

# Biodegradation of Polyethylene Using *Lysinibacillus Macroides*: Isolation, Characterization, and Evaluation

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## Abstract:

Urbanization and economic development has led to increase in production of plastics. The increased production of plastics has resulted in the accumulation of plastics in the environment which leads to plastic pollution. The plastics are exposed to various weathering process and undergoes decomposition and leads to formation of microplastics. The Polyethylene is one of the microplastics which contributes to the maximum share of pollution and is very hazardous. The safe degradation of polyethylene can be done by microbial degradation. This study contemplated the extensiveness of plastic degradation by the use of microbes. The species of bacterium were isolated from Plastic dumping grounds in Karad. The isolated and screened microbes were assessed further in the terms of their degradation potential. The evaluation of polyethylene degradation potential was executed by weight loss method, FTIR analysis and scanning electron microscopy. One bacterial isolate showed positive results and the screening results showed growth which measured 7mm around the inoculated well. The screened out isolate degraded 40% of the polyethylene which was evaluated by weight loss method. The Scanning electron microscopy showed the pits and holes which were formed by degradation . The promising isolate was later identified by 16S rRNA gene sequencing as *Lysinibacillus macroides*.

Key Words	Polyethylene, Microplastic, Biodegradation, Evaluation, <i>Lysinibacillus macroides</i>
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## Introduction:

The lengthy carbon chains that make up the backbone of plastic molecules are organic polymers commonly called as plastics which are developed as a result of polymerization (Koushal *et al.*, 2014). In addition to carbon and hydrogen, nitrogen, sulfur, and other diverse organic and inorganic components produced from fossil fuels, make up plastics (Kumari N.A *et al.*, 2013). The production of plastics has increased to another limit due to increased demand as a result of urbanization. The plastics are causing detrimental effects by accumulating in the environment and causing pollution. Plastics on the land are exposed to UV light and weathering

processes such as photodegradation, thermal oxidation, hydrolysis, biodegradation, and fragmentation which leads to its formation of fragments and pieces. (Bakht *et al.*, 2020). This leads to formation of microplastics. The microplastics affects various forms of lives by its deleterious impact. Microplastics affect the atmosphere as well as various living creatures such as animals, aquatic ecosystem and humans. There are numerous varieties of microplastics such as Polyethylene, Polypropylene, Polyethylene terephthalate and many more. Polyethylene contributes to maximum share of pollution. The inert synthetic polymer known as polyethylene is made up of a lengthy chain of ethylene monomers. One of the microplastics the one present in highest percentage which is polyethylene accumulates in the environment and causes hazardous effects to multiple life forms. There are various methods used for plastic management like incineration, land filling (Bakht *et al.*, 2020). However, these techniques have several drawbacks which affect the environment as they are not ecofriendly procedures. Thus, we need to adopt some method which is safe, ecofriendly and useful. Biodegradation is the safe and useful method in which microorganisms are employed for the degradation of plastics. Polyethylene is a hydrophobic polymer with a high molecular weight, making it resistant even to microbial degradation. Additionally, microbial enzymatic system is unable to recognize functional groups on polyethylene. These characteristics of polyethylene makes it tough to degrade. Hence, polyethylene needs to be targeted for the degradation to manage the pollution. However, various microorganisms are isolated which have the potential to degrade polyethylene upto certain extent. There are certain reports *Pseudomonas* sp., *Bacillus* sp., *Mycobacterium* sp., and *Nocardia* sp. have the potential to degrade PE. It was reported that *Lysinibacillus* sp. is capable to degrade polyethylene and polypropylene by 4% within 26 days of evaluation period (Jeon *et al.*, 2021). *Lysinibacilli* forms a biofilm on the polyethylene and targets for degradation (Oliveira *et al.*, 2021). Among the known microplastic degrading microbes *Lysinibacilli* shows promising results in degradation of polyethylene.

In the current investigation, an attempt is made for microorganisms that breakdown polyethylene and evaluation of the degradation potential of the isolate and isolate *Lysinibacillus macroides* is promising one.4

There are many reports proving the potential of *Lysinibacillus* to degrade microplastics. There are reports of degradation of polyethylene and polypropylene degradation by *Lysinibacillus* sp. which was investigated by GC-MS, SEM, XRD and FTIR. According to a research study by Jeon *et al.*, 2021 it is reported that *Lysinibacillus* sp. reduced weight of polyethylene by 9% over 26 days. Similarly, in a research study by Mukherjee *et al.*, 2016 it was found that, *Lysinibacillus fusiformis* degraded  $2.97 \pm 0.05\%$  within 1 month of incubation. It was also reported that mixed culture of *Lysinibacillus* and *Aspergillus* sp. have the potential to degrade 29.5% and 15.8% for the UV-irradiated and non-UV-irradiated films, respectively. It is clear from this study that the performance of *Lysinibacillus macroides* to degrade polyethylene is simply astounding which degraded 40% of polyethylene within 40 days of incubation. *Lysinibacillus* leads to enzymatic degradation by synthesizing enzymes which results in plastic degradation. There are reports of various enzymes hydrolysing plastic which eventually leads to plastic degradation. Enzymes such as cutinase, lipase, esterase, protease, laccase, peroxidase are involved in degradation of plastics (Kaushal *et al.*, 2021)

## **Material and methods:**

### **1) Collection of samples: (Divyalakshmi and Subhashini, 2016)**

Microorganisms are known as the most adaptable living beings to environmental changes. Soil, water, and wastes are teeming with diverse groups of microorganisms. When they get exposed to recalcitrant material like plastics, they develop an enzyme system to degrade it. Hence, various samples were collected from diverse sources/sites (contaminated with polyethylene-based plastics and hence possible source of polyethylene degrading microorganisms) like marine water, mangrove sediment, soil from plastic dumping grounds, soil from coastal regions, and waste treatment plant effluents. Additionally, small pieces of polyethylene were buried in the soil 5-10 cm deep and were allowed to be there for three months and periodically soil from that site was sampled after 20, 40, 60, 80 and 90 days surrounding the polyethylene pieces. The collection of samples from such sites was carried out as there is high chance of presence of microbes which degrade multiple forms of microplastics which is a natural process. Later these microbes are tested for their potential to degrade polyethylene.

### **2) Enrichment, isolation and screening of polyethylene degrading microorganisms: (Rani *et al.*, 2021; Patil *et al.*, 2015)**

#### **a) Enrichment of polyethylene degrading microorganisms:**

The samples acquired from various sources, such as marine water, mangrove sediment, soil from plastic dumping grounds, and coastal regions were inoculated in 1g/100mL amount and subjected to enrichment of microplastic degrading microorganisms using Mineral Salt liquid culture medium with polyethylene as the sole carbon source. For increasing the microbes and enriching the microbes that break down plastics from different soil and water samples, the samples were infused separately into different flasks containing liquid culture nutrient medium supplemented with polyethylene at 0.1% W/V concentration and were kept incubating at 30°C for a duration of 30 days on incubator shaker with shaking speed of 147 rpm. The samples from enrichment flasks were then kept ready for isolation of organism using solid media.

#### **b) Isolation of microorganisms from enriched samples: (Divyalakshmi and Subhashini, 2016)**

The enriched samples were diluted in distilled water ( $10^{-1}$ - $10^{-3}$ ). 0.1mL of each dilution from enrichment culture flask were spread on solidified medium with mineral salts supplemented with polyethylene and were kept incubating at 30°C for a duration of 30 days. In the control plate the standard culture degrading polyethylene (*Bacillus megaterium*) was used.

Subculturing of the representative colonies was done on solidified medium with mineral salts and polyethylene and preserved at 4°C in triplicates. The growth on MSM agar with polyethylene was compared with control.

#### **c) Screening of polyethylene degrading microorganisms: (Divyalakshmi and Subhashini, 2016)**

The screening of microorganisms that breakdown polyethylene was performed by following method. The isolated bacteria were assessed to ascertain their skills to breakdown polyethylene

using medium infused with mineral salts and the composition of the mineral salt medium is listed as follows: (per litre of D/W) 0.1g each of Dipotassium phosphate, sodium chloride and ammonium sulphate; Potassium dihydrogen phosphate (0.2g), Calcium chloride dihydrate(0.002g), Boric acid(0.005g), Magnesium sulphate heptahydrate (0.5g), Copper sulphate (0.001g), Zinc sulphate heptahydrate(0.001g), Manganese sulfate(0.001g) and Ferrous sulphate heptahydrate(0.01g). Polyethylene was infused in the mineral salt medium at 0.1% W/V concentration followed by autoclaving at 121°C for 20 min. The prepared media was then transferred into plates and after solidification wells were cut and 20µL culture of separate organisms were infused in the well. Plates were then kept for incubation at 30° C for 4 weeks and growth around the wells was observed. The experiments were run in triplicates and the average of triplicates were recorded as results. In the control sterile distilled water was used in place of culture of isolates in the wells as negative control and culture of standard organism (*Bacillus megaterium*) as positive control.

### **3) Evaluation of Bacterial Degradation of polyethylene:**

Following methods will be used for evaluation of degradation of plastics:

- a. Weight Reduction Method. (Divyalakshmi and Subhashini, 2016)
- b. FTIR Analysis (Divyalakshmi and Subhashini, 2016)
- c. Scanning Electron Microscopy (Auta H.S. *et al.*, 2017)

#### **a. Weight loss method: (Divyalakshmi and Subhashini, 2016)**

The polyethylene bags were trimmed to size, weighed initially, and then cleaned with distilled water that was sterile. After that, they were allowed to soak in unrefined black phenol for thirty minutes, and then they were allowed to dry for fifteen minutes under laminar air flow. 50 milliliters of sterile mineral salt medium were infused with weighed polyethylene strips. 0.1 mL (at 10<sup>9</sup> CFU/mL) of each screened isolate was infused into separate flasks containing polyethylene film and mineral salt media. One flask was kept as a negative control without any inoculated microbe and other with standard culture as positive control (polyethylene degrader). For 4 weeks, flasks were incubated at 30°C in an incubator shaker. Following incubation, the polyethylene strips were rinsed with sterile distilled water, sprayed with alcohol, allowed to air dry properly under Laminar air flow, and then weighed to determine their final weight. The following calculation was used to determine the percentage of polyethylene degradation:

$$\text{Percentage(\%) Degradation} = \frac{\text{Initial weight of milk cover} - \text{Final weight of milk cover}}{\text{Initial weight of milk cover}} \times 100$$

#### **b. FTIR Analysis:**

In FTIR, samples were exposed to infrared radiation. There was some IR transmission and some IR absorption. The obtained spectrum showed the molecular absorption and transmission of sample. FTIR analysis was performed on samples with high percentage(%) deterioration, and the findings were noted. When percentage transmission is increased and percentage absorption was decreased in case of treated polyethylene sample after exposure to test organism, it was taken as distortion of molecular structure of polyethylene sheet and it was taken as degradation and was compared to control sheets.

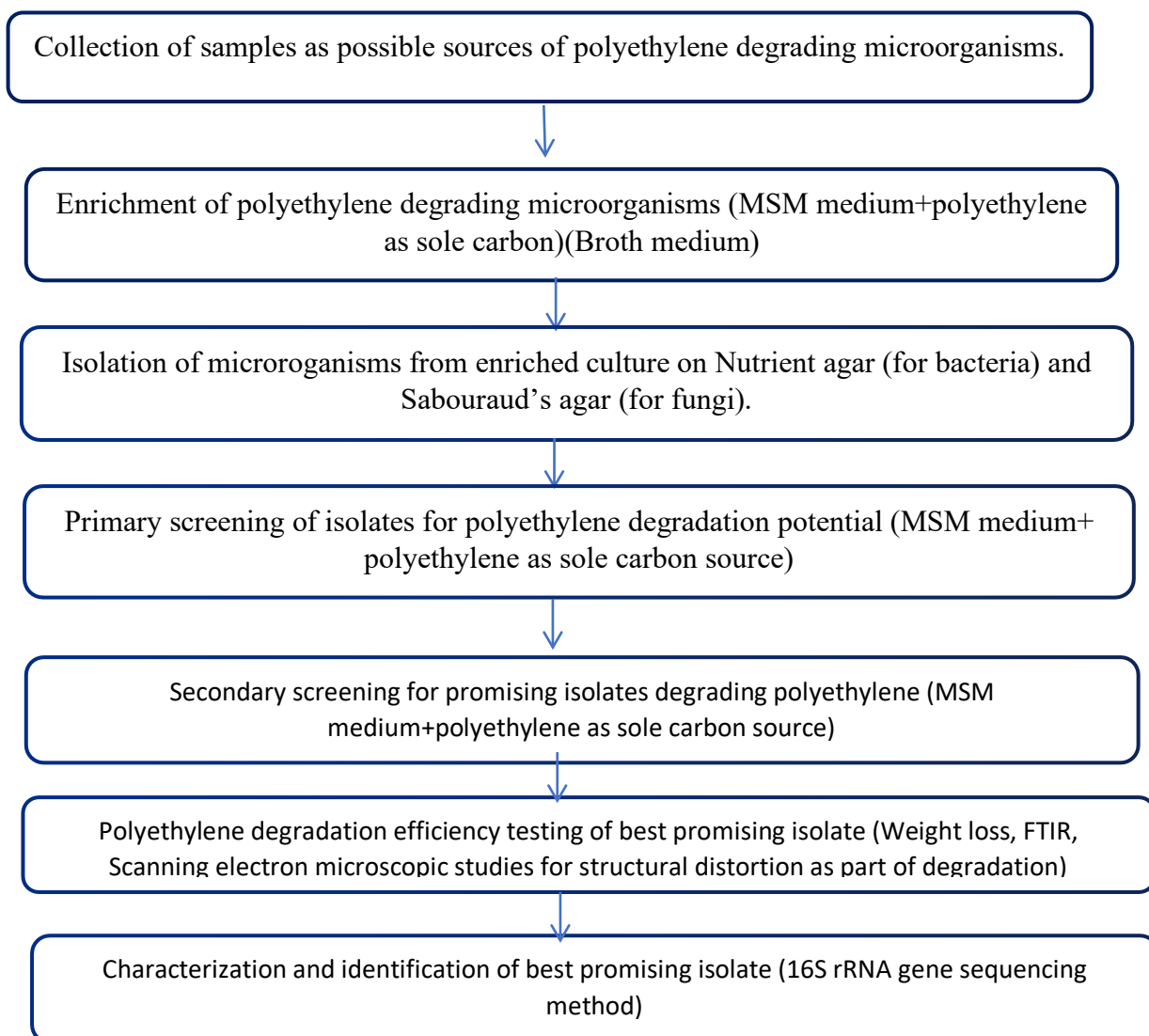
### c. Scanning Electron Microscopy:

A scanning electron microscope was used to examine the structural changes in the plastic surface before and after degradation at various time intervals. Following a brief washing with 2% (V/V) SDS and distilled water, and a flush with 70% ethanol to eliminate the inoculated microbial cells, the treated samples were examined using a scanning electron microscope for distortion of structures. When polyethylene sheets upon exposure to test organisms are distorted as compared to control sheet, it was taken as sign of polyethylene degradation.

### 4) Characterization and identification of promising Isolates:(Divyalakshmi and Subhashini, 2016):

The identification of selected potential organism at molecular level was done by 16 S rRNA gene sequencing. The obtained genome sequence was then employed for generation of phylogenetic tree by BLAST analysis and similarity was examined to identify the organisms to species level. The genome sequence was then deposited to NCBI and accession number was obtained. This characterization study was carried out at Esonowa Innovations private Ltd., Nagpur. The methodology at glance is depicted in the flow chart form (Fig.1) below:

**Fig.1: Methodology at glance:**



## Results and Discussion:

### 1) Collection of samples:

Different soil samples from plastic dumping grounds was collected from Malkapur plastic dumping ground and Karad plastic dumping ground. The samples were collected in zip-lock bags and immediately processed in the laboratory (Table 1).

**Table 1: Source wise sample collection:**

Sr. No.	Types of samples with pH and temperature.	Source of samples.	Number of samples collected.
1	Soil sample pH- 7.8; Temperature-31°C	Soil sample collected from plastic dumping ground, Karad	1
2	Soil sample pH- 7.8; Temperature-30°C	Soil samples collected from landfill with plastic waste, Malkapur.	1
3	Marine water pH- 8.0; Temperature-30°C	Water samples collected from coastal regions, Mumbai.	1
4	Soil from coastal regions. pH- 8.0; Temperature- 32°C	Soil samples collected from coastal regions, Mumbai.	1

Slight alkaline pH (7.0-8.0) and temperature at mesophilic range (30-32°C) will allow flourishing of mesophilic microorganisms which will be slightly alkalophilic to growing at neutrality.

### 2) Enrichment, Isolation and Screening of polyethylene degrading microorganisms:

**a) Enrichment of polyethylene degrading microorganisms:**

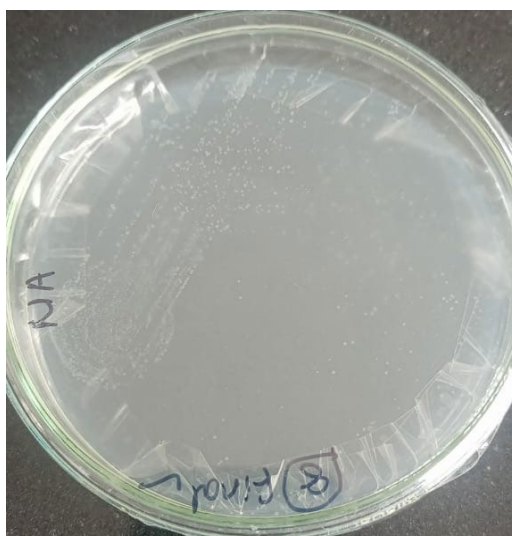
Enrichment of the polyethylene degrading microorganisms was obtained in Mineral Salt medium with polyethylene where polyethylene was the sole carbon source. These enriched samples were then used for isolation of microorganisms (Fig.2).



**Fig.2: Tube showing enrichment of polyethylene degrading microorganisms.**

**b) Isolation of microorganisms from enriched samples:**

Isolation of microorganisms from enriched sample was carried out on Nutrient Agar Medium. Nutrient agar supplies nutrients all those required for growth of polyethylene degraders and non-degraders. Some polyethylene degraders may be slow growers. To avoid exclusion of any polyethylene degraders nutritionally rich nutrient agar was used. The fig.3 depicts growth of microorganisms (colonies) from enriched sample. Number of isolates (bacterial and fungal) were obtained and were preserved in triplicates on nutrient agar slants at 4°C till further use. The appearance of the bacterial colonies on Nutrient Agar medium is depicted in Fig.3.



**Fig. 3: Isolate from enriched sample on Nutrient Agar medium at 30°C for 48h incubation.**

### c) Screening of polyethylene degrading microorganisms:

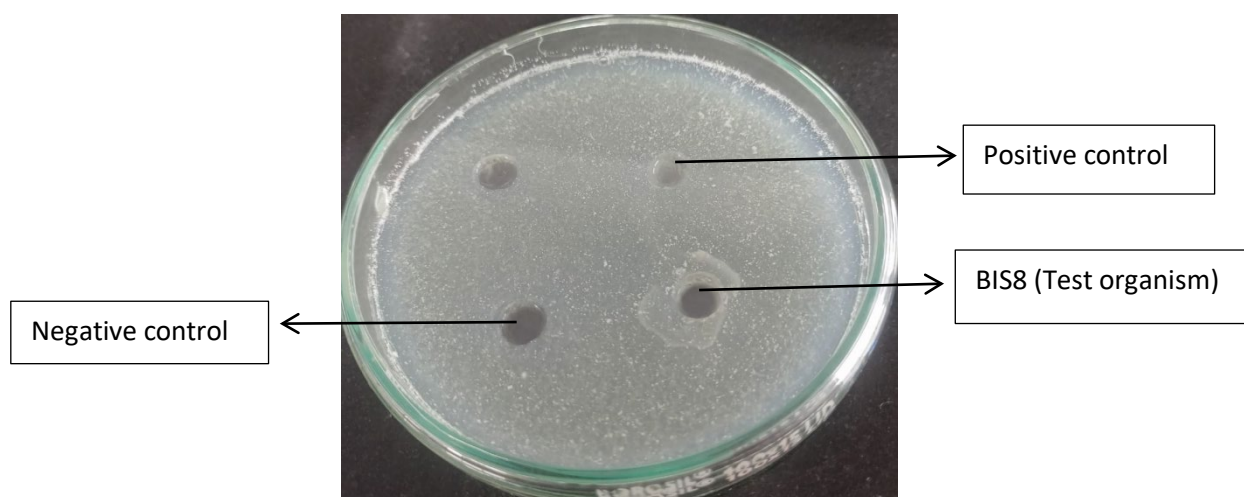
The nutrient agar isolates were grown on mineral salt medium with polyethylene as sole carbon source and they were grown on mineral salt medium with polyethylene as sole carbon source and they were taken as polyethylene degraders and compared with positive and negative controls. The weightloss evaluation method showed that isolate BIS8 was efficient in polyethylene degradation and its growth on agar well media is depicted in Table-2 and fig.4.

The results of screening of isolate which was named as BIS8 is summarized in Table 2:

**Table 2: Screening of polyethylene degrading microorganisms:**

Sr. No.	Isolate no.	Degradation activity	Size of growth
1.	BIS8	++	7 mm

As shown in Table 2 and fig-4, the isolate BIS8 showed growth around the inoculated well in the media containing polyethylene as the sole carbon source.



**Fig.4: Growth of isolate BIS8 and positive control wells and no growth in negative control well.**

### 3) Evaluation of polyethylene degradation by BIS8 isolate:

The screened isolate BIS8 was evaluated for degradation potential. The polyethylene sheets were treated with the isolate and its degradation was examined by Weight loss method, FTIR analysis and Scanning electron microscopy.

#### a. Weight loss method:

**Table 3: Percentage degradation of polyethylene with BIS8 isolate, positive control and negative control:**

Set of experiments in triplicates.	Initial Weight(Average results of triplicate)	Final weight(Results of triplicate)
BIS8 set	0.05	0.03



Positive control set	0.05	0.035
Negative control set	0.05	0.05

I) Percentage degradation of polyethylene by BIS8.

$$\text{Percentage(\%) Degradation} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

$$= \frac{0.05 - 0.03}{0.05} \times 100 = 40\%.$$

**Percentage degradation of polyethylene by isolate BIS8 is 40%.**

II) Percentage degradation of polyethylene by positive control =  $\frac{0.05 - 0.035}{0.05} = 30\%$

**Percentage degradation of polyethylene by positive control is 30%.**

III) Percentage degradation of polyethylene in negative control set =  $\frac{0.05 - 0.05}{0.05} = 0\%$

**Percentage degradation of polyethylene by negative control set is 0%.**

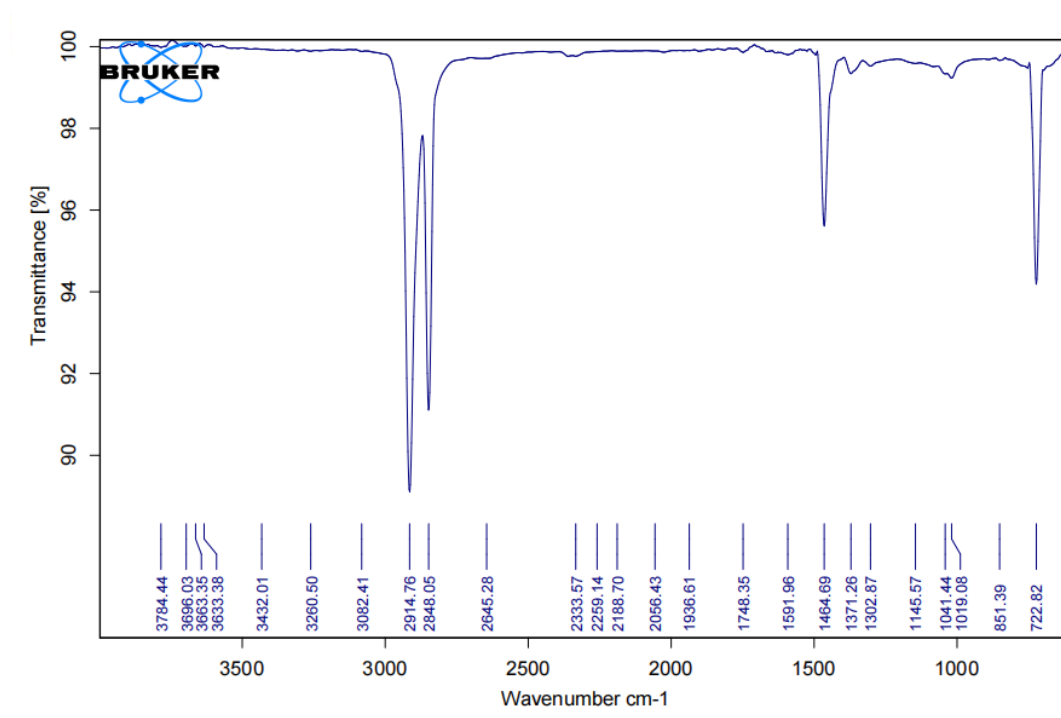
The degradation results (Table-3) depicted that BIS8 isolate showed polyethylene degradation (40%) in comparison with positive control (30%) which is 10% more than standard test organism while in negative control expected 0.0% degradation was obtained.

It was found that the control polyethylene sheet didn't cause any change when weighed after evaluation period.

There are certain reports of biodegradation of polyethylene by *Lysinibacillus* with *Aspergillus* and it was 29.5% within 126 days of incubation period (Atefeh *et al.*, 2013).

#### **b) FTIR analysis of polyethylene treated with isolate BIS8.**

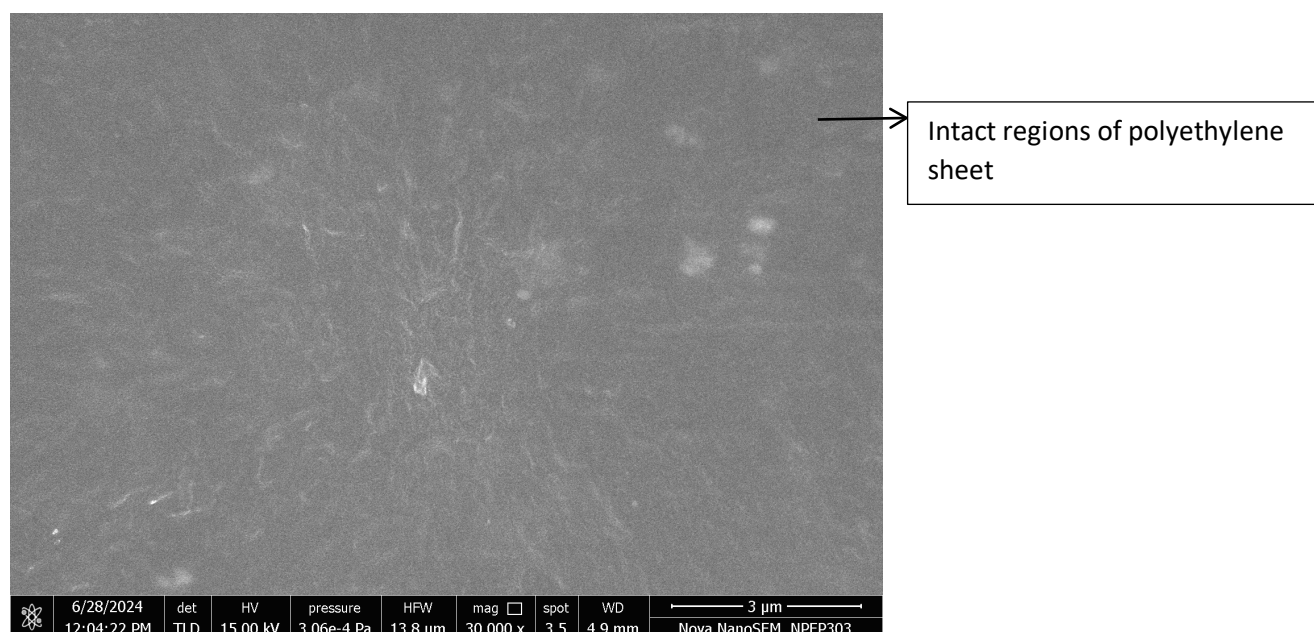
The polyethylene sheet which was treated with BIS8 was analysed by FTIR. It is clear from the graph (Fig.5) that the peaks at 2914, 2848, 1464, 722 corresponded to CH stretch, CH stretch, CH bend and CH rock bonds respectively and alkanes functional group which indicated the degradation of polyethylene. In a research study, no extra functional groups were formed, just the intensity in many groups were changed. (Divyalakshmi and Subhashini, 2016). Here decrease in absorption and increase in transmission in FTIR was noted.



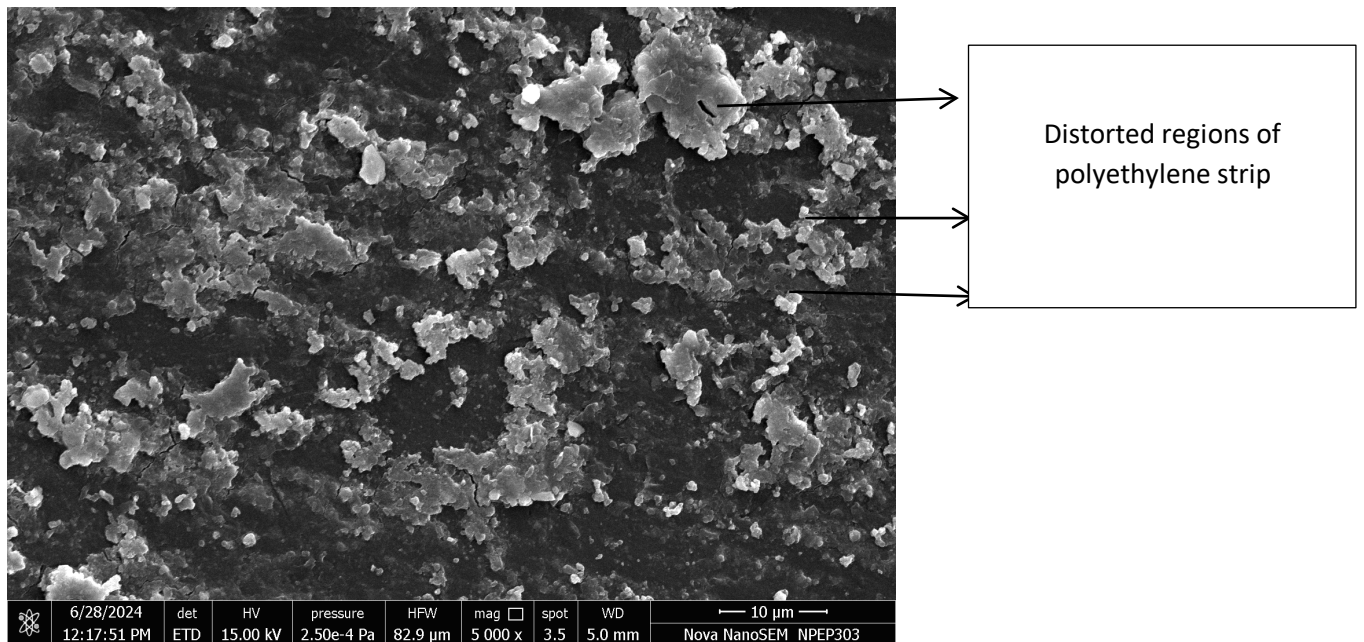
**Fig.5: FTIR analysis of polyethylene sheet treated with isolate BIS8**

**c) Scanning electron microscopy:**

The promising isolate was used for treating polyethylene. Then, further the isolate was found promising to degrade polyethylene. The microplastic sheets after the treatment were analysed by SEM. The change in morphology and structure was examined by SEM. (Distortion of polyethylene sheet) (Fig.6 and 7)



**Fig.6: SEM of Polyethylene control**



**Fig. 7: SEM of Polyethylene sheet treated with BIS8**

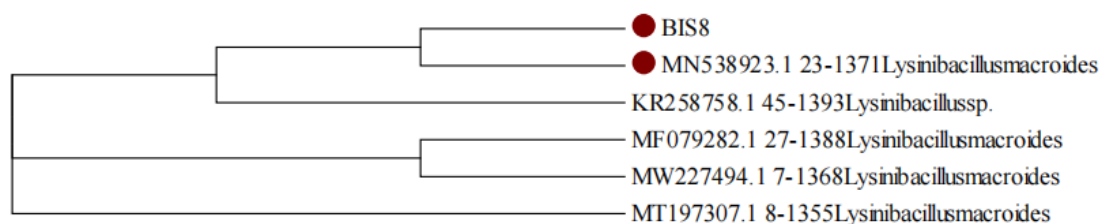
As depicted in Fig. 6 and 7, it is clear that there is a change in the morphology and structure. The control sheet is plain and unaffected while the treated sheet has pits and holes (distortion) on it which proves the potential of the isolate to degrade polyethylene. The formation of pits and holes as shown in Fig.7 depicts the initiation of degradation of process while the control sheet is plain without any change in morphology. The isolate BIS8 has been found promising to degrade polyethylene which is evident in Fig.7.

The isolate BIS8 has carried out the weightloss of 40%.

In a research study polypropylene degradataion was analysed by SEM. The plastic weight when treated with *A. fumigatus* degraded upto 9.5% of plastic over a period of 6 months. The SEM of the treated sample resulted in formation of wrinkles and foldings with development of streaks and rifts (Oliya et al., 2020). While in this study large pits and holes were formed which indicated the promising property of *L. macroides*.

#### 4) Characterization and identification of promising Isolates:

The isolate BIS8 was identified by 16S rRNA gene sequencing and it was found that it was *Lysinibacillus macroides*.



**Fig. 7: Phylogram of *Lysinibacillus macroides* based on 16S rRNA gene analysis**

The promising isolate to degrade polyethylene was found to be *Lysinibacillus macroides*. There are reports of *Lysinibacillus* sp which was isolated from soil showing promising results in degradation of polyethylene and polypropylene. There are reports that *Lysinibacillus* when combined with *Aspergillus* gives promising results in degradation of microplastics by degradation of 29.5% for UV-irradiated films and 15.8% for non-UV-irradiated films (Esmaeili *et al.*, 2013). There are certain reports of *Lysinibacillus* degrading polyethylene by 9% within 26 days of incubation without any pretreatment (Jyoti *et al.*, 2021). The isolate *Lysinibacillus macroides* showed better results of degradation i.e. 40% in 30 days(4 weeks). The positive control *Bacillus megaterium* showed 30% degradation in 30 days. Better results may be owing to proper enrichment and adaptation. The employment of *Lysinibacilli* for degrading polyethylene gives promising results. And these bacteria along with other fungus such as *Aspergillus* carry out the degradation of polyethylene and gives remarkable results . The use of *Lysinibacillus* for biodegradation on lab-scale gives amazing results but use of this bacterium on-field is quite a challenging task and has limitations. The involvement of other microbes and their enzymes can influence the action of *Lysinibacillus*. Thus, research must be done on application of microbes on field. The use of consortia of bacteria and molds can be done to carry out onfield degradation. The consortia of compatible organisms gives better results than the single isolate (Bardaji *et al.*, 2020; Gao and Sun, 2021). The present isolate BIS8(*Lysinibacillus macroides*) can be further used in consortia for better results.

### **Conclusion:**

The natural sources such as plastic dumping areas have the microorganisms which are a natural process contributing to reduce the problem of plastic pollution. The microorganisms on the plastic dumping grounds carry out the biodegradation. The samples were collected and the microorganisms were isolated and multiplied for employing in biodegradation process in the laboratory. The isolate BIS8 showed promising results by degrading 40% of the polyethylene in Mineral Salt medium with polyethylene as the only carbon source and identified as *Lysinibacillus macroides*. This depicts that the microorganisms have the potential to degrade microplastics. The degradation potential of the bacteria can be employed to degrade polyethylene on large-scale. However, there is a chance of alteration of activity of the bacteria by other microbes, several measures can be adopted for successful remediation process. We can use consortia of compatible organisms for fast and promising results. Even the promising isolate can be genetically engineered to withstand various environmental changes and outdoor effects . Even various enzymes produced by the isolate to carryout enzymatic degradation can be extracted and used on-field. The use of microbes on-field is not a good idea as the interference of other microbes and their enzymes may affect the activity of promising microbe. Hence, isolation of enzymes from promising microbe and treatment of plastic with the enzyme will give remarkable results. This will give remarkable results without any interference of other factors.

### **Conflict of interest:**

There was no conflict of interest amongst the authors.

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