Research Article

Isolation and Characterization of Polyethylene-Degrading Microbes from the Digestive System of *Perionyx excavatus*

Kannabiran. R[1], Logeshwaran Vellingiri[2*], Dr.P.Chidambararajan[3], Abilash. G[4]
[1]M.Sc Student, [2]Research Scholar, [3] Professor & Head, [4]Research Scholar
PG and Research Department of Biotechnology, Dr. N. G. P Arts and Science College, (Autonomous) Coimbatore-48, India.
***Corresponding author's E-mail:** drchidambararajan@drngpasc.ac.in

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ABSTRACT

Background: Polyethylene's resistance to degradation and adverse environmental impacts necessitate urgent intervention. This study explores microbial degradation, focusing on earthworms, particularly *Perionyx excavatus*, as potential agents for efficient plastic degradation.

Methodology: 11 bacterial and 5 fungal strains isolated from *P. excavatus* were characterized for their potential in polyethylene breakdown. Polyethylene underwent pre-treatment with 30% H2O2 under UV light, inducing observable changes. Microbial degradation experiments compared bacterial and fungal rates. Microscopic imaging and FTIR analysis validated structural changes, and weight loss measurements quantified degradation.

Results: Fungal strains exhibited higher polyethylene degradation rates than bacteria. Microscopic imaging and FTIR analysis confirmed successful degradation by both. Weight loss measurements quantified substantial compromise in polyethylene tensile strength.

Conclusion: Microbial-mediated degradation, exemplified by bacterial isolate 6 (BAC 6), offers a sustainable approach to polyethylene waste management. BAC 6, a novel species related to Bacillus cereus strain JCM 2152, This research highlights the efficacy of earthworm-associated microbes in polyethylene degradation, offering a sustainable waste management solution with global implications for addressing plastic pollution. The findings contribute to interdisciplinary approaches for a sustainable future.

Keywords: Polyethylene Biodegradation, Earthworm, Sustainable Plastic Management, Environmental Remediation.

INTRODUCTION

Polyethylene, a widely used synthetic polymer, was first discovered in 1933 by Eric Fawcett and Reginald Gibson at Imperial Chemical Industries (ICI) in the UK. Over the years, plastic production has skyrocketed, contributing to a significant environmental issue. Plastic waste, particularly polyethylene, is a major pollutant, originating from fossil fuels and causing adverse effects on ecosystems globally. Plastic waste generation per person is alarming, with 0.34 kg produced daily on average. In India, this figure is slightly lower, at 0.01 kg per person per day. 380 million metric tons of plastic were produced worldwide in 2019 alone. Unfortunately, the recycling rates remain relatively low, with only about 9% of all plastic waste generated being recycled.

Plastic pollution poses a grave threat to biodiversity, impacting over 270 marine species and disrupting the natural life cycles of organisms, from sea birds to marine mammals. Beyond aquatic ecosystems, microplastics infiltrate terrestrial environments, affecting soil properties, nutrient uptake, and plant health. Plastic contaminants, especially persistent organic pollutants (POPs), cause internal damage to aquatic life, leading to behavior changes, growth abnormalities, and health issues, including cancer. The primary challenge in addressing plastic pollution lies in the durability of plastics, compounded by processes like photodegradation and biodegradation[2]. Plastic additives, such as endocrine-disrupting chemicals, further contribute to health concerns, and the need for urgent alternatives.

Recent efforts have focused on the natural degradation of polyethylene, exploring the hidden properties of microorganisms. Earthworms, particularly *Perionyx excavatus*, show promise in bioremediation4. With their ability to convert organic waste into biofertilizers and their association with plastic-degrading microbes, earthworms can play a crucial role in mitigating plastic pollution. Plastic pollution remains a global challenge with far-reaching ecological and health consequences. The alarming statistics underscore the urgency of adopting sustainable practices[21]. Earthworm-mediated bioremediation emerges as a potential solution, offering a natural and effective way to address plastic pollution and promote environmental sustainability. Continued research and proactive measures are essential to mitigate the impacts of plastic pollution and ensure a healthier planet for future generations.

MATERIALS AND METHODS

Organism for Study:

Perionyx excavatus specimens were collected from Selvam Organic Farm, Udumalpet, Tiruppur, at coordinates 10.5810° N, 77.2040° E. Authentication was performed by

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Dr. Kathireshwari from the Department of Zoology, Kongunadu Arts and Science College, Coimbatore. Earthworms were incubated in 15-day pre-compost cattle manure (urine-free) for adaptation[1,5].

Test Condition:

Twenty earthworms were grown in laboratory conditions within 14x10x4 cm-sized containers, and acclimatized for one week. Two replicates were maintained, with a moisture content of 76–83%, a temperature of 25±1°C, and a pH of 7. Earthworms were incubated in a 12L/12D photo period using 300 g of pre-composted urine-free cattle manure. Earthworms weighing 800–1000 grams after one week were utilized for experiments[1,41].

Preparation of Sample (PE, LDPE&HDPE):

Three polyethylene variants - Polyethylene (PE), Low-Density Polyethylene (LDPE), and High-Density Polyethylene (HDPE) - were utilized. Samples were sliced to 0.5mm, and LDPE and HDPE were pretreated with 30% H_2O_2 and UV light for 15 days [22, 45].

Preparation of Earthworm Extract:

Earthworm extracts were prepared from two earthworms per replicate, either through the homogenized or sliced method. The extracts underwent serial dilution up to 10-7 after sterilization with 30% and 70% ethanol. Extracts were prepared for both methods for further microbial isolation[1,4].

Isolation of Microbes:

Extracts were inoculated on nutrient agar and sabouraud dextrose agar for bacterial and fungal growth, respectively. Incubation was performed at 33±2°C for 4-8 days for bacteria and at 24±2°C for 7-14 days for fungi. Isolated colonies were purified using the quadrant streaking method and subcultured on nutrient agar slant and sabouraud dextrose agar.

Identification of Microbes:

Identification involved colony morphology observation for bacteria and lactophenol cotton blue staining for fungi. For bacteria, Gram's staining and biochemical tests (Indole, Methyl red, Voges Proskauer, Triple sugar iron test, and Simmons citrate agar) were conducted. For fungi, initial identification was based on lactophenol cotton blue staining.

Colony Morphology:

Colony morphology was assessed based on margin, color, elevation, and shape.

Gram's Staining:

A smear of organisms was prepared and subjected to crystal violet, gram's iodine, 95% ethanol (Decolourizer), and saffranine treatment for microscopic observation[32].

Biochemical Analysis:

Indole Test: Inoculation into tryptophan broth followed by Kovac's reagent addition after 24-28 hours at 37°C.

Methyl Red Test: Inoculation into MRVP broth followed by 0.02% methyl red solution addition after 24 hours at 37°C.

Voges Proskauer Test: Inoculation into MRVP broth followed by 5% alpha-naphthol and 40% potassium hydroxide addition after 24 hours at 37°C.

Simmons Citrate Agar Test: Streaking on simmon's citrate Agar slant followed by incubation at 35-37°C for 4-7 days.

Triple Sugar Iron Test: Streaking on TSI agar slant followed by incubation at 35-37°C for 18-24 hours.

Screening of Polyethylene Degrading Microbes:

Isolated microbes were acclimatized with polyethylene samples for 24 hours for bacteria and 3-4 days for fungi. Biodegradation was assessed with a 1mg/ml (25mg/25ml) concentration of a combination of LDPE and HDPE over 15 days. Additional assessments were made without pretreatment, and glucose was periodically added to bacterial inoculation. Polyethylene-degrading bacteria were isolated based on weight loss, microscopic observation, and FTIR analysis (PE, LDPE &HDPE). Screened microbes underwent further analysis by 16S rRNA sequencing.

RESULT AND DISCUSSION

Primary treatment of polyethylene

The polyethylene (LDPE&HDPE) is pre-treated with 30% H_2O_2 under UV light for 15 days to weaken the strength of the polyethylene due to its complex structure. During the exposure period, the polyethylene (LDPE&HDPE) can attain some chemical and physical modifications. The pre-treatment of polyethylene (LDPE&HDPE) both gives an unpleasant smell after 2-3 days of exposure of 30% in H_2O_2 under UV light. The color of LDPE changes from white to eggshell white color, and the passing of light intensity is high compared to control LDPE[44]. The HDPE color changes from white to pale yellow, which indicates the LDPE and HDPE are weakened by the pre-treatment of 30% in H_2O_2 under UV light[2, 35].

Isolation of microbes

Homogenized was carried by Samanta et al.[1], in the presence of the study, the homogenization and slicing method was carried out to compare the presence of microbes in the gut and to isolate the gut microbes for the study. In both the methods, the 11 bacterial isolates and 5 fungal isolates have been isolated, and compared. It is found that by both the methods, same isolate was found.

Initial identification of microbes

The initial identification is done by colony morphology, gram staining, and biochemical tests.

Colony morphology (BAC- Bacteria, FUN- Fungi)

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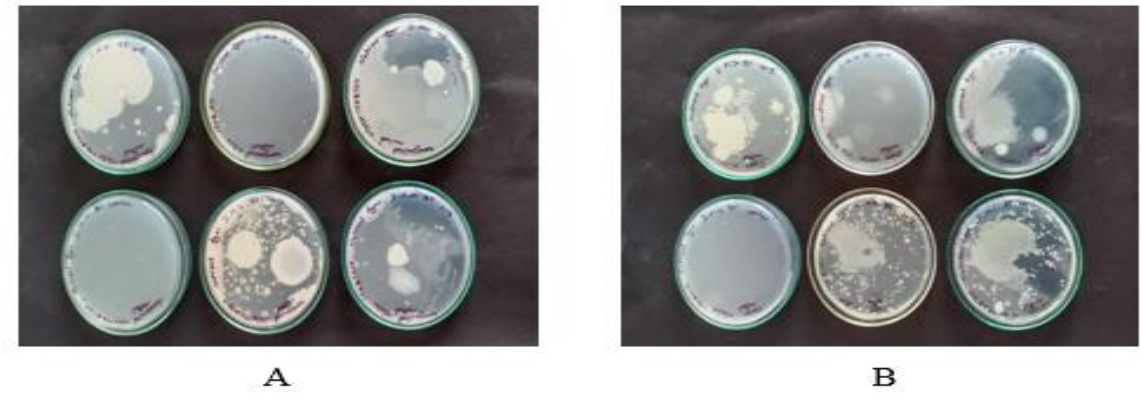

(A - homogenized method, B – sliced method)

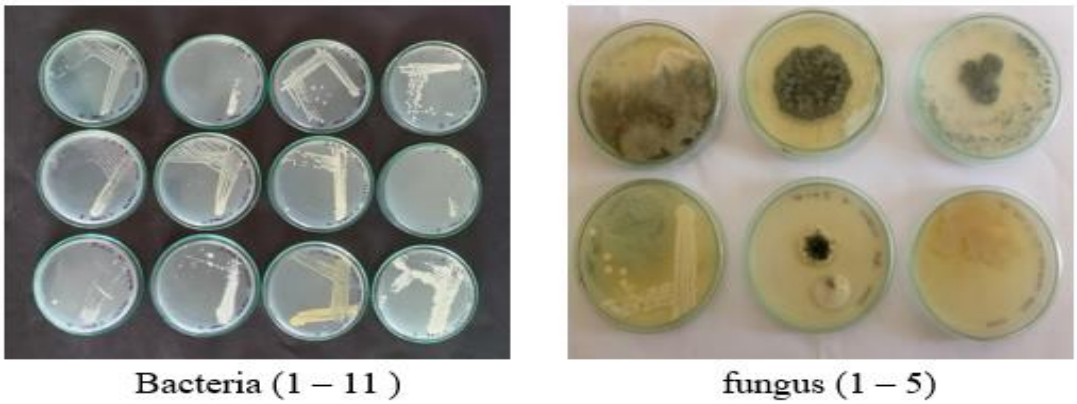

Figure 1: Isolation of microbes

Table 1: Colony Morphology

Isolate	Margin	Color	Elevation	Shape
1	Entire	White	Flat	Round
2	Entire	Milky White	Convex	Round
3	Filiform	White	Flat	Filamentous
4	Entire	White	Convex	Round
5	Entire	White	Flat	Round
6	Entire	White	Convex	Round
7	Entire	Milky White	Flat	Punctiform
8	Entire	White	Flat	Punctiform
9	Entire	Orange	Flat	Punctiform
10	Filiform	White	Flat	Filamentous
11	Entire	White	Flat	Punctiform

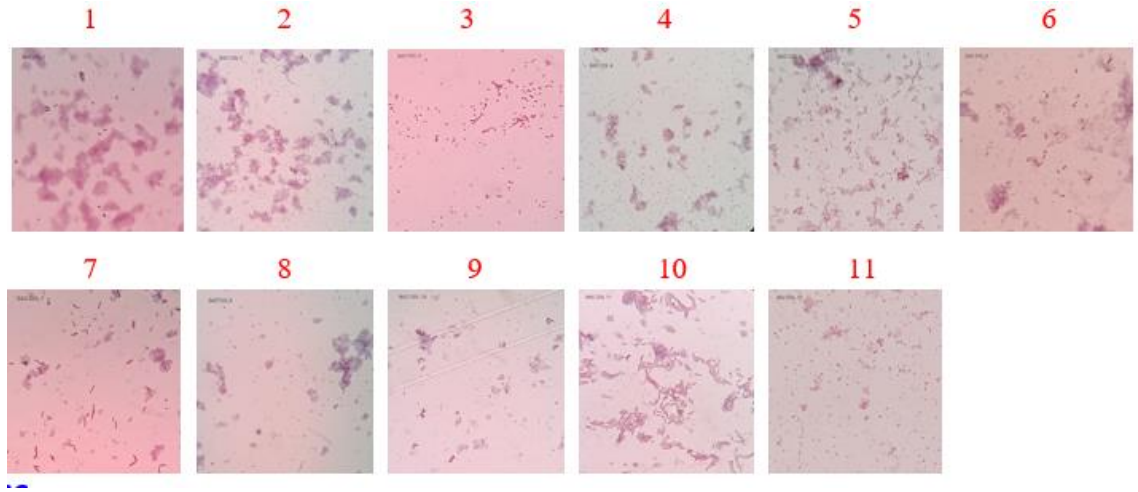

Figure 2: Gram's staining

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The morphological identification of bacterial isolates revealed consistent entire margins, with exceptions in BAC 3 and BAC 10, exhibiting filiform margins. colors varied. The result is shown in Table 1.

Gram's staining

Bacterial isolates were done for gram staining, 9 organisms were shown gram-positive due to their purple color, they are BAC 1, BAC 2, BAC 3, BAC 4, BAC 5, BAC 6, BAC 8, BAC 9, and BAC 10. Whereas 2 organisms are Gram-negative due to pink color, they are BAC 7 and BAC 11. The results are shown in Figure 2 and Table 2.

Biochemical test

The biochemical tests conducted on bacterial isolates included Indole, Methyl Red, Voges Proskauer, Triple Sugar Iron, and Simmons Citrate tests.

Indole Test: Only BAC 2 showed a positive result, indicating the production of tryptophanase.

Methyl Red Test: BAC 1 and BAC 2 exhibited a positive result, while BAC 6 showed a negative result due to the production of unstable acids.

Voges Proskauer Test: BAC 3 and BAC 6 yielded a positive result, indicating fermentation to produce acetoin and butylene glycol.

Triple Sugar Iron Test: BAC 5 and BAC 9 gave positive results, changing media color from reddish-brown to yellow, while others were negative.

Simmons Citrate Test: BAC 3, BAC 5, BAC 6, BAC 7, and BAC 10 showed a positive result, indicating the ability to utilize citrate as a source of energy.

Overall, these tests contribute to the initial identification of bacterial isolates based on their biochemical characteristics.

Table 2: Gram's staining

Isolate	Gram Stain Result	Shape of bacteria
1	Gram-Positive	Rod
2	Gram-Positive	Cocci
3	Gram-Positive	Diplococci
4	Gram-Positive	Diplococci
5	Gram-Positive	Cocci
6	Gram-Positive	Rod
7	Gram-Negative	Rod
8	Gram-Positive	Rod
9	Gram-Positive	Rod
10	Gram-Positive	Rod
11	Gram-Negative	Rod

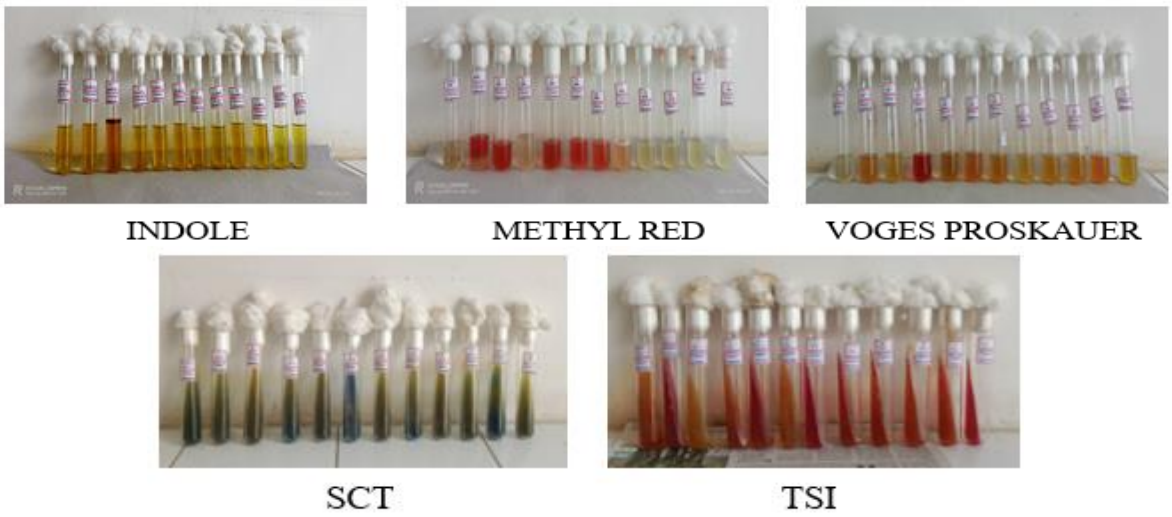

Figure 3: Biochemical Test

Table 3: Biochemical test

Isolates	Indole	Methyl red	Voges proskaur	T.S.A test	S.C A test
1	_	+	_	RS, RB	-
2	+	+	-	YS, YB	-
3	-	-	+	RS, RB	+
4	-	-	-	RS, RB	-
5	-	-	-	YS, YB	+
6	-	+	+	RS, RB	+
7	-	-	-	RS, RB	+
8	-	-	-	RS, RB	-
9	-	-	-	RS, RB	-
10	-	-	-	RS, RB	+
11	-	-	-	RS, RB	-

(RS – Red Slant, RB – Red Butt, YS – Yellow Slant, YB – Yellow Butt)

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Weight loss of polyethylene

Polyethylene degradation is analyzed by weight loss. Degradation of polyethylene, LDPE, and HDPE is calculated by the following formula.

$$\%\text{Biodegradation} = \frac{\text{Initial weight-Final weight}}{\text{Initial weight}} \times 100$$

1mg/ml concentration of polyethylene is incubated with a nutrient broth medium. A combination of both LDPE and HDPE with a 1mg/ml concentration of sample is inoculated with medium (12.5mg of LDPE and 12.5mg of HDPE) (25/ 25mg). After 15 days, the polyethylene, LDPE, and HDPE are gently washed with tap water and again washed with saline solution to remove bacterial cells on the surface of the polyethylene, LDPE, and HDPE. Polyethylene degradation was observed effectively with bacterial isolate 6 having 14% of degradation and bacterial isolate 10 had the limited capability of degradation of about 2.80%. Bacterial isolate 6 gives a good result on LDPE and HDPE, Bacterial isolate 6 degrades the combination of LDPE and HDPE by 6%. Followed by bacterial isolate 6, bacterial isolates 5,8 and 11 also have the capability of degrading the LDPE and HDPE at 1.20%, 3.20%, and 1.60% respectively. The result is shown in chart 1-PE, LDPE &HDPE

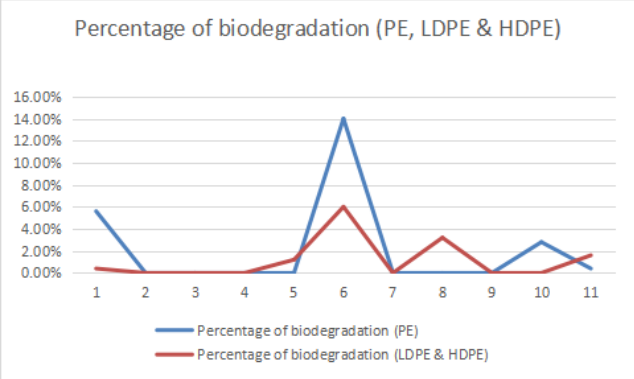

Chart :1

Fungal isolates also have the capability of degrading polyethylene higher than bacteria. The same bacteria fungal also incubated with Polyethylene, LDPE, and HDPE with sabouraud dextrose broth. The fungal degradation is higher than bacterial degradation. Fungal isolate 1 gives high degradation of polyethylene at 26%. Followed by fungal isolates 2,3,5 and 6 also have the capability of degrading polyethylene, at 8%, 2%, 12%, and 20% respectively[2,49,50].

Fungal isolate 1,5 effectively degrades the LDPE and HDPE at 22%. Followed by Fungal isolates 2,4,6, also capable of degrading the LDPE and HDPE at 5%,13%, and 21% respectively. The result is shown in chart 2-PE, LDPE &HDPE

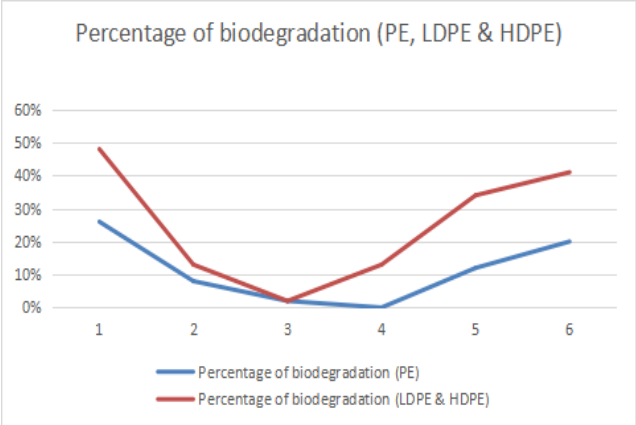

Chart :2

Morphological analysis of polyethylene, LDPE, and HDPE

The biodegradation of polyethylene has been further confirmed by using a simple microscope at 45X magnification. And saw the border and edges of the polyethylene. After that, viewed light intensity. Compared to the control, the intensity of the light is gradually increasing, like UV LDPE, BAC 6LDPE, and FUN 1LDPE. Microbial attachment spaces are viewed, which indicates the microbial attachment on the surface of the polyethylene, LDPE, and HDPE. The images are shown in Figure 4.

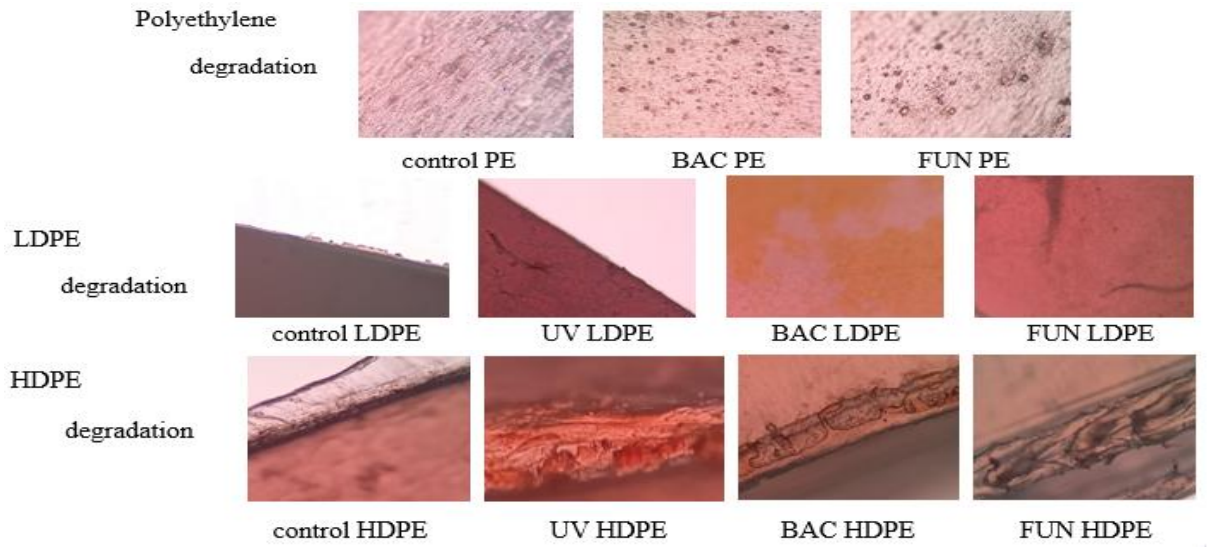

Figure 4: Microscopic imaging of polyethylene, LDPE, and HDPE (BAC 6 & FUN 1)

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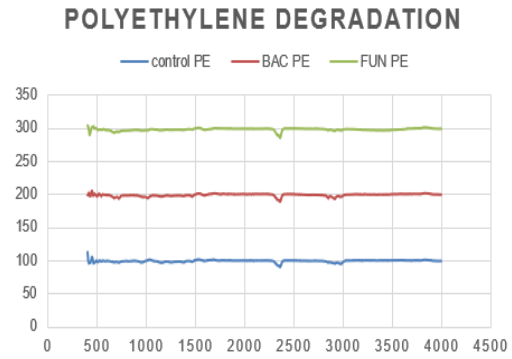
When treated with UV the polyethylene degradation is noted by slight damage on the surface of the polyethylene. Similarly, bacteria and fungi are gradually increasing the damage of polyethylene. The LDPE and HDPE degradation is done through the passing of light intensity and microbial attachment[2].

FTIR analysis of Polyethylene, LDPE, and HDPE

The pre-treated LDPE and HDPE effect by UV light and hydrogen peroxide is confirmed by the FTIR analysis. Polyethylene, LDPE, and HDPE without any treatment is used as a control. The primary weakening of LDPE and HDPE is confirmed by the FTIR analysis. PE, LDPE & HDPE were analyzed by FTIR for the supporting evidence for the biodegradation.

FTIR analysis of polyethylene

The FTIR results showed polyethylene degradation band was formed and compared to the control polyethylene. In bacterial degradation polyethylene, four bonds are observed in the spectra due to the vibration and stretching of atoms. The bacterial isolate 6 (BAC6) of presence toward the biodegradation of polyethylene was analyzed by FTIR, and the presence of BAC 6 new bands was assigned in different spectrums. The bands are 2021.40 cm-1 (diazo compounds), 1897.95 cm^{-1} aromatic compounds,1751 cm^{-1} (alkyl carbonate) and 1010.70 cm^{-1} (cyclohexane). the diazo compounds vibrate and stretching is the diazo compound to form carbene or nitrene reactivates[46]. The result is shown in Graph 1 and Table 4 The polyethylene degradation by FTIR analysis (BAC 6 & FUN 1)



Graph 1: polyethylene degradation

Microbes	FREQUENCY	ABSORBTION cm-1	APPEARANCE	GROUP	COMPOUND CLASS
Bacterial	2050 - 2000 cm^{-1}	2021.40	Very strong	C – N – N stretching vibration	Diazo compounds
Bacterial	2000 – 1660 cm^{-1}	1897.95	weak	Ortho overtone vibration	Aromatic compounds
Bacterial	1805 – 1755 cm^{-1}	1751	Very strong	C = O stretching vibration	Alkyl carbonate
Bacterial	1015 - 950 cm^{-1}	1010.70	Weak	C – C skeleton vibration	Cyclohexanes
Fungus	1150 – 1085 cm^{-1}	1134.14	Strong	C – O stretching	Aliphatic ether

Table 4: polyethylene degradation

FTIR analysis of BAC 6 shows a different spectrum, it indicates the presence of Dia 20, aromatic compound, and alkyl carbonate with absorbance of 2021.40 cm^{-1}, 1897.95 cm^{-1}, and 1751 cm^{-1} respectively. Also, cyclohexanes at 1010.70 cm^{-1}, and diazo compounds indicate vibrating and stretching to carbene or nitrene reactives [48].

In fungal degradation of polyethylene, one bond was observed in spectra due to stretching of C-O, the band formed the 1150-1085 frequency range and the absorption band was 1134.14 cm^{-1}.

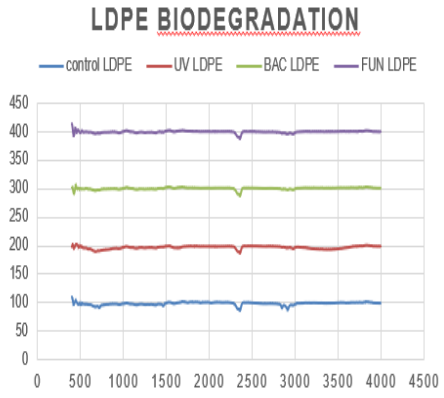
LDPE- FTIR analysis

The LDPE was pre-treated with UV light and hydrogen peroxide, the UV light caused the LDPE to break down the internal band, and absorption at 3371.57 was formed due to O-H stretching. The formation of alcohol on the surface of the LDPE is due to thermo-oxidative and photooxidative degradation. Pre-treated LDPE shows 2 bands at 3371.57, and 1635.64 due to the O-H stretching and C=C stretching and vibration[9].

In bacteria (BAC 6), one band is observed at 3726.47, due to the O-H Stretching, and breakdown of the inner band by the free alcohol.

In fungus (FUN 1), one band is observed at 1257.59, it is formed due to the c-o stretching the compound is an aromatic ester[46]. The result is shown in Graph 2 and Table 5.

Biodegradation of LDPE (BAC 6 & FUN 1)



Graph 2: LDPE degradation

Microbes	FREQUENCY	ABSORBTION cm-1	APPEARANCE	GROUP	COMPOUND CLASS
UV Treated	3550 – 3200 cm^{-1}	3371.57	Strong	O - H stretching	Alcohol (intra molecular bonded)
UV Treated	1640 – 1630 cm^{-1}	1635.64	Strong	C = C stretching vibration	Vinyl Ether
Bacterial	3700 – 3584 cm^{-1}	3726 .47	Strong	O – H stretching	Alcohol (free)
Fungal	1310 – 1250 cm^{-1}	1257.59	Strong	C -O stretching	Aromatic ester

Table 5: LDPE degradation

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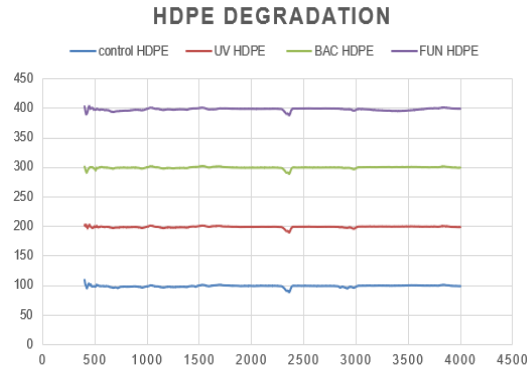
HDPE – FTIR analysis

The FTIR analysis of HDPE is treated with UV light and Hydrogen peroxide. Due to the formation of a band between 825 and 810, the absorption frequency is 810.1, this band indicates that the deformation of CH_2 can happen in vinyl ether [18].

In bacteria (BAC 6) two band is deformed due to the C-H stretching in the ketone (alkene) group in the HDPE, Bands 2916.37 and 1465.9, both indicate the deformation of the alkene group in the HDPE[46].

In fungal (FUN 1) two absorptions were observed. These are the 1257.59, due to stretching C-O, this compound is secondary alcohol and aromatic ester[48]. The result is shown in Graph 3 and Table 4

Biodegradation of HDPE (BAC 6 &FUN 1)



Graph 3: HDPE degradation

Treatment/Microbes	FREQUENCY cm-1	ABSORBTION cm-1	APPEARANCE	GROUP	COMPOUND CLASS
UV Treated	825 – 810	810.1	Strong	CH2 out-of-plane deformation vibration	Vinyl ether
Fungal	1310 – 1250	1257.59	Strong	C -O stretching	Aromatic ester
Fungal	1124 - 1087	1087.85	Strong	C – O stretching	Secondary alcohol
bacterial(D)	3000 - 2840	2916.37	Medium	C - H Stretching	Alkene
bacterial(D)	1465	1465.9	Medium	C - H bending	Alkene

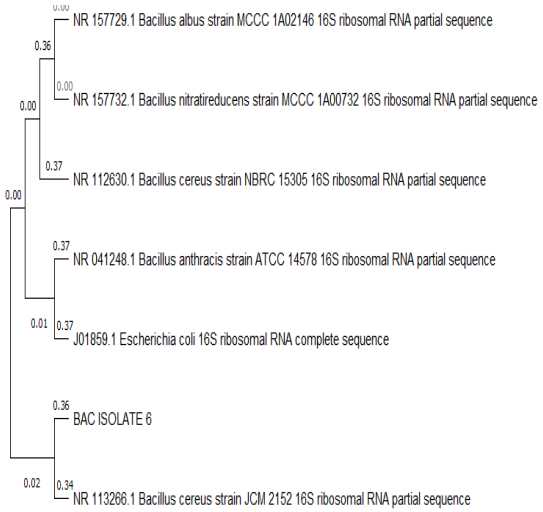
Table 6: HDPE degradation

Sequencing of screening organisms

The isolated organisms from the gut of the *Perionyx excavatus*. In that bacterial isolate 6 (BAC 6) and fungal isolate 1(FUN 1) had high capability to degrade the polyethylene, LDPE, and HDPE. The 16s rRNA sequencing is performed for the bacterial 6 isolates (BAC 6). Based on the sequencing, the data applied in the mega software isolated organism is a novel species based on the phylogenetic tree analysis of the isolate. From sequencing data, the phylogenetic tree was constructed by using mega software

The Bacterial 6 isolate (BAC 6) is similar to *Bacillus cereus* strain JCM 2152, with 99.89 % sequence similarity identified.

PHYLOGENETIC TREE



CONCLUSION

Microbial isolates, both bacterial and fungal, exhibited varying degrees of PE degradation. Weight loss measurements confirmed the degradation process, with fungal isolates generally outperforming bacteria. Microscopic imaging and FTIR analysis provided visual and structural evidence of microbial action on PE. Bacterial isolate 6 (BAC 6) and fungi 1 showed high efficacy, identified as novel species closely related to *Bacillus cereus*. This study showcases the potential of earthworm-associated microbes, particularly BAC 6, in degrading PE. The findings contribute valuable insights to the academic discourse on sustainable waste management practices, emphasizing the importance of interdisciplinary approaches for addressing the global plastic pollution crisis. Further research and proactive measures are essential for implementing effective solutions and ensuring a sustainable future.

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