RESEARCH ARTICLE

Sustainable Biodegradation of Recalcitrant HDPE Plastics using *Bacillus pseudomycoides*: A Microbial Approach towards Plastic Waste Mitigation

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ABSTRACT: High Density Polyethylene (HDPE) plastic is one of the most resistant manmade polymers, posing significant environmental challenges. In this study, microbes capable of growing on this recalcitrant plastic were isolated from soil samples collected from plastic contaminated areas. The isolated *Bacillus pseudomycoides* utilized High-Density Polyethylene (HDPE) of 75 microns as the sole carbon source under controlled conditions (neutral pH; temperature: 30 - 37°C) for growth. Biosurfactant production by *Bacillus pseudomycoides* was confirmed, and its cell surface hydrophobicity was measured via BATH assay. The efficacy of biodegradation was assessed by Gravimetry, SEM, FTIR and GC-MS. Gravimetric analysis showed a significant weight loss of 31% in the HDPE plastics after one year by *Bacillus pseudomycoides* when compared to the control in similar conditions without any microbes. Scanning Electron Micrographs showed bacterial adherence and fissures on HDPE surface, indicating degradation. FTIR showed major changes in polymer structure, such as appearance of hydroxyl (-OH), carbonyl (C=O), and alkane/alkene groups and possible synthesis of carboxylic acids, in consistence with microbial fermentation. GC MS analysis showed the presence of diverse HDPE degradation products. Control samples indicated no significant changes, ruling out any abiotic factor influence. These results demonstrate *Bacillus pseudomycoides* has the ability to degrade HDPE plastics.

KEYWORDS: Biodegradation, FTIR, GC-MS, PCR, Plant growth study, Sequencing

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1. INTRODUCTION

Plastic products have gained popularity globally due to their practicality, durability, versatility and low-cost. The global annual production of plastics is greater than 400 million tons [1]. A greater part of this plastic is accumulated as waste in environments, mainly due to the same unique properties. Global plastic waste generation is estimated to be about 360 million tons per year [2]. These plastics fragment into micro- and nanoplastics and contaminate the environment and food chain [3].

Of all the plastics used, HDPE plastic is one of the most recalcitrant types of plastics, known for its resistance to degradation. It is a crystalline, linear thermoplastic made from ethylene monomers with high density, great strength, and hydrophobicity and lacks functional groups, which makes it resistant to degradation. Due to its durability, it is being used in various fields like packaging, piping, films etc. However, its chemical inertness and resistance to degradation-two qualities that make it valuable - have also contributed to environmental pollution. Conventional methods of plastic waste disposal like landfilling, incineration and mechanical recycling pose major health and

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environmental risks [4] [5]. Hence, there exists a persistent demand for sustainable waste management procedures for HDPE wastes.

Microbial biodegradation has become a viable and environment friendly method to address plastic contamination [6]. Several bacterial and fungal species have been identified with the ability to degrade different varieties of plastics. Bacteria such as *Ideonella*, *Pseudomonas*, *Bacillus* and *Rhodococcus* are widely recognized to degrade plastics by producing degradative enzymes. Fungi such as *Aspergillus, Penicillium*, *Zygomycota* and *Fusarium* species and even algae have been found to degrade plastics [7]. Microbes metabolize carbon polymers in plastics, thereby yielding non-toxic byproducts and provide energy or convert polymers into other beneficial compounds [8]. They adhere to the plastic surface forming biofilms, secreting extracellular enzymes or generating reactive species that initiate polymer-chain oxidation and fragmentation, thereby enabling microbial assimilation of smaller oligomers. The key enzymes secreted by microbes are esterases, hydrolases, oxidases and laccases [9]. Biosurfactants produced by plastic degrading bacteria facilitate their surface colonization on plastics [10]. The enzymatic activity and biosurfactant production together enhance the bioavailability of the substrates for the adhered microbes.

Microbial degradation can serve as an ecofriendly and cost-effective solution for HDPE waste management too. Fungi such as *Aspergillus fumigatus*, *A. flavus*, and *Fusarium sp*. have been reported to have the potential to degrade HDPE [11]. Previous studies have identified bacteria such as *Staphylococcus spp*., *Pseudomonas spp*. and *Bacillus cereus*, *B. megaterium*, *B. parapsilosis*, *B. velezensis* and *Brevibacillus parabrevis* capable of HDPE degradation. These strains have been isolated from diverse habitats including landfill soils, agricultural land, marine environments, and even bovine feces. These environments exert selective pressure for microbes to evolve the ability to utilize the available waste materials as carbon sources, which may be effective against HDPE. In addition, landfill-derived consortia and mixed cultures combining *Bacillus* and *Pseudomonas species* have been documented in these studies [12] [13] [14]. Therefore, identification of microbes, which are able to degrade HDPE, is an important step towards environmental sustainability and may help us to tackle the emerging problem of HDPE wastes.

Members of the genus *Bacillus* are noted for their metabolic versatility and ability to degrade hydrophobic organic compounds, particularly petroleum hydrocarbons [15]. Their ability to form stress-resilient endospores makes them especially suitable for survival and metabolic activity in harsh municipal solid waste dumps, where long-term exposure to plastics likely evolves microbes capable of initiating biodegradation processes. Hence, the present study is hypothesized to collect soil samples from plastic dumped municipal wastes that may serve as a good source of HDPE degrading bacterial species, especially *Bacillus sp*. This research is aimed at isolating bacterial strain(s), particularly from the genus *Bacillus species*, capable of degrading HDPE (75 microns thickness). Further, it intends to determine the efficacy of bio-degradation by various analytical techniques that include Growth curve, weight loss determination, Scanning Electron Microscopy (SEM), Fourier-Transform Infrared Spectroscopy (FTIR), and Gas Chromatography-Mass Spectrometry (GC-MS), which will enhance the clarity of the findings. This approach is innovative in identifying HDPE-degrading *Bacillus*, linking biosurfactant production and cell surface hydrophobicity and validating degradation through a comprehensive suite of techniques (Gravimetry, SEM, FTIR, GC-MS).

2.0 MATERIALS AND METHODS

2.1 Soil Sample Collection and Physicochemical Characterization

Sample collection was carried out by using a convenience sampling approach from plastic waste dumped municipal waste dumping sites, in Pudussery Panchayat, Palakkad District, Kerala, India (Lat 10.767448° and Long 76.68853°). This site was chosen, since the tropical climate of the region may promote microbial diversity and metabolism and may contain indigenous microbial communities that foster plastic-adapted microorganisms. The sites contained mixed solid waste, enriched with HDPE plastics. Three points rich in plastic debris were selected. Soil samples were collected in sterile containers in triplicates from a depth of 5–15 cm, to minimize surface contamination. All samples were aseptically transported and processed within 24 hours to preserve microbial viability [16].

Soil samples were analyzed for physicochemical properties to assess the impact of different environmental factors on the soil microbial diversity. Assessed parameters included pH, electrical conductivity, organic carbon, total nitrogen, essential macronutrients like calcium, potassium, and phosphorus, micronutrients such as copper and zinc. Different techniques followed for the measurements are listed in Table 1.

Table 1: Methods followed for the Physicochemical Characterization of the collected soil samples

S. No	Parameters	Method	References
1.	pH	Electrometry	[17]
2.	Conductivity	Conductometry	[18]
3.	Carbon	Elemental Analysis	[19]
4.	Nitrogen	Kjeldahl Method	[20]
5.	Carbon Nitrogen Ratio	Calculation	
6.	Phosphorous	Olsen Method	[21]
7.	Potassium	Flame Photometry	[22]
8.	Calcium	Atomic Absorption Spectroscopy	[23]
9.	Zinc		[24]
10.	Copper		[25]

2.2 Isolation of HDPE Plastics Degrading Bacteria

Bacterial strains were isolated using the spread plate and serial dilution techniques from the soil samples. Morphologically distinct colonies were sub-cultured to produce pure cultures that were grown in Minimal Salt Medium. Commercially available HDPE carry bags with 75-micron thickness were used in the study. HDPE were cut (3cm x 3cm), washed with ethanol and cleaned with distilled water. No pretreatment with heat or UV was carried out. To identify bacterial strains capable of degrading HDPE plastics, pure cultures were grown in minimal salt medium containing pre-weighed pieces of HDPE as sole carbon source in triplicates. The cultures were incubated at 37°C for a period of 45 days. Negative control (in triplicates) was maintained in the same combination without microbial inoculation to adjust for HDPE degradation from abiotic factors. The OD readings were measured at 600 nm at 5-day intervals and the growth curve was plotted [26]. Bacterial strains with good growth were further assessed for long term (365 days) as mentioned in section 2.7

2.3 Biochemical and Morphological characterization of the bacterial isolates

Gram's staining was employed to identify the cell wall morphology of the bacterial isolates. *Staphylococcus aureus* and *Escherichia coli were used* as reference controls for Gram positive and Gram-negative organisms respectively. Motility was assessed using semi-solid motility agar (0.3–0.4% agar) by stab inoculation and incubation at 37°C for 24–48 h; diffuse growth away from the stab line indicated motility, while growth restricted to the stab line indicated non-motility. *E. coli* (motile) and *Klebsiella pneumoniae* (non-motile) served as controls [27].

The bacterial isolates were grown in substrate specific media such as Starch agar and Casein agar to screen for extracellular enzyme production. *Bacillus* Isolation Agar was used to identify *Bacillus* species, which are known for the production of enzymes involved in polymer degradation [28]. Biochemical characterization was carried out using IMViC tests, catalase test, and hydrolysis assays following standard protocols [29] [30]. The Indole test was considered positive upon formation of a red ring after addition of Kovac's reagent, indicating tryptophan breakdown. Positive Methyl Red test gave a stable red color developed after adding methyl red indicator primarily due to acid fermentation. Voges–Proskauer test showed positivity by the appearance of pink to red color after addition of α-naphthol and potassium hydroxide showing acetoin production. Citrate utilization was confirmed with a color change from green to blue on Simmons citrate agar. Catalase activity was indicated by immediate bubble formation after adding 3% hydrogen peroxide. Hydrolytic enzyme production was determined by clear halo formation on starch agar after iodine flooding (amylase activity), clear zones on skim milk agar (casein hydrolysis), and liquefaction of gelatin after refrigeration (gelatinase activity) [31].

2.4 Oil Spreading Assay

Oil Spreading Assay is a simple and rapid qualitative method used to detect biosurfactant production by microorganisms. In this method, surface tension is decreased using biosurfactants and oil is transferred on to a water surface, which forms a clear zone known as the oil movement diameter. It is extensively useful for identification of bacteria involved in degradation of hydrocarbons and plastics in environmental microbiology. When a drop of culture supernatant containing biosurfactant is added onto an oil layer, the biosurfactant pushes the oil aside, and the size of the resulting clear zone is proportional to its concentration. In the present study, 10 µL of culture supernatant was added to the center of the oil layer, and the diameter of the clear zone was observed and measured in millimeters using a scale [32]. Rhamnolipid and distilled water were used as positive and negative controls respectively.

2.5 Evaluation of Cell Surface Hydrophobicity

The BATH (Bacterial Adhesion to Hydrocarbons) Assay quantifies cell-surface hydrophobicity by calculating the percentage of bacterial cells that partition into a hydrocarbon phase, consequently lowering the optical density of the aqueous phase. Higher hydrophobicity is seen in stronger bacterial adherence to hydrophobic polymers in plastic-degradation and has a higher potential for plastic biodegradation. In this procedure, bacterial cells reaching the mid log phase were collected, cleaned and suspended in PBS (Phosphate-buffered saline) to a specific optical density (A_0) at 600 nm. In to a glass tube containing 4 mL of this suspension, 0.5 mL of hydrocarbon (Hexadecane) was added and vortexed vigorously for 2 minutes. Allowed phase separation for 15 to 30 minutes. Aqueous phase was carefully removed and the final OD (A_1) is measured. The formula $[(A_0 - A_1)/A_0]$ is used to calculate Cell hydrophobicity [33].

2.6 Molecular identification of HDPE degrading bacteria

One bacterial isolate with maximum growth in HDPE media was identified for advanced research. The conventional phenol–chloroform technique was used to isolate genomic DNA [34], 16S rRNA genes were amplified by means of the universal oligonucleotide primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The purification and categorization of PCR product was done. The EzBio-Cloud 16S database was used to analyze the similarity with the nearest species. The Jones-Taylor-Thornston matrix-based model and MEGA 11 software were used in the construction of phylogenetic tree. Using the Banklt protocol, sequences were uploaded to the GenBank database of the National Centre for Biotechnology Information with the accession number OR388540.

2.7 In vitro biodegradation assay

The ability of microorganisms to degrade synthetic polymers under controlled laboratory conditions is evaluated using an *in vitro* biodegradation assay**.** HDPE plastic were cut (3 × 3 cm), cleaned with ethanol, rinsed and dried at 50–60°C. Initial weights (W_0) were recorded. Mid-log phase cultures were inoculated into sterile minimal salt medium (MSM), and the optical density was adjusted to 0.5 at 600 nm Each flask received one pre-weighed HDPE piece and the microbial inoculum (1–5% v/v), along with appropriate controls: an abiotic control (MSM + HDPE) and a biotic control (MSM + inoculum). Flasks are incubated at 37°C for a period of 365 days. At fixed interval time points, triplicates were retrieved, the HDPE pieces were recovered, washed to eliminate biofilm, dried out, and their final weight (Wt) was documented to assess the degree of biodegradation through weight loss [35].

2.8 Determination of Total viable Count

In plastic-biodegradation studies, Standard Plate Count or, Total Viable Count (TVC), is used to calculate feasible microorganisms settling on polymer surfaces. This is estimated by CFU per HDPE piece or per unit area. From the *in vitro* bio degradation assays, HDPE pieces were removed, microbes attached were dispersed into PBS. Serial tenfold dilutions were prepared. 0.1 mL of suitable dilutions were inoculated onto nutrient agar, in triplicates. Plates were incubated at 37°C for 24–72 hours, after which colonies were counted. TVC was calculated using CFU = (mean colony count × dilution factor) / volume plated, and were expressed per HDPE piece or per cm^2 of plastic surface. Controls, such as blanks and inoculum controls, were included for authentication [36].

2.9 Measurement of Weight Loss

The HDPE degradation by the selected bacterial isolate was quantified using measurement of loss

in weight [37]. The weight loss percentage was calculated by the formula:

$$\text{Weight Loss} = \frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}} \times 100$$

2.10 Characterization of HDPE Plastics (Before and after biodegradation Assay)

Several methods were applied to assess the plastic samples from the culture after 12 months of *in vitro* biodegradation.

2.10.1 Scanning Electron Microscopy (SEM)

The plastic samples were retrieved from the medium and examined to evaluate bacterial colonization and surface morphology using FE-SEM. The treated plastic samples were cleaned using Phosphate Buffered Saline (0.01M) to remove adhering medium and loosely attached bacterial cells. The cleaned HDPE pieces were then fixed for 120 minutes at 4 °C in a 4% glutaraldehyde solution, dehydrated for 30 minutes in 50% ethanol, dried, and layered with a thin film of gold (~10 nm) by means of a sputter coater for 30 seconds to enhance conductivity. Imaging was carried out at an accelerating voltage of 10 kV using a scanning electron microscope. High magnifications ranging from 500X to 10,000X were used to record the micrographs. Assessment of the degradation was done by comparing the surface of the treated samples with that of the untreated controls, focusing on the presence of pits, cracks, erosion patterns, fissures and increased surface roughness [38].

2.10.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is used to identify chemical and functional group changes in polymers—such as oxidation, hydroxylation or carbonyl formation, during biodegradation. HDPE pieces were retrieved from the biodegradation flasks, gently rinsed with sterile distilled water to remove loosely attached biomass, and dried at 50–60°C, cooled in a desiccator. Chemical bond modifications in the HDPE samples were examined using a Shimadzu FTIR-8200 spectrometer. Spectra were verified at a resolution of 4 cm^{-1} over the range of 400–5000 cm^{-1} using 32 scans for every sample. The changes in the functional groups such as appearance, disappearance and intensity variations of the characteristic peaks were identified by comparing the treated sample with the spectra of control HDPE [39].

2.10.3 GC-MS Analysis

After the completion of *in vitro* biodegradation assay, the HDPE pieces were removed and the culture media was centrifuged. The supernatant was stored at -20°C, whereas the pellet was removed. After thawing, the samples were acidified to pH 2.0 with 1 N HCl and allowed to rest for 2 hours. Thrice the quantity of ethyl acetate was used to extract the sample; the combined extracts were first dried using anhydrous sodium sulfate and then using rotary evaporation, it was condensed. GC–MS analysis was performed using an Agilent 7890B GC coupled with a 5977A MSD equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The program is designed to elute both lower molecular weight compounds and long chain fatty acids and other alkanes. The initial temperature is 50 °C, (hold for 2 min) to capture volatile fragments. Ramp 1 temperature is 10°C/min to 150°C (hold for 0 min). Ramp 2: 7°C/min to 300°C (hold for 10–15 min) to elute high-retention-time components. The Injector Temp is 280 °C in splitless mode (for trace degradation products). Ionization Mode: Electron Ionization (EI) at 70 eV. The compounds were identified by comparison with the NIST mass spectral library, and matches with similarity indices ≥85% were considered significant. The retention times were compared with that of the control samples to confirm degradation-specific peaks [40] [41].

2.11 Statistical analysis

All the quantitative assays were performed in triplicates and data are presented as Mean ± Standard Deviation. Comparisons were carried out with the paired two-tailed Student's t test performed using Graph Pad Prism software. Statistical significance is reported at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Physicochemical Characteristics of Soil Samples collected from plastic waste dumped municipal wastes

Majority of putative bacteria capable of biodegrading plastics were isolated from polluted locations, like landfills [42]. Hence, in the present study too, landfill sites were chosen for identifying bacteria capable of degrading

HDPE. Soil pH, conductivity and micronutrients are critical for the health and survival of the microbiome. Hence, they were assessed in the collected samples (Table 2). The composition of soil microbiomes is significantly influenced by pH [43]. All the soil samples had a slightly alkaline pH. This may be helpful in the growth of alkaliphilic bacteria such as *Bacillus sp.*, while suppressing the growth of Fungi and acidophiles.

Table 2: Physicochemical characteristics of the collected soil samples

S. No	Parameters	S1	S2	S3
1	pH	7.6 ± 0.02	7.9 ± 0.015	7.8 ± 0.02
2	Conductivity (µs/cm)	2 ± 0.10	4 ± 0.45	2 ± 0.50
3	Carbon (%)	6.6 ± 0.20	7.3 ± 0.06	6.7 ± 0.10
4	Nitrogen (%)	1.6 ± 0.15	1.5 ± 0.01	1.5 ± 0.03
5	Carbon Nitrogen Ratio	4.1 ± 0.4	4.8 ± 0.1	4.6 ± 0.3
6	Phosphorous (mg/kg)	1842 ± 5.5	1845 ± 0.6	1832 ± 1.7
7	Potassium (mg/kg)	1476 ± 5	1397 ± 2	1349 ± 2
8	Calcium (mg/kg)	246 ± 13	300 ± 1	274 ± 3
9	Zinc (%)	11.4 ± 0.25	11.9 ± 0.05	11.3 ± 0.06
10	Copper (mg/kg)	190 ± 4.0	194 ± 2.0	193 ± 0.6

*** The values represent mean ± SD of triplicates**

All the soil samples exhibited a conductivity < 5 µs/cm indicating they are non-saline, healthy soils optimal for diverse microbial growth [44]. All the three soil samples had high carbon levels. Carbon levels >5 % often boost microbial biomass and diversity, providing abundant energy and food supplies for bacteria and fungi. When balanced with other nutrients, these carbon levels promote robust microbial communities and increased soil biological activity [45]. In tune with the carbon levels, Nitrogen levels were also higher than typical soils. At 1.5 – 1.6 %, nitrogen encourages strong microbial development and activity [46]. S2 and S3 had better carbon: nitrogen ratio compared to S1. The ideal C:N ratio of soil organic matter range from 10:1 to 24:1, with 10:1 ideal for microbial decomposition, and 24:1 being ideal for balanced microbial activity [47]. Samples in the present study had lower Carbon levels in comparison.

Extremely high Phosphorus contents of around1800 mg/kg are observed in all the three samples. This may be due to the landfill nature, may be due to the dumping of waste materials. These levels are far higher than the normal P ranges (~10–40 mg/kg). High P has been shown to increase microbial growth, diversity and enzymatic activities [48]. Potassium levels were also high (>1300 mg/kg), compared to the usual ranges of 100 to 250 mg/kg. Osmoregulation, microbial growth support, and microbial enzyme activation all depend on potassium [49]. Such elevated levels of Potassium point to nutrient-rich soils that are ideal for microbial growth. Calcium content of 250–300 mg/kg, observed in all the samples, is considered moderate. It indirectly supports microbial growth and activity by stabilizing soil structure and buffering pH [48]. Zn levels of about 11% are significantly higher than normal soil Zn levels of 1–50 mg/kg or 0.001–0.005%. Typically, soil containing 10–50 mg/kg of zinc promotes microbial development [50]. Such high percentages could be a sign of organic contamination. Similarly, normal copper values in soils generally range from about 2–109 mg/kg, with most agricultural soils falling below 50 mg/kg [51]. The observed copper levels in the present soil samples in range of 190–194 mg/kg are significantly higher than natural background and recommended limits. The landfill characteristics of the soil sources may have resulted in the high levels of

micronutrients. Protracted and uncontrolled waste deposition might have caused the excessive P, K, Zn, and Cu concentrations in landfill soils. However, due to developed tolerance mechanisms, specialized microbial communities continue to exist in toxic landfill sites, supporting essential soil activities and demonstrating the possibility of both natural and engineered bioremediation [52]. Hence, we tried to isolate microbes from these samples.

3.2 Isolation of Soil Bacteria, morphological and biochemical characterization

From the three samples of soil used, 26 bacterial strains were isolated in total, (10 from sample I, 8 each from samples II & III) and screened for hydrolytic enzyme production using Casein agar, Starch agar, and *Bacillus* Isolation Agar. The bacterial isolates were inoculated in selective media. Bacteria capable of producing amylase were identified by Starch agar medium [28]. Isolates 4 and 5 were found to produce amylase. Isolates 1 and 5 produced clear zones on Casein agar medium indicating the production of caseinase enzyme. Caseinase enzyme does not directly degrade plastics, but its proteolytic process may help in the process of biodegradation. All the five isolates produced colonies of varying morphology in *Bacillus* Isolation Agar medium. This growth suggests that the genus *Bacillus*, producing hydrolytic and oxidative enzymes were capable of plastic biodegradation and they survive even in harsh environments through the formation of spores [53]. These results highlighted the potential of the bacterial isolates for extracellular enzyme secretion and plastic biodegradation. Consequently, for further biochemical and morphological categorization, these five isolates were chosen. The biochemical and morphological characteristics of the selected isolates are specified in Table 3.

Table 3: Morphological and Biochemical Characterization of Bacteria Isolated from the collected soil sample

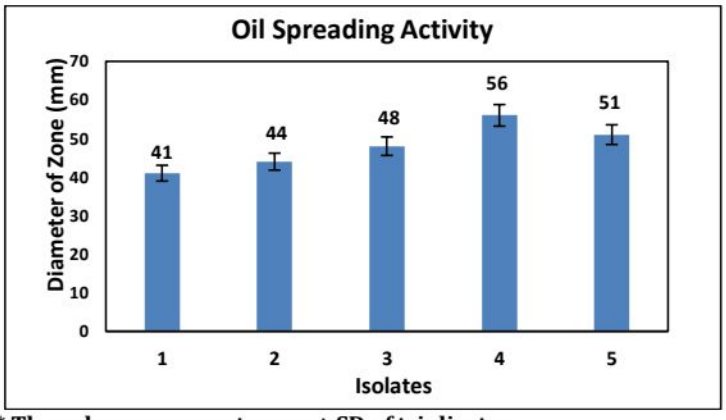
Characteristics	Isolates				
	1	2	3	4	5
Colony	Cream	Cream	White	White	Cream
Form	Circular	Circular	Round	Rhizoidal	Circular
Margin	Entire	Entire	Entire	Hair like	Entire
Colour	White	White	White	White	Transparent
Elevation	Raised	Raised	Convex	Raised	Raised
Morphology	Rods	Rods	Rods	Rods	Rods
Gram Staining	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Rod
Endospore	+	+	+	+	+
Motility	+	+	+	+	+
Biochemical					
Indole	+	+	+	–	+
MR	+	+	+	–	–
VP	+	+	+	+	+
Citrate	+	+	+	+	+
Catalase	+	+	+	+	+
Starch	+	+	+	+	+
Casein	+	+	+	+	+
Gelatin	+	+	+	+	+
Motility	+	+	+	–	+
Casein hydrolysis	+++	+	++	+	+++
Starch hydrolysis	+	+	+	+++	+++

3.3 Production of Biosurfactant of Bacterial Isolates

The surface-active compounds produced by bacteria are Biosurfactants. They are amphiphilic compounds, which lower surface and interconnected tension among solids, liquids, and gases. They include both hydrophilic and hydrophobic moieties. Bacteria secrete biosurfactants such as glycolipids, lipopeptides, phospholipds, and other derivatives on the cell

surface or secrete extracellularly [54]. These biosurfactants reduce surface tension, promoting biofilm growth, increase enzyme activity and change surface morphology contributing to plastic biodegradation [55]. Combination of enzymatic and surfactant activities from the microbes could improve the rate of HDPE degradation.

Oil-spreading test was used to assess the biosurfactant production. Of all the five isolates, isolate 4 showed excellent biosurfactant activity with an oil-spreading diameter of 56 mm (Figure 1). In a study from coastal soils, a *Bacillus simplex* strain was identified producing a novel biosurfactant with an oil-spreading diameter of 54 mm [56]. Isolate 4 demonstrated biosurfactant productions on par with the *B simplex strain*. In addition, Bacterial strains exhibiting oil-spreading diameter above 35 mm are recognized as good biosurfactant producers [57]. Therefore, all the five isolates in the present study demonstrates a promising biosurfactant production potential, which enhance their biodegradation efficacy.

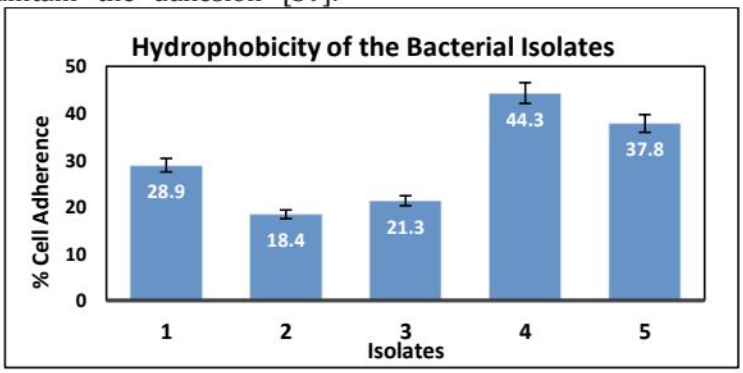


* **The values represent mean ± SD of triplicates**

Figure 1: Oil spreading activity of bacterial isolates

3.4 *Cell Surface Hydrophobicity of Bacterial Isolates*

The extent of biofilm formation on polymeric surfaces like HDPE is determined by the intricate interplay between hydrophobic or hydrophilic properties of the surface of bacterial cell and the substrates [58]. Adherence of bacteria to surfaces is sustained largely by the cell surface hydrophobicity (CSH). The bacteria need to be hydrophobic to maintain the adhesion [59]. Therefore, CSH was assessed by the BATH assay. Out of the five isolates, Isolate 4 showed the highest degree of cell surface hydrophobicity of 44.3% and better cell adhesion, which may lead to effective degradation of HDPE (Figure 2). This property of Cell Surface Hydrophobicity may promote initial adhesion and persistent colonization on hydrophobic HDPE surfaces. Earlier studies on *Pseudomonas* also showed comparable results [60].



* **The values represent the mean ± SD of triplicates**

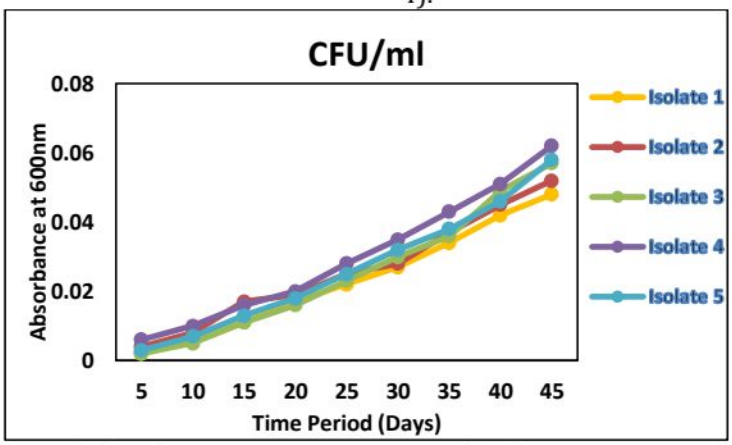
Figure 2: Hydrophobicity of the bacterial isolates

3.5 HDPE degradation and Molecular Identification of HDPE degrading bacteria

The growth curve of the five isolates on HDPE plastics was determined using the optical density at 600 nm for every 5 days up to 45 days (Figure 3).

The sole carbon source in the medium was HDPE. All the Isolates were observed to multiply over the time period. However, isolate 4 showed highest growth when compared to the other isolates. This may be due to the high biosurfactant production evidenced in Isolate 4. All the other isolates also showed comparable growth in the HDPE medium in tune with their biosurfactant production.

From all the above findings, isolate 4 was found to be effective and had a greater degree of degradative ability when compared with other Isolates. Hence, for further analysis, isolate 4 was selected. 16srRNA gene sequencing and phylogenetic analysis of Isolate 4 revealed *Bacillus pseudomycoides*. Phylogenetic analysis of bacterial strains was performed using MEGA 11 software suite. The phylogenetic tree and evolutionary relationships of the strain Isolate 4, was constructed using the sequence of closely related strain. Isolate 4 was identified as *Bacillus pseudomycoides* by phylogenetic analysis (Figure 4).



*** The values represent mean ± SD of triplicates**

Figure 3: Growth curve of the Bacterial Isolates during 45 days of incubation

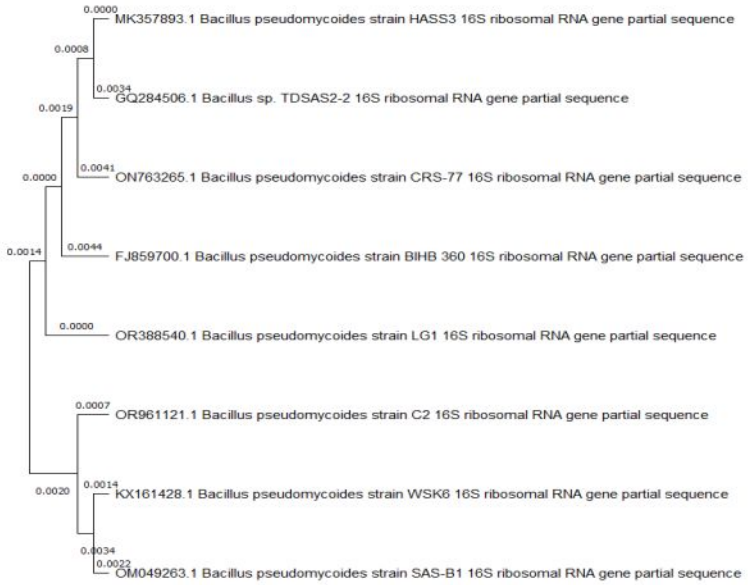


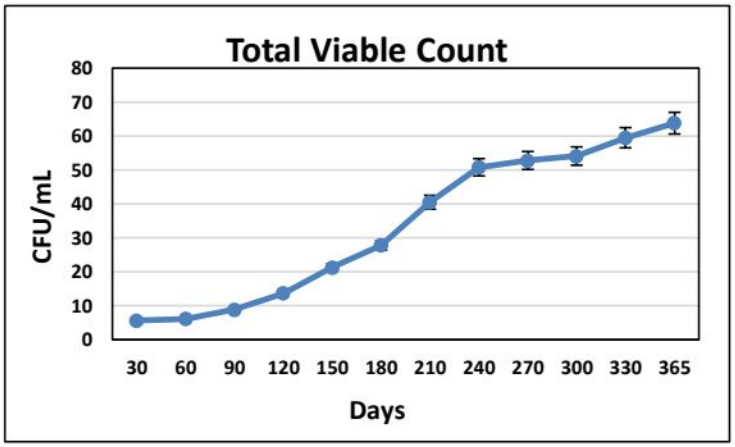
Figure 4: Phylogenetic tree based on 16 S rRNA gene sequences using maximum likelihood method of *Bacillus pseudomycoides* OR388540 and closely related strains

3.6 In vitro biodegradation of HDPE plastic

The isolated *Bacillus pseudomycoides* was cultured in a medium comprising HDPE as the sole carbon source for a period of one year.

3.6.1 Total Viable Count of Bacillus pseudomycoides

The Total Viable Count showed gradual increase over the time (Figure 5). This showed that *Bacillus pseudomycoides* utilized the HDPE plastics as the sole carbon source. After 12 months, *Bacillus pseudomycoides* indicated active metabolism and continued to degrade HDPE. This sustained growth at low but measurable density underscores the strain's adaptation and utilization of HDPE, a polymer widely recognized for its recalcitrance due to high hydrophobicity, crystallinity, and absence of readily hydrolysable groups. In spite of the modest CFU/ml, the ability of the isolated *Bacillus pseudomycoides'* to survive in nutrient-poor and demanding environment for 12-months demonstrates its intrinsic ability to biodegrade HDPE and utilize the products for growth. This ability may be enhanced in complex natural environments in the presence of additional inorganic and organic substrates as seen in the source soil. By shifting metabolic fluxes towards pathways engaged in polymer breakdown, or supplementing with specific cofactors or co-metabolites, and improving medium composition, its ability of HDPE may be further accelerated in real time environments.

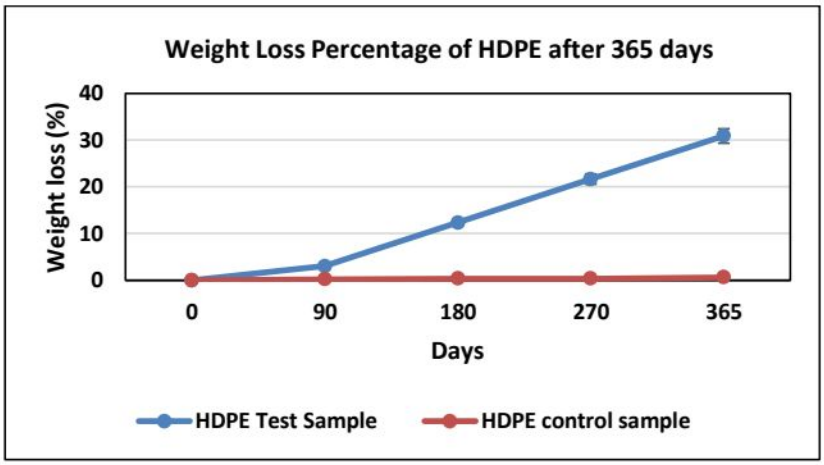


*** The values represent mean ± SD of triplicates**
Figure 5: Total Viable Count

3.6.2 Weight reduction of HDPE plastic sample treated with Bacillus pseudomycoides

The isolated *Bacillus pseudomycoides* was capable of decreasing the weight of HDPE plastic over time, by about 6% loss in the first month. After a period of one year, this extended up to 30.9% (Figure 6), significantly ($p<0.001$) higher than that of negative controls (0.6%). The gradual weight loss over time indicates that the bacterial isolate may both colonize the HDPE surface and start oxidative and enzymatic reactions that break down the polymer chain. This breaks down may release nitrogenous byproducts and low-molecular weight organic molecules into the ecosystem, which may aid in the cycling of nutrients in the soil. Earlier studies have reported modest HDPE degradation abilities by individual strains. For example, 1.78 % decrease in HDPE weight over a period of 30 days by *Bacillus cereus* were reported in a study [14], whereas 3.78 % of weight loss by *Brevibacillus parabrevis* over a period of 40 days was reported by another study [12] - maximum weight reduction achieved by a single species in previous studies. Comparatively, *B. pseudomycoides* isolated in the present study, reduced HDPE weight by 3 % in 90 days. In contrast, mixed consortia of microbes have exhibited higher efficiencies. It was observed, that 17 - 23 % of weight loss was observed by a variety of bacterial sp [67], whereas a consortium of microbes can cause a weight loss of up to 31 %, both over 60 days [5]. *Fusarium sp*. was able to achieve a maximum degradation of 2.65% in 60 days. Notably, in the present study, a single, well-characterized strain capable of substantial HDPE biodegradation, achieving 31% weight loss after one year was identified, thereby surpassing

previously reported efficiencies for individual bacterial isolates and highlighting its promise for bioremediation applications.



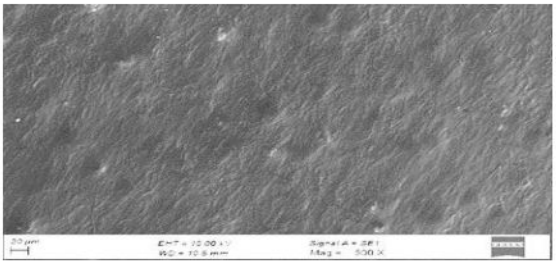
*** The values represent mean ± SD of triplicates**

Figure 6: Weight loss percentage of HDEP after 365 days of incubation

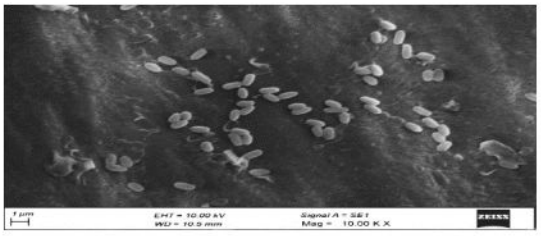
3.7 Characterization of HDPE Plastics before and after biodegradation

3.7.1 Scanning Electron Microscopy (SEM) of Control and Bacillus pseudomycoides treated HDPE Plastic sample

HDPE degraded by *Bacillus pseudomycoides* showed substantial variations in surface morphology after 12 months. Scanning Electron Micrographs (Figure 7) showed bacteria on HDPE surface. In addition, irregularities, pits and fissures along with the clusters of microbes were observed. Previous studies also identified microbial biofilms, which promoted surface erosion and polymer chain cleavage by enzymatic hydrolysis on HDPE [68]. Biofilms decreased polymer hydrophobicity, increased microbial accessibility and augmented biodegradation [69]. The versatility of the *Bacillus species* in degrading HDPE polymers resulted in surface deterioration such as cracks and pits [70]. In a similar way, the results of the present research showed that *Bacillus pseudomycoides* had good adherence to HDPE surfaces that lead to degradation.



7(a): SEM micrograph of Control



7(b): SEM micrograph of Treated sample

Figure 7: SEM micrograph of HDPE plastic treated with *Bacillus pseudomycoides* (a) Control (b) Treated

3.7.2 Fourier Transform Infrared Spectroscopy (FT-IR) of Control and Bacillus pseudomycoides treated HDPE Plastic sample

FT-IR spectroscopy is an excellent analytical tool for identifying molecular changes in polymer degradation [71]. In the present study, FTIR analysis of HDPE after 1-year exposure to *Bacillus pseudomycoides* (Table 4; Figure 8) showed major changes in polymer structure. The transmittance of the O–H stretching band, between 3200–3600 cm^{-1} decreased by 5% over one year, confirming oxidative functionalization of HDPE during microbial degradation [72]. Similar broadening of this band and formation of hydroxyl groups have been reported in other polyethylene degradation studies [73] [74]. The characteristic C=C alkene stretching vibration at 1640 cm^{-1} also showed a reduction in transmittance from 80% to 75%, reflecting increased absorbance and the accumulation of unsaturated bonds in the polymer backbone. This observation is consistent with structural modifications reported in earlier microbial degradation studies [75]. Furthermore, the C–Cl stretching band at 600–700 cm^{-1} increased by approximately 5%, indicating substitution reactions and structural modification of HDPE, in agreement with previous findings [76]. Collectively, these spectral changes establish that *Bacillus pseudomycoides* induces oxidative and substitution reactions in HDPE, leading to significant structural alterations of the polymer backbone. Comparable FTIR shifts—such as elevated carbonyl and hydroxyl intensities—have been documented during LDPE degradation by other Bacillus species, suggesting a similar degradative mechanism is operative in the present study.

Table 4: FT-IR Results

Wavelength	Functional Groups	Intensity		
		0 day	180 days	365 days
~3200 cm^{-1} to 3400 cm^{-1}	O-H	+	+	++
~1640 cm^{-1}	C=C (alkene)	+	+	++
~600 cm^{-1} To 700 cm^{-1}	C-Cl	+++	+++	++

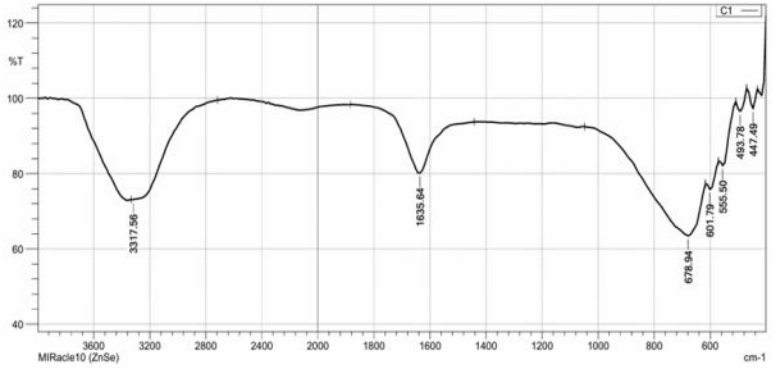


Figure 8a: FT-IR spectra of untreated HDPE sample

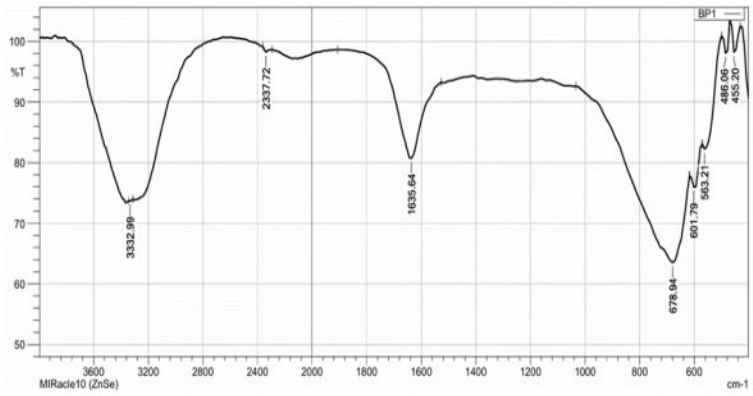


Figure 8b: FT-IR spectra after 180 days

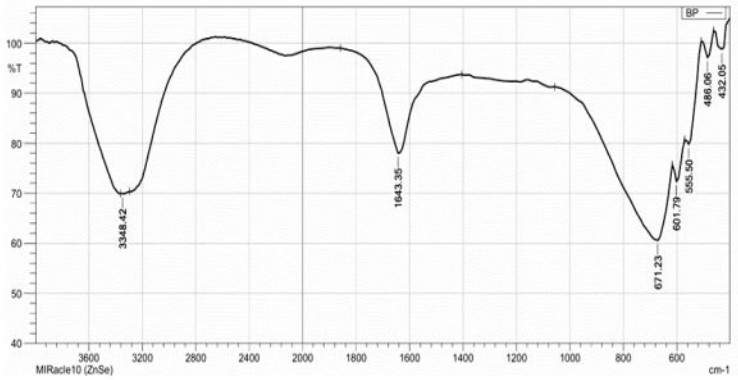


Figure 8c: FT-IR spectra after 365 days

Figure 8: FTIR Spectrum of HDPE plastic before and after biodegradation (8a) untreated (8b and 8c) treated (after 180- and 365-days incubation respectively)

In addition, presence of new peaks was observed at 1642, 671.23, 601.79, 555.50, 486.06, and 432.05 cm^{-1}, equivalent to functional groups like C=C (alkene), C–Cl, C–Br, and C–I. This may be due to microbial change of HDPE into low-molecular-weight compounds. From the results, it can be confirmed that *Bacillus pseudomycoides* carry out oxidative and hydrolytic cleavage and also halogenation-associated changes. These FT-IR findings highlight the ability of the isolated *Bacillus pseudomycoides* to chemically alter HDPE by forming new functional groups and modifying existing ones. Such transformations suggest an advanced ability to break down HDPE.

3.7.3: Gas Chromatography-Mass Spectrometry (GC-MS) *of Control and Bacillus pseudomycoides treated HDPE Plastic sample*

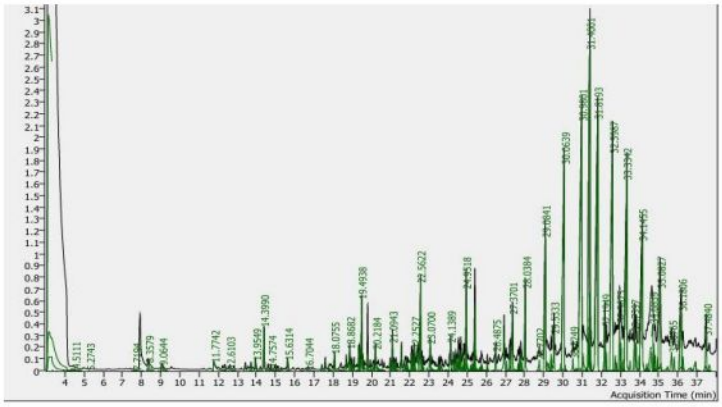
Gas Chromatography - Mass Spectrometry (GC-MS) is a systematic procedure to identify and quantify the breakdown products formed during the biodegradation of plastics using microbes [77]. In the present study, the analysis of *Bacillus pseudomycoides* degradaed HDPE using GC-MS showed the presence of thirty-eight diverse compounds, mostly HDPE degradation products. Among these products, phthalic acid di (oct-3-yl) ester was the major compound. Other byproducts with high levels include dibutyl phthalate, pentadecane, octadecane, heneicosane, tetracosane, tricosane, and pentacosane and phthalic acid derivatives (Figure 9).

The GC–MS chromatogram of untreated HDPE showed characteristic high molecular weight hydrocarbon peaks between 29–33 min, corresponding to long-chain alkanes typical of polyethylene. In contrast, the treated HDPE exhibited a significant increase in the number and intensity of peaks in the 15–28 min range, indicating the formation of low molecular weight hydrocarbons and oxygenated degradation products. These changes confirm the transformation of HDPE by *Bacillus pseudomycoides* into a different array of hydrocarbon chains and oxygenated intermediates. The compounds represent a homologous series of alkanes, ranging from Octane (C11) to Triacontane (C30), which provide a direct evidence of backbone depolymerization and also chain scission. Crucially, the intense peak detected at 31.483 minutes was identified as Phthalic acid, which is a key bio-oxidation product that signifies the enzymatic conversion of hydrophobic hydrocarbon chains into more polar, biodegradable molecules [40]. The appearance of the low-to-medium molecular weight fragments, which is absent in the HDPE control, validates the role of *B. pseudomycoides* in the structural breakdown of the polymer [78].

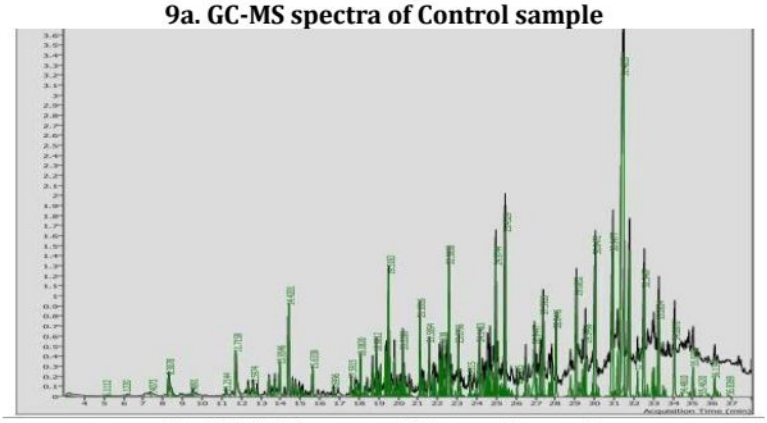
A similar study was conducted where *Bacillus cereus* and *Lysinibacillus fusiformis* were used for degradation of polythene. 1, 2, 3, 4 - tetra methylbenzene was the main degradative end product in the culture supernatant of *L fusiformis* and 1, 2, 3 trimethyl phtalate for *B. cereus* strain [79,80]. HDPE degradation by *Bacillus pseudomycoides* also showed comparable findings.

Bacillus pseudomycoides grew steadily over a period of 12 months with HDPE as the sole carbon source, causing chemical rearrangement,

surface degradation, polymer oxidation, and detectable weight loss (~31%). The chemical, structural and physical variations in HDPE and generation of various low-molecular-weight degradation intermediates were confirmed by balancing SEM, FTIR, and GC-MS examinations. These results clearly demonstrate *B. pseudomycoides'* capacity to degrade one of the most resistant polymers.



9a. GC-MS spectra of Control sample



9b: GC-MS Spectra of Treated sample

Figure 9: GCMS Spectra of HDPE plastic (a) Control and (b) Treated

CONCLUSION

The present study demonstrates the ability of a newly isolated *Bacillus pseudomycoides* to initiate biodegradation of untreated HDPE (75-micron thickness) under aerobic conditions. After one year of incubation, *B. pseudomycoides* caused a significant weight reduction of 31% in HDPE plastic samples when compared to un-inoculated controls. Multiple techniques such as SEM, FTIR and GC-MS were used to establish the surface erosion, structural modifications and presence of diverse degradation products by *Bacillus pseudomycoides.* These findings highlight the novelty of this strain as a promising candidate for HDPE bioremediation, given its biosurfactant production that facilitates colonization of hydrophobic substrates. Further studies are warranted to elucidate the degradation pathways, optimize culture conditions, and explore large-scale applications for effective plastic waste management and recovery of value-added products.

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Declaration

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of interest

The authors of this manuscript have no conflict of interest to declare.

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