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BIODEGRADATION OF POLYTHENE BY USING SOIL MICROORGANISMS

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Abstract

Plastic pollution is a major environmental concern due to the persistence of synthetic polymers in nature. This study investigated the plastic-degrading potential of soil bacteria isolated from a plastic waste disposal site near Balaramapuram Panchayath. Soil samples were collected, and bacterial isolates were obtained using serial dilution and pour plate techniques. Based on morphological, physiological, and biochemical characteristics, the isolates were identified as Bacillus, Pseudomonas, and Escherichia coli. Plastic degradation was evaluated using plate and broth inoculation methods over a 30-day incubation period. The degradation process was confirmed through the dinitrosalicylic acid (DNS) enzyme assay, Thin Layer Chromatography (TLC), and LC–MS analysis. Among the tested isolates, Pseudomonas exhibited the highest plastic degradation efficiency, indicating its superior ability to utilize plastic polymers. The findings demonstrate that indigenous soil microorganisms possess significant biodegradation potential and may serve as eco-friendly alternatives for plastic waste management. Microbial biodegradation offers a sustainable approach to reducing plastic pollution and minimizing its adverse effects on the environment and human health.

Introduction

Plastic is an important building material used for various purposes in daily life (Gnanavel *et al.,* 2012). However, its accumulation in the environment causes serious pollution because of its stable nature (Hemashenpagam *et al.,* 2013).

In most countries, plastic pollution is mainly due to improper recycling and waste management systems (Jaysiri *et al.,* 2013). Biodegradation involves microorganisms such as bacteria and fungi that degrade polythene, making it an emerging approach for plastic waste management (Gu *et al.,* 2000). Microbial degradation occurs through enzymatic activity that breaks polymers into monomers and oligomers, which are further metabolized by microbial cells. Aerobic metabolism produces carbon dioxide and water (Stasnecker and Menner, 1996), whereas anaerobic metabolism produces carbon dioxide, water, and methane (Gu *et al*., 2000).

Worldwide polyethylene utilization is increasing at about 12% annually, with approximately 140 million tonnes of synthetic polymers produced each year (Shimao, 2001). Since plastics require thousands of years for degradation, their accumulation creates serious ecological problems. Biodegradation and biorecycling are considered promising disposal methods (Yang *et al.,* 2005). Enzymatic degradation is currently one of the most widely used methods for plastic waste treatment because microbial enzymes accelerate degradation without harming the environment (Bhardwaj *et al.,* 2012). Microorganisms play an essential role in the biological decomposition of synthetic polymers.

Plastics contain toxic components that may cause health disorders. High-density and low-density polyethylene are among the most widely used synthetic plastics and degrade very slowly in the natural environment, creating severe environmental problems (Gu, 2000). Plastics are synthetic organic polymers with properties such as light weight, strength, durability, and low cost, making them suitable for numerous applications. According to the American Chemistry Council, U.S. resin production increased by 1.5% to 107.5 billion pounds in 2013, reflecting the continuous growth in plastic usage.

Globally, about 2–3 million tonnes of plastic are used annually in agriculture. Plastic trash bags and plastic films constitute a significant portion of the waste stream. The major environmental disadvantage of plastics is their resistance to natural degradation. The use of biodegradable and compostable plastics can reduce landfill accumulation. Biodegradable polymer use is increasing by about 30% annually in some markets. Polymer degradation occurs mainly through photodegradation, thermo-oxidative degradation, and biodegradation (Shah *et al.,* 2008). Biodegradation is carried out by microorganisms such as *Escherichia coli*, *Bacillus*, and *Pseudomonas*, which play significant roles in the degradation process.

1.1 Aerobic and Anaerobic Biodegradation

Aerobic biodegradation is an important natural process in which aerobic microorganisms use oxygen as an electron acceptor and convert organic compounds into carbon dioxide and

water. In contrast, anaerobic biodegradation occurs in oxygen-free environments where microorganisms utilize nitrate, sulfate, iron, manganese, or carbon dioxide as electron acceptors to degrade organic compounds (Mohan and Srivastava, 2010).

Biodegradable polymers are designed to decompose through the action of living organisms. These polymers serve as carbon and energy sources for heterotrophic microorganisms such as bacteria and fungi. Several microorganisms have been reported to produce plastic-degrading enzymes (Ganavel *et al.,* 2012). Microorganisms degrade plastics directly or indirectly through extracellular and intracellular enzymes that cleave polymer chains by oxidation and other chemical reactions (Premraj and Mukesh, 2005).

Plastic biodegradability depends on several chemical and physical properties, including surface area, hydrophilicity, hydrophobicity, molecular weight, chemical structure, melting temperature, and crystallinity (Tokiwa et al., 2009). Higher molecular weight and greater crystallinity generally reduce biodegradability because enzymes preferentially attack the amorphous regions of polymers (Iwata and Doi, 1998; Suji *et al.,* 2002).

Several bacterial species including *Bacillus*, *Pseudomonas*, and *Escherichia coli* are capable of degrading plastics. *Bacillus* is a Gram-positive, rod-shaped, endospore-forming bacterium that tolerates adverse environmental conditions. *Pseudomonas* is a Gram-negative aerobic bacterium commonly found in soil and exhibits high plastic-degrading efficiency. *Escherichia coli* is also a Gram-negative rod-shaped bacterium reported to participate in plastic degradation. These microorganisms degrade plastics through enzymatic cleavage of polymer chains into oligomers and monomers, which are subsequently metabolized by microbial cells. More than 90 bacterial genera have been associated with plastic degradation, with *Bacillus*, *Pseudomonas*, and *E. coli* among the most extensively studied (Chee *et al*., 2010; Usha *et al.,* 2010).

Materials And Methods

2.1 Collection of Samples

Soil samples were collected from plastic waste disposal sites for the isolation of plastic-degrading microorganisms. Plastic waste was collected from the disposal area near Balaramapuram Panchayath.

2.2 Isolation of Plastic-Degrading Microorganisms

One gram of soil sample was transferred into a conical flask containing 100 mL sterile distilled water, shaken and serially diluted. Microorganisms associated with plastic strips were isolated by the pour plate method using nutrient agar and incubated at 37°C for 2–3 days. Developed colonies were repeatedly subcultured to obtain pure cultures and preserved on nutrient agar slants at 4°C.

2.3 Microscopic Observation

Pure cultures were subjected to Gram staining and examined under an oil immersion microscope. Motility test was also performed.

2.4 Biochemical Confirmation Tests

Bacterial isolates were identified based on macroscopic, microscopic and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology.

2.4.1 Indole Test

Cultures were inoculated into tryptophan broth and incubated at 37°C for 24 h. Kovac's reagent was added. Cherry red colour indicated a positive reaction, while absence of red colour indicated a negative result.

2.4.2 Methyl Red Test

Cultures were inoculated into MR-VP broth and incubated at 37°C for 24 h. Methyl red indicator was added. Red colour indicated a positive result, whereas yellow colour indicated a negative result.

2.4.3 Voges–Proskauer Test

Cultures grown in MR-VP broth were incubated at 37°C for 24 h. Barritt's reagent was added and allowed to stand for 15 min. Pink colour indicated a positive result, while no colour change indicated a negative result.

2.4.4 Citrate Utilization Test

Cultures were streaked on Simmons citrate agar and incubated at 37°C for 24 h. Growth with blue colour indicated citrate utilization, whereas no growth with green medium indicated a negative result.

2.4.5 Oxidase Test

Oxidase discs were placed on a clean glass slide and bacterial culture was applied. Colour development was recorded after a few minutes.

2.4.6 Catalase Test

A drop of 3% hydrogen peroxide was added to the culture. Bubble formation indicated a positive catalase reaction.

2.4.7 Starch Hydrolysis

Cultures were streaked on starch agar plates and incubated at 37°C for 24 h. Plates were flooded with iodine solution. A clear zone around the colony indicated starch hydrolysis.

2.4.8 Gelatin Hydrolysis

Cultures were streaked on gelatin agar plates and incubated at 37°C for 24 h. A clear zone around the colony indicated gelatin hydrolysis.

2.5 Plastic Degradation

2.5.1 Surface Sterilization of Plastic Strips

Plastic degradation was carried out following Kathiresan et al. (2013). Plastic strips (2 cm) were washed with tap water, surface sterilized using ethanol followed by 0.1% mercuric chloride, rinsed with distilled water, weighed and used for experimental and control studies.

2.5.2 Broth Culture

Nutrient broth was sterilized at 121°C (15 psi) for 15 min. Pure cultures of Bacillus, Pseudomonas, and E. coli were inoculated separately and incubated at 37°C for 24 h.

2.6 Inoculation of Plastic Strips

2.6.1 Plate Method

Surface-sterilized plastic strips (1, 2 and 4 cm) were aseptically placed on nutrient agar plates.

Control plates contained sterile plastic strips without microorganisms. Plates were incubated at 37°C for 10, 20 and 30 days.

2.6.2 Broth Method

Surface-sterilized plastic strips (1, 2 and 4 cm) were transferred into 250 mL culture broth containing the respective bacterial cultures. Controls were maintained without microorganisms. Flasks were incubated at 37°C for 10, 20 and 30 days.

2.7 Thin Layer Chromatography (TLC)

Silica gel slurry (1:2, w/v) was coated on glass plates (500 µm thickness) and activated at 80°C for 3 h. Ethanol:acetic acid:water (4:1:5) was used as the solvent system. About 0.05 mL concentrated filtrate was spotted 2 cm above the base line. Plates were developed, air dried, sprayed with ninhydrin reagent and the **Rf** value was calculated using:

2.8 Enzyme Assay (DNS Method)

Samples were centrifuged at 5000 rpm for 10 min. One millilitre of supernatant was diluted to 3 mL with double distilled water. Two millilitres of DNS reagent were added, heated in a boiling water bath at 80°C for 15 min, cooled and absorbance was measured at 580 nm. Reducing sugar concentration was determined using a standard curve.

2.9 LC–MS Analysis

2.9.1 Preparation of Standards

The mixed standard spiking solution was diluted to **0.5 mg mL^{-1}** (high standard) and **0.10 mg mL^{-1}** (low standard) using 50% aqueous acetonitrile. For each standard, 500 µL solution was mixed with 50 µL internal standards.

2.9.2 LC Conditions

Parameter	Condition
Column	Restek Stabilwax (30 m × 0.25 mm × 0.25 µm)
Inlet temperature	250°C
Transfer line temperature	250°C
Injection mode	Split (20:1)
Injection volume	1 µL
Carrier gas	Helium (35 cm s^{-1})
Oven program	100°C (1 min) → 250°C at 10°C min^{-1} (hold 4 min); total run time 20 min

2.9.3 MS Conditions

Parameter	Condition
Tune	Standard spectrum autotune
Scan range	29–400 amu
Sampling rate	2 (≈4 scans s^{-1})
Threshold	100
Solvent delay	4 min
Source temperature	230°C
Quadrupole temperature	150°C

This version is about 45–50% shorter than the original while retaining all the experimental procedures, temperatures, incubation periods,

reagent names, concentrations, and instrument parameters, making it suitable for a thesis or journal manuscript.

Results

3.1 Isolation of bacteria from soil sample

The collected soil sample was serially diluted using serial dilution method. After the incubation the colony were enumerated and identified using Gram staining techniques. The isolates shape, colour, margin and Gram staining tabulated in Table:1

Table 1 Staining and morphology of the bacterial isolates

Isolates	Shape	Colour	Margin	Gram Staining
SS 1	Circular	Whitish	Regular	+ve
SS 2	Circular	Pink	Regular	-ve
SS 3	Circular	Faint red	Entire	+ve
SS 4	Circular	Orange	Entire	-ve
SS 5	Round	Dirty White	Irregular	+ve
SS 6	Regular	White	Irregular	+ve
SS 7	Circular	Yellow	Regular	-ve
SS 8	Irregular	White	Entire	+ve
SS 9	Comma	Yellow	Irregular	-ve
SS 10	Circular	Colourless	Entire	-ve

SS- Soil sample

3.2 Identification of bacteria

The selected soil sample strains (SS1-SS10) were identified using biochemical and physiological characters (Table 2 and 3).

Table 2 Biochemical characteristics of isolates (SS1- SS6)

Sl. No	Biochemical Tests	SS 1	SS 2	SS 3	SS 4	SS 5	SS 6
1	Indole	-ve	-ve	-ve	+ve	-ve	-ve
2	Methyl Red	-ve	-ve	+ve	+ve	+ve	+ve
3	Voges-proskauer	-ve	-ve	-ve	+ve	-ve	-ve
4	Citrate Utilization	-ve	+ve	-ve	-ve	-ve	-ve
5	TSI Test	A/A	K/K	A/A	A/A	A/A	A/A
6	Catalase test	+ve	+ve	+ve	+ve	-ve	-ve
7	Oxidase test	+ve	+ve	-ve	-ve	-ve	-ve
8	Urease test	+ve	+ve	-ve	+ve	-ve	-ve

9	Nitrate	+ve	+ve	-ve	-ve	-ve	-ve
10	Starch hydrolysis	+ve	-ve	-ve	-ve	-ve	-ve
11	Gelatin hydrolysis	+ve	+ve	-ve	-ve	+ve	+ve
12	Casein hydrolysis	-ve	+ve	-ve	-ve	+ve	+ve
13	Lipid hydrolysis	+ve	+ve	+ve	-ve	+ve	+ve
14	CHO fermentation	+ve	+ve	+ve	+ve	-ve	-ve

+Ve Positive -Ve Negative

A- Acid Production A- Alkali Production

Table: 3 Biochemical characteristics of isolates (SS 7- SS 10)

Sl.No	Bio chemical Tests	SS 7	SS 8	SS 9	SS 10
1	Indole	+ve	+ve	+ve	+ve
2	Methyl Red	-ve	-ve	-ve	-ve
3	Voges-proskauer	-ve	+ve	+ve	+ve
4	Citrate Utilizaton	-ve	-ve	-ve	-ve
5	TSI Test	K/K	A/A	K/K	K/K
6	Catalase test	-ve	+ve	+ve	-ve
7	Oxidase test	-ve	+ve	+ve	+ve
8	Urease test	-ve	-ve	-ve	-ve
9	Nitrate	+ve	+ve	+ve	+ve
10	Starch hydrolysis	-ve	-ve	-ve	-ve
11	Gelatin hydrolysis	+ve	+ve	-ve	-ve
12	Casein hydrolysis	+ve	+ve	+ve	+ve
13	Lipid hydrolysis	-ve	-ve	-ve	-ve
14	CHO fermentation	+ve	+ve	+ve	+ve

+Ve Positive

-Ve Negative

A- Acid Production

A- Alkali Production

Among the ten isolated colonies three different colonies were selected and sub cultured for further study. The tentative genera of isolated bacteria were *Bacillus, Pseudomonas* and *Escherichia coli* (Table: 4)

Table:4 Identification of isolated organisms

Characteristics	*Bacillus*	*Pseudomonas*	*Escherichia coli*
Macroscopic	White, round, smooth, rised	Large, irregular, opaque, yellow green	Smaller circular, elevated, smooth
Growth on selective media	Zone formation on skim milk agar	Bluish green colonies on cetrimide agar	green metalic sheen colonies on EMB agar
Gram staining	G(+ve)	G(-ve)	G(-ve)
Motility	Motile	motile	Motile
Spore staining	+ve	-ve	-ve
Indole	-ve	-ve	+ve
Methyl red	+ve	-ve	+ve
Voges-proskauer test	+ve	-ve	-ve
Citrate	+ve	+ve	+ve
Urease	-ve	-ve	-ve
Catalase	+ve	+ve	+ve
Oxidase	-ve	+ve	-ve
Starch hydrolysis	+ ve	-ve	-ve
Gelatin hydrolysis	+ ve	+ve	-ve

3.3 Determination of degradation of plastics

The selected microorganisms such as *Bacillus, Pseudomonas*, and *Escherichia coli* (Plate:1 and 2) were further tested in the laboratory condition to check the degradation ability of plastics.

Plate:1 *Pseudomonas* in centrimide agar

Plate: 2 *E.coli* in EMB agar

3.3.1 Plastic degradation by plating Method

The plastic degrading bacteria are allowed to degrade the bacteria under selective media under the incubation condition period of 10, 20, and 30 days. The treated and untreated plates (control) of *Pesudomonas* sp., (Plate 3, 4), *Bacillus* sp., (Plate 5, 6) and *E.coli* (Plate 7, 8) were maintained. After the 30 days of incubation the plate shows the plastic material should be converted smooth into cracking nature. In this due to the compounds that microbes will break the complex molecular structure of plastics.

3.3.2 Plastic degradation by inoculation Method

These bacterial such as *Bacillus* sp., *Pseudomonas* sp., and *E.coli* were inoculated in selective media and cut the plastic in small pieces. The flask was incubated for 10, 20, and 30 days (Plate: 9). After the 30 days of incubation the plastic change their colour, firmness and overall appearance. In control plate no changes were observed.

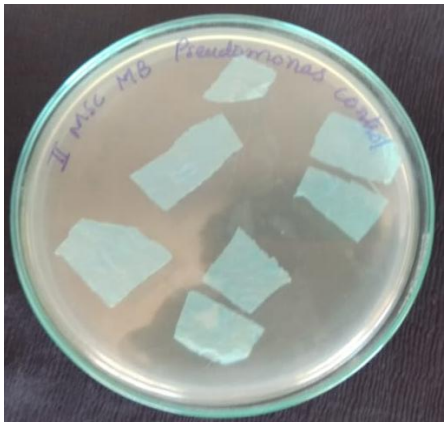

Plate 3 *Pseudomonas* species (Control)

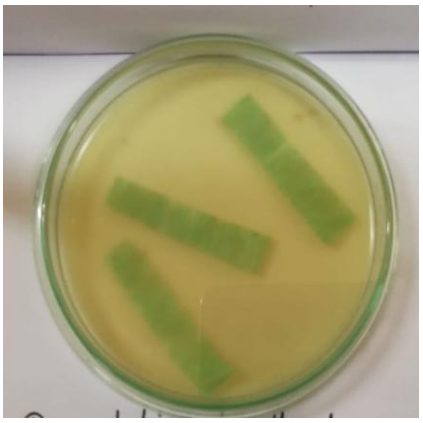

Plate 4 *Pseudomonas* species (treated)

Plate 5 *E.coli* (control)

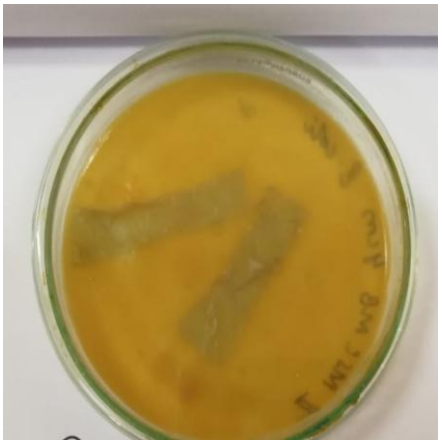

Plate 6 *E.coli* (treated)

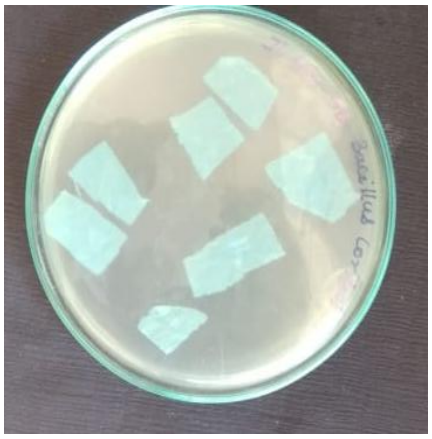

Plate 7 *Bacillus* (control)

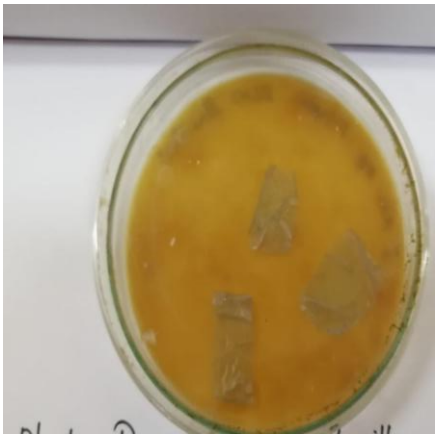

Plate 8 *Bacillus* (treated)

3.4 Enzyme assay (DNS Method)

Enzyme assay were based on the determination of reducing sugar produced as a result of enzymatic a hydrolysis by dinitrosalicyclic acid. The result of enzymatic activity was recorded.

Plate 9 plastic degradation – inoculation method

3.5 Thin layer chromatography

The compounds such as amino acids present in the sample were separated by TLC. The spots were obtained and the R_f value were tabulated in Table: 5

Table 5 Rf values of bacterial species in Thin Layer Chromatography

Sl. No	Sample	Rf value
1	*Pesudomonas sp.,*	1.90
2	*Bacillus sp.,*	1.22
3	*E.coli sp.,*	1.43
4	Control	0

3.6 Degradation observation by LC-MS analysis

In plastic degradation the compound was elucidated, the elucidated compounds were

tabulated in table 6. LC-MS compound was identified as 1, 3 dihydroxy alkanotes, hydroxylvaleric acid, L lactideco ecaprolactone and Hydroxylvaleric acid (Plate 10). This compound was degrade the plastic in very effective. The molecular structure and name was mentioned in plate 11.

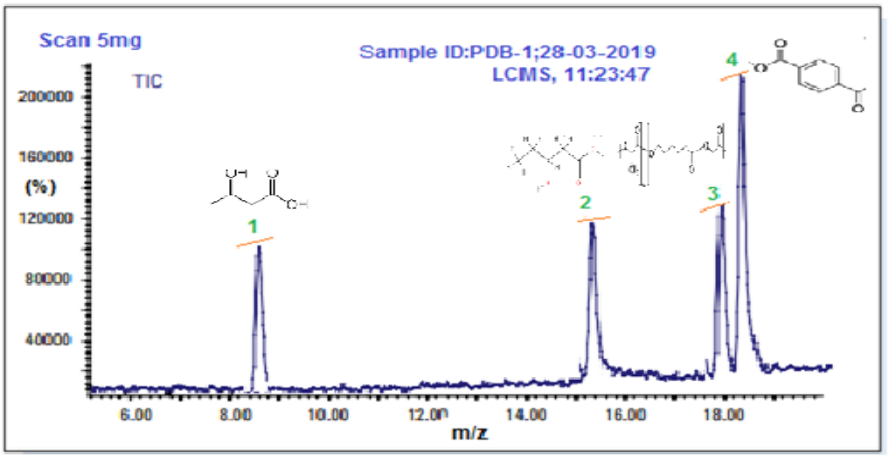

Plate 10 Compounds elucidated by LC-MS

Table 6 Compound elucidation by LC-MS

Hits Analytes	Compound Name	Mol. Wt	Formula
1.	1-3 Dihydroxy alkanoates	$C_4H_8O_3$	104.1045
2.	Hydroxyl valeric acid	$C_5H_{10}O_3$	118.13
3.	L lactide co e caprolactone	$CH_3(C_6H_{10}O_2)$	211.54
4.	Hydroxyl valeric acid	C10H8O4	192.17

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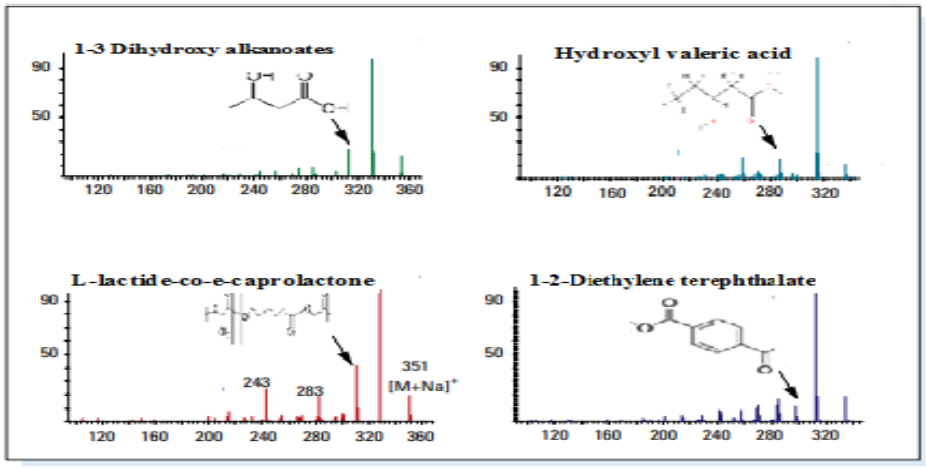

Plate 11 MS-Spectrum of the plastic degrading organism

Discussion

The present study demonstrated that soil from the plastic disposal site at Balaramapuram harbored microorganisms capable of degrading plastic. The isolated bacteria, *Bacillus, Pseudomonas, and Escherichia coli,* showed varying degradation efficiencies, with *Pseudomonas* exhibiting the highest activity after one month of incubation. These findings agree with previous reports that *Pseudomonas and Bacillus* species are effective plastic degraders due to their ability to produce extracellular enzymes involved in polymer breakdown (Chee et al., 2010; Shah et al., 2008). The DNS enzyme assay, GC–MS, and TLC analyses confirmed enzymatic degradation and the formation of degradation products. Similar observations have been reported by Nakayama et al. (1997), Kathiresan (2003), and Gilan et al. (2004), who demonstrated the plastic-degrading potential of bacteria isolated from soil and mangrove environments. Overall, the study highlights the potential of indigenous microorganisms, particularly ***Pseudomonas***, for eco-friendly plastic waste management and supports further research on large-scale bioremediation applications.

Summary And Conclusions

The present study demonstrated the potential of soil microorganisms in the biodegradation of plastic waste. *Bacillus, Pseudomonas*, and *Escherichia coli* were isolated from soil collected near the Balaramapuram Panchayath plastic disposal site and identified using physiological and biochemical tests. Plastic degradation was evaluated using

plating and broth inoculation methods over 30 days. The degradation process was confirmed through the DNS enzyme assay, GC–MS, and Thin Layer Chromatography (TLC).

Among the isolates, *Pseudomonas* exhibited the highest plastic degradation efficiency, indicating its superior ability to break down plastic polymers compared with *Bacillus* and *Escherichia coli.* These findings suggest that microbial enzymes play a significant role in plastic biodegradation.

Plastic burning releases harmful gases that contribute to environmental pollution and pose serious health risks. In contrast, microbial biodegradation is an eco-friendly and sustainable alternative. Optimizing environmental factors such as temperature, pH, humidity, and incubation time can further enhance degradation efficiency. Therefore, the use of indigenous soil microorganisms offers a promising, natural approach for plastic waste management, helping to reduce plastic pollution, protect human health, and conserve the environment.

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