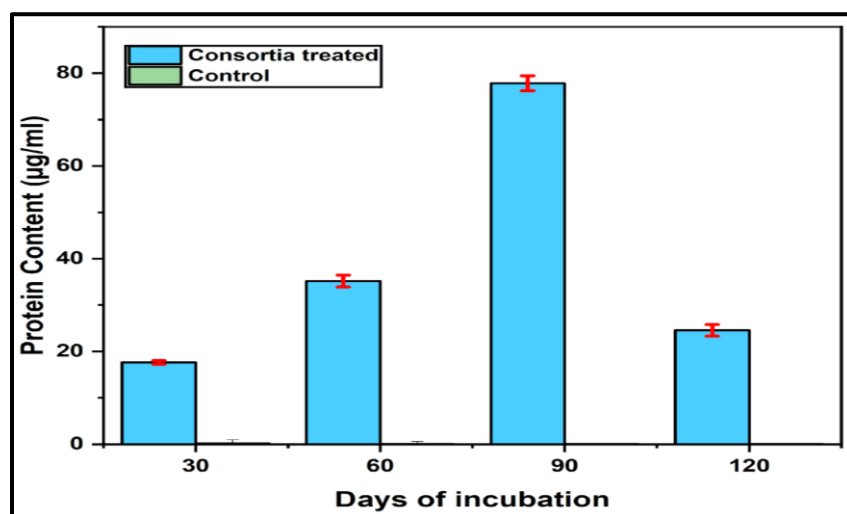


Fig.5.6 Result of estimation of protein content from the biofilms attached to LDPE films inoculated with artificial bacterial consortia for four months by Bradford assay.

The maximum protein content of the consortia was reported at 90 days of incubation ($77.81 \pm 1.62 \mu\text{g/mL}$). The extractable protein content

from the polymer surface indirectly indicates the adaptation of bacterial communities on the polymer film. Compared to the control LDPE film without bacterial inoculation, all treated conical flasks in the incubation showed a significant increase in protein content.

5.5.4.4 Viability of the In-vitro consortium treated LDPE film by FDA analysis

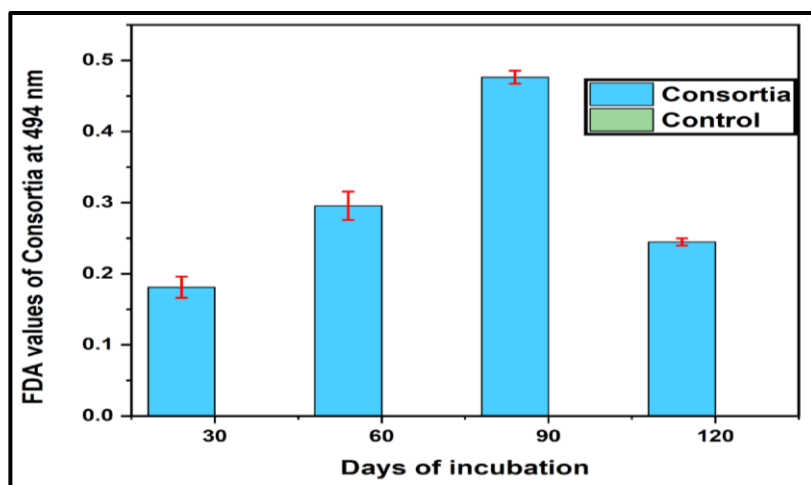


Fig.5.7 Viability of consortia on LDPE film for 120 days in CFLMSM.

Like other efficacy tests used to ensure biofilm formation in consortia, the FDA (Fluorescein diacetate test) demonstrated the efficiency of bacterial strains in our in vitro consortia in adhering to LDPE films. In our analysis, the strain's maximum viability appeared at 90 days of incubation.

5.5.5 Enzyme activities of bacterial consortium treated with polymer for 120 days in CFLMSM

We estimated enzymes from the biofilm-forming bacterial strains to confirm the growth consortia on the surface of LDPE films and their catalytic activities using the hydrocarbon chain as a carbon source. The escalated enzyme activities in the biofilm confirm their efficacies in degrading the polymeric compound (Results depicted in Fig.5.8).

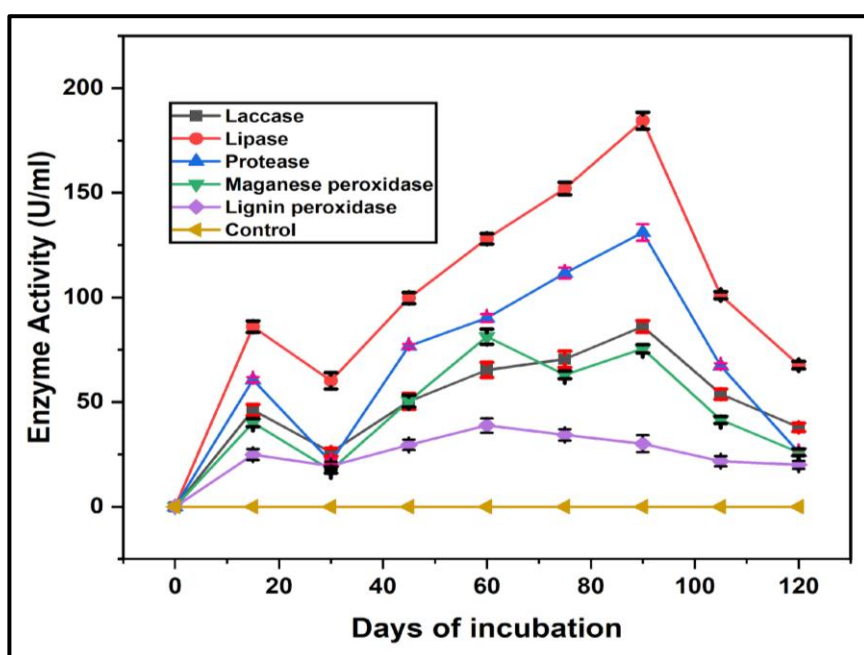


Fig.5.8 Estimating laccase, lipase, protease, Manganese peroxidase and lignin peroxidase enzymes in the biofilm consortia on LDPE films.

The maximum production of laccase, lipase and protease enzymes was observed at 90 days of incubation. However, the manganese peroxidase and lignin peroxidase enzyme production rate was maximum at 60 days of incubation. The result indicates the capabilities of all the bacterial species to work as a practical consortium. The

Pseudomonas species-producing enzymes, such as manganese peroxidases and lignin peroxidase production rates, were highest at 60 days of incubation due to the highest biomass of this strain exhibited at this incubation period. Furthermore, the *Micrococcus* species demonstrated an elevated production of enzymes such as esterases in the consortium-treated biofilms (Fig.5.9).

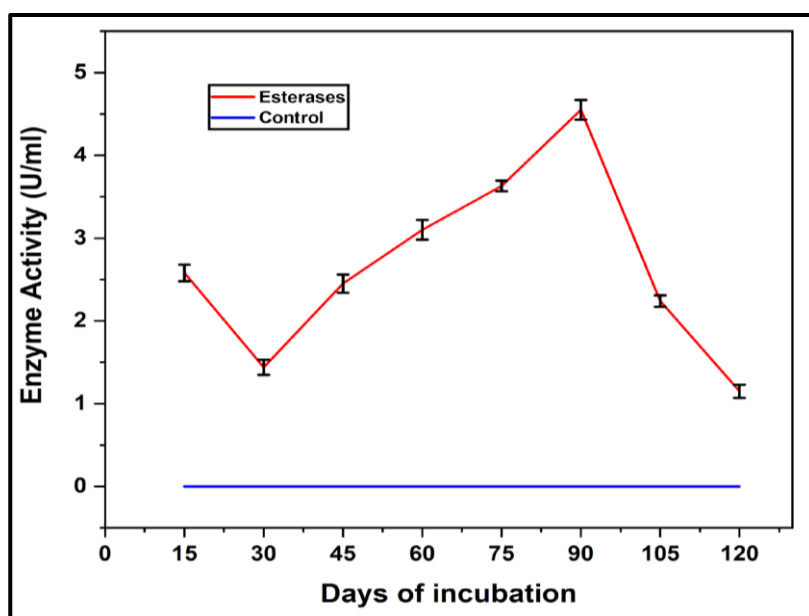


Fig.5.9 Esterase enzyme production by the consortium. The maximum esterase production was observed at three months (90 days) of incubation.

All the enzyme assays manifest the growth of all strains in the consortium.

5.5.6 Validation experiments for LDPE biodegradation using the formulated consortia on LDPE films, with an incubation period of 120 days in a carbon-free medium.

5.5.6.1 Field Emission Scanning Electron Microscopy

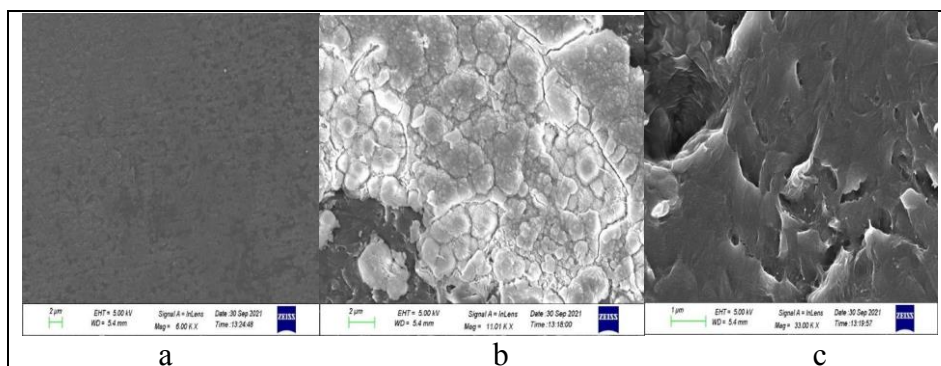


Fig.5.10(a) Micrograph of FE-SEM analysis of LDPE films placed in a carbon-devoid synthetic medium for four months. The morphology of the control film (a) shows a plane surface devoid of cracks, clefts, furrows, and inner-layer colonisation. Specimen LDPE film treated with a bacterial consortium (b) of *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, and *Micrococcus luteus* NJD3. It displays fine cracks, peel-off surface layers, notches, and surface friction. A magnified FE-SEM image of the bacterial consortium confirms the formation of grooves or pits after 120 days of incubation.

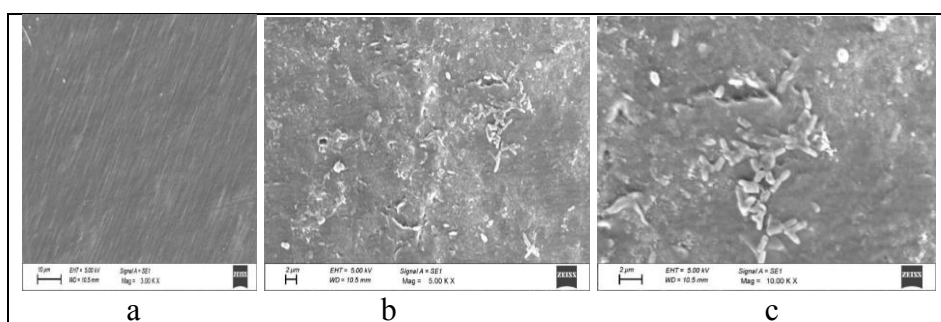


Fig.5.10(b) Conformation of biofilm formation at 90 days of incubation of LDPE films in mineral salt medium with or without adding in-vitro consortia. FE-SEM image (a). Control LDPE film without treating it with bacterial strains; it has no bio-attachment. Image b. Surface roughness and bio-attachment of bacterial consortia at 90 days after inoculation. Image c. Magnified image of biofilm formation by bacterial strains on LDPE film.

Our investigation revealed significant topographical changes in the consortia-treated LDPE films, including voids, cracks, inner-layer bio-attachments, cavity formations, and surface oxidations. These changes may be due to the catalytic activities of the strains adhered to the polymer surface. We have established robust biofilm development and surface irregularities, including minor groove formation, during the 90-day incubation of LDPE films in a carbon-devoid mineral medium. Further incubation of LDPE films up to 120 days proved the degradation abilities of bacterial strains.

5.5.6.2 SEM-EDAX analysis

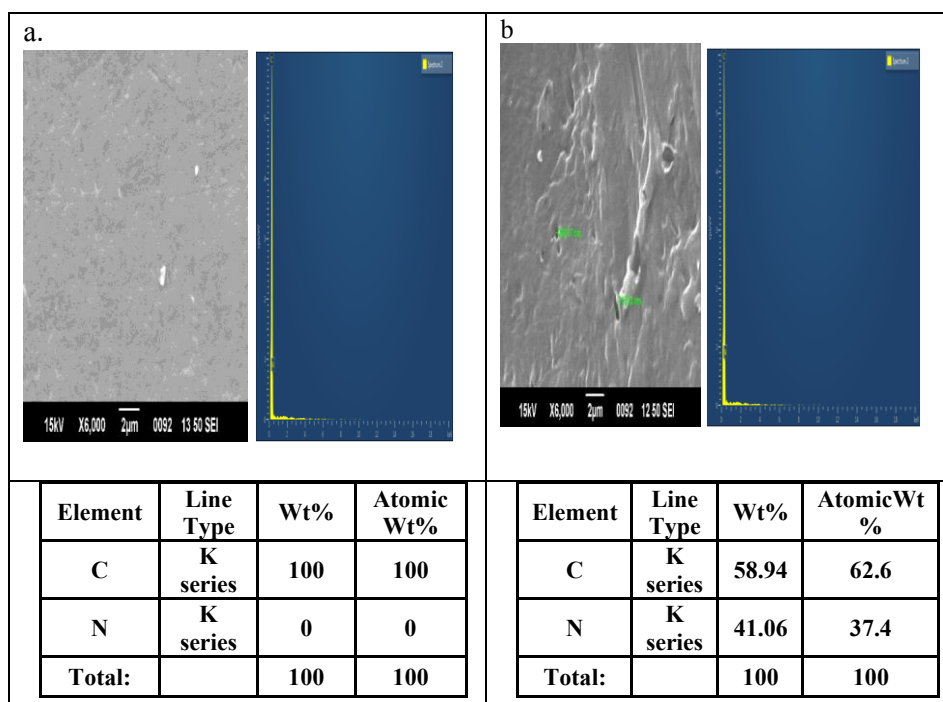
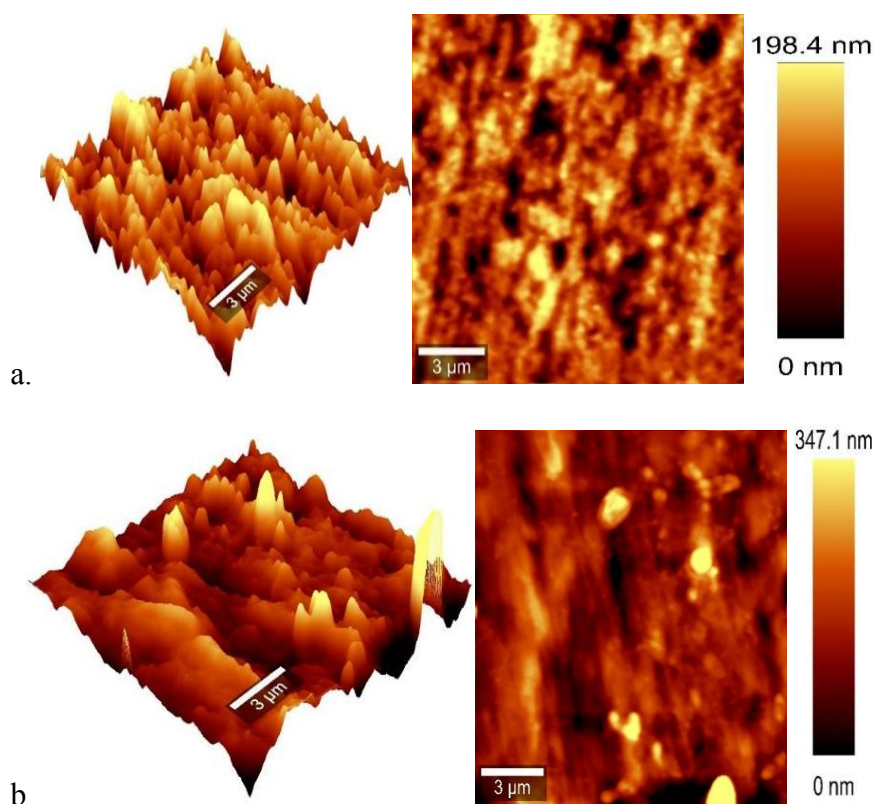


Fig.5.11 Micrographs from SEM-EDAX investigations documenting the elemental alterations in LDPE films with (image b) or without (image a) bacterial inoculation. It recorded the changes in elements (carbon and nitrogen) on the surface of polymer strips in terms of a K series line type (George et al., 2022).

SEM-EDS micrograph of consortia-treated LDPE film showed a notable reduction in the carbon, an increase in nitrogen elements (Elemental changes depicted in the image) and many surface alterations. The carbon element's weight percentage (Wt%) and atomic weight percentage (Wt%) were reduced from 100% to 58.94% and 62.6%, whereas the nitrogen element's weight percentage (Wt%) increased from 0% to 41.06% and 37.4%. Compared to prior scientific investigations (Chamas et al., 2020; Sathish et al., 2020), the current research showed distinct chemical changes in the LDPE film. It gives us a shred of solid evidence to prove the efficacy of consortia.

5.5.6.3 AFM analysis



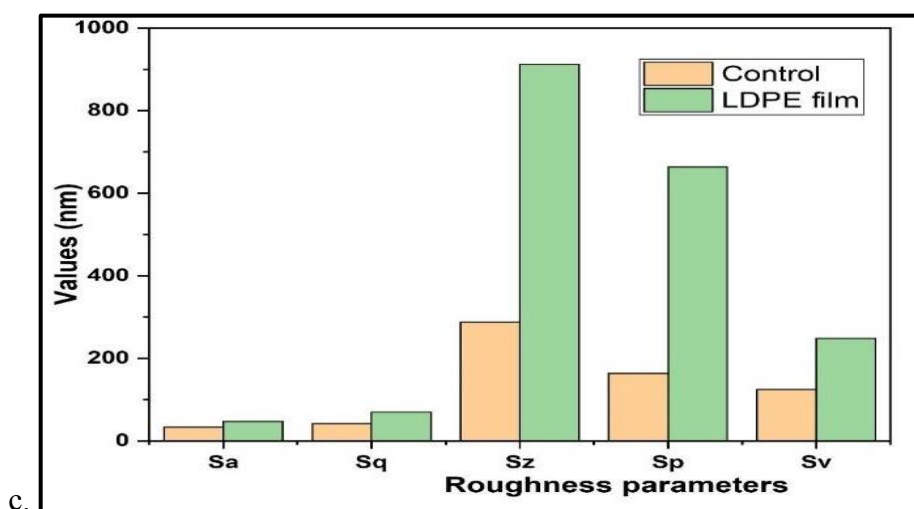


Fig.5.12 The high-resolution AFM images (3D-2D images) reveal the change in surface texture of the control film without bacterial inoculation (image a) and bacterial consortia constructed in our laboratory (image b). The surface roughness profile (at the nanoscale) of the control and consortium-treated LDPE film was calculated and displayed in a graph (image c).

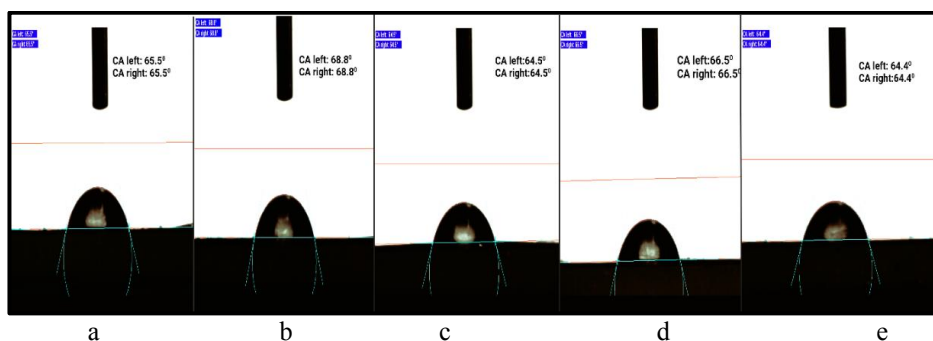
The AFM instrument's accurate imaging and quantification of surface roughness assisted our investigation in proving the efficacy of in vitro consortia for disintegrating LDPE films (Khandare et al., 2021). The 3D-2D AFM images revealed significant topography changes, including pits, fissures, grooves, and erosions, in the bacterial-treated LDPE film compared to the control film. Moreover, the cantilever tip of the instrument gently oscillated over the entire surface of the polymer strips. It quantified the surface roughness profile in the nanoscale (the mean average surface area- (Sa), root mean square of average surface roughness-(Sq), maximum valley roughness-(Sz), and peak height- (Sp) or pit height- (Sv)), represented in Fig.5.12 (Dey et al., 2020; Iaculli et al., 2017). These measurements helped to visualise the topographic changes exhibited by the bacterial consortia for 120 days. In contrast with control films, the sample films showed a marked

rise in Sa (43%), Sq (68%), Sz (217%), Sp (229%), and Sv (100%) values.

5.5.6.4 Contact Angle Analysis

The contact angle measurement, using water droplets (WCA), quantifies the degree of wettability imparted to the surface of LDPE film after four months of incubation with bacterial consortia (Rong et al., 2024). In our work, the artificial consortia we formulated exhibited a water contact angle of $65.94 \pm 0.81^\circ$ compared with the control film's WCA of $90.88 \pm 1.22^\circ$. These significant changes in contact angles demonstrated the consortium's ability to introduce hydrophilicity into the polymer.

a. Bacterial treated



b. Control film

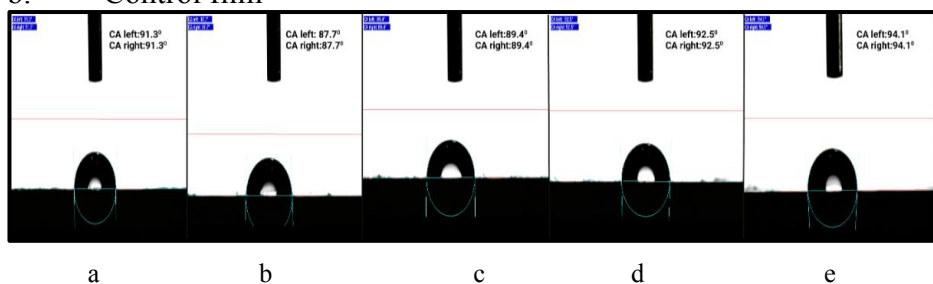


Fig.5.13 Water-based Contact angle (WCA) images of LDPE strips added with lab-formulated consortia (a. 65.5° b. 68.8° c. 64.5° d. 66.5° e. 64.4°) compared with the control LDPE film without bacterial consortia (a. 91.3° b. 87.7° c. 89.4° d. 92.5° e. 94.1°).

5.5.6.5 Tensile testing

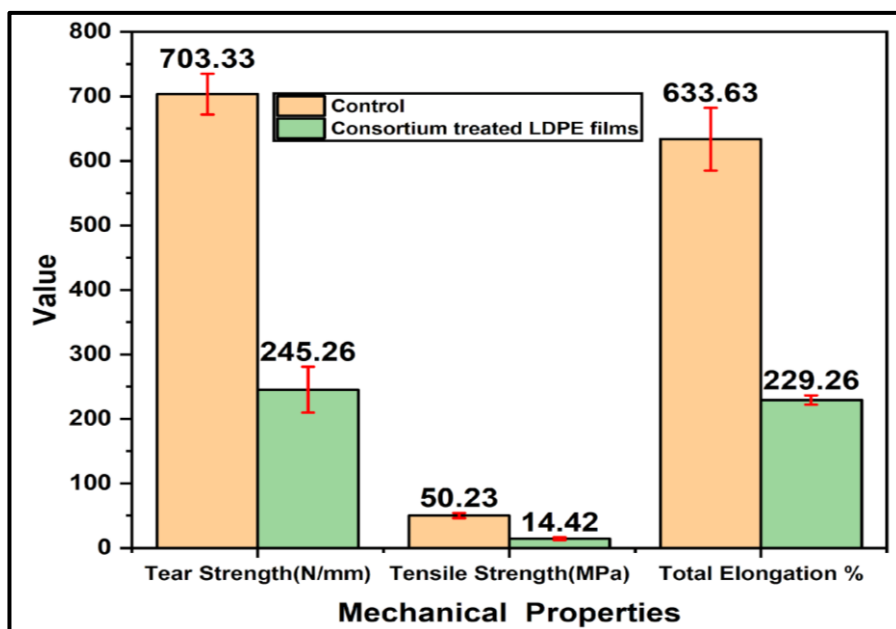
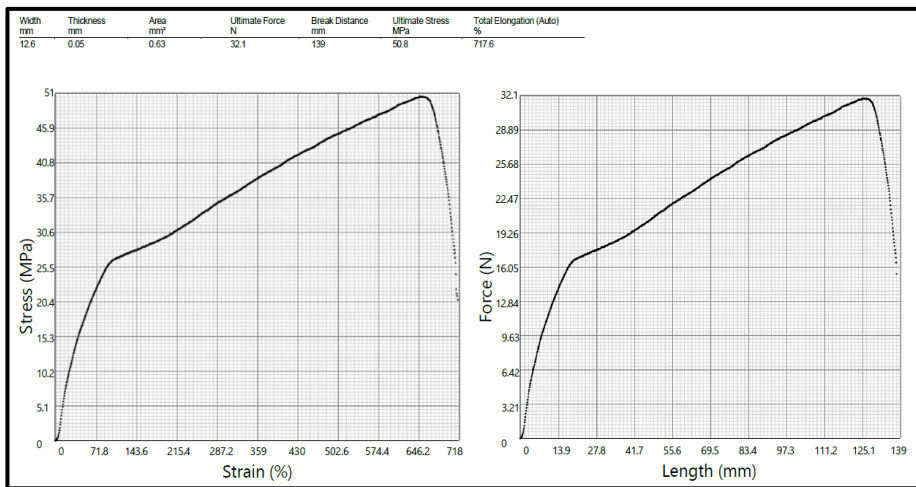


Fig.5.14(a) Results of tensile testing of consortia-treated LDPE films.

In contrast with the control film, the treated film reduced the tensile properties of LDPE strips. This manifests the consortia's capabilities in breaking hydrocarbon bonds through their catalytic activities. Control LDPE films-maintained baseline tensile properties, confirming bacterial-induced reductions.

a.



b.

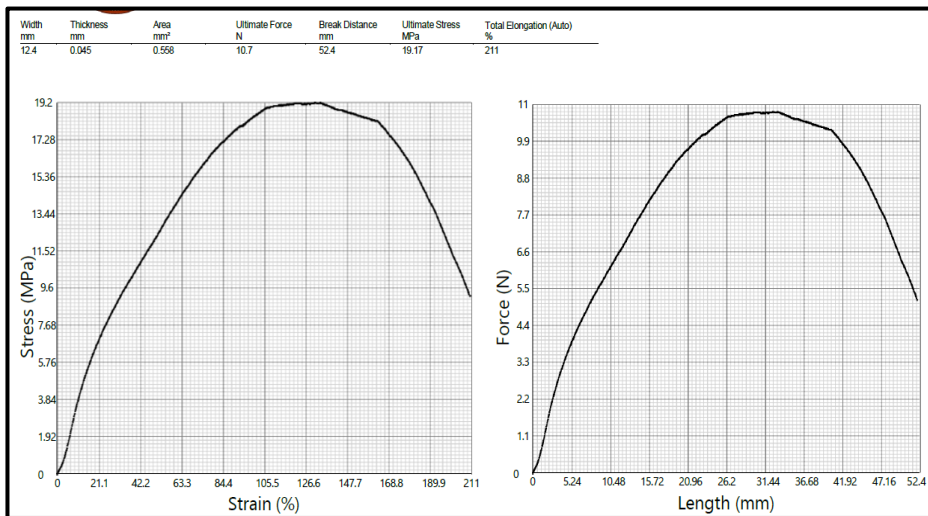
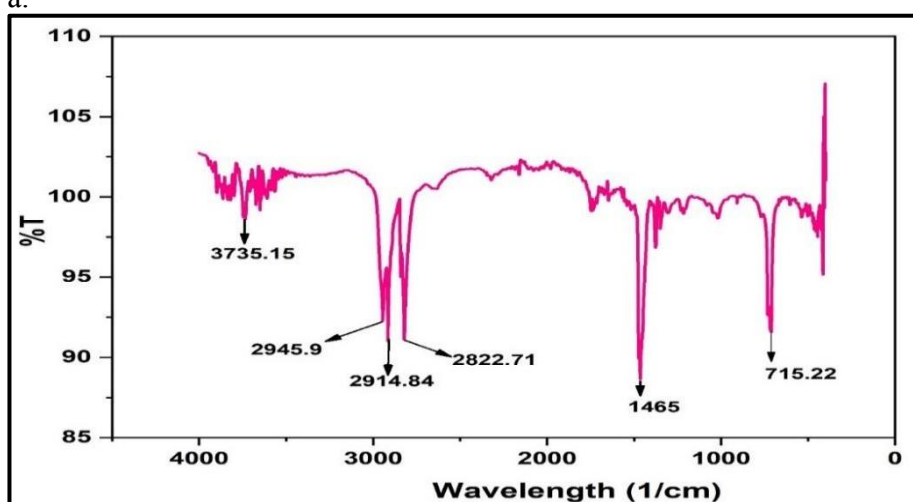


Fig.5.14(b) The graph exhibits the differences in tensile properties of a control (a) and bacterial-treated film (b).

5.5.6.6 FTIR analysis of LDPE films treated with formulated consortia

5.5.6.6.1 Spectrum analysis

a.



b.

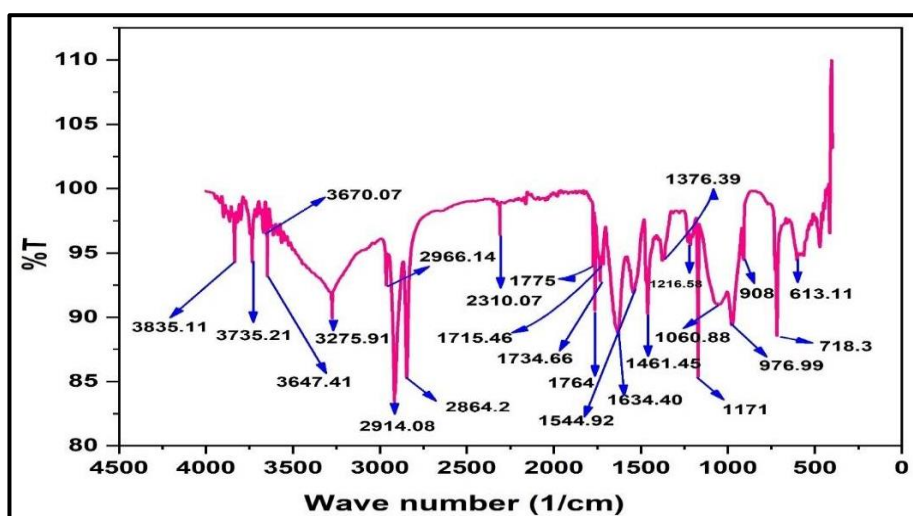


Fig.5.15 FTIR absorption fingerprint of control LDPE film without any bacterial species addition (a) and LDPE films in the mineral salt medium added with a bacterial consortium of *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Micrococcus luteus*.

The control LDPE film FTIR-absorption spectra mainly consist of only the polymer-based fingerprint region (C-H stretching at 2945.9 cm^{-1} , 2914.84 cm^{-1} , 2822.71 cm^{-1} , C-Br stretching at 715.22, and C-H bending at 1465 cm^{-1}). The slight oxidation (OH group at 3735.15 cm^{-1}) may be due to the prolonged incubation of LDPE films in the carbon-free mineral salt broth.

S. No	Wave number cm^{-1}	Peak Nature	Interpretation
1	3835.11 cm^{-1} , 3735.21 cm^{-1} , 3670.07 cm^{-1} and 3647.41 cm^{-1}	O-H stretching	Strong sign of oxidative degradation (Formation of alcohols/ Carboxylic acids)
2	2864.2 cm^{-1} and 2914.08 cm^{-1}	C-H stretching	Typical stretching of LDPE backbone (Romero-Sánchez & Martín-Martínez,2003)
3	2310.07 cm^{-1}	C $\equiv$ N stretching	It may be due to the presence of the nitrile group.
4	1775.01 cm^{-1}	C=O stretching	Indicates ester groups
5	1764 cm^{-1} , 1734.66 cm^{-1} and 1715.46 cm^{-1}	C=O stretching	Suggests the presence of aldehydes/vinyl group
6	1634.4 cm^{-1}	N-H bending	Maybe due to the amide group on the films
7	1216.58 cm^{-1}	C-N bending	implies the presence of an amine group
8	1461.45 cm^{-1}	C-H bending	From CH ₂ /CH ₃ groups (Romero-Sánchez & Martín-Martínez,2006)
9	1376.39 cm^{-1}	O-H bending	Illustrates the presence of phenol groups
10	1171.01 and 1060.88 cm^{-1}	C-O stretching	Alcohol/ester formation
11	976.99, 908, 718.3, 613.11 cm^{-1}	Aromatic compound bending, C-Br stretches, and out-of-plane C-H bending	Polymer-specific fingerprint region

Table 5.4 FTIR spectra show peaks after 120 days of incubation with consortia-treated LDPE film.

5.5.6.6.2 FTIR indices calculations

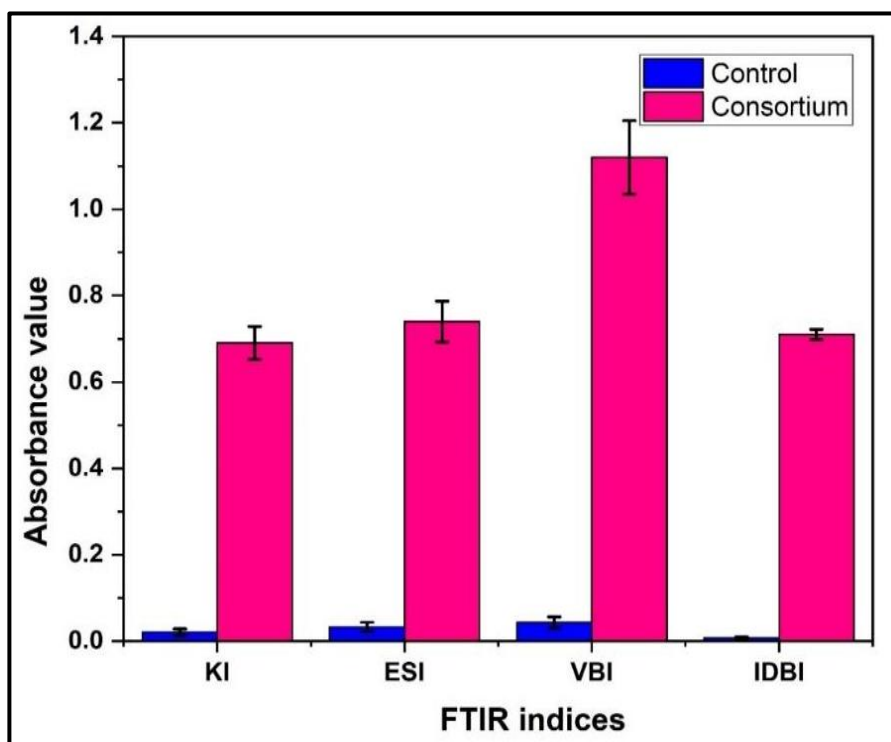


Fig.5.16 The FTIR indices graph of the consortium illustrates the calculated values of carbonyl, ester, vinyl and internal double bond indices derived from FTIR spectral data. The blue bar diagram shows the computed absorption values of the control LDPE film. The pink bar diagram showcases the chemical modification values of bacterial consortia-treated LDPE films.

The FTIR and indices graph demonstrated the consortium's effectiveness in mineralising complex molecules into simpler ones (Peixoto et al., 2017). Our data indicated a significant increase in all FTIR index values. All the FTIR indices of consortium-treated LDPE films showed a considerable rise in values compared to the control LDPE films not treated with bacterial consortia. In our analysis, the Vinyl bond indices (VBI) of treated LDPE films showed the highest

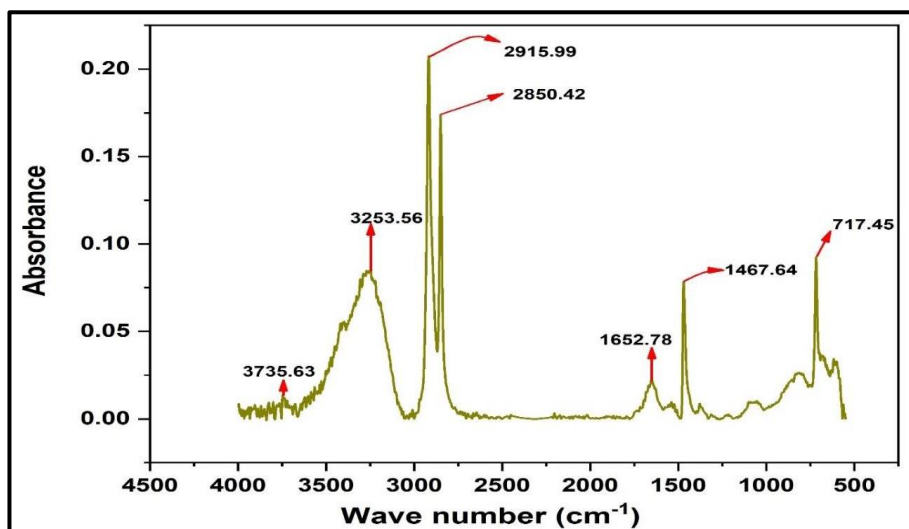
index values compared to the control. It is followed by ester bond indices (ESI), internal double bond indices (IDBI) and carbonyl bond indices (KI). These differences helped us validate the biofilm formation and its catalytic activities on the film compared to the control.

5.5.6.6.3 FTIR-ATR-FPA analysis

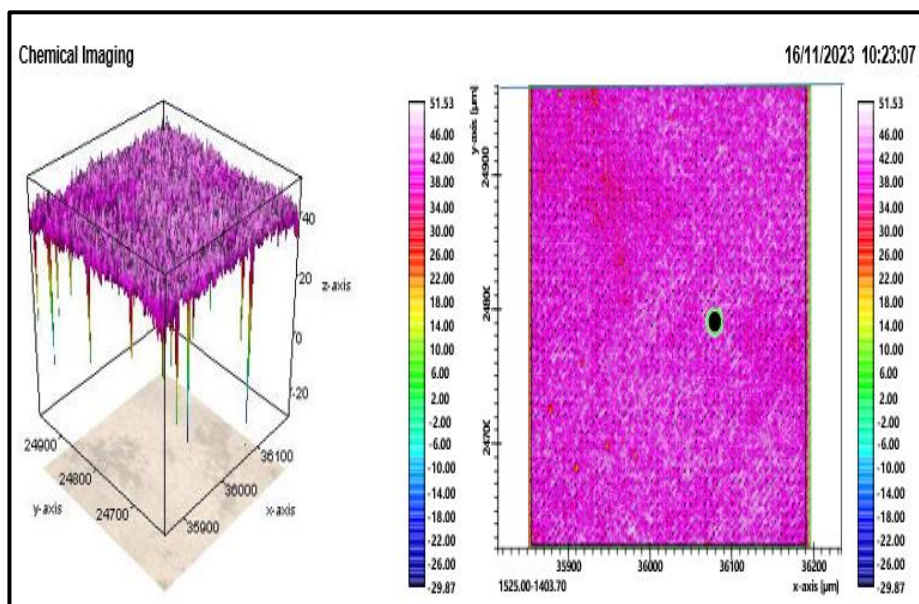
This study confirmed C=C bending (at 1652.82 cm^{-1} , 1646.99 cm^{-1} , 977.28 cm^{-1} , 912 cm^{-1} and 717.96 cm^{-1}), C-H bending (at 1467.99 cm^{-1} , 1373.14 cm^{-1} and 818.48 cm^{-1}), N-O stretching at 1552.49 cm^{-1} and C-N stretching at 1207.28 cm^{-1} , which strongly proves the biofilm formation and catalytic activities of strains in the consortium. The results of this experiment were like those of the FTIR analysis. This experiment further confirmed the chemical changes on the surface of the films.

Our investigation performed the FPA analysis in the $1000\text{-}2000\text{ cm}^{-1}$ range, where maximum biodegradation peaks appeared in FTIR and FTIR-ATR data. The micrograph changed the colour from pink (in control films without bacterial biofilm) to blue (in the bacterial consortia attached to LDPE film). It emphasised the chemical modifications on the surfaces of LDPE films inoculated in the mineral salt medium.

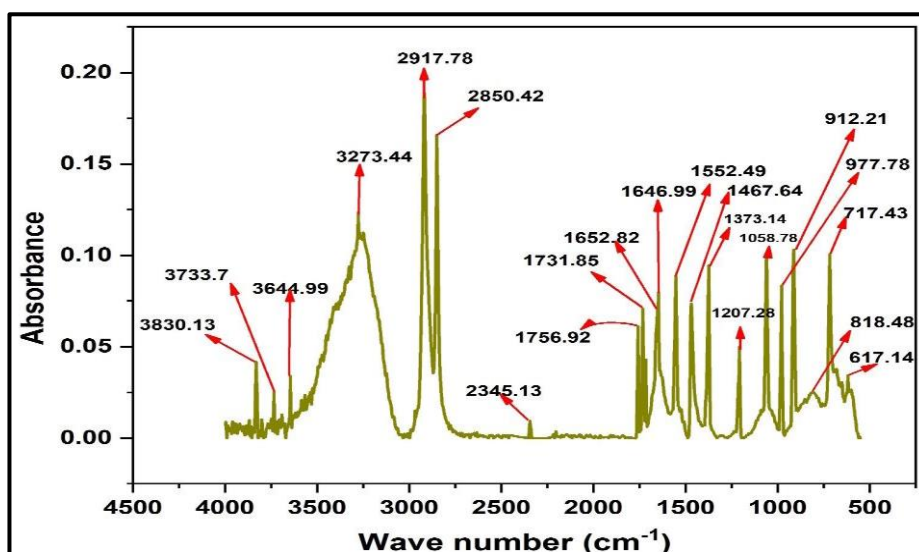
- a. FTIR-ATR of LDPE film without bacterial treatment for four months



- b. FPA analysis of LDPE strips without bacterial consortia



- c. FTIR-ATR of LDPE strips inoculated with the bacterial consortium for four months



- d. FPA analysis of LDPE strips inoculated with bacterial consortium

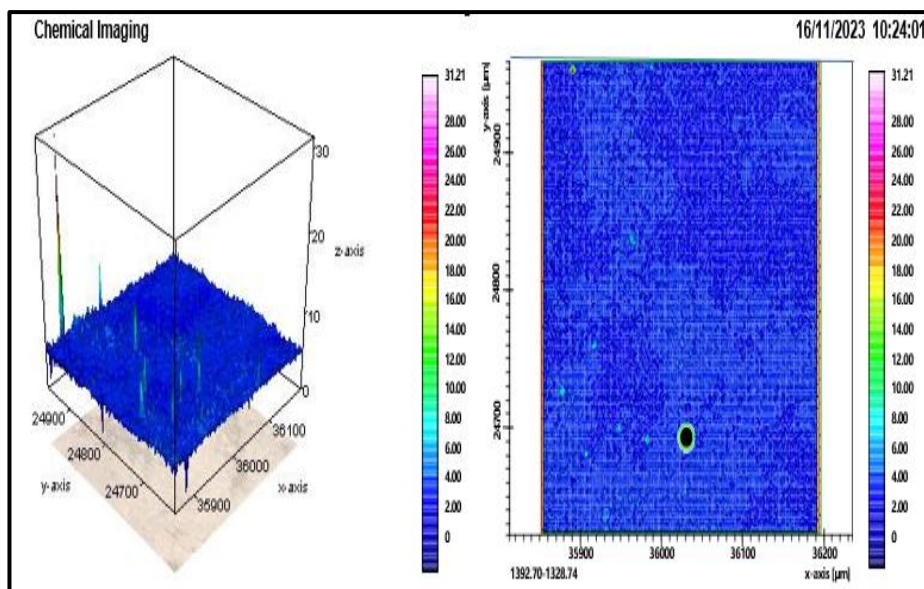
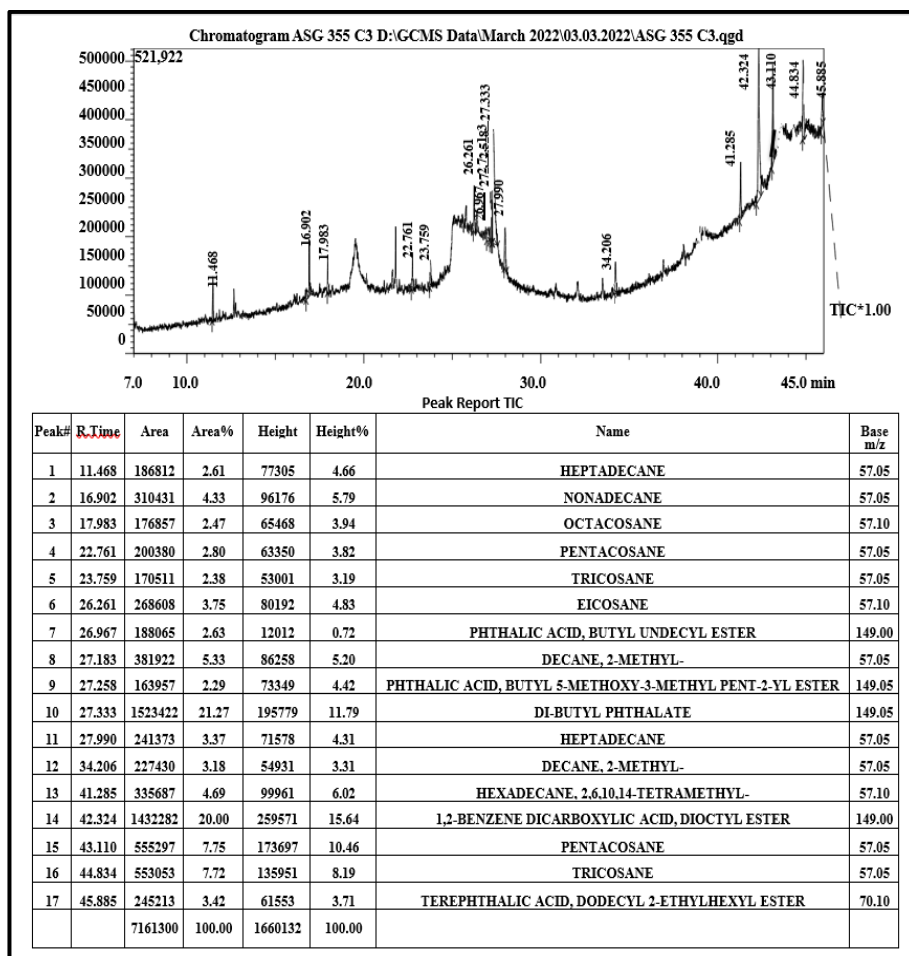


Fig.5.17 FTIR-ATR with Focal plane array analysis of control without bacterial inoculation (a, b) and with bacterial consortium inoculation (c, d).

5.5.6.7 GC-MS analysis

- a. The GC-MS chromatograph of LDPE film and the peak illustration table of the control LDPE film devoid of consortia addition



- b. GC-MS chromatogram treated with the artificial consortium formulated under laboratory conditions

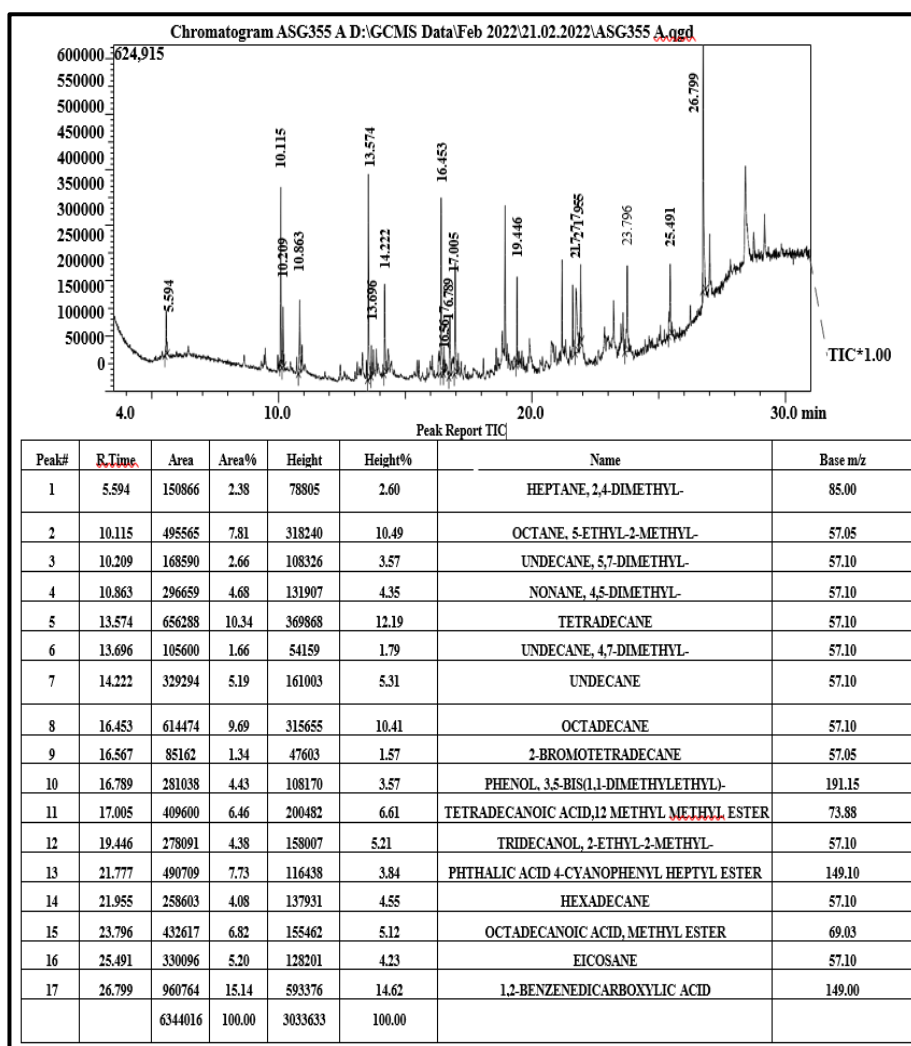


Fig.5.18 Demonstration of volatile compounds released from the LDPE film, both with and without bacterial inoculation, after 120 days of prolonged incubation in the mineral salt medium.

Unique volatile compounds in the chromatogram of consortia-treated films compared to the control.

- a. **2,4-Dimethylheptane** is a medium-chain alkane in which the hydrogen atoms at positions 2 and 4 have been replaced with methyl groups. It is a 9-carbon compound (Empirical formula C_9H_{20}). In a study by (J. Ji et al., 2020), the compound was reported as a non-toxic intermediate produced from 2,6-dimethylphenol, a toxic compound released from plastic dumps.
- b. **Octane,5-ethyl-2-methyl**, is an 11-carbon hydrocarbon (Chemical formula $C_{11}H_{24}$). A recent study reported this compound as a volatile alkane released from LDPE film following microbial attack (Cabanés et al., 2020).
- c. **Undecane 5,7-dimethyl ($C_{13}H_{28}$) and Undecane 4,7-dimethyl ($C_{13}H_{28}$)** are methylated medium chain alkanes (Eregie & Jamal-Ally, 2023). Similar hydrocarbons were observed in microbial-treated polyethylene films treated with *Lysinibacillus* Species JJY0216, isolated from Soil.
- d. **Nonane 4,5-dimethyl ($C_{11}H_{24}$)** is a methylated, volatile, medium-chain alkane.
- e. **Dodecane,2,6,11-trimethyl ($C_{15}H_{32}$)**, is a branched medium-chain alkane with three methyl substitutions at positions 2,6, and 11. Similar compounds have been reported in the biodegradation of polyethylene compounds by (Paulraj & Narayanasamy, 2025; Uddin et al., 2025). Additionally, the biodegradation of multiple methylated alkane compounds, such

as squalene, demonstrates the effect of alkane dehydrogenases and the activity of esterases.

- f. **Phenol 3,5-bis(1,1-dimethyl ethyl) (C₁₄H₂₂O)** is an intermediate compound synthesising long-chain hydrocarbons in LDPE film (Abraham et al., 2017).
- g. **Tetradecanoic acid,12-methyl ester (C₁₄H₂₈O₂)**, is a metabolic intermediate compound that appears in the biodegradative pathway of LDPE film (Zampolli et al., 2023).
- h. **Octadecanoic acid, methyl ester (C₁₉H₃₆O₂)** – It is one of the fatty acid methyl esters in the chromatogram. The biodegradation rate of fatty acid methyl esters was significantly higher than that of long-chain hydrocarbons (Lacalle et al., 2021).
- i. **Tridecanol,2-ethyl-2-methyl (C₁₆H₃₄O)**, is a fatty alcohol. It indicates the oxidation of long-chain hydrocarbons by microbial attack. Similar compounds were demonstrated by (Yuan et al., 2023) in their work on *Raoultella* species in polyethylene films.
- j. **Tetradecane (C₁₄H₃₀), Undecane (C₁₁H₂₄), Hexadecane (C₁₆H₃₄) and Octadecane (C₁₈H₃₈)** are medium carbon alkanes. Similar compounds have been reported in microalgae, specifically *Uronema africanum* Borge (Sanniyasi et al., 2021).
- k. **Phthalic acid 4-cyanophenyl heptyl ester (C₂₂H₂₂NO₄)** was a unique volatile compound reported in the chromatogram, containing a nitrogen element, due to microbial attack on LDPE

film. It may be an intermediate compound produced during the biodegradation of phthalates (Montazer et al., 2020).

1. **1,2-Benzene dicarboxylic acid** (phthalic acid) is a breakdown product of certain phthalate esters commonly used as plasticisers in LDPE film. The presence of esterases or lipases may be the reason behind the formation of 1,2-benzene dicarboxylic acid (Sun et al., 2022). Many previous works have identified the presence of such by-products in their GC-MS images, demonstrating microbial degradation of LDPE films (DSouza et al., 2021).

Overall, the production of alcohol, acid, methyl esters, and short-chain alkane compounds demonstrates the potential of the consortium added to LDPE film for its biodegradation.

5.6 Discussion

The current study aimed to formulate artificial consortia of isolated bacterial strains from long-run landfills. In our investigation, we effectively enriched the pellets of isolated cultures (*Bacillus* sp., *Pseudomonas* sp., and *Micrococcus* sp.) to an optical density of 0.7 to 0.9. It may be the first study report to demonstrate a weight loss of $54.67 \pm 1.28\%$, achieved by treating static culture consortia enriched in carbon-free medium with non-pretreated LDPE film. In this investigation, we have recorded a daily consumption rate of polymer strips as 0.0066 ± 0.0023 g and a reduced half-life span of 105 ± 3.7 days. Similarly, a recent scientific investigation using a novel bacterial consortium of three species of *Brevibacillus* and one species of *Anerobacillus*, isolated from Landfill soil, demonstrated a 58% weight

loss at 50°C after 140 days of incubation (Skariyachan et al., 2018). The efficiencies of bacterial consortia in forming biofilm were studied using the BATH test, the FDA test, and the CO₂ test.

The results of the BATH test demonstrated an increase in cell surface hydrophobicity in the bacterial strains of the consortium, as well as their ability to migrate to the surface of the hydrocarbon compound. Elevated cell surface hydrophobicity and favourable interactions of bacterial consortia at high pressure may facilitate their adaptation to the hostile environment of the polymer (Gaylarde et al., 2023). Consequently, the aqueous phase exhibited a decrease in hydrophobicity percentage or an enhancement in turbidity reduction. Several microbial strains with high cell surface hydrophobicity on LDPE films showed similar results (Deng et al., 2020; Soleimani et al., 2021). Our FDA analysis demonstrated maximum cell viability and metabolic activity on the LDPE films inoculated in a carbon-free synthetic medium during a 30-day incubation period. In this test, the high metabolic activity of bacterial cells in consortium converted 3,6-diacetyl fluorescein to fluorescein, a yellow-green-coloured compound (Ziegler et al., 1975). In addition, we conducted a gravimetric analysis of CO₂ as an initial test to demonstrate the strains' capabilities to break the C-H bonds under pressurised aerobic conditions for 120 days (Pathak & Navneet, 2018). Our study reported that CO₂ evolution rates were significantly higher than those in earlier similar reports (Ameen et al., 2015). Many researchers have illustrated that potential strains can utilise oxygen for metabolic activities and produce CO₂ as end products (S. H. Ji et al., 2023; L et al., 2024).

The robust biofilm formation over an extended period of 120 days was studied using EPS production by bacterial strains, pH changes in the inoculation medium, protein estimation in the biofilm, and viability tests of the bacterial strains. The EPS analyses revealed the presence of polymeric substances secreted by bacterial colonies that adhere to solid surfaces (Costa et al., 2018). They constitute more than 50% of the biofilm's total organic matter. It comprises water and polymeric substances, including polysaccharides, proteins, and DNA (Di Martino, 2018). Several earlier investigations have demonstrated that firm biofilm formation on recalcitrant surfaces can lead to their effective degradation ((Orr) et al., 2004). Moreover, several recent analyses have demonstrated increased EPS production from bacterial attachment (Agarwal et al., 2024; Harrat et al., 2024). In addition, the medium's pH change results validated the strains' metabolic activities in carbon-depleted media on the polymer strips (Chigwada et al., 2023). The significant reduction in pH in the current study may be due to the acidic metabolites produced by bacterial strains in the consortia, utilising LDPE strips as an energy source (Alamer et al., 2024). The previous work of (M. P. Das & Kumar, 2015) reported similar results in the carbon-free media inoculated with *Bacillus amyloliquefaciens* on LDPE strips for biodegradation. Protein estimation is an indirect method for identifying robust biofilm formation on polymer films. A few researchers have recently estimated the protein content of biofilms on LDPE strips inoculated in a carbon-free medium (Kavitha & Bhuvaneswari, 2021; Suresh et al., 2023). The bio-attachments on LDPE strips were further confirmed by viability tests (FDA test). This

test indirectly demonstrates the strain's ability to produce hydrolytic enzymes for assimilating LDPE films (Sridharan et al., 2021).

The consortia exhibited all the enzymes produced by individual bacterial strains, including lipases, proteases, laccases, lignin peroxidases, manganese peroxidases, and esterases. The consortium has shown a higher production of lipase enzymes. It indicates the efficacy of bacterial strains in breaking down long-chain insoluble substrates by hydrolysing ester bonds (Fojan et al., 2000). Numerous researchers have demonstrated that *Bacillus* and *Pseudomonas* species produce elevated lipase when exposed to LDPE-like hydrocarbons, facilitating their degradation (Abubakar et al., 2024; Restrepo-Florez et al., 2014).

We conducted scanning electron microscopy (SEM) experiments to validate the preliminary analysis. Many collateral research activities have recently yielded similar SEM results, demonstrating surface morphological changes and microbial attachments in the internal layers of the polymer after treatment with natural or in vitro consortia (Mohammadi et al., 2022; Negi et al., 2011). SEM-EDAX analysis of the current study revealed the elemental differences in carbon and nitrogen between the control and consortia-treated film. These changes may be due to the enzymatic activities of the biofilm or other chemical reactions induced by specific structural features on the LDPE films (Chen et al., 2022). FTIR analysis in this study further confirmed the presence of nitrogen elements, demonstrating the efficiency of the strains in the consortium.

Our current study demonstrates changes in surface roughness using AFM analysis. The topographic changes in LDPE films by the bacterial consortium after 120 days of incubation show their ability to attach to the compound and break down long hydrocarbon chains into simpler compounds (Kale et al., 2015). Although many previous studies have reported changes in surface roughness textures due to microbial attack (Gomez-Mendez et al., 2018), the current study provides a meticulous path to create biotechnological remediation for recalcitrant compounds, such as low-density polyethylene.

Typically, a WCA below 90° implies that the solid surface is hydrophilic, whereas a WCA above 90° emphasises the polymer's hydrophobicity (Law, 2014). Compared to the control, the significant changes in the WCA of bacterial consortia-treated polymer pieces demonstrated the ability of bacterial strains to reduce the hydrophobicity of polymer surfaces. Several similar studies have validated our results (Lomwongsopon et al., 2025; P. Wang et al., 2023).

Beyond the surface changes, we observed a notable reduction in the mechanical strength of LDPE strips after 120 days of incubation. The results of our investigation suggested the strong depolymerisation of the polymer backbone by the catalytic activities of bacterial strains in our formulated consortia. Multiple earlier comparable results obtained by oxidising LDPE films using bacterial strains in a consortium helped validate our findings.

Our study's FTIR examination showed several intermediates in the LDPE degradation process. The absorption spectra analysed by our

consortium revealed several O-H peaks (3835.11 cm^{-1} , 3735.21 cm^{-1} , and 3647.41 cm^{-1}), indicating the oxidative breakdown of the polymer strips. The oxidation of LDPE films provides compelling evidence of the biodegradation of LDPE films (S. H. Ji et al., 2024). Bacterial enzymes can act on LDPE, potentially resulting in the oxidation or hydrolysis of long hydrocarbon chains and the creation of new functional groups. It enhances the hydrophilicity and degradability of the polymer (Nademo et al., 2023). In our work, we observed numerous new functional groups, including C=C, N-H, C≡N, and N-O bonds, as well as changes in the intensity of existing functional groups and variations in the position of these groups. The carbonyl groups may be either keto-carbonyl (1764 cm^{-1}) or vinyl carbonyl (1715.46 cm^{-1}). The simplest form of carbonyl groups (C=O), such as aldehydes (1734.66 cm^{-1}) and esters (1775.01 cm^{-1}), indicates the consortium's efficacy in biodegrading the polymer compound. Several previous studies have demonstrated that these sites can be easily accessible to bacterial enzymes for further assimilation (Kopecká et al., 2022; Mohanan et al., 2020). The nitrogen-containing peaks in the FTIR fingerprint of the treated film (such as the peaks at 2310.07 cm^{-1} (C-N bending-amide group), 1634.4 cm^{-1} (N-H bending-amine group) and 1216.58 cm^{-1} (C-N bending-amine group) may be due to the metabolic activities of strains attached to the polymer surface (Yao et al., 2022b). The peak between the $1025\text{--}1380\text{ cm}^{-1}$ absorption range indicates the presence of alcohol/ester functional groups (Gawali et al., 2025). It manifests the biodegradation of LDPE polymer through an enzymatic reaction. Our spectrum identified an O-H bending peak at 1376.39 cm^{-1}

and two C-O stretching peaks at 1171.01 cm^{-1} and 1060.88 cm^{-1} (T. Zhang et al., 2025). These chemical changes demonstrated the presence of LDPE biodegradation intermediates, such as alcohols or esters (Tirkey & Upadhyay, 2025). The C=C peaks, located between 715 cm^{-1} and 995 cm^{-1} , signify the presence of alkene groups, indicating the breakdown of large polymers (Sathiyabama et al., 2024). In addition, spectral changes, such as C-H stretching and bending vibrations, were commonly observed in the FTIR fingerprints of LDPE strips without added bacterial strains. This FTIR result showed similar chemical modifications to those obtained by applying an individual bacterial strain in a consortium to the LDPE film for 120 days. The C-Br peak in the $700\text{-}720\text{ cm}^{-1}$ range indicates that the same polymer films were used for control and consortium treating experiments. The overall chemical modifications on the films may further lead to compound degradation.

The FTIR indices of consortia-attached LDPE films showed a marked increase in values compared to individual strains inoculated to LDPE films and control films without bacterial inoculation. The elevated FTIR values in our treated LDPE films indicate that the microbial strains in the consortium are catalysing the biodegradation of long-chain hydrocarbons through robust biofilm formation. The rise in FTIR indices provides a precise measurement of oxidation processes that trigger the breaking of chemical bonds (Jebashalomi et al., 2024). Numerous preceding scientists have documented the enzymatic activities, including those of oxidoreductases, in synthesising keto and ester carbonyl compounds on LDPE films affixed to the polymer

substrate (Albertsson et al., 1998; Sudhakar et al., 2008). Moreover, bacterial adherence to LDPE films may cause terminal double bond formation (through the Norrish mechanism) or ester bond formation, thereby increasing the index values (Balasubramanian et al., 2010). The elevated vinyl bond indices in the FTIR data of bacterial-treated LDPE films may be attributed to the increased amount of proteins, lipids, and polysaccharides on the polymer surface, resulting from the formation of strong biofilms.

FTIR-ATR confirms the introduction of alcohols, acids, esters, and aldehydes in the LDPE films. These compounds have been identified as intermediates in the biodegradation of LDPE (Llorente-García et al., 2020). Moreover, the microbes' primary fragmentation of polymer chains is the most challenging and time-consuming stage in the decomposition process. Therefore, a longer incubation duration (120 days in the current study) may result in substantial amounts of carbonyl, ester, vinyl, and other modifications on LDPE films, thereby increasing the degradation rate (Montazer et al., 2019). The FTIR and FTIR-ATR results were corroborated by FPA analysis, which facilitated the demonstration of the micrograph of the highly deteriorating region of LDPE film. Several subsequent researchers employed analogous strategies to facilitate the biodegradation of polymer surfaces (Chen et al., 2020).

In the current investigation, the volatile compounds released from the control LDPE film without bacterial inoculation showed long-chain hydrocarbons and complex plasticisers. However, the chromatogram of the bacterial consortia-treated film displayed the medium-chain

alkanes (tetradecane, undecane, hexadecane, and octadecane) and methylated medium-chain volatile alkanes. Moreover, it consists of alcohols, acids and methyl ester derivatives. Previous studies demonstrated that methylated hydrocarbons can be oxidised to primary alcohol, aldehydes, secondary alcohols, and acids by following the sub-terminal oxidation pathway (Sowani et al., 2019). It can be accomplished with enzymes such as laccases, esterases, lipases, and alkane hydrolyses (Ndahebwa Muhonja et al., 2018). Since the production of branched hydrocarbon compounds is unusual, we hypothesise that they could have originated from the original structure of LDPE.

5.7 Conclusion

In our research, we developed an in vitro consortium utilising three potential bacterial strains, *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, and *Micrococcus luteus* NJD3, isolated from long-term plastic waste sites in different parts of Kerala, for the bioremediation of low-density polyethylene strips in a carbon-free medium. This consortium was applied to LDPE strips under static culture conditions at room temperature for four months. This resulted in a substantial reduction in the weight of non-pre-treated polyethene films by approximately 55%. Moreover, we have assessed the daily consumption rate as 0.007 ± 0.002 g and the compound's half-life as 105 ± 3.7 days. Preliminary confirmatory tests, including the BATH test, FDA test, CO₂ evolution measurements, P^H change estimations in media, and protein content assessment of strains on the polymer and EPS production analyses during incubation, were conducted to validate

dense biofilm formation under challenging conditions. Moreover, our research demonstrated the presence of enzymes such as laccases, lipases, proteases, manganese peroxidases, lignin peroxidases and esterases. It reflects the proliferation of all strains on the polymer surfaces by utilising it as an energy source. SEM, SEM-EDAX, and AFM analyses confirmed the alterations in the topography of LDPE strips in the CFLMSM during 120 days of incubation with the potential consortium. Further, the study of change in water contact angle enabled us to demonstrate the introduction of hydrophilicity on the surface of the polymer. It may be due to the catalytic activities of the biofilm attached to the LDPE compound. We also observed profound variations in mechanical strength measures in these bacterial consortia-adhered LDPE films. Compared to the control LDPE films, the FTIR analysis of polyethylene films supplemented with a bacterial consortium helped identify chemical modifications in the polymer chain, such as oxidation, esterification, stretching in alkane or alkene bonds, and the introduction of novel bonds to the hydrocarbon chains. It suggests the possible mechanism by which bacterial strains form robust biofilms and break the polymer chain into smaller molecules, which are subsequently utilised for their metabolic processes. Moreover, we validated the FTIR spectrograph results for both sample and control LDPE films using FTIR-ATR coupled with FPA imaging. Furthermore, we conducted GC-MS analyses to identify the intermediate compounds, including short-chain alkanes, acids, alcohols, and other volatile intermediates, during the biodegradation of LDPE. All the validation experiments demonstrated that this

artificially formulated consortium could be utilised for further research to develop a feasible bioprocess for degrading recalcitrant compounds, such as LDPE films.

CHAPTER 6

EXAMINATION OF THE BIOREMEDIATION EFFICACIES OF ISOLATED STRAINS FROM THE LONG-RUN PLASTIC DUMP INTO GARDEN SOIL CONTAMINATED WITH LDPE FILMS THROUGH THE BIO-AUGMENTATION METHOD

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6.1 Introduction.

Conventional methods for extracting noxious components from recalcitrant compounds in our surroundings primarily suffer from inherent drawbacks, including high operational costs, the frequent generation of by-products, and the slow degradation of plasticisers. Therefore, many novel investigators have depicted the efficiencies of natural isolates or their consortia evolved in laboratory settings for biofilm formation and subsequent degradation under high-stress environments (Sanniyasi *et al.*,2021; Skariyachan *et al.*,2021).

In situ bioremediation methods are considered environmentally beneficial, affordable, and sustainable among microbial remediation procedures. Two site-specific in-situ bioremediation mechanisms are involved in the bioremediation of recalcitrant compounds: bioaugmentation and biostimulation. The bioaugmentation process utilises specific strains to effectively address a naturally contaminated site. In contrast, biostimulation enriches indigenous strains by adding extra nutrients to the contaminated site, thereby bioremediating the polymeric compounds. Previous investigations demonstrated that the effects of bio-augmentation and bio-stimulation are case-specific, yielding results that may vary and are contingent upon the selection of inoculants and nutrients (Wu *et al.*,2019).

Bio-augmentation is the most effective in-situ bioremediation technique for enhancing microcosm biomass in the soil where toxic polymer-contaminated soil disrupts the ecological balance. The fundamental idea behind this intervention is that an exogenously boosted genetic variety will enhance the metabolic capacities of the native microbial population already present in the biotope scheduled

for cleanup, resulting in a broader range of productive biodegradation reactions. The success of bioaugmentation depends on various factors, including strain characteristics, environmental conditions, and culture growth patterns (Tyagi *et al.*,2011). The most common form of bioaugmentation involves using enriched microbes or their consortia, genetically engineered microbes, and gene cloning mechanisms to convert non-potential strains into potential strains for biodegradation (El Fantroussi & Agathos,2005).

Biostimulation is an equally vital technique to degrade polymeric compounds deposited in soil. Earlier research has demonstrated the enhanced ability of fungal strains to degrade polyurethane polymeric components buried in soil samples through biostimulation with aqueous yeast extract (Cosgrove *et al.*,2010). In addition, a recent investigation by (Kavitha & Bhuvaneswari,2021) demonstrated that the cumulative effect of bio-augmentation and bio-stimulation can enhance the microcosms of polyethylene-polluted garden soil.

6.2 Aim and Objectives

1. Collect a garden soil sample and analyse its physical properties for use in bioaugmentation studies.
2. Set up garden soil-microcosm treatment set-ups.
3. Chemical analyses of soil collected from each experimental set after 120 days of incubation.
4. Total heterotrophic count of soil samples before and after soil treatments.
5. Demonstrate biofilm formation on LDPE films by calculating the total biomass count, performing the FAD test, estimating protein content, and conducting the CO₂ evolution test.

6. Demonstrate the efficacy of strains in adhering to LDPE film through a weight loss experiment.
7. Validate the biodegradation of LDPE films buried in garden soil for four months using SEM, AFM, FTIR, and GC-MS analysis.

6.3 Review of Literature

Research conducted at field and pilot scales on bio-augmentation for pollutant removal provides crucial information for addressing plastic waste removal from landfills. To enhance plastic breakdown, laboratory-scale research should evaluate the landfill plastisphere's capacity to absorb plastic waste using an experimental design that replicates field conditions, such as microcosm studies (Baskaran & Sathiavelu,2022). A few earlier studies proved the efficacy of bio-augmentation for the degradation of polyethene compounds (Listed in Table 6.1).

S. No	Microbial strains used for augmentation	Efficacies	References
1	<i>Enterobacter cloacae</i>	Five-fold microcosms compared to non-bio-augmented soil	(Sarker <i>et al.</i> ,2021)
2	<i>Pseudomonas sp.</i>	Enhanced microcosms compared to non-bio-augmented soil.	(Tribedi & Sil,2013)
3	<i>Pseudomonas aeruginosa</i> and <i>Pseudomonas allopuntida</i>	Increased microcosms in augmented soil	(Salinas <i>et al.</i> ,2023)

Table 6.1 Bacterial strains isolated from the LDPE-polluted soil sample.

Bioaugmentation may increase biomass at the pollution site, which depends on various environmental factors, including the physicochemical properties of the dump site soil, the synergistic effects of microbial strains, the bioavailability of nutrients, predation, and contaminant concentrations (Saha *et al.*,2021). Adding organic or inorganic bio-stimulants, particularly those with high nitrogen or phosphorus content, may enhance the growth of indigenous strains, leading to improved biodegradation of polymers in the natural environment (Gaylarde *et al.*,2023). The cumulative or individual in-situ bioremediation mechanism that has to be developed for better bioremediation is still under research. Many strains that exhibit better results in laboratory conditions do not show improved degradation in in situ conditions.

6.4 Methodology

6.4.1 Collection of Garden Soil

The garden soil was collected 5cm below the surface and placed in a zip-lock polyethylene bag. It was mixed and thoroughly sieved to remove stones, gravel, soil macrofauna, large roots, and plant residues. The soil samples were brought to the lab in a polyethylene cover for further analysis. The physical parameters of the garden soil, including temperature, pH, and moisture content, were measured using a HASTHIP Professional Soil Meter 3-in-1 (India) at the sample collection site.

6.4.2 Set-ups of Soil Microcosm Experiments

Each soil microcosm treatment setup includes the following experiment sets,

Experiment set 1

1. Garden soil sample mixed with LDPE films (Control 1-E1C1).
2. A garden soil sample mixed with LDPE films and augmented with each bacterial strain (*Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, *Micrococcus luteus* NJD3) or its consortium. The experiments were designated as E1T1, E1T2, E1T3, and E1T4.

Experiment set 2

1. Garden soil samples with LDPE film mixed with carbon-free liquid mineral salt medium (CFLMSM) (Control 2-E2C2).
2. Garden soil samples supplemented with mineral salt and LDPE films (bio-stimulated) and augmented with each bacterial strain (T1, T2, and T3) or its consortium, T4. The experiments were represented as E2T1, E2T2, E2T3, and E2T4.

The bacterial cultures (individually or as a consortium) for augmentation were previously enriched in mineral salt media for 2-5 days to achieve a final optical density (OD) of 0.6-0.9. 12 mL of enriched cultures were transferred into 6 Eppendorf tubes and spun at 12,000 rpm for 7 minutes (Remi-CM-12-PLUS Microcentrifuge). The pellets were washed three times with distilled water, mixed in distilled water, and added to bio-augmenting and bio-stimulating setups. All the pellets were mixed with 1.5 mL of CFLMSM for the bio-stimulation set-up and added to the garden soil. Additionally, 16 mL of CFLMSM was added to 100 g of soil. Then, all the soil samples (250 g each wrapped in aluminium foil) were mixed with 240 mg of LDPE films

and stored for four months. The LDPE films buried in the soil were surface-sterilised using an SDS solution and ethanol (Kyaw *et al.*, 2012) before being added to each experiment setup.

6.4.3 Chemical analysis of garden soil samples

After 120 days, the chemical components, including available nitrogen, phosphorus, potassium, and organic content, of the collected soil and soil samples in the experiment setups, as outlined in sections 6.4.3.1, 6.4.3.2, 6.4.3.3, and 6.4.3.4, were analysed.

6.4.3.1 Available nitrogen in the soil estimation by following the protocol of (Prasad, 1965)

The available nitrogen in the garden soil collected can be estimated using alkaline potassium permanganate (KMnO_4), a mild oxidising agent. This distillation method utilises a mixture of sodium hydroxide solution, potassium permanganate solution, and a soil sample. The liberated ammonia gas during heating is collected into the boric acid solution and converted into ammonium borate. Furthermore, the oxidised compound-containing solution is titrated against a standard H_2SO_4 solution, and the available amount of nitrogen is calculated.

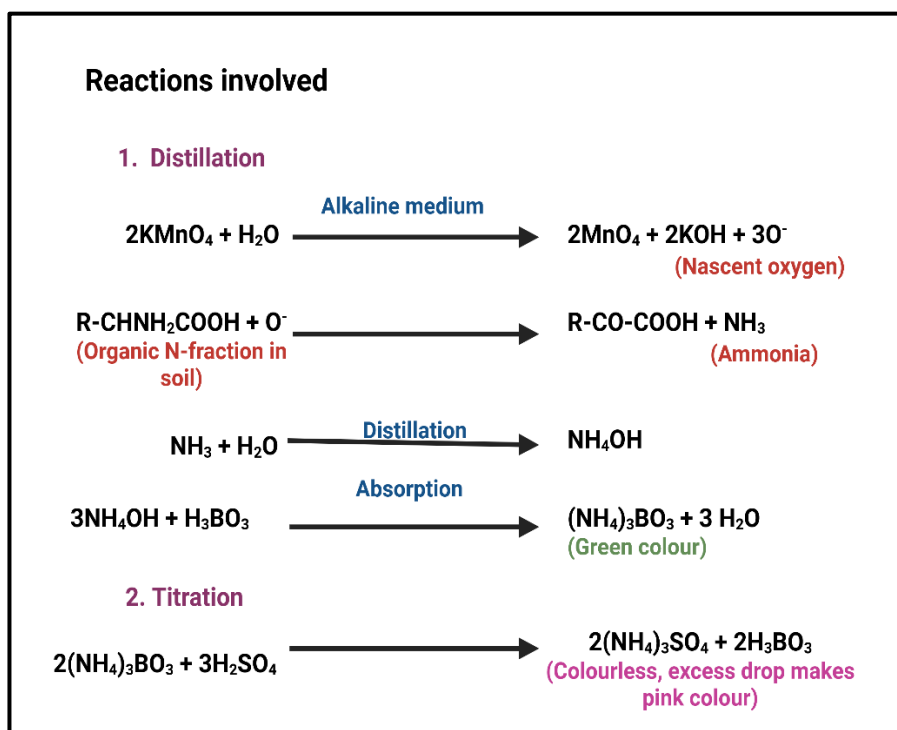


Fig.6.1 Reactions involved in the estimation of available nitrogen in the garden soil collected for our study

Reagents

1. Potassium permanganate solution (0.32% KMnO_4)
2. Alkali solution (2.5% NaOH)
3. Boric acid, H_3BO_3 (4%)
4. Standard H_2SO_4 (N/50)
5. Mixed indicator: bromocresol green powder (0.5g) and methyl red powder (0.1g) were dissolved in 100mL of ethyl alcohol.

Procedure

1. Weighed out 20 g of the garden soil sample after sieving and put it in a distillation flask.
2. Moisten the sample with distilled water and secure the distillation assembly in place.
3. The distillation flask was charged with 100 mL KMnO_4 and 100 mL NaOH solution.

Precautions

- Before adding NaOH to the distillation, the delivery tube must be dipped in the receiver filled with boric acid to minimise the escape of ammonia gas.
 - All the connections should be tight after adding NaOH to the distillation flask. Otherwise, the produced ammonia will not oxidise to ammonium borate.
 - We can add a small quantity of liquid paraffin after adding the KMnO_4 and NaOH solution to the distillation flask to prevent frothing and bumping during boiling.
4. The complete contents of the distillation apparatus were distilled at a moderate rate, and the released ammonia was collected in a 250 mL conical flask. To this, 20 mL of boric acid and four drops of mixed indicator were added. The receiver flask is initially filled with a pinkish boric acid solution containing a mixed indicator solution that changes

colour from pink to green upon ammonia absorption. (Note: Shortly after an hour, the solution's colour turned blue).

5. In about 30 minutes, nearly 100-150 mL of the distillate will be collected, and the distillation connection tubes will be disconnected.
6. The boric acid solution was back-titrated with 0.02 N sulfuric acid.
7. At the endpoint, the solution's blue colour turns colourless, and one drop of excess turns pink.

Blank correction should follow the above protocol without adding a garden soil sample to the distillation flask.

Calculations

1 mL of 0.01N H₂SO₄ can release 0.00014g of nitrogen

Therefore, 1 mL of 0.02N H₂SO₄ can release 0.00028g of nitrogen

Available Nitrogen in 100g of soil

$$= \left(\frac{Z \times 0.00028 \times 100}{20} \right)$$

20 = Dry weight of garden soil

Final titre value, $Z = Y - X$

Where X Blank correction (Control) titer value (mL),

Y = Titre value of test sample (mL)

6.4.3.2 Estimation of available phosphorus in Garden soil (De Silva *et al.*,2015)

Phosphorus (P) in soil exists in organic and inorganic forms. The inorganic phosphorus forms are more readily accessible for organic and inorganic soil plant absorption than other forms. This approach quantifies the relative absorption of inorganic ortho-phosphate ($\text{PO}_4\text{-P}$) in soils spanning a neutral to alkaline pH range. Hydroxide and bicarbonate molecules efficiently remove phosphate from soil particles by competing with other molecules. Additionally, elevated pH levels reduce the extent of secondary absorption. Under acidic conditions, the inorganic ion reacts with reagent A to form a complex. This intricate molecule is reduced by the reagent B in the presence of ascorbic acid, forming a blue-coloured compound measured at a wavelength of 660 nm. The absorbance level is directly correlated with the orthophosphate content in the soil sample.

Reagents for estimating inorganic orthophosphates in the soil sample

1. 1.0 M Sodium bicarbonate solution with pH 8.6 (Olsen's reagent): Prepare it by dissolving 46.2 grams of NaHCO_3 in 1,000 mL of deionised water and adjusting the pH with 1 M NaOH.
2. Darco G60 (activated charcoal) or activated carbon
3. Reagent A
 - a. Component I (Ammonium Molybdate Solution): 12 grams of ammonium molybdate in 250 mL of deionised water.

- b. Component II (Antimony potassium tartrate solution): Add 0.3 grams of antimony potassium tartrate powder to 100 mL of the solution.
- c. Component III: Combine 5N H_2SO_4 with 30 mL of deionised water, mix thoroughly, and dilute to a final volume of 100 mL with deionised water.
- d. Mix the above three components to create a 2-litre solution using deionised water.

Dissolve 1.1 grams of ascorbic acid in 200 mL of reagent A to make reagent B (Ernstberger *et al.*, 2004).

Reagents for the standard phosphate curve

Stock solution for phosphate estimation: Prepare a 50ppm primary stock solution (Dissolve 0.22 g of $\text{K}_2\text{H}_2\text{PO}_4$ in 400 mL of distilled water, then add 25 mL of 1 N sulfuric acid and dilute to 1000 mL). A 5ppm solution was prepared from the stock to plot the phosphorus standard curve. Different volume of 5ppm stock, 5 ml Olsen's reagent and 4 ml of reagent B were mixed and upon blue colour development, absorbance values at 660nm recorded to plot the standard curve.

Procedure

1. Weighed 5 g of the garden soil and transferred it to a 250 mL Erlenmeyer flask.
2. To decolourise the extract, 60 mL of sodium bicarbonate (1.0M) and a pinch of Darco 60 were added to the Erlenmeyer flask.

3. Shook the flasks in a rotatory shaker at 250 rpm for 30 minutes.
4. Filtered the contents (using Whatman No.40 filter paper) to obtain a colourless filtrate.
5. Equal amounts of filtered solution and reagent B (5 mL each) were transferred to a 50 mL Erlenmeyer flask, and the total volume was adjusted to 25 mL with distilled water.
6. Using a spectrophotometer, shake the contents for a few minutes to develop a blue colour and measure the absorbance at 660 nm.

Calculation

Available Phosphorous in the garden soil (Kg / ha) =

$$\left(\frac{X \times \text{total volume of soil extractant in NaHCO}_3 \times \text{Final volume of sample taken} \times 2.24 \times 10^6 \times 2.29}{10^6 \times \text{Weight of the garden soil} \times \text{Volume of aliquot}} \right)$$

X = Concentration of phosphorus read from the standard curve

6.4.3.3 Estimation of available potassium in garden soil (Stanford & English, 1949)

Principle

Alkali metals, such as potassium, in garden soil dissociate into atoms when introduced to a flame. In this technique, ammonium ions (NH_4^+) are used to leach potassium ions (K^+) that were previously present on exchange sites in the soil.

This process can be depicted as follows:



Reagents

Ammonium acetate, pH 7.0 (Neutral NH_4OAC)

Standard potassium stock: Prepare a stock solution of 1000 ppm potassium by diluting the appropriate volumes to obtain working standards of 0, 2.5, 5, 7.5, 10, 12.5 and 15 ppm potassium in 100 mL volumes.

Protocol

Five grams of soil were taken from the garden, sieved to remove rocks, and then transferred to a 250 mL Erlenmeyer flask. Then, a 25-mL ammonium acetate solution was added to the soil sample, and it was shaken continuously with the help of a shaker at 250 rpm for 5 minutes. The whole content was filtered successively using Whatman No.40 filter paper and collected in fresh tube, and it further used to test the potassium content with a flame photometer. The amount of potassium was determined using the potassium concentration obtained from the standard curve of the flame photometer reading (Systronic Flame Photometer 128). Blank ammonium acetate solution was analysed to record baseline potassium readings.

Calculations

Available potassium in garden soil (Kg / Ha)

$$= \left(\frac{R \times \text{Volume of extractant (25 ml)} \times 2.24 \times 10^6 \times 1.2}{10^6 \times \text{Dry weight of the garden soil (5g)}} \right)$$

R = Concentration of K from the standard graph

6.4.3.4 Evaluation of organic carbon by following the protocol of (Walkley & Black,1934)

Potassium dichromate ($K_2Cr_2O_7$) can oxidise organic carbon in garden soil when combined with concentrated sulfuric acid (H_2SO_4). Potassium dichromate generates nascent oxygen, which undergoes a series of chemical reactions with the organic carbon, resulting in the formation of CO_2 . In the presence of phosphoric acid (H_3PO_4), the excess amount of $K_2Cr_2O_7$ is titrated with a standardised ferrous ammonium sulfate solution using a ferroin indicator or diphenylamine. The organic carbon content of the garden soil was calculated using the chromic acid titration method.

Reagent used

1. 1N Potassium dichromate: Prepare a $K_2Cr_2O_7$ solution by dissolving 49 grams of potassium dichromate in 400 mL of distilled water, mixing well, and then diluting to 1000 mL with distilled water.
2. Concentrated H_2SO_4 (99.5%)
3. Ortho-phosphoric acid
4. Sodium fluoride
5. 0.5N Ferrous ammonium sulphate ($FeSO_4(NH_4)_2SO_4.6H_2O$)
6. Ferroin indicator

Protocol

1. A 500-mL round-bottom flask was filled with 1 g of sieved garden soil.

2. The soil sample was then vigorously spun five or six times after being combined with 15 millilitres of 1N $K_2Cr_2O_7$ solution and 25 millilitres of concentrated H_2SO_4 solution. The mixture was allowed to stand on an asbestos sheet for 30 minutes. (The asbestos sheets were used to prevent the table from burning due to the heat generated by H_2SO_4).
3. Add 250 mL of distilled water to the mixture to dilute the solution.
4. After adding 0.25 g of sodium fluoride and 12 mL of 80% orthophosphoric acid, the mixture was left to stand for 90 minutes (Yadav *et al.*, 2022).
5. One mL of ferroin indicator was added to the conical flask before titration and mixed well.
6. The excess $K_2Cr_2O_7$ was titrated against a 0.5 N ferrous ammonium sulfate ($FeSO_4$) solution until the colour transitioned from yellowish green to dark green and, ultimately, brownish red at the endpoint. Concurrently, blank titration was carried out without soil in the conical flask.

Calculation

Organic carbon percentage in the garden soil collected (OC%)

$$= \left(\frac{(X - Y) \times N \times 0.003 \times 100}{W} \right)$$

X and Y were mL of 0.5 N ferrous sulfate required for the blank and sample runs.

N = Normality of $FeSO_4$

W = Dry weight of the garden soil sample

6.4.4 Microbial count in soil samples

The heterotrophic microbial populations in the soil microcosms were quantified by taking a 1g soil sample from the control and treatment samples. To assess the total viability of microbes in the contaminated environment through microbial inoculation, soil samples were collected intermittently throughout 15 to 120 days from each soil treatment setup. For the total population count, soil aliquots in serial dilution set-ups of saline solution were spread onto Luria-Bertani agar for 24-hour incubation at 30 °C. Colony counts were performed, and CFU/g was calculated.

6.4.5 Biofilm formation on LDPE films in soil samples

6.4.5.1 Biomass count

Quantified the microbial populations on the LDPE films buried in the soil by the protocol suggested by (Joseph *et al.*, 2001). The LDPE films were collected from both the control and experimental setups after 120 days of the experiment, placed in a saline solution, and then serially diluted. The remaining aliquots were plated on Luria-Bertani agar medium and incubated (at 37°C for 2 days). The total biomass count was calculated in colony-forming units (CFU) per square centimetre (cm²). Control LDPE films without bacterial inoculation were plated to account for background microbial growth.

6.4.5.2 FDA test

LDPE films were buried in the soil samples after 120 days, and their control without bacterial adhesion was solubilised in 60 mL of fluorescein diacetate sodium phosphate buffer (FDSPB) (15 mL). For

this analysis, 0.1 mL of FDA (3 mg/mL in acetone) was added at room temperature. After incubation, a 2 mL solution of each sample was centrifuged, and the absorbance of the supernatants was recorded at 494 nm. Control LDPE films without bacterial inoculation were incubated in FDSBPB to baseline absorbance at 494 nm.

6.4.5.3 Estimation of protein

LDPE films from all the microcosm films and their control without any microbial addition were mixed with a saline solution and centrifuged (8,000 rpm for 16 minutes) to collect the supernatants. Then, each supernatant was mixed with Bradford reagent (at a 1:3 ratio) and incubated for 5 minutes. Thereafter, a spectrophotometric reading was taken at 595 nm, and the protein content of the biofilms on LDPE film was estimated using a Bovine serum albumin (BSA) standard curve. Control LDPE films without bacterial inoculation were assayed for baseline protein content.

6.4.5.4 pH measurement

The pH of the soil samples was tested before and after bacterial addition at 0, 30, 60, 90, and 120 days using a GroLine soil digital pH meter (HANNA, India, model H1981030). Before the analysis, the pH meter was standardised with pH 7 and pH 4 solutions. For this analysis, 10 g of each soil sample was placed in sterile beakers, and the contents of each beaker were mixed with 40 mL of distilled water. The mixtures were then incubated for 5-7 minutes. The beaker was kept on the lab slab for 15 minutes without shaking, and then the pH of the soil sample was recorded.

6.4.5.5 CO₂ evolution test

Respirometric analyses were performed to evaluate CO₂ evolution and investigate the metabolic activities induced in each microcosm using a modified protocol (Colla *et al.*, 2014). For the study, carbon dioxide produced during microbial activities was captured in 50 mL flasks, the receiver flask of the respirometer setup, containing a 1M KOH solution (20 mL) in the respirometric flasks. Every 30 days of incubation, the KOH solution from the respirometer flask was replaced, and 1 mL of 20% BaCl₂ solution and four drops of 1% phenolphthalein solution were added. Then, a 1M HCl solution was taken in a burette and titrated against a white-coloured residual KOH solution. The carbon dioxide released can be found in the following equation:

$$\text{CO}_2 \text{ generated (mg / Kg)} = \left(\frac{(B - T) \times 6 \times M_{\text{HCl}}}{M_c} \right)$$

[B and T were mL of HCl used to titrate for blank and treatments;

6 is the equivalent weight of carbon;

M_{HCl} = the molarity of HCl; and M_c = the dry weight of soil.

In our analysis, to compare the CO₂ evolution from all the soil experiment setups, we conducted CO₂ evolution from four treatment setups (designated as Treatment Setup 1, Treatment Setup 2, Treatment Setup 3, and Treatment Setup 4) for 30 and 120 days of incubation. Each treatment set consists of a control soil sample mixed with garden soil only (C), garden soil mixed with LDPE films (C1), garden soil mixed with nutrients (C2), garden soil mixed with LDPE films augmented with one of the bacterial strain (*Bacillus cereus* NJD1,

Pseudomonas aeruginosa NJD2, and *Micrococcus luteus* NJD3) or its consortium, and garden soil mixed with LDPE films augmented with one of the bacterial strain or its consortium and stimulated with nutrients. Before commencing the experiment, the glassware used for the estimation was sterilised. We used soil samples from each treatment setup for titration during the experiment.

6.4.6 Weight reduction of LDPE films buried in soil samples

LDPE films were collected from each soil experiment setup, surface-sterilised, and dried. Then, the LDPE films were weighed using a high-precision balance (Shimadzu ATX224R), and the percentage of weight reduction was calculated (Dey *et al.*, 2020). Additionally, we calculated the daily uptake of bacterial strains and the half-life of LDPE films. All the results were compared with the LDPE films without bacterial inoculation.

6.4.7 Validation studies to demonstrate the efficacy of potential strains to attach to LDPE films, mixed in soil experiment setups

6.4.7.1 Demonstration of biofilm formation using scanning electron microscopy

For this analysis, surface-sterilised LDPE films were dried with hot air at 55°C (overnight incubation), sliced into 1 cm × 1 cm films, and used for ZEISS-Sigma field-emission scanning electron microscope analysis. Both treated and untreated LDPE films in garden soil stimulated by nutrients for 120 days, either with or without bacterial inoculation, retrieved from CFLMSM, were used for this analysis.

6.4.7.2 Surface of alterations of the LDPE by atomic force electron microscopy technique

The topographic alterations on LDPE films due to microbial biofilms were studied after 120 days of incubation of polymer films in soil samples with or without the addition of isolated strains. This analysis utilised surface-sterilised and dried films to capture 2D and 3D images of LDPE using the AFM instrument with WITec Project Plus software. The surface roughness parameters of LDPE films were calculated and plotted using OriginPro-2018.

6.4.7.3 FTIR analysis to demonstrate chemical alterations by the biofilm on the surface of LDPE films

FTIR spectrum analyses were performed on LDPE films mixed in soil samples after 120 days using a PerkinElmer two-spectrum FTIR spectrometer (L1600000A with LiTaO₃ detector) with IR Spectrum 10.6.0 version. The chemical modifications in the bacterial-treated films were compared to those in the LDPE films buried in the soil samples without bacterial adhesion (Control LDPE films were incubated in CFLMSM-augmented soil without bacteria for 120 days to check the baseline chemical alterations).

6.4.7.4 GC-MS analyses of LDPE films buried in the soil samples

LDPE films were harvested from the soil experiments (controls and treatments) and then surface-sterilised. The LDPE films were cut and introduced into chloroform solutions, which were then centrifuged and filtered for spectral analysis using a Shimadzu gas chromatography-mass spectrometry instrument (model QP2010S). The instrument

features a column Rxi-5SilMS with helium as the carrier gas, and the in-built software GC-MS solution was utilised to visualise the metabolic products produced by microbes.

6.4.8 Statistical analysis

All the tests were analysed statistically significantly ($p < 0.05$) using ANOVA analysis in GraphPad Prism, and the graphs were plotted by OriginPro 2018.

6.5 Result

6.5.1 Soil samples collection and analysis of physical properties for the augmentation studies

We collected arable soil samples with a loamy sand texture from three different locations in our garden in the morning before 9 o' clock. We analysed their physical properties at the time of sample collection using a digital soil meter, which yielded the following results: p^H - 7.52 ± 0.27 , temperature - $28.5\text{ }^{\circ}\text{C} \pm 0.3\text{ }^{\circ}\text{C}$, and moisture content - $43.8\% \pm 1.2\%$.

6.5.2 Microcosm developments in the experimental setup

All the soil set-ups were incubated for four months at room temperature under static culture conditions. During incubation, the aluminium wraps were kept out of sunlight, and the soil samples were intermittently moistened (every 15 days) with distilled water to maintain microbial growth in the samples. Following the specified incubation time, soil samples and polymer films were extracted from

the microcosms independently to assess the level of biofilm formation and polymer degradation.

6.5.3 Chemical properties of the collected soil samples

We analysed the chemical properties of garden soil before and after bacterial inoculation, specifically after 120 days of inoculation. The chemical changes in the soil sample indirectly help us determine the development of the microcosm in the garden within the experimental setups of the soil. Due to these additions, the LDPE films in the soil experimental set-up showed an initial reduction and a slow increase in elements such as nitrogen and organic carbon in bio-augmenting and bio-stimulating experimental set-ups, resulting from microcosm development in the harsh environment. However, the elements such as phosphorus and potassium levels decrease in the bio-augmentation soil-experiment set-ups (due to the assimilation of such elements by the microbes growing in soil) and increase in bio-stimulation setups (may be due to the addition of minerals in such experimental setups) (see the chemical analysis report in Table 6.2).

S. No	Chemical analysis (Kg/ha)	Garden soil before the addition of bacterial species	Soil experimental set-up after 120 days of bacterial inoculation			
			Garden soil with LDPE films	Bio-augmented soil with LDPE films	Stimulated garden soil with LDPE films	Bio-augmented and stimulated garden soil with LDPE films
1	Available nitrogen	229.81 ± 32.7	175.12 ± 11.68	192.72 ± 16.78	197.68 ± 22.68	252.62 ± 26.71
2	Available Phosphorous	32.11 ± 11.24	28.34 ± 8.08	22.28 ± 16.24	30.85 ± 8.81	19.38 ± 13.5
3	Available potassium	780 ± 15.81	779 ± 21.28	716.82 ± 18.64	784 ± 17.28	702.64 ± 22.65
4	Organic carbon %	0.62 ± 0.08	0.48 ± 0.02	0.58 ± 0.09	0.53 ± 0.06	0.64 ± 0.015

Table 6.2 Chemical analyses in collected garden soil samples and soil samples in experimental setups.

6.5.4 Heterotrophic count of microbial population in the soil samples in each soil treatment

The increased degradation of LDPE by augmented microcosms (E1T1 and E2T2) may be attributed to the higher abundance of microorganisms, which includes potential strains capable of LDPE degradation. To assess the increase in microcosms of soil samples collected from a semi-arid area under more severe summer conditions after bio-augmentation and bio-stimulation, we conducted a heterotrophic count test in all experimental setups.

S. No	Soil setup	Heterotrophic count (CFU/g)
1	Garden soil	$2.8 \times 10^7 \pm 1.852$
2	E1C1	$1.37 \times 10^4 \pm 2.671$
3	E1T1	$2.2 \times 10^5 \pm 2.217$
4	E1T2	$1.8 \times 10^5 \pm 1.418$
5	E1T3	$2.0 \times 10^5 \pm 1.09$
6	E1T4	$9.2 \times 10^6 \pm 3.11$
7	E2C2	$1.78 \times 10^5 \pm 2.122$
8	E2T1	$6.6 \times 10^6 \pm 1.452$
9	E2T2	$5.3 \times 10^6 \pm 2.328$
10	E2T3	$5.9 \times 10^6 \pm 1.688$
11	E2T4	$9.9 \times 10^7 \pm 3.791$

Table 6.3 Increase microbial populations in the augmented and bio-stimulated experiment setup.

Our experiment found an initial decrease in microbial populations in the soil experiments after adding LDPE, a toxic material for the normal flora. However, the microbial population increased significantly due to bacterial augmentation and stimulation of mineral nutrients. This result showed that microbial populations added to the soil experiment setup multiplied steadily in the toxic environment.

6.5.5 Demonstration of biofilm formations on the polymer films mixed in the soil samples of microcosm experiments after 120 days

6.5.5.1 Biomass count

To identify the microbial biofilm formation on the LDPE film, the biomass on the surface of the LDPE film was counted. It represents the

total count of organisms in various stages of growth and their metabolically active stage.

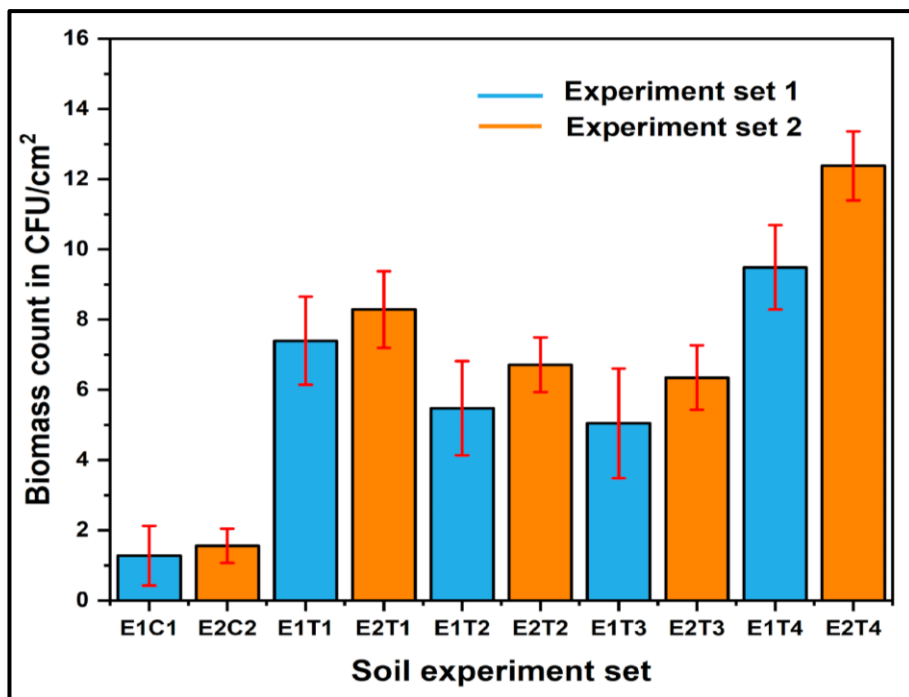


Fig.6.2 Biomass count of LDPE films in all soil experiment setups.

6.5.5.2 FDA Analysis Test

The FDA analysis of the current study indicated the presence of living bacterial species on the LDPE films buried in soil experiments (E1 and E2). Many enzymes, including lipases, proteases, and esterases, can hydrolyse fluorescein diacetate. In our experiment, all the bacterial-treated LDPE films, after 120 days, showed significantly higher absorbance values than the LDPE films in the control soil setup. It proved the efficacy of added bacterial strains in forming biofilms on the polymer films.

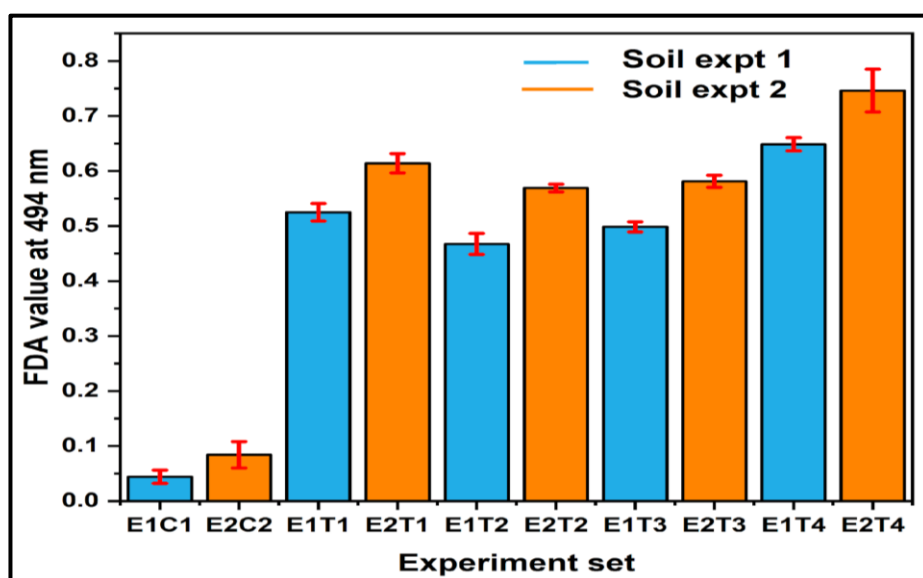


Fig.6.3 FDA analysis of LDPE films buried in soil samples for 120 days.

Our analysis revealed that, among the potential individual strains, the *Bacillus cereus* NJD1-inoculated soil samples buried in LDPE films exhibited the highest number of living organisms in experiments 1 and 2. Moreover, the LDPE films inoculated with the consortium showed the highest FDA values in the bio-augmented cum stimulated soil setups (E1T4 and E2T4). These results further confirmed the organisms' adherence to the LDPE films.

6.5.5.3 Protein analysis on the biofilm

Even after prolonged incubation, we got protein content in our analysis, indicating the potential of bacterial strains to adhere to LDPE films.

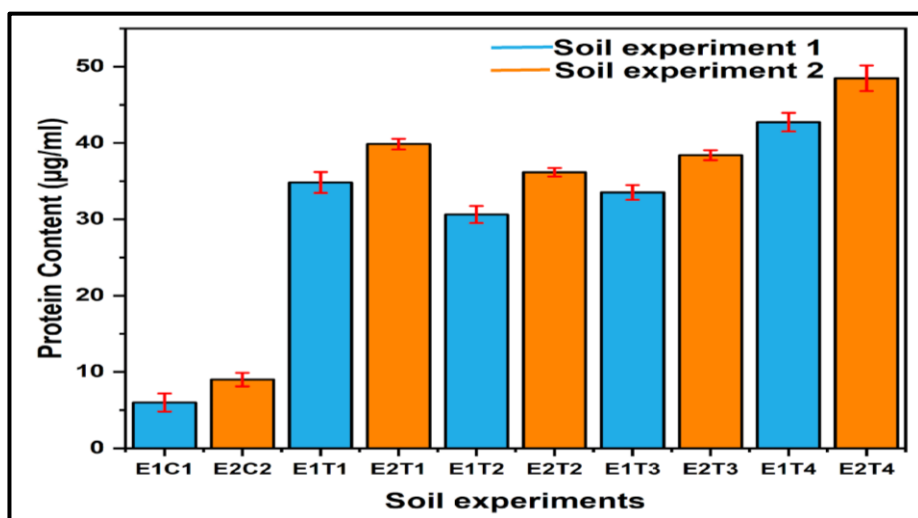


Fig.6.4. Estimation of protein using bacterial biofilms on polymer strips

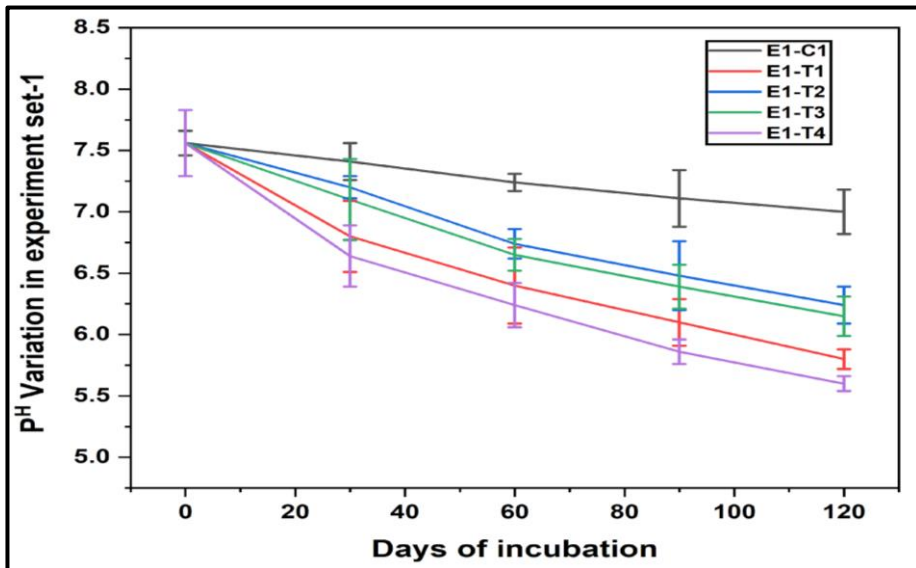
The protein content of LDPE-adhered microbes was highest in the *Bacillus cereus* NJD1-inoculated soil experiment compared to *Micrococcus luteus* NJD3 and *Pseudomonas aeruginosa* NJD3-inoculated soil experiments. A previous study on the protein content assessment of bacterial biofilms on LDPE strips demonstrated comparable efficacy in degrading the polymer (Mehmood *et al.*, 2016).

6.5.5.4 pH measurements of the soil samples in each experiment set up to 120 days of incubation.

The pH change of the soil samples inoculated with bacterial species and the uninoculated control soil samples indicates microbial activity in the soil samples mixed with LDPE films. Compared with other experiments in our analysis, the pH variations in control soil samples exhibited significant variations. Among the bacterial species, *Bacillus cereus* NJD1 inoculated soil samples showed the highest pH difference compared to other culture-inoculated soil experiments. Moreover, the bacterial consortium showed a pH below 5.5 in augmented cum stimulated soil experiments. The potential microbes in soil samples

may cause biofilm formation on LDPE films, which are incorporated into each soil experiment for biodegradation purposes.

a.



b.

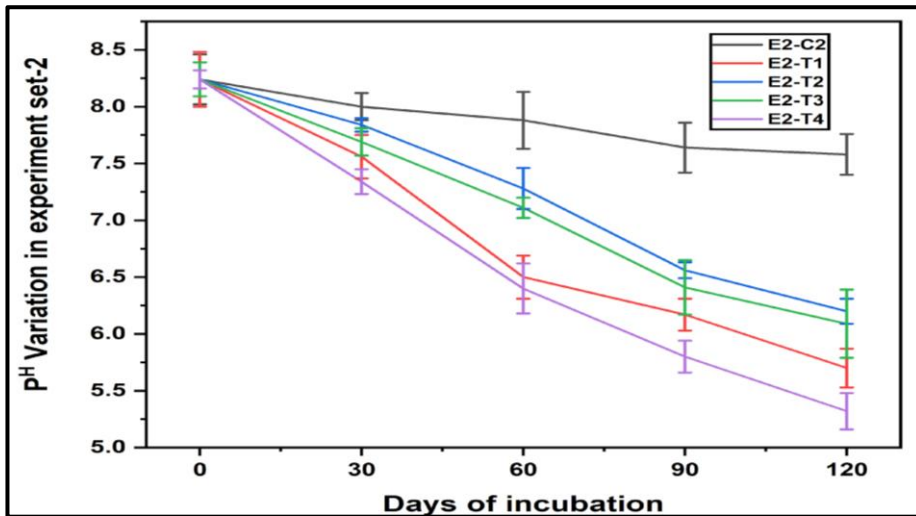
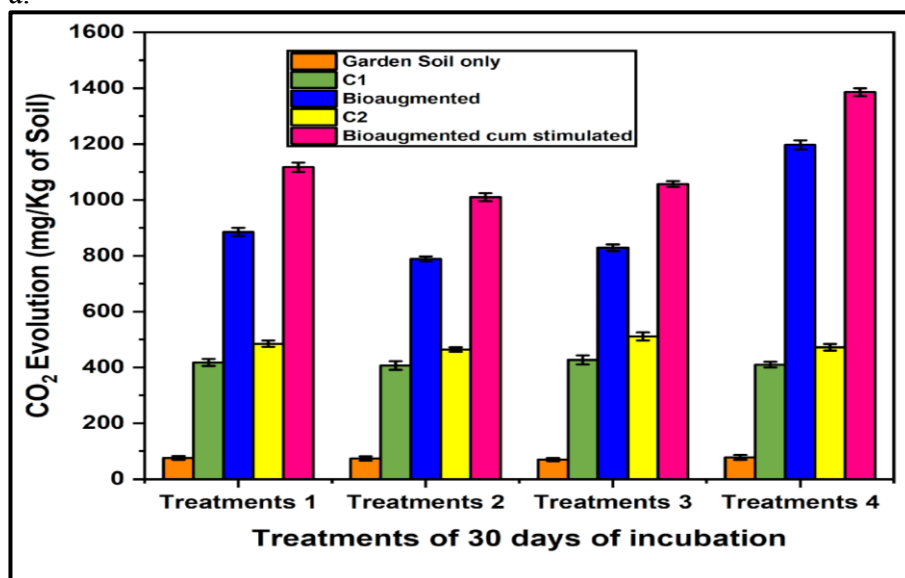


Fig.6.5 pH variation in the soil samples: a. Soil experiment setup-1, b. Soil experiment setup-2.

6.5.5.5 CO₂ evolution

The current test verified the CO₂ evolution for 30- and 120-day incubations with bacterial species and stimulants. In all these experiment setups, we found an escalated rate of CO₂ release due to the combined addition of bacterial strains and stimulants for degrading LDPE films. In our mesocosm experiments, among the individual strains, the *Bacillus cereus* NJD1-treated soil samples recorded the highest rate of CO₂ in both 30-day (1117 ± 16.4 mg/Kg of soil) and 120-day (548 ± 9.2 mg/Kg of soil) incubations compared to the control. Similarly, the consortium of isolated bacterial strains exhibited a significant increase in its rate of CO₂ evolution at 30 (1386 ± 13.9 mg/Kg of soil) and 120 days of incubation (615 ± 11.8 mg/Kg of soil) (see the results of treatment 4, Fig.6.6).

a.



b.

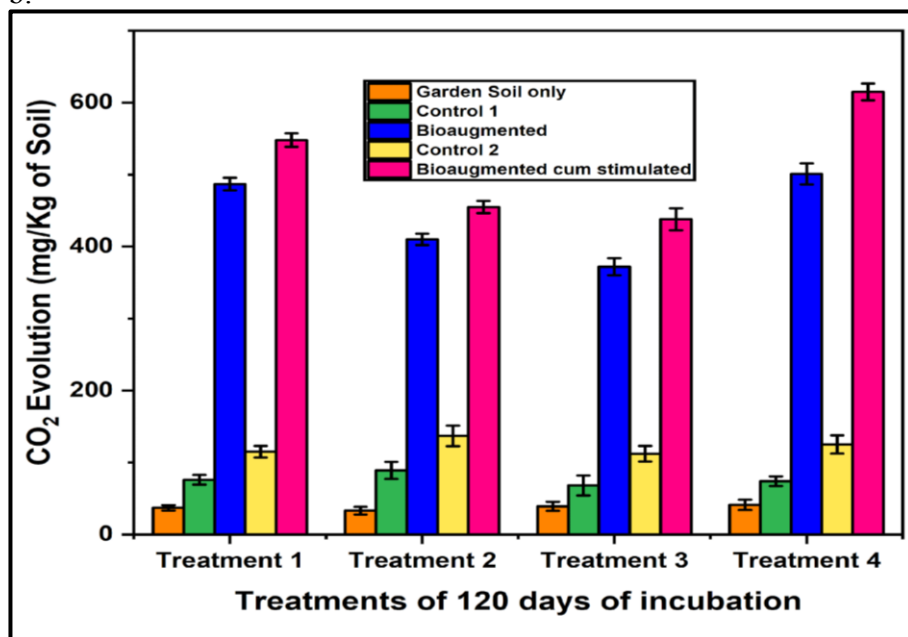


Fig.6.6 CO₂ evolution from soil treatment sets incubated for (a) 30 days and (b) 120 days.

The biodegradation of pollutants depends on the integrated activity of physical, chemical, and biological factors, whose specific regulation and kinetics vary with space and time (Bala *et al.*, 2022). Hence, our analysis revealed a substantial decrease in CO₂ production in all treatments for 30- and 120-day incubation periods with potential strains.

6.5.6 Weight reduction experiment of LDPE films buried in soil samples

Weight reduction experiments are a preliminary validation test for the degradation of LDPE films. It demonstrates the strain's efficacy in adhering to the polymer film and disrupting the hydrocarbon chain.

S. No	Name of the Soil treatments	Weight drop percentage (W%)	Daily consumption rate (g/day) (K rate)	t _{1/2} (half-life span of the polymer (in days)
Experiment I (Bioaugmented treatments with their control)				
1	E1C1	2.23±0.393	0.0002±0.00001	3834.64±724.86
2	E1T1	25.4±0.49	0.0024±0.00001	283.59±6.37
3	E1T2	17.4±0.46	0.0016±0.00001	434.14±13.36
4	E1T3	23.87±1.27	0.00228±0.00014	306.77±18.52
5	E1T4	29.99±0.973	0.00297±0.0001	233.79±8.86
Experiment 2 (Bioaugmented cum stimulant added treatments and their control)				
6	E2C2	4.7±0.88	0.00043±0.00001	1753.24±359.563
7	E2T1	32.1±1.99	0.00327±0.0002	214.654±16.77
8	E2T2	24.1±0.49	0.0025±0.00021	281.54±21.09
9	E2T3	28.65±0.9	0.0029±0.0001	242.674±9.10
10	E2T4	34.1±0.49	0.0035±0.00001	199.492±3.48

Table 6.4 Weight loss of LDPE films in augmented soil treatments (E1T1, E1T2, E1T3, and E1T4) and their control (E1C1) of experiment 1 and bacterial species augmented cum stimulated with mineral salt broth (E2T1, E2T2, E2T3, and E2T4) and control (E2C2) of experiment 2.

Weight loss experiments of LDPE films mixed with garden soil treatments proved the efficacy of isolated strains for polymer biodegradation. It is a primary validation test. The test also demonstrated the potentialities of the combined effect of bio-augmentation and stimulation with nutrients. Among the bacterial species, *Bacillus cereus* NJD1. Proved a maximum weight loss of

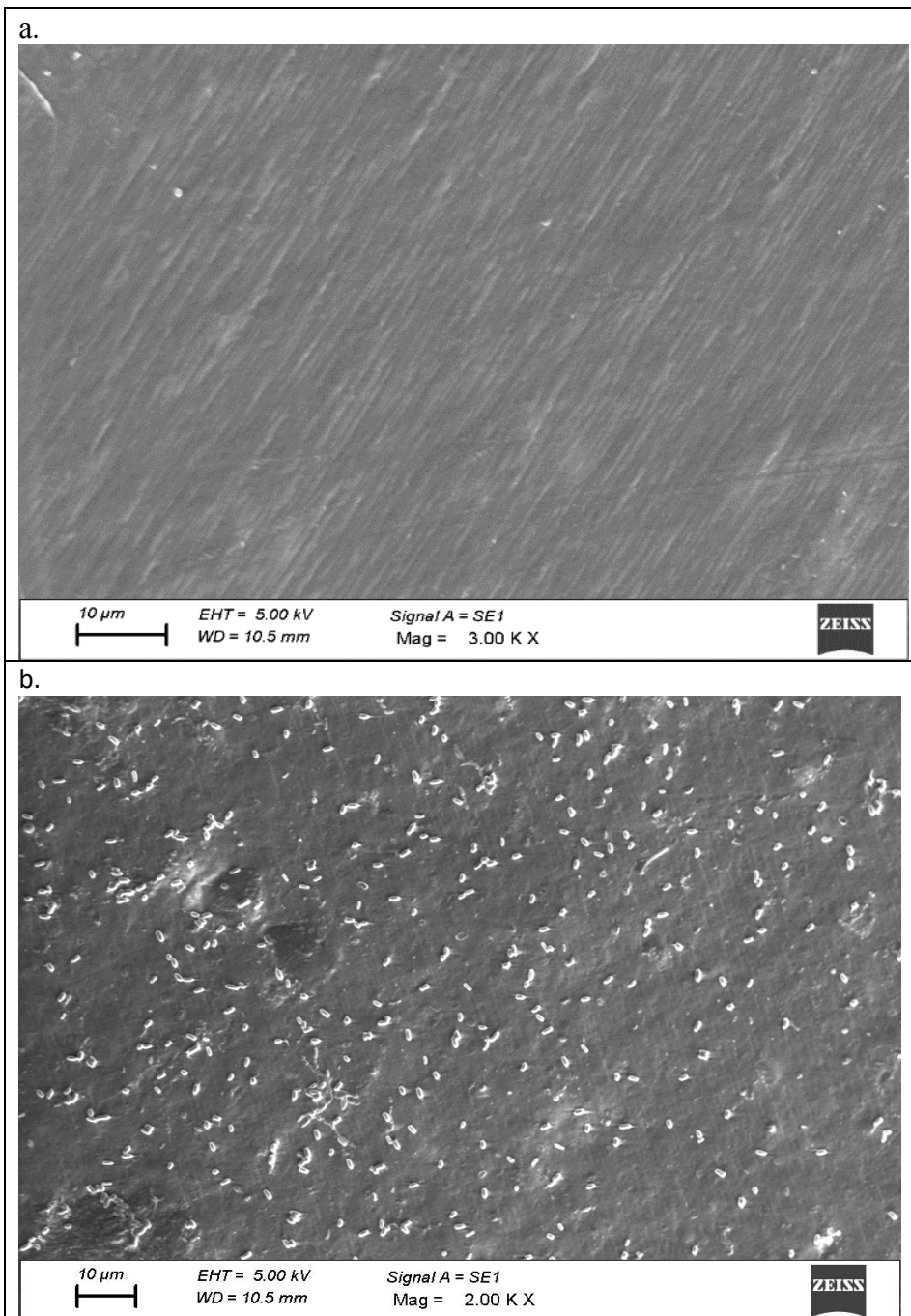
32.1±1.99%. Notably, the bacterial consortium induced the maximum degradation in the E2T4 treatment, with mineral-augmenting bacteria contributing to a $34.1 \pm 0.49\%$ reduction in weight.

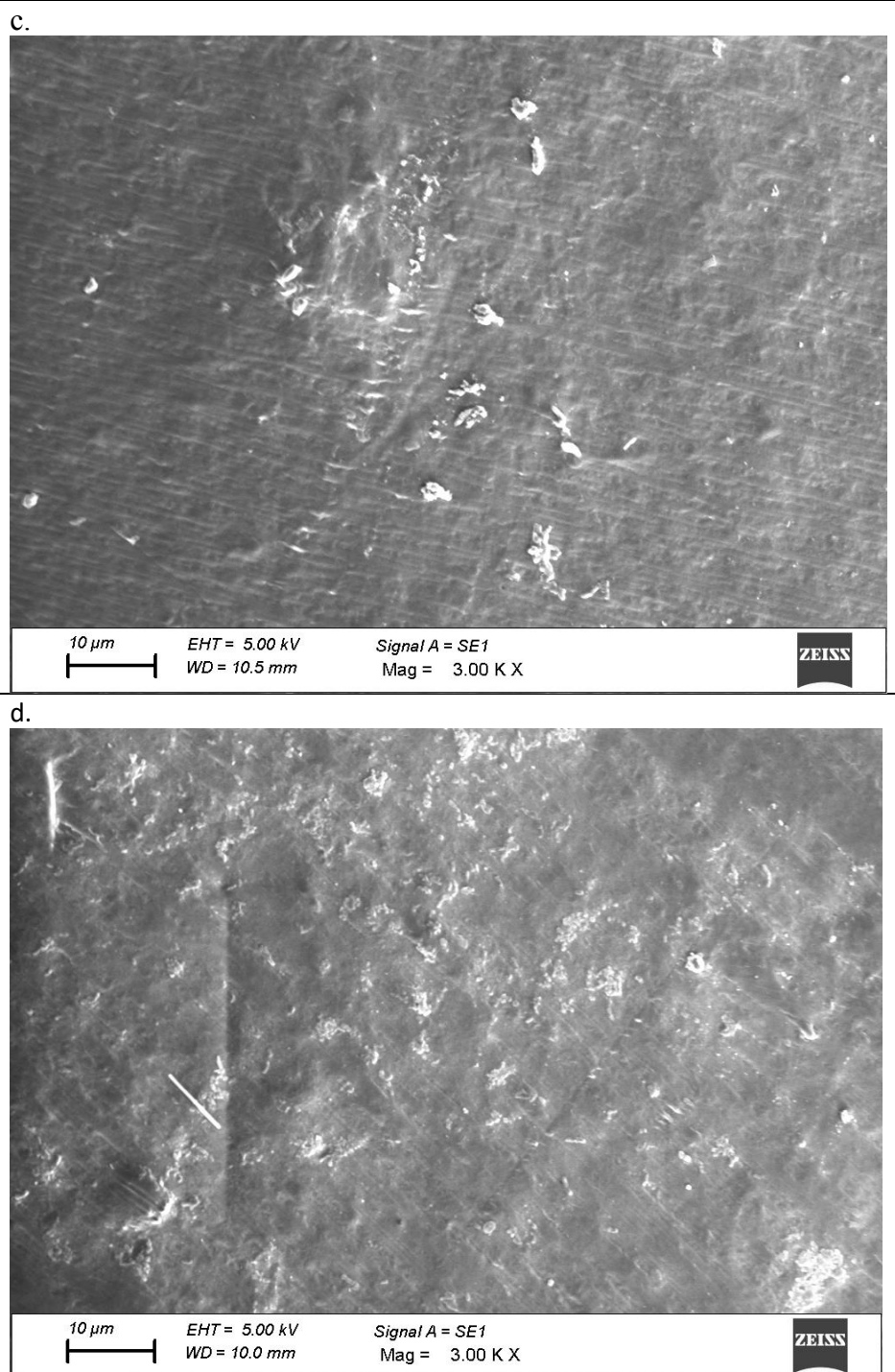
6.5.7 Validation of the efficacies of microbial strains and stimulants added to each soil experiment

We collected LDPE films from augmented bacterial species and mineral-stimulant-added treatment setups for validation experiments.

6.5.7.1 SEM analysis

For this analysis, LDPE films were collected from soil samples treated with isolated potential bacterial strains, and a control LDPE film was sterilised without bacterial inoculation and then surface-sterilised. All the bacterial species inoculated in LDPE films showed robust biofilm formation compared with the control LDPE inoculated in a soil treatment setup without adding the isolated strain (Results displayed in Fig.6.7). The formulated bacterial consortia treated LDPE films showed biofilm formation and surface cracks on the polymer film after 120 days of incubation. All the bacterial-treated LDPE films exhibited significant surface changes, including surface erosions, pits, and fissures. In our images, a few strains appeared in the inner layers of the polymer strips. Overall, the oxidative reactions and firm bacterial attachments, even after surface sterilisation, proved the inoculated strain's ability to degrade the polymer for 120 days.





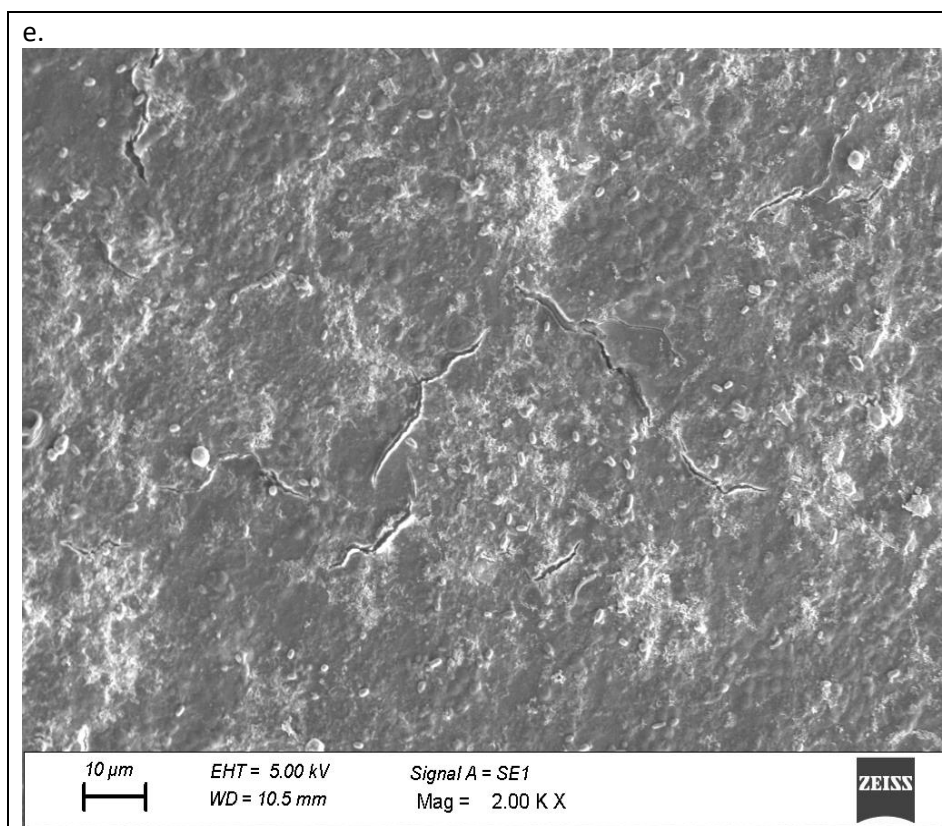
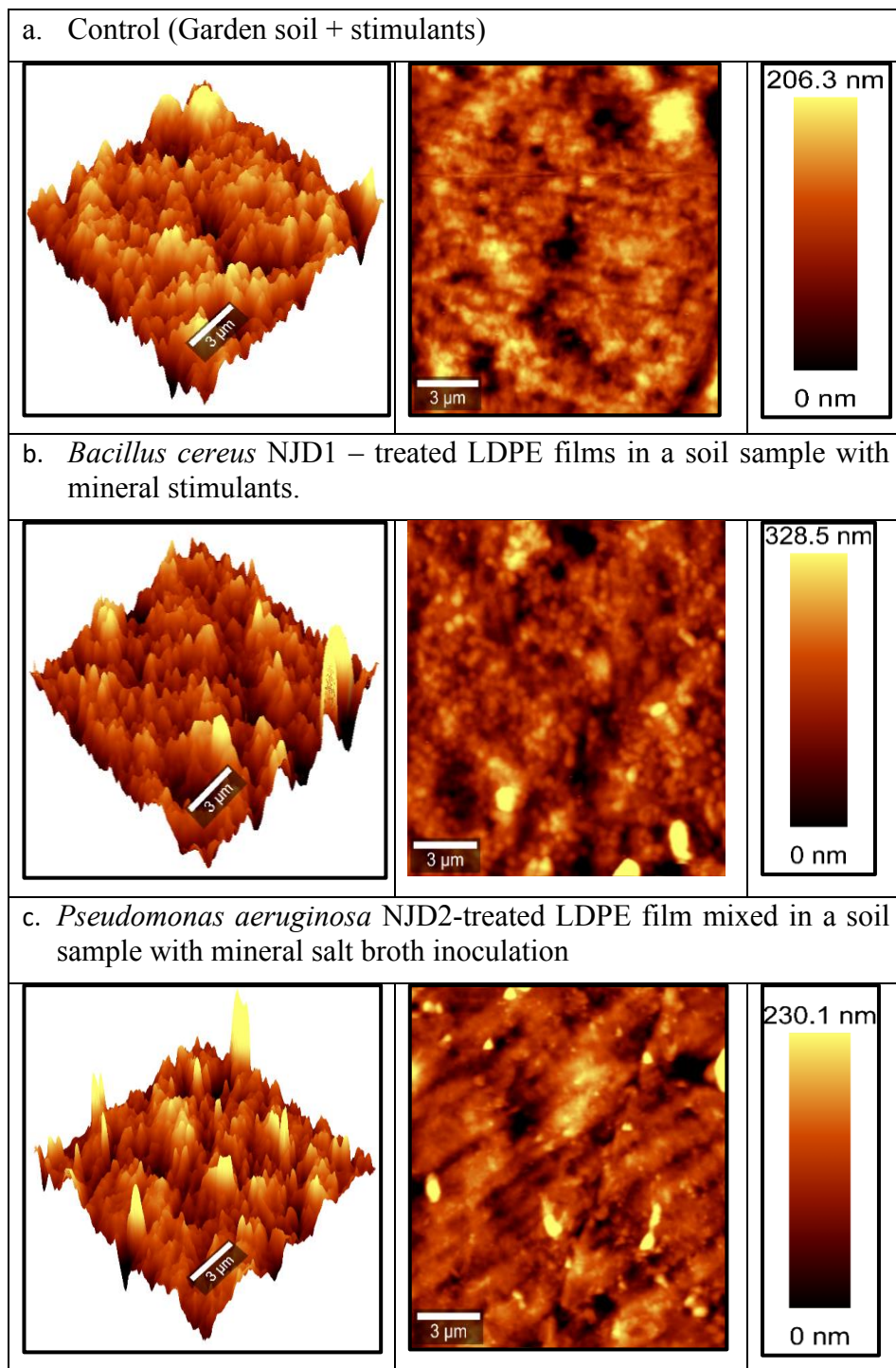


Fig.6.7 SEM analysis of LDPE films in garden soil stimulated with mineral stimulants for 120 days of incubation, without or with bacterial species inoculation. a. Control. b. *Bacillus cereus* NJD1-treated c. *Pseudomonas aeruginosa* NJD2-treated d. *Micrococcus luteus* NJD3-treated e. Consortium of all isolated strains-treated.

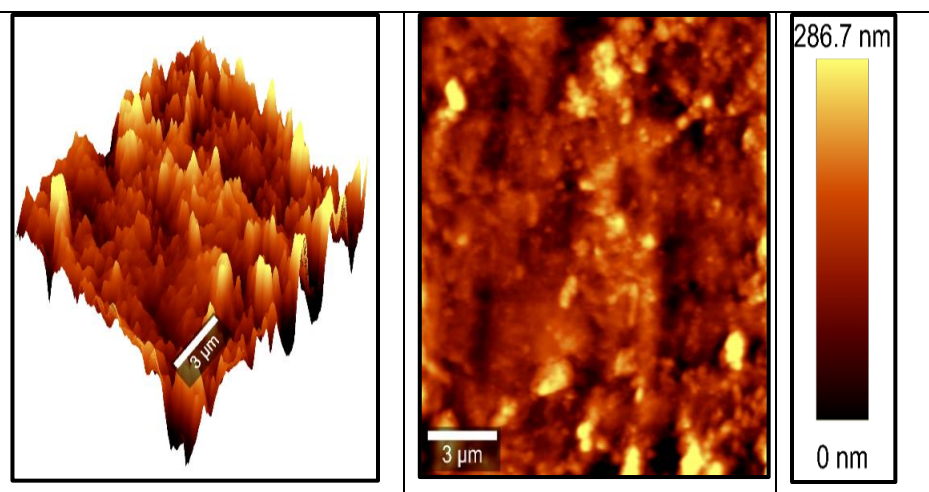
Our current study reported significant biofilm formation by all isolated strains and ambient surface changes compared to previous studies conducted over 120 days of incubation without pre-treatments. Moreover, in the current study, all experiment setups were kept at room temperature under static culture conditions. *Bacillus cereus* NJD1 induced the most pronounced surface morphological alterations on LDPE films among the soil augmented bacterial strains stimulated with mineral salt broth. The isolated strain consortium showed several cracks and strong biofilm on the surface and inner layer of the polymer.

6.5.7.2 AFM analysis

The topographic analysis of LDPE films, studied by atomic force microscopy (AFM), revealed surface deterioration due to the cleavage of C-H bonds. It indicates the potentialities of microorganisms added to the soil setups of the enriched experiments. All the results were compared with control soil setups (garden soil mixed with mineral stimulants) that did not include bacterial addition. In addition to the topographic images (3D and 2D), the statistical values of roughness parameters obtained from the AFM instrument (using WITEC software) helped identify changes in the surface due to the catalytic activities of bacterial strains added to each microcosm setup enriched with mineral salt broth. All the isolated strain-treated microcosm mixed LDPE films showed a significant increase in roughness parameters, even after surface sterilisation (see Fig.6.8).



d. *Micrococcus luteus* NJD3-treated LDPE films were buried in the garden soil sample with mineral stimulants.



e. A consortium of isolated strains formulated in lab-treated LDPE films was buried in a soil sample mixed with mineral salt medium.

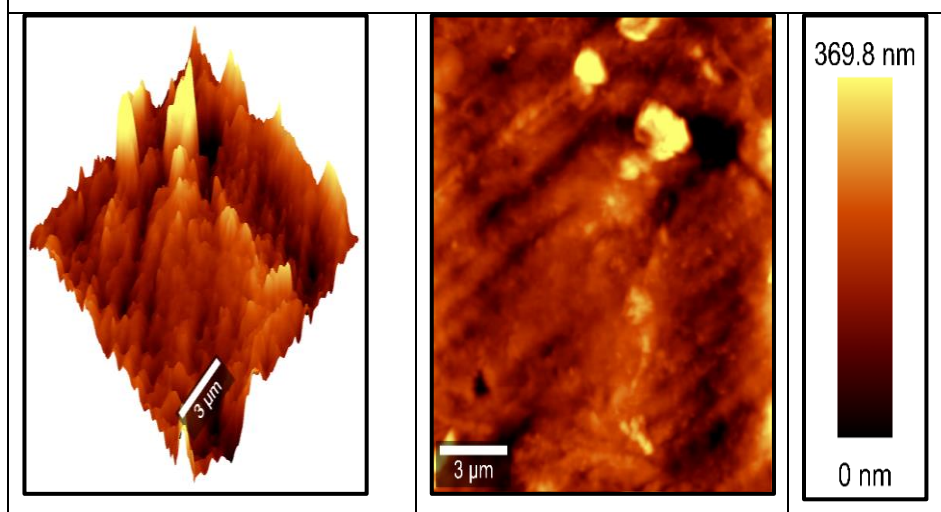


Fig.6.8(a) AFM micrograph of surface sterilised LDPE films collected from various microcosm experiments with their control. The first column depicts 3-D images, and the second shows 2D images.

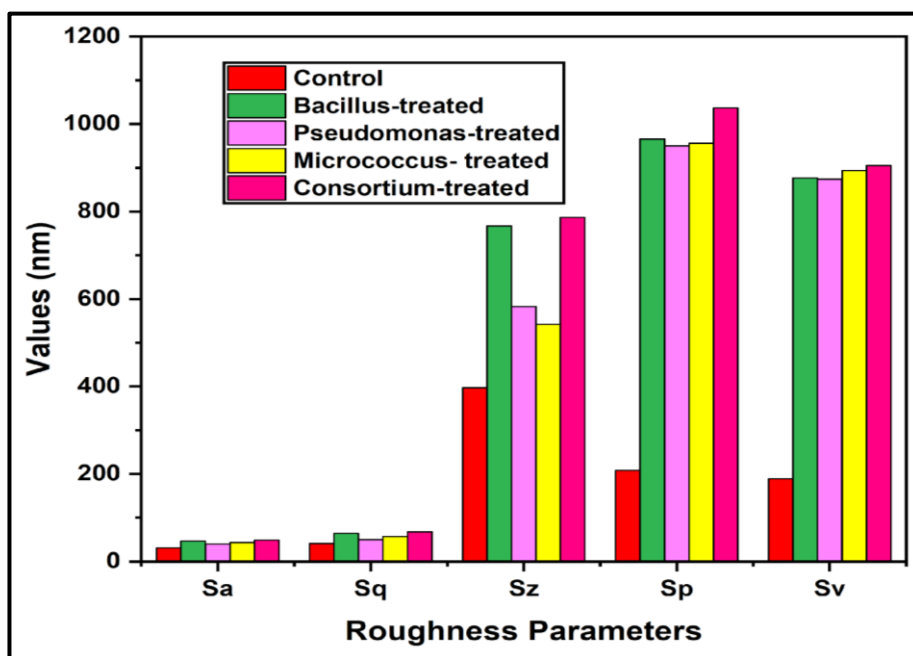
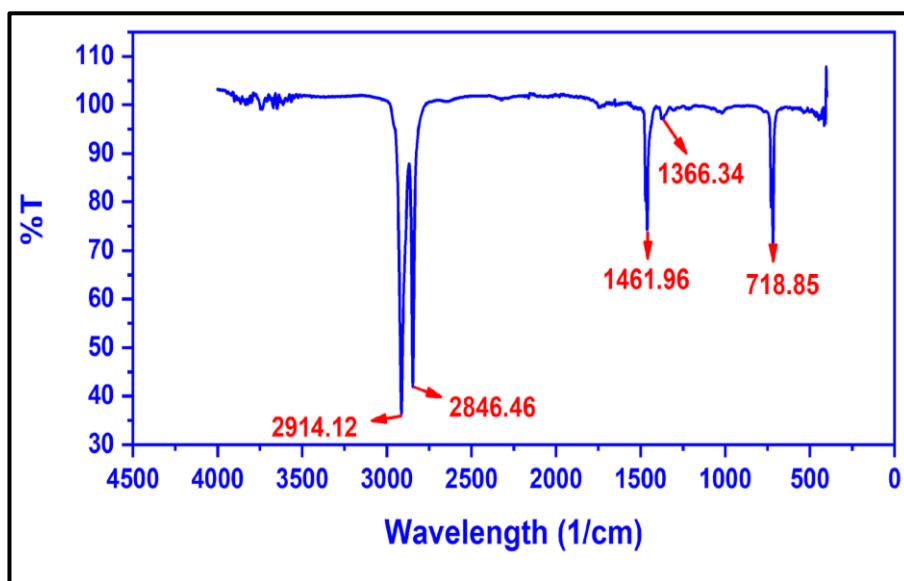


Fig.6.8(b) Surface roughness parameters (Sa, roughness mean data; Sq, root mean square data; Sz, maximum height of surface; Sp, peak-maximum height; Sv, pit-maximum height) of surface-sterilised LDPE films collected from each soil microcosm enriched with mineral salt broth. Results were compared with the control.

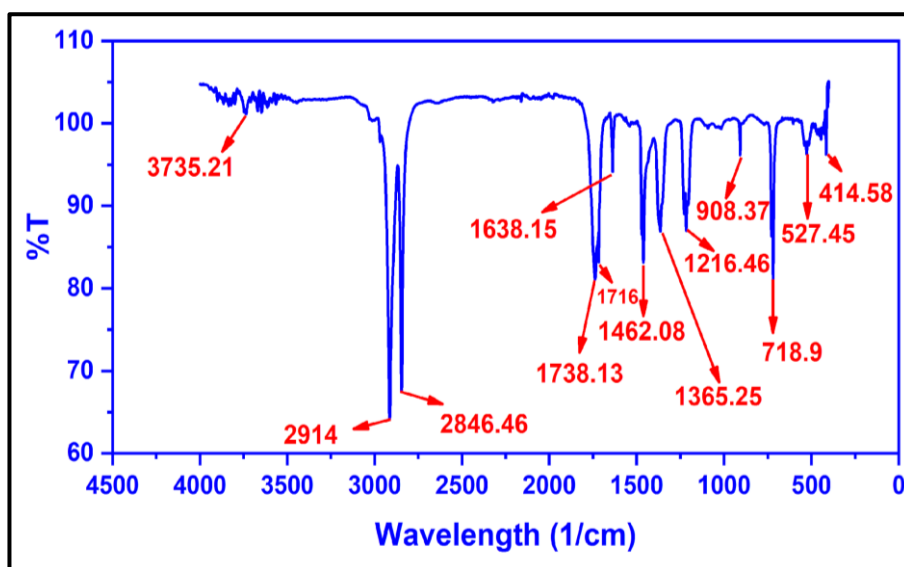
Among the individual strains, *Bacillus cereus* NJD1-treated LDPE films showed the highest roughness changes compared to *Pseudomonas aeruginosa* NJD2 and *Micrococcus luteus* NJD3-treated polymer strips. The consortium of all the strains showed the highest roughness parameter values compared to individual strains. It may be due to the strains' catalytic activities, which enable them to form robust biofilms on polymers and utilise the hydrocarbon chain as their carbon source.

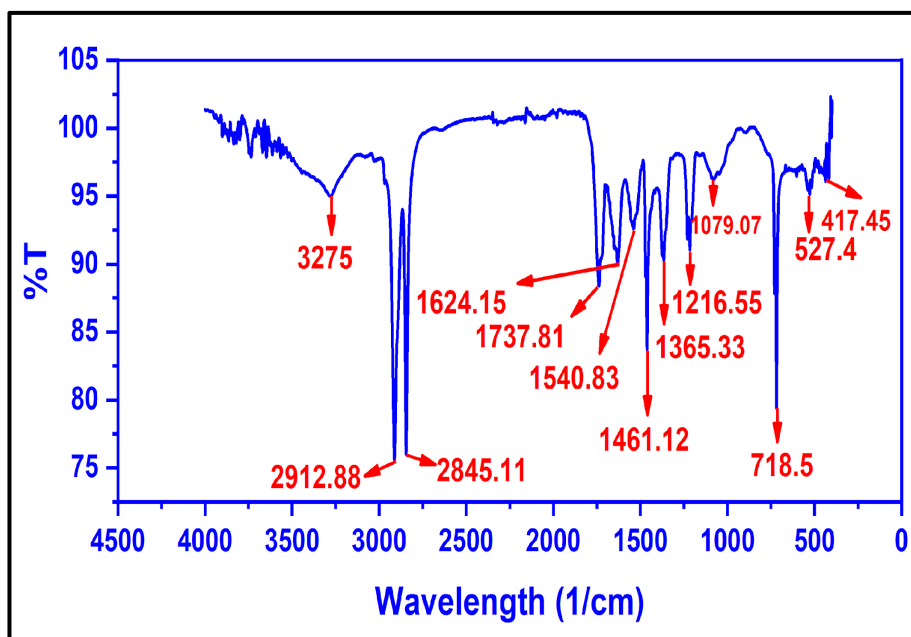
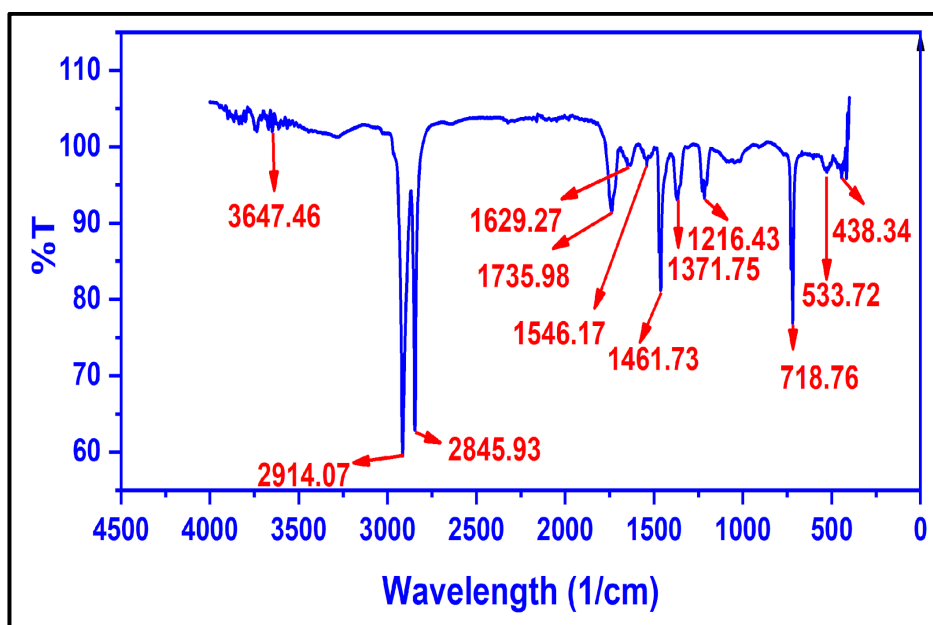
6.5.7.3 FTIR analysis of LDPE films incubated in soil samples for four months

a. Control



b. *Bacillus cereus* NJD1-treated



c. *Pseudomonas aeruginosa* NJD2-treatedd. *Micrococcus luteus* NJD3-treated

f. Consortium-treated

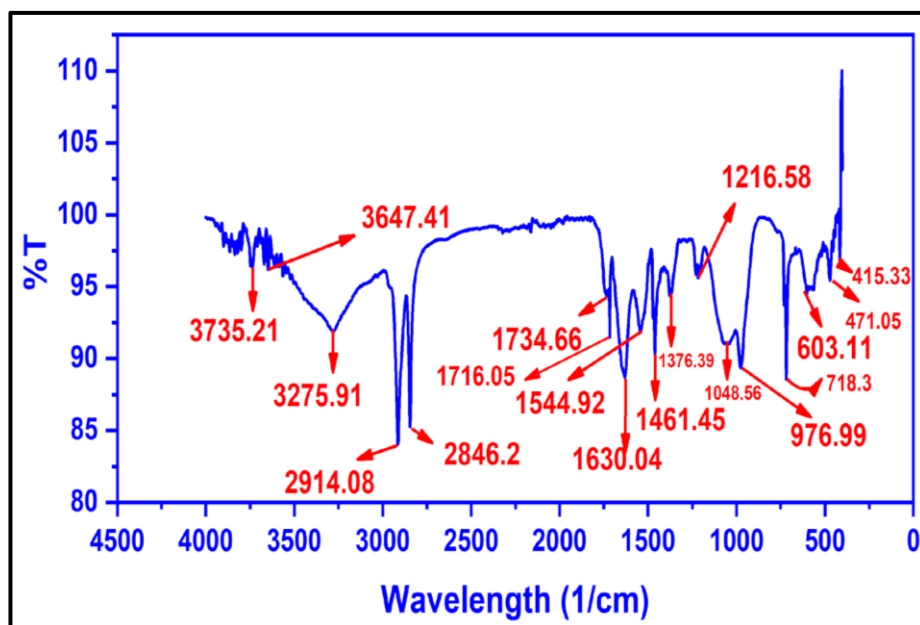


Fig.6.9 FTIR chromatograph of LDPE films buried in stimulated soil samples: without (a) and with bacterial treatments (b, c, d, and e).

Compared to control LDPE films without the addition of isolated bacterial species in the soil augmentation, the isolated strain-treated films showed many similar peaks in the 1200-1800 cm^{-1} range. The FTIR chromatograph confirmed the chemical changes in the LDPE films and the metabolic products on the polymer surface. The presence of the O-H group in all bacterial-treated LDPE films indicates the oxidation products due to bacterial attack. Moreover, all the peaks in the FTIR spectrum in the soil augmentation experiments were similar to the absorption bands in the LDPE strips treated with isolated bacterial species in the strenuous condition of a carbon-free medium.

S. No	Stretching/bending peak with band range	FTIR spectrum absorption peak at cm^{-1} of each microcosm experiment				Functional group
		<i>Bacillus cereus</i> NJD1-treated	<i>Pseudomonas aeruginosa</i> NJD2-treated	<i>Micrococcus luteus</i> NJD3-treated	Consortium	
1	C=O stretching (1734-1750 cm^{-1})	1738.13	1737.81	1734.98	1734.6	Ester
2	C=C stretching (1626-1665 cm^{-1})	1638.15	1624.15	1629.27	1630.04	Keto
3	N-O stretching (1500-1550 cm^{-1})	-	1540.83	1546.17	1544.92	Nitro-group
4	C-H bendings (1365-1395 cm^{-1})	1365.25	1365.33	1371.75	1376.39	Alkane (Methyl) group
5	C-N Stretching (1020-1250 cm^{-1})	1216.46	1216.55	1216.43	1216.58	Amine group
6	C-Br stretching (400-600 cm^{-1})	527.45 414.58	527.4 417.45	533.72 438.34	603.11 471.05 415.33	Bromine-substituted compounds

Table 6.5 A few similar absorption peaks appeared in the FTIR spectrum of LDPE films buried in each garden soil experiment setup, incubated for 120 days.

In addition to the comparable peaks in bacterial fingerprints (Table 6.5), each species-treated LDPE film exhibited distinct absorption bands. For instance, *Bacillus cereus* NJD1-treated LDPE films showed unique absorption peaks at 3725.21 cm^{-1} (hydroxyl group stretching), 1716 cm^{-1} (carbonyl group stretching), and 908 cm^{-1} (alkene group bending), *Pseudomonas aeruginosa* NJD2-treated LDPE films in soil showed a specific peak at 3275 cm^{-1} (hydroxyl group stretching) and 1079 cm^{-1} (Carbonyl group stretching). The *Micrococcus luteus* NJD3-treated LDPE films in the soil microcosm exhibited a unique peak at 3647.46 cm^{-1} , corresponding to the N-H stretching vibration. The

pronounced peak in the bandwidth of 3200-3700 cm^{-1} manifests the hydroxyl group stretching, characteristic of alcohol groups. In contrast, the moderate peak in this region confirms the presence of N-H groups, corroborating the oxidative reactions on the polymer surface and the formation of a resilient biofilm (Zhang *et al.*, 2022). Moreover, the bacterial consortium's FTIR spectrum demonstrated the efficacy of each bacterial strain in forming a biofilm on LDPE and imparting chemical alterations. All bacterial-treated LDPE films exhibit peaks at 2910-2914 cm^{-1} , 2840-2850 cm^{-1} , 905-980 cm^{-1} , and 410-610 cm^{-1} . It indicates the backbone absorbance bands of LDPE film. As demonstrated in our study, these alterations in functional groups (stretching or bending), shifts in the intensities of the IR spectrum, the introduction of novel peaks, and differences in peak breadth signified degradation-induced transformations.

The FTIR analysis of all isolated individual bacterial strain or their consortium-augmented LDPE films verifies the effective degradation of LDPE, with oxidative and microbial pathways yielding carbonyls, hydroxyls, and esters. These chemical alterations reduce the polymer's hydrophobicity and increase its biodegradability, facilitating its conversion into simpler, environmentally friendly compounds.

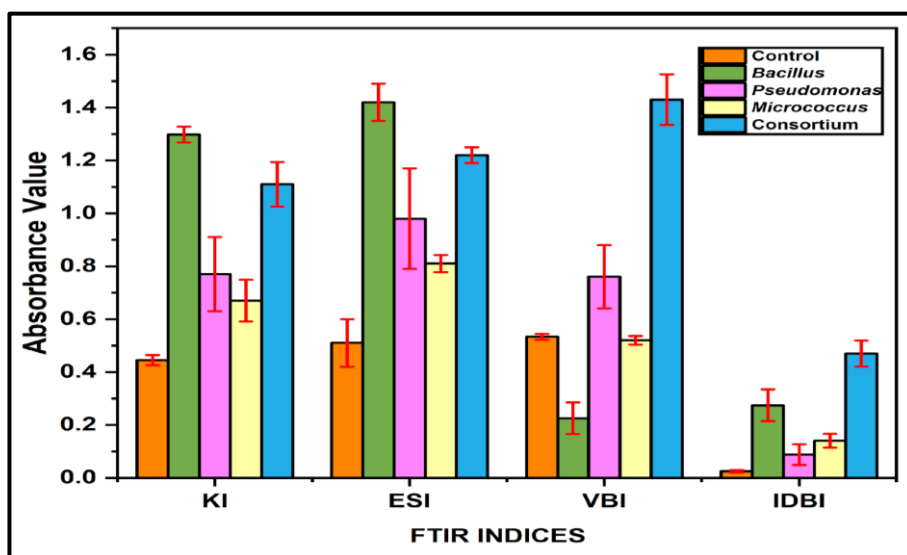


Fig.6.10 FTIR indices (carbonyl indices, KI; ester bond indices, ESI; vinyl bond indices, VBI; internal double bond indices, IDBI), calculated using FTIR data of LDPE films mixed in each soil experiment setup. The FTIR indices were computed using FTIR data of LDPE films mixed with potential strains screened from the long-run depot after 120 days of incubation.

While calculating the FTIR indices using the FTIR data of LDPE films mixed with soil samples containing bacterial strains and stimulants, the carbonyl and ester carbonyl indices were higher for the *Bacillus cereus* NJD1-treated LDPE films compared to those of other isolated strains. However, the consortium showed broader-spectrum metabolic activities. It indicates its efficacy in biodegradation and bacterial transformation activities. *Pseudomonas aeruginosa* NJD2 and *Micrococcus luteus* NJD3 have a moderate effect on biodegradation in the soil-augmented scenario. The control films exhibited minimal chemical bonding or changes in functional groups.

6.5.7.4 GC-MS analysis

a. Control

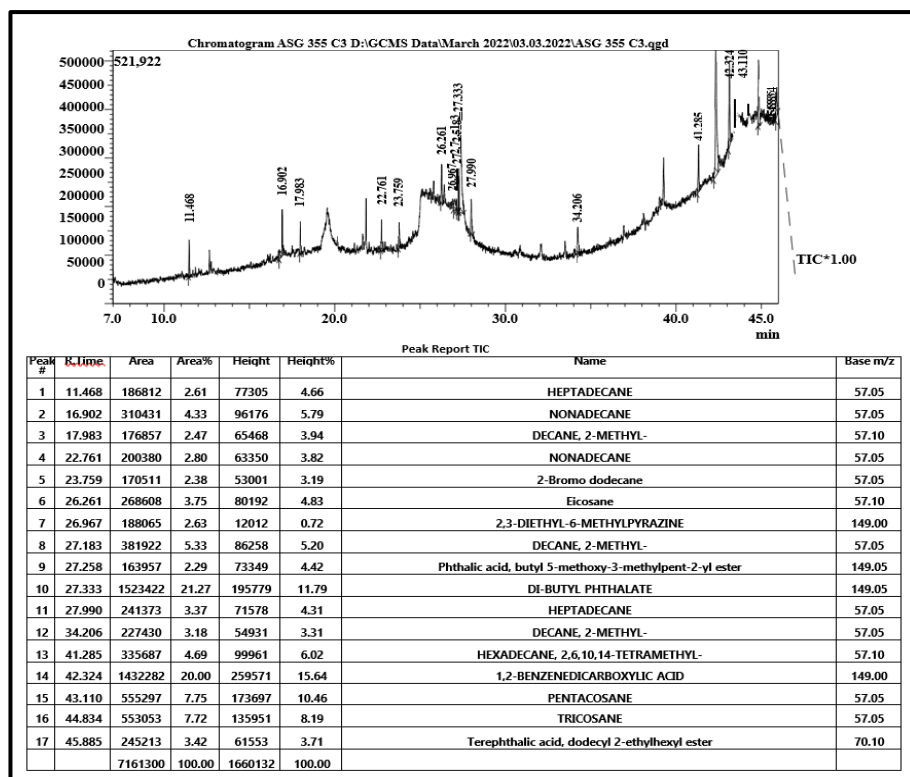


Fig.6.11(a) GC-MS Spectrograph of control LDPE film.

This profile of LDPE film indicates a chemically stable and intact structure. It includes long alkane chains (Heptadecane, nonadecane, pentacosane, and tricosane), additives (decane-2-methyl, 2,3-dimethyl-6-methylpyrazine), and plasticisers (phthalic acid esters).

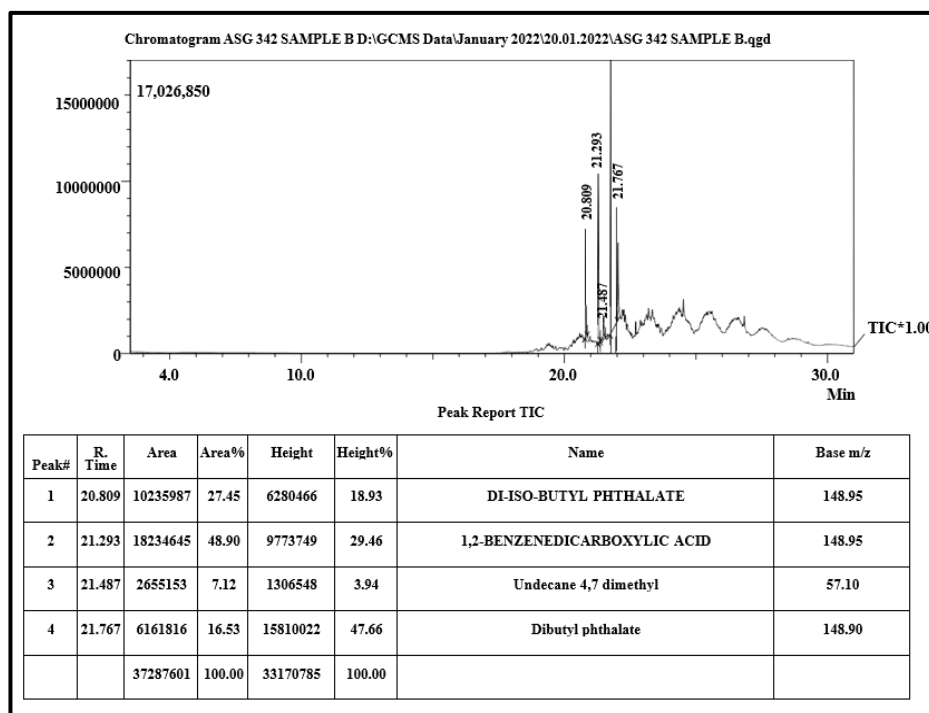
b. *Bacillus cereus* NJD1 –treated

Fig.6.11(b) GC-MS spectrometric analysis of *Bacillus cereus* NJD1-treated LDPE films.

Compared to the control, the *Bacillus cereus* NJD1-treated LDPE films exhibited fewer peaks and more degradation by-products, indicating the formation of new metabolites. This spectrograph indicates the presence of degraded plasticiser, namely phthalic acid or 1,2-benzenedicarboxylic acid, and plasticiser degradation products such as di-iso-butyl phthalates. Additionally, it reveals the presence of aliphatic hydrocarbons, including undecane-4,7-dimethyl, a by-product of alkane degradation.

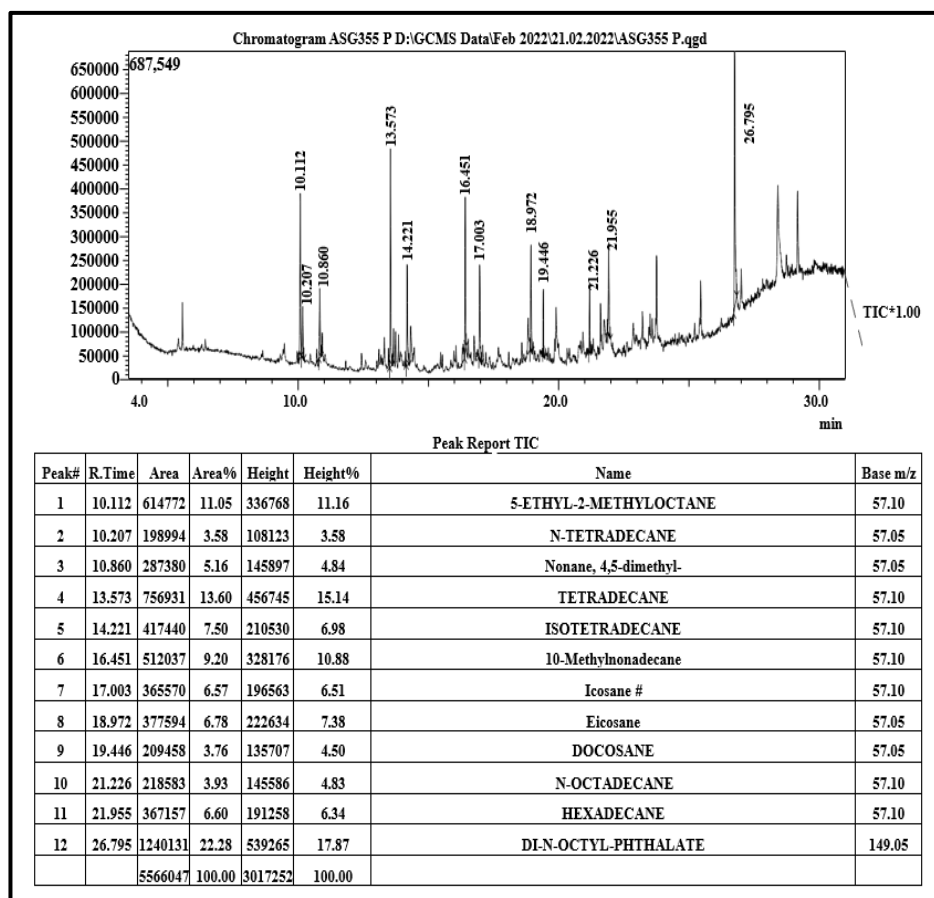
c. *Pseudomonas aeruginosa* NJD2 -treated

Fig.6.11(c) GC-MS analysis of *Pseudomonas aeruginosa* NJD2-treated LDPE film in a soil microcosm experiment after 120 days.

The *Pseudomonas aeruginosa* NJD2-treated LDPE films consist of multiple media (tetradecane and n-octadecane) or branched hydrocarbons (5-ethyl-2-methyl octane and iso-tetradecane) in the GC-MS spectrum, suggesting the fragmentation and formation of various degradation products of the polymer. The presence of a volatile compound, specifically nonane 4,5-dimethyl, indicates that the enzyme is acting on long-chain hydrocarbon chains. The presence of Di-n-octyl

phthalates demonstrated partial degradation of the plasticiser in the LDPE film by the bacterial action

d. *Micrococcus luteus* NJD3-treated

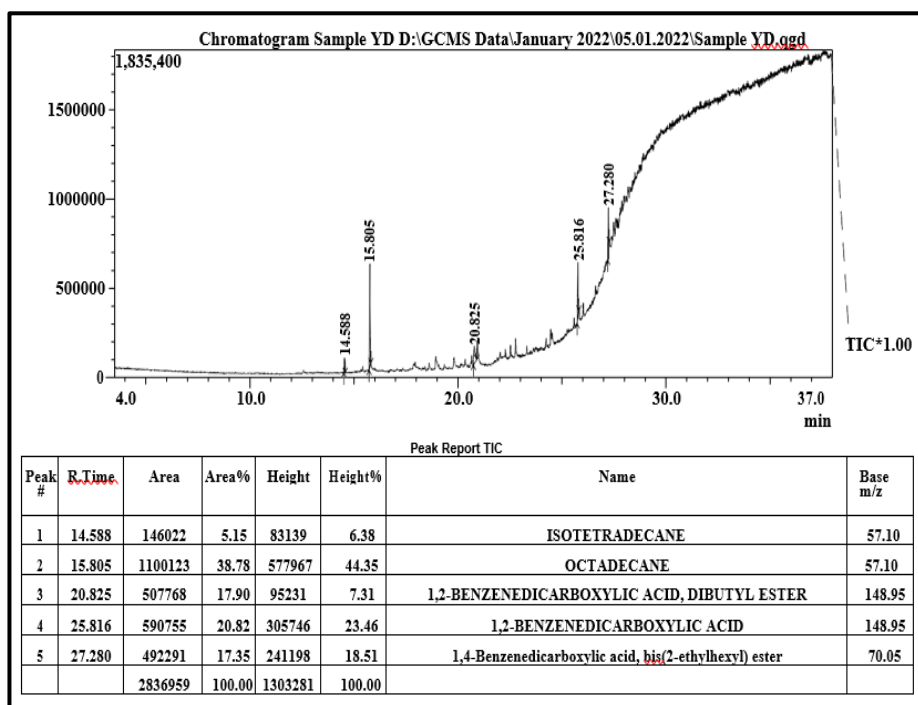


Fig.6.11(d) GC-MS image of *Micrococcus luteus* NJD3-treated LDPE film in a soil microcosm experiment after 120 days.

Compared to the control, the *Micrococcus luteus* NJD3-treated LDPE films showed the presence of octadecane and iso-tetradecane in the spectrum, indicating the release of hydrocarbons, a robust sign of LDPE chain scission. In addition, detecting both intact esters (DBP, DEHP) and their acid counterparts (benzene dicarboxylic acid) indicated partial microbial degradation of plasticisers through oxidation or ester hydrolysis.

e. Consortia-treated

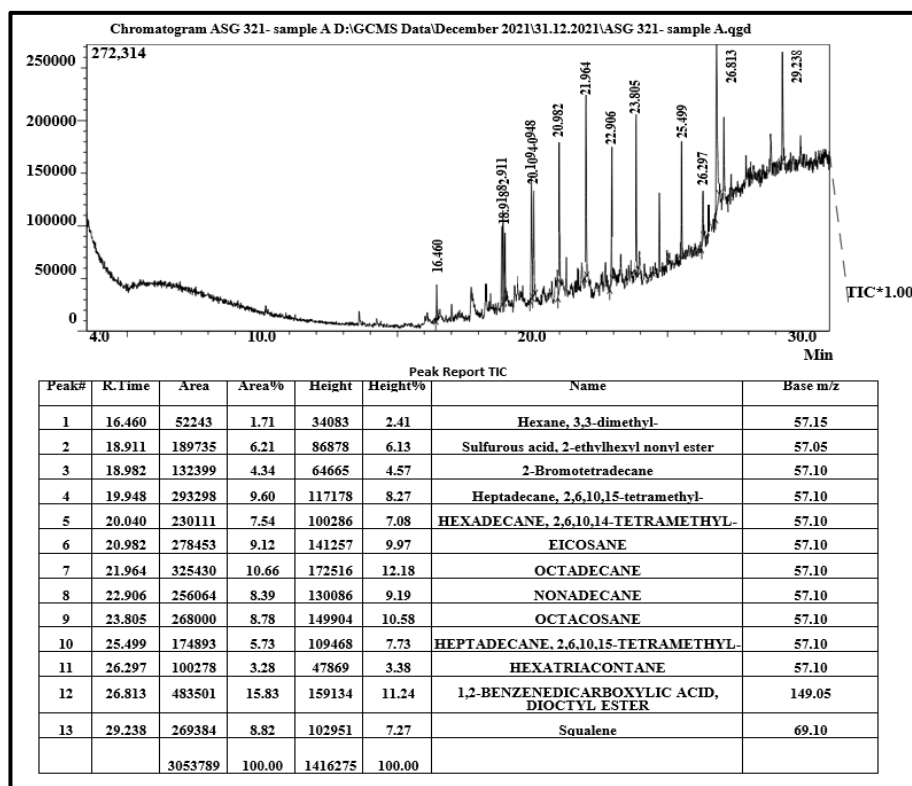


Fig.6.11(e) GC-MS spectra of LDPE films from consortia-treated LDPE films in mineral-stimulated soil samples incubated for four months.

The consortia-treated LDPE film consists of several branched alkanes, long-chain alkane byproducts, plasticiser-degraded, and additive-degraded products.

S. No	Peak name	Interpretation
1	Hexane 3,3-dimethyl	Branched alkane- Possible LDPE breakdown product
2	Sulfurous acid, 2-Ethylhexyl nonyl ester	Suggest the bio-surfactant degradation
3	2-Bromotetradecane	It may result from halogenated degradation
4	Hexadecane 2,6,10,14-tetramethyl	Branched long-chain hydrocarbons- Typical LDPE degradation products
5	Eicosane, Octadecane, Nonadecane and Nonacosane	Long-chain alkanes-LDPE polymer backbone fragments
6	Heptadecane 2,6,10,15-tetramethyl	Indicates the repeated presence of LDPE byproducts
7	Hexatriacontane	High molecular weight hydrocarbon- Slowly degraded LDPE fragment
8	1,2-Benzenedicarboxylic acid, dioctyl ester	Plasticiser leached or partially degraded
9	Squalene	Hydrocarbon is often obtained Through microbial biosynthesis

Table 6.6 The list of GC-MS peaks depicts the degraded LDPE by-products by the action of bacterial consortia.

6.6 Discussion

The current investigation analysed the bioaugmentation potential of individual bacterial strains isolated or their consortium formulated in lab conditions in LDPE mixed garden soil. Bioremediation treatments can affect soil physicochemical properties differently (Thapliyal *et al.*, 2024). Thus, we analysed the physical and chemical parameters of soil samples collected from garden soil before augmenting the isolated

strains. Many studies have demonstrated that microorganisms requiring carbon, hydrogen, nitrogen, oxygen, and phosphorus require additional components to synthesise macromolecules (Cai *et al.*,2023; Merchant & Helmann,2012). Fertilising facilitates the addition of critical components by these microbes in the soil, allowing them to reproduce and flourish (Ahmed *et al.*,2023). Subsequently, microorganisms in the soil may be unfavourably influenced by fluctuations in temperature, pH, nutrient availability, heavy metal contamination, oxygen saturation, and other variables, which can affect the significant ecological phenomena associated with nutrient cycling (Abdul Rahman *et al.*,2021; Stroud *et al.*,2007). It has been observed that, in severely contaminated environments, combining two or more remediation techniques is crucial for enhancing bioavailability and efficacy. The growth of potential individual microbes or their consortium is crucial in the treatment techniques.

However, several microbial strains can be found naturally in contaminated soils and may not possess the appropriate metabolic capacities to degrade target pollutants. It may be due to the contaminant's overall toxicity. Numerous prior studies have shown that the breakdown of contaminants relies on the combined actions of organisms and diverse environmental conditions (Bhatt *et al.*,2021; Maglione *et al.*,2024). Thus, our current study incorporated biostimulants to sustain bioactivities (Jia *et al.*,2024) on polymer surfaces buried in soil samples.

In the present investigation, we demonstrated biofilm formation and metabolic activities in all soil experiment setups (experiments 1 and 2)

using biomass count, FDA analysis, protein estimation tests, analysis of pH changes in the medium, and a CO₂ evolution test.

The rise in microbial populations on LDPE films buried in bio-augmented and stimulated environments with nutrients can be attributed to their ability to facilitate strong biofilm formation, which may result in the depolymerisation of the polymer compounds. A recent scientific investigation of *Enterobacter cloacae* in soil samples yielded similar results, indicating strong biofilm formation on polymer strips (Sarker *et al.*,2020). A recent FDA analysis of LDPE films buried in agricultural soil revealed similar biofilm formation attributed to prolonged incubation with potential bacterial strains isolated from plastic dumpsites (Pathak & Navneet,2017).

The protein estimation enabled us to determine the presence of bacterial cultures in the logarithmic phase on LDPE films buried in the soil samples (Ji *et al.*,2024). It complements the biomass count and FDA results.

The pH change of the soil samples was an indirect measure of biofilm formation on the LDPE films mixed in each experimental setup. Compared to the control soil setup, the reduction in pH in other experiment setups indicates the catalytic activities of microbes utilising LDPE films as an energy source (Bhattacharya & Gupta,2021).

Numerous scientific investigations have demonstrated that plastic components in the soil can affect the soil's organic carbon pool and accelerate its conversion to CO₂, in addition to producing end products such as CH₄ and H₂O (Guo *et al.*,2023). Therefore, Scientists regarded

the rate of CO₂ release as an indirect measure of LDPE degradation (Ali *et al.*,2016). This study assessed the metabolic activities of periodic soil microcosm studies by respirometric analyses. The CO₂ evolution in our research can be attributed to microbial activities on soil organic carbon (SOC) and inorganic carbon (SIC), or the microbial mineralisation of LDPE films mixed with the soil samples (Cao *et al.*,2022; Janzen,2004). Adding isolated microbes to the soil samples can also alter LDPE films buried in soil by modifying the soil structure and microbial interactions (Romano *et al.*,2024). Several earlier scientific reports have demonstrated that the mineral stimulation of soil samples can enhance the biodegradation of polymeric compounds (Escalante *et al.*,2015; Hou *et al.*,2019). Consequently, our study revealed the maximum CO₂ generation in the soil experimental setup alongside LDPE sheets treated with simulated bacterial species and minerals. Several prior research studies have demonstrated that the CO₂ emissions from LDPE films buried in soil are consistent with the findings of our microcosm experiment (Muliadi *et al.*,2021; Xiong *et al.*,2023).

Another important analysis to establish the efficacy of bacterial strains in different soil experiment setups was the weight loss of LDPE film experiments after 120 days of incubation. Our soil mesocosm tests for weight loss produced far larger percentages than those documented in numerous prior studies (Montazer *et al.*,2020; Sarker *et al.*,2021).

Later, the robust biofilm formation and biodegradation on LDPE films in the bacterial augmented cum mineral mineral-stimulated soil experiment were demonstrated through various validation experiments,

including SEM, AFM, FTIR, and GC-MS analyses. The scanning electron micrographs of LDPE films from each soil experiment revealed the exfoliation of LDPE films and the formation of biofilms on their inner surfaces, characterised by pits, cracks, grooves, and fissures. These can reduce the structural integrity of the polymer by exposing crystalline regions, thereby facilitating further microbial catalytic activities (Harrat *et al.*,2024). Multiple prior investigations have demonstrated that surface modifications caused by bacterial strains combined with soil samples persist for over six months (Basim *et al.*,2022). Conversely, a few studies have examined the effectiveness of microbial strains in soil biodegradation trials lasting 100 to 120 days (Barral & Stenson,2020; Nademo *et al.*,2023).

The present study's AFM micrograph and surface parameter analyses proved the efficacy of isolated bacterial strains on the polymer surface. The current study yielded significantly higher roughness data than previous studies on LDPE films buried in soil (Hou *et al.*,2019; Sridharan *et al.*,2021).

Many recent FTIR studies of LDPE films augmented with bacterial strains in soil and stimulated with mineral salt broth have revealed several chemical alterations on the polymer surface (Roy *et al.*,2021; Zhu *et al.*,2021). The production of carbonyl groups marks the initial stage in biodegradation, ultimately leading to the synthesis of carboxylic acid groups (Sharma & Neelam,2023). This compound can be broken down into simpler compounds through the citric acid cycle. Moreover, the peaks between 900 and 1350 cm^{-1} indicate the presence of oxygen functional groups, such as ethers, alcohols, or esters. It

manifests the bacterial enzymatic reaction on the LDPE films, and these moieties further degrade into new chemical compounds (Khan *et al.*,2023). The nitro and amine groups are novel functional groups in the polymer surface spectrum. It may be due to bio-adherence and its enzymatic reaction, which utilises the compound as its energy source.

Carbonyl and internal double bonds on polymer surfaces indicate the catalytic activities of microbial strains (Lokesh *et al.*,2023; Moharir & Kumar,2021; Rong *et al.*,2024). The presence of oxidoreductase enzymes in the isolated strains may be the reason behind the formation of keto and ester bonds on the LDPE films (Albertsson *et al.*,1998). The significant changes in FTIR indices of LDPE films treated with bacterial strains, compared to those of control LDPE films, validate the disruption of the ordered arrangement of the polymer, which results in a reduction of its crystallinity. These high rates of chemical changes in LDPE films validate the robust biofilm formation and chemical reactions, such as oxidation, hydrolysis, internal bond formation, and esterification, which may eventually lead to the biodegradation of polymer films (Mohanani *et al.*,2020). The significant difference in the FTIR index value may be due to the increased bio-attachment of strains to the polymer surface.

Chemical and molecular changes in polymer films can reduce crystallinity and introduce amorphous surfaces. These amorphous surfaces with unstable carbon chains have been proven to be specific targets for bacterial degradation (Lear *et al.*,2021). It severely affects the three-dimensional structures of LDPE film crystallinity.

All the GC-MS spectra of bacterial-treated films showed intermediates of polymer degradation, such as alkanes and fatty acid ester-degrading components. In contrast to the control spectrum, the *Bacillus cereus* NJD1-treated LDPE film showed a reduced number of peaks in its chromatogram. However, the other strains treated with LDPE films exhibited chemical changes resulting from the catalytic activities of the microbial strains. Moreover, the chromatograph of consortium-treated LDPE films revealed the presence of acid compounds and alkane intermediates involved in the biodegradation of the LDPE's essential chain and stabilisers. The intermediate compounds detected in the LDPE films in the soil microcosm experiments demonstrated an identical pathway to those generated by mixing the polymeric strips in the mineral salt media with the same bacterial strains or consortium for 120 days of incubation. Similar intermediates have been identified in numerous earlier investigations as indicators of LDPE biodegradation (Mazaheri & Nazeri,2023; Redko *et al.*,2024; Sangale *et al.*,2019).

6.7 Conclusion

Our current analyses evaluated the efficacy of isolated strains from long-term depots in bio-augmenting LDPE films mixed into garden soil. The bacterial strains for the in situ studies were *Bacillus cereus* NJD1, *Pseudomonas aeruginosa* NJD2, and *Micrococcus luteus* NJD3. Moreover, we checked the potential of the consortium of these strains, formulated in laboratory conditions, on LDPE strips. Bacterial-augmented soil microcosm setups, both with and without mineral stimulation, were evaluated for enhanced remediation of polymers. We observed improved bacterial biofilm formation in LDPE films

incorporated into a mineral-stimulated soil microcosm compared to a bacterial-augmented soil sample mixed with polymer strips. SEM, AFM, FTIR, and GC-MS analyses validated this. Hence, it has demonstrated the comprehending functions of isolated bacterial strains for a cost-effective, eco-friendly, and sustainable mechanism to bio-remediate xenobiotic compounds, such as LDPE films, in mineral-stimulated soils.

CHAPTER 7

CONCLUSION AND MAJOR OUTCOMES

White pollution due to plastic materials has become a daunting threat to our environment. Low-density polyethylene material, the most common plastic material in the packaging industry, after use ends up in plastic depots in the ecosystem. Now, the current world is struggling to properly manage and recover resources from such a huge amount of plastic waste. Primary contributing factors such as the absence of technical skills to manage toxic waste, deficient infrastructure for recycling and recovery, and a general unawareness of legal guidelines are fuelling the escalating plastic waste burden. The contemporary research by scientists for the cost-effective and environment-friendly degradation of LDPE-like plastic compounds proved the accelerated pace of microbial inocula. Therefore, in our current study, we aimed to isolate and identify potential bacterial strains from the natural depot. Further, we tried to formulate an effective consortium in lab conditions for the enhanced breakdown of toxic and recalcitrant polymeric chains to non-toxic intermediate compounds, which can further mineralise to CO₂ and H₂O. Moreover, our study focused on to augmentation of the isolated strain or consortium in garden soil and enhancing its activities in the presence of nutrients.

This study systematically evaluated the biodegradation potential of indigenous bacterial strains isolated from long-term landfill soils of

Kerala (Alathur, Laloor, and Bhramapuram). The key outcomes are as follows:

1. Isolation and Identification of Bacterial Strains

- ❖ From enriched landfill soils, three potent LDPE-degrading strains were identified:
 - *Bacillus cereus* NJD1 (OLA823002.1)
 - *Pseudomonas aeruginosa* NJD2 (OL823063)
 - *Micrococcus luteus* NJD3 (OL823065)
- ❖ Identification was confirmed through morphological, biochemical, 16S rDNA sequencing, and MALDI-TOF analyses.

2. LDPE Degradation Efficiency

- ❖ Individual strains demonstrated significant LDPE biodegradation under static culture conditions in carbon-free mineral salt medium (CFLMSM).
- ❖ *Bacillus cereus* NJD1 showed the highest degradation efficiency ($42.73 \pm 1.105\%$), followed by *Micrococcus luteus* NJD2 ($40.22 \pm 2.04\%$) and *Pseudomonas aeruginosa* NJD3 ($39.7 \pm 1.27\%$) over 120 days.

3. Biofilm Formation and Enzymatic Support

- ❖ All strains formed robust biofilms, confirmed by biomass quantification, viable counts, protein estimation, EPS analysis, and pH variation.
- ❖ Enzyme assays revealed production of laccases, lipases, and proteases in all strains. Additionally, *Pseudomonas* biofilms exhibited lignin peroxidase and manganese peroxidase activity, while *Micrococcus* biofilms demonstrated esterase activity.
- ❖ Biosurfactants were detected in all isolates and characterised as lipoproteins via FTIR analysis.
- ❖ Among all strains, *Bacillus cereus* NJD1 consistently showed the highest degradation efficacy and strongest biofilm formation.

4. Consortium Formulation and Synergistic Activity

- ❖ An artificial consortium comprising the three strains demonstrated enhanced degradation performance.
- ❖ The consortium achieved $54.67 \pm 1.28\%$ LDPE weight reduction, surpassing individual strain performance.
- ❖ Degradation was supported by robust biofilm formation and multi-enzyme activity, validated through FTIR, AFM, SEM-EDAX, UTM-tensile testing, and GC-MS analyses.

5. Soil Microcosm Experiments

- ❖ Bioaugmentation and biostimulation strategies were tested in garden soil microcosms supplemented with LDPE films.
- ❖ After 120 days, control setups with LDPE alone (ET1) showed nutrient depletion (N, P, K, organic carbon), while inoculated setups exhibited nutrient enrichment due to microbial activity.
- ❖ Bacterial adherence to LDPE in soil was confirmed via biomass counts, protein estimation, pH changes, CO₂ evolution, and weight loss.
- ❖ The environmental relevance of these bacterial strains was evaluated using bioaugmented cum biostimulated soil microcosm setups, with validation provided by SEM, AFM, FTIR, and GC-MS investigations.

In conclusion, these strains—individually or as consortia—represent promising candidates for the eco-friendly bioremediation of LDPE plastic waste, offering a potential sustainable solution for mitigating plastic pollution in our ecosystem.

RECOMMENDATIONS

This experiment effectively isolated indigenous bacterial strains from plastic landfill environments to break down LDPE films. In the current study, we demonstrated the catalytic activity of bacterial strains in a medium devoid of carbon sources under room-temperature static culture conditions. Through multiple preliminary analyses, we demonstrated the efficacy of bacterial strains in adhering to and degrading refractory substances such as LDPE strips. Additionally, we confirmed their degrading efficiency via weight loss, FTIR, SEM, AFM, WCA measurement, tensile testing, and GC-MS analysis. Furthermore, we utilised the most potent bacterial strains isolated from plastic landfill spots to formulate an effective bacterial consortium in lab conditions. Finally, we proved the efficacy of strains in garden soil mixed with LDPE strips to degrade the polymer pieces. This method demonstrated the eco-biotechnological utilisation of Indigenous bacteria to mitigate the dreadful effects of white pollution.

Further, this detailed investigation proposes the following recommendations to extend our research to eradicate LDPE accumulation around the globe.

- **Genomic and Enzymatic Profiling:**

Future studies should focus on whole-genome sequencing and bioinformatics analysis of the most efficient bacterial strains to identify key genes and enzymes responsible for LDPE degradation. This can help understand the metabolic pathways

and aid in potential genetic engineering for enhanced degradation.

- **Enzyme Isolation and Characterisation:**

Isolate and purify LDPE-degrading enzymes from the effective strains. Characterising these enzymes under different environmental conditions can facilitate their direct application in waste treatment or industrial processes.

- **Scale-up and Pilot Studies:**

Implement pilot-scale biodegradation units using the selected strains or consortia in landfill-mimicking conditions to evaluate their practical applicability and scalability in real-world plastic waste management systems.

- **Bioreactor Design and Optimisation:**

Design bioreactors tailored for continuous or batch LDPE degradation, integrating optimal parameters such as temperature, pH, moisture, and aeration for maximum microbial activity.

- **Eco-Toxicological Assessment:**

Assess the environmental impact of biodegradation by-products to ensure that the process is eco-friendly and does not lead to secondary pollution.

- **Integration with Waste Management Systems:**

Collaborate with municipal waste management authorities to integrate microbial degradation with existing plastic recycling or disposal systems, especially in regions with high LDPE waste accumulation.

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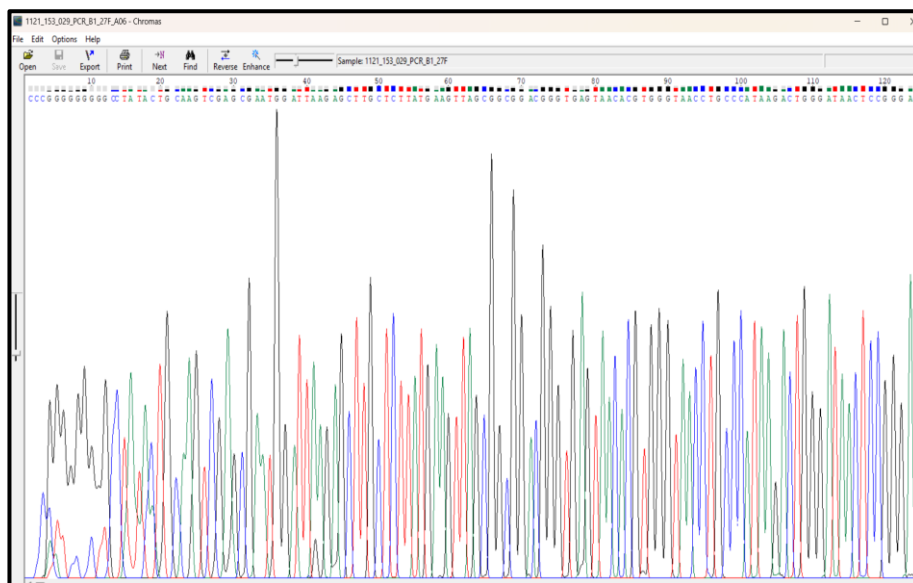
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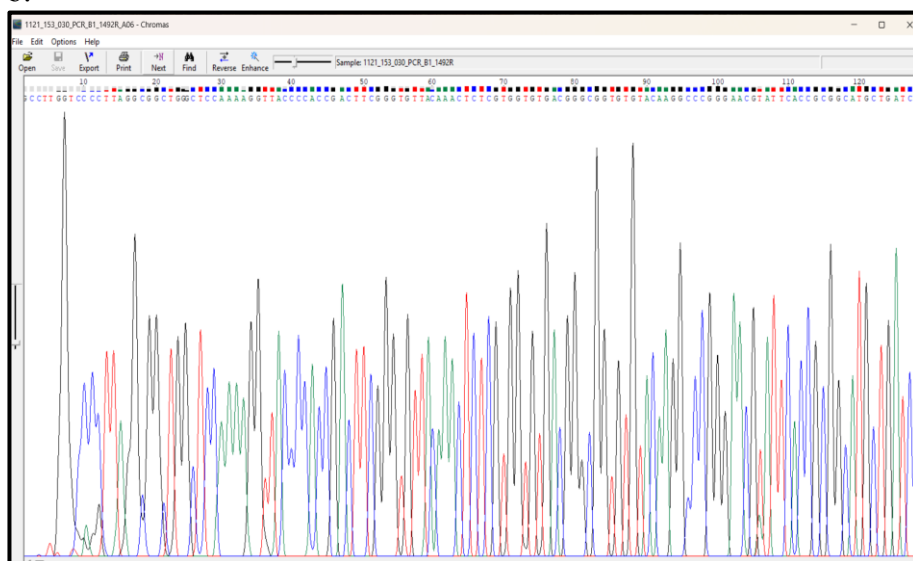
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APPENDICES

a.

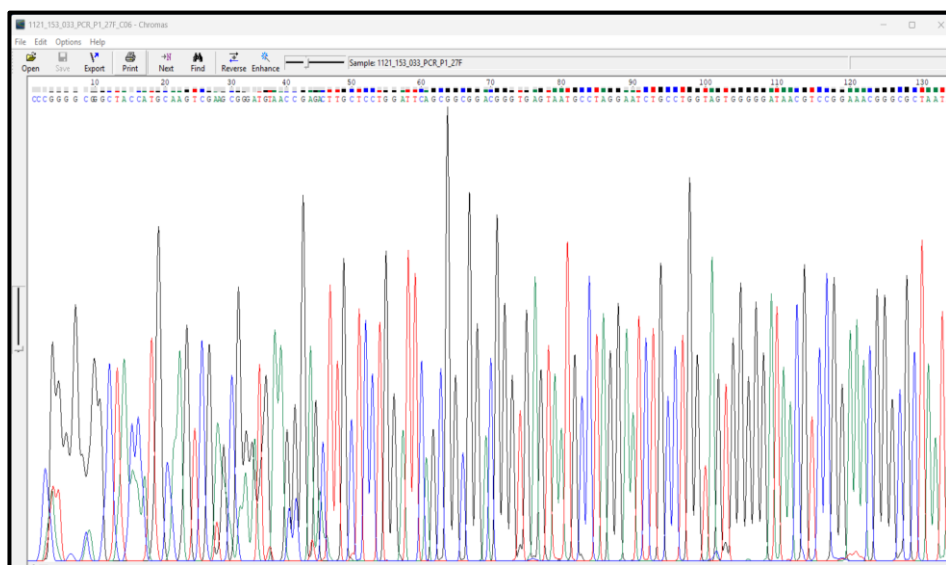


b.

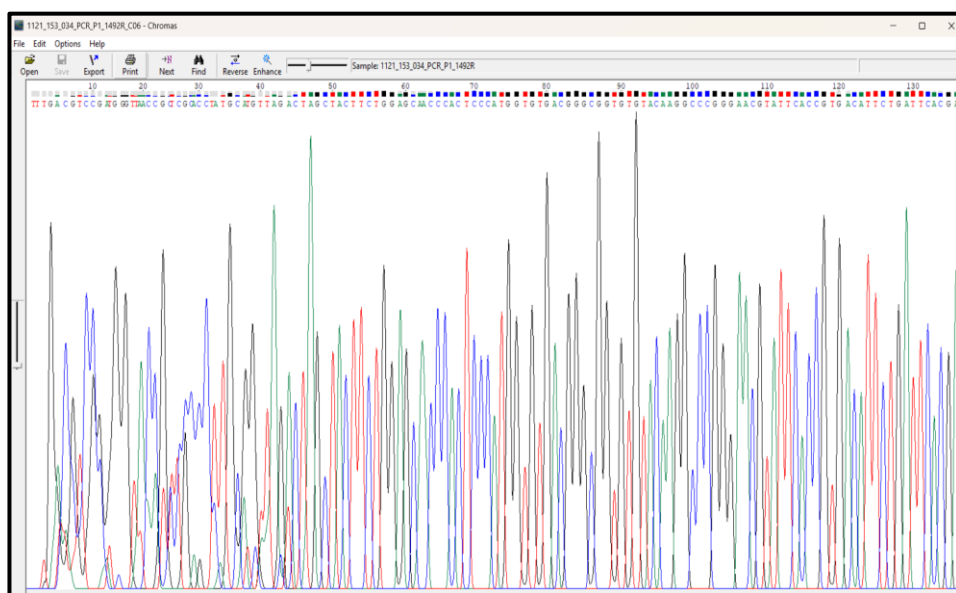


16S rDNA sequence chromatogram of *Bacillus cereus* NJD1. a. Forward sequence b. Reverse sequence.

a.

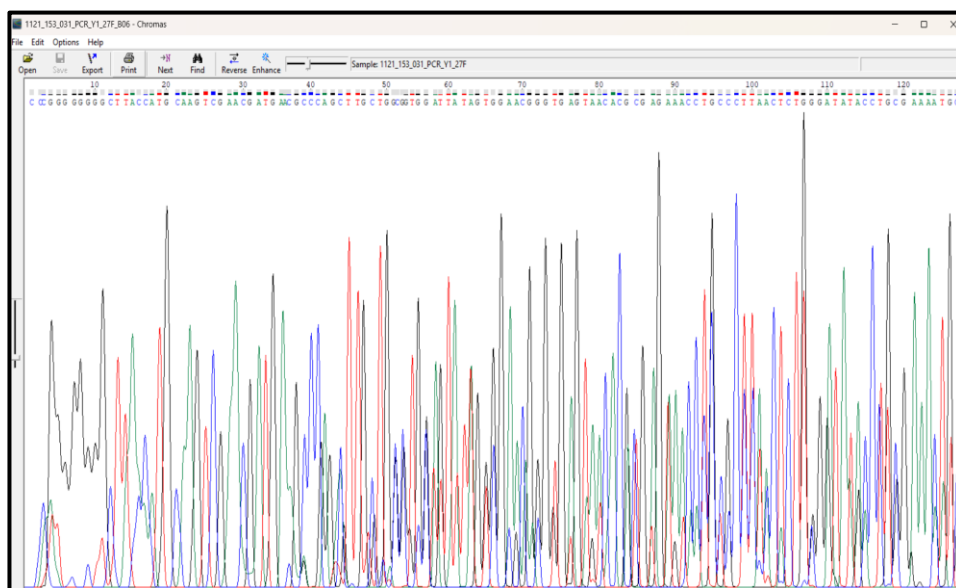


b.

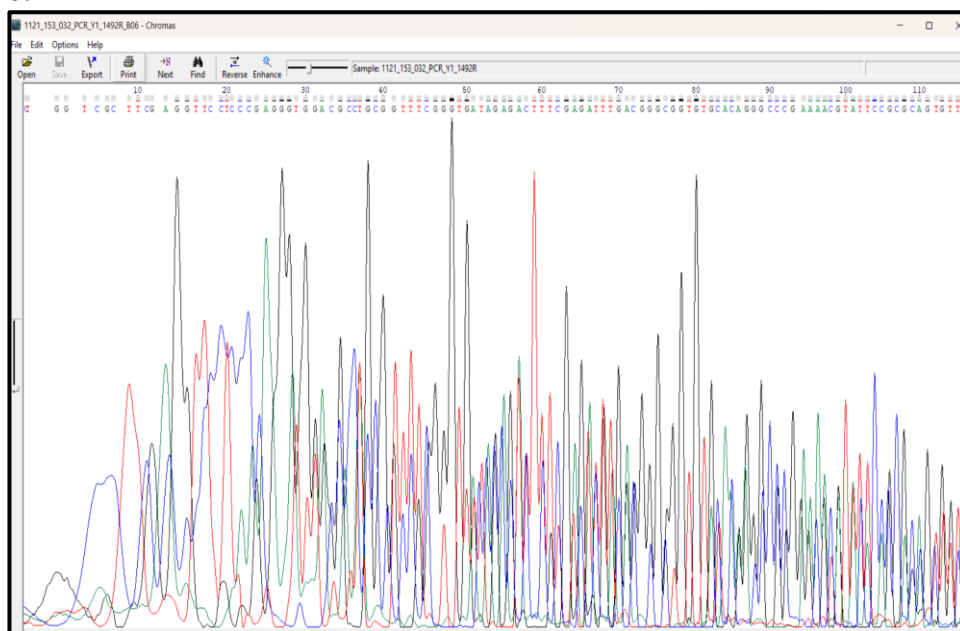


16S rDNA sequence chromatogram of *Pseudomonas aeruginosa* NJD2. a. Forward sequence b. Reverse sequence.

a.



b.



16S rDNA sequence chromatogram of *Micrococcus luteus* NJD3. a. Forward sequence b. Reverse sequence.

DETAILS OF PUBLICATIONS

Research Paper

1. Nithya Jayan, Sinosh Skariyachan, Denoj Sebastian. The escalated potential of the novel isolate *Bacillus cereus* NJD1 for effective biodegradation of LDPE films without pre-treatment. *Journal of Hazardous Materials*. <https://doi.org/10.1016/j.jhazmat.2023.131623>.

Oral Presentation

1. Nithya Jayan, Dr.Denoj Sebastian. 'Microbial biodegradation of LDPE film using *Bacillus* sp.' in the International seminar on Global warming vs Alternative Development strategies on 5th and 6th May, 2022, organised by Fathima Arts and Science College, Moothedam, Nilambur, Kerala, in collaboration with ARIO (Association of International Researchers of Indian Origin).
2. Nithya Jayan, Dr.Denoj Sebastian. 'Mysterious ability of fungal species to degrade low-density polyethylene bags': Two-days National seminar on 'Recent trends in Microbiology' conducted by the Department of Life Sciences, University of Calicut on 26-27th September 2017.
3. Nithya Jayan, Dr.Denoj Sebastian. 'Can a microbe eat plastic?': National seminar on Go Green with Microbes held on 11th and 12th October 2017, at Govt Arts and Science College, Kozhijampara, Palakkad, organised by the Department of Microbiology.

Best oral presentation award

1. Nithya Jayan is conferred the best oral presentation award for the paper entitled 'Mysterious ability of fungal species to degrade low-density polyethylene bags' presented at the Two-days National seminar on 'Recent trends in Microbiology' conducted by the Department of Life Sciences, University of Calicut on 26-27th September 2017