Research Article

Detection of Nano Plastic Particles Produced by Gram Negative Plastic Degrading Bacteria Isolated from *Galleria mellonella* Larvae and Soil Samples in Mosul City: A Sustainable Approach to Plastic Waste Management

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Abstract | Plastic production increased dramatically, affecting both human and animal health when plastic waste aggregate in the environment, due to the hardness of polymers and non-solubility in water, natural deterioration of plastic is very slow, where bacteria play vital role in degradation various types of plastic polymer, it is the safe and sustainable solution for ecosystem. The capacity of degradation of polymer including polyethylene terephthalate (PET) low density polyethylene(LDPE) using bacterial isolates was detected. Nine soil samples were collected from (landfill places, agricultural soil- oil polluted soil), and three samples from *Galleria mellonella* were used. After 4 months of incubation (12) Gr - bacterial strain that have ability to degrade polyethylene were identified based on 16srRNA sequence, Six of them degraded PET, while the other six isolates degraded LDPE. The utilization of PET by bacterial isolates under study was better than LDPE, according to the results of FTIR spectrum, GCMS ,weight loss, and SEM techniques. FTIR analysis showed variation in peaks, form new chemical bond in polymer, the most potent ability to degraded (PET) was achieved by *Enterobacter hormaechei* ZEV5.iq and *Enterobacter kobei* ZEV4.iq (isolated from soil) at 56% and 55% respectively, and *Enterobacter cloacae* (isolated from *Galleria mellonella*) at 56%. Whereas, the ability to degrade (LDPE) was achieved by *Achromobacter piechaudii* with 50%. GC-MS analysis indicated to formation of fatty acid, alcohol, nitrile, Aldehyde and other new compounds. SEM appeared to be altered in surface of plastic, EDS detect high atomic ratio of oxygen and disappearance in atomic ratio of carbon content. The non-toxicity of final product of polyethylene was proved by using it as supportive and growth enhancing of *Lepidium sativum* seeds with germination rate at (80-90)%.

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Introduction

The improvement of technology led to increase consumption of plastic in the world, the first advanced plastic, Bakelite, made in 1907. Numerous procedures advanced for the mass of plastic, plastic polymer utilized in different industries and commerce institutions. Plastic have appropriate properties such as durability, non-reactivity with other material, low cost, flexibility, and resistance to corrosion, which make request for plastic developed day to day (Hale *et al.*, 2020). Polyethylene (PE) is a large molecule made from long chain monomers of ethylene (C2H4), made by polymerization, PE made of carbon, hydrogen, sulfur, nitrogen, and many organic and inorganic resources, most wastage of polyethylene products are water bottle which consist of polyethylene terephthalate PET and shopping bag which consist of Low density polyethylene LDPE. Polyethylene has non-polar nature, therefore plastic need longer time for decomposition, waste build up in landfills for many years, to decrease them waste incineration that release toxic gases in ecosystem, sustainable practice through biodegradation of plastic waste (Faisal and Al-Saffar, 2023). Biodegradation employing bacteria, fungi that exist in soil, and gut microbes of larvae insects considers as a good choice (Mishra *et al.*, 2020). These organisms have enzymatic activity for hydrolyzing polymer by attacking polyethylene, breaking it into smaller fragments. Bacteria metabolize end product of polymer decomposition and utilize carbon compound which present in plastic as a source of energy. This solution is eco-friendly and more benefits than other chemical and physical methods which need expensive treatment and may cause serious collapses to environment (Sheldon and Norton, 2020).

The concentrated studied of *Galleria mellonella* began in 2007 and paved the importance way in plastic deterioration by insects. Insect larvae and intestinal microorganisms have a mutually valuable symbiotic relationship therefore the larvae consumed low density polyethylene LDPE and polyethylene terephthalate PET in their nutrition, because there is a structural similarity between beeswax and Polyethylene. Scientists isolated bacteria from the intestine of insects which depolymerize long chain of polyethylene and advance mineralize the metabolites substance to CO_2 (Geyer, 2020). Based on mentioned above, this study aimed to detection of the ability of bacterial species isolated from soil and Wax Moth *Galleria mellonella* to degradation and consumption of polyethylene terephthalate, and low density polyethylene.

Materials and Methods

Media used for isolation

Bushnell Hass broth was used to study the capability of bacterial isolates to degrade polyethylene as a source of carbon and energy, which was prepared according to Bukht *et al.* (2020). The media was distributed in 26 flasks, each one contained 100ml, then flasks were divided in to two groups. First group including 12 flasks, (1g) of local polyethylene terephthalate was added to each flask. As for the second group, (1g) of local low density polyethylenec. After that all flasks autoclaved in autoclave.

Soil samples collection and preparation

Nine samples of soil were collected from October to December 2024 in Mosul city, involving: Agricultural soil, landfills, and oil polluted soil at depth of 20 cm. After removing impurities, soil samples dried at room temperature. One gram of each dried soil added to 9 ml of sterile distilled water to prepare serial dilutions, up to (10^{-3}) which considers as a source of bacteria that can be used to analyze polymer. Then (100µl) of (10^{-3}) dilution added to 20 flasks, two of them used as control, All flasks incubated in shaking incubator at 28 °C with 150 rpm for (120) days (Feil and Pretz, 2020).

Bcterial isolation from Galleria mellonella

Three samples obtained from *Galleria mellonella*, the surface of larvae sterilized by immersing them in 70% ethanol, rinsing with sterile distilled water, then the larvae dissected under sterile condition. The gut tissue homogenized in a sterile saline solution to create a microbial suspension, which then inoculated in (6) flasks of Bushnell Hass broth (3) of them containing 1 g of local polyethylene terephthalate and the other (3) containing 1 g of local low density polyethylene as the sole carbon source. All flasks Incubate at 28 °C for (120) days (Brandon *et al.*, 2021).

Isolation of bacteria

After the end of incubation period, 1 ml of each flask was taken to make serial dilution up to 10^{-3} and (0.1 ml) of each dilution was spread on nutrient agar plate and incubated at (28 °C) for 48h (Al-Omari *et al.*, 2024).

Identification of bacteria

Diagnosis of bacterial isolates depending on its phenotypic characteristics involved (colony's shape, color and Gram stain). The diagnosis confirmed by using *16srRNA* gene (Al-Jarjary and Alsammak, 2023).

Molecular identification of bacteria species

DNA extraction

DNA extraction was conducted using kit provided from Geneaid /Taiwan, according to company instructions. The concentration and purity of DNA extracted was measured using Nano-drop Spectrophotometer (Bio Drop/ England) in Research laboratory of Department of Biology, College of Science (Khalaf and Altaii, 2020). Bacterial isolates identified using universal primers provided from Macrogene company listed in Table 1 (Hasan and Al-Sammak, 2024).

Table 1: *Primers used to amplify the 16S rRNA gene.*

Primer	Primer sequence (5' to 3')	Size of product (bp)
27F	AGAGTTTGATCTGGCTCAG	1465
1492R	GGTTACCTTGTTACGACTT	

Sequence for 16s rRNA gene

The sequence of all PCR product conducted in Psomagene company in Maryland, USA, Genetic congruence of 16s rRNA gene determined by using Basic Local Alignment Search Tool (BLAST) which is available in National Center Biotechnology Information (NCBI) (Younis and Faisal, 2024).

Fourier-transform infrared spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (Bruker_ alpha, Germany) used to determined changes of functional structural in plastic polymer. FTIR was utilized after (120) days of incubation by taking apart of cultures and centrifuged at 3000 rpm for 10 min. Plastic pieces removed, washed with distilled water, dried at room temperature (25±2 °C) for 3 days, then recorded their infrared spectra on a FTIR spectrometer in the region 4000–400/cm (Elsamahy *et al.*, 2021). FTIR spectrometer available in Central Laboratory College of Science University of Mosul.

Weight loss experiment

Plastic pieces removed from broth culture, washed and dried at room temperature were weighted. The percentage of weight loss calculated by using the formula: (de Monte *et al.*, 2022).

$$Weight\ loss = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100$$

Gas chromatography mass spectrometry (GC-MS)

About 10ml of each culture was centrifuged at 6500 rpm for 10 min to remove cell debris. The supernatant dried at room temperature for 3 days and used for GC–MS analysis, which available in the Environmental Research Center in Baghdad University (Bryan *et al.*, 2020).

Phytotoxicity of cell-free culture supernatant

To study of phytotoxicity (10) seeds of *Lepidium sativum* (bought from a local market) were placed on plate containing filter paper. About (4) ml of supernant, that prepared by cenrifugation each culture at 6500 rpm for 10 min, was added to to each plate, control plate was used by addition (4) ml of water. All plates were preserved at room temperature 25°C, and seed germination was determined after 5 days of incubation in the dark place. The Relative seed germination calculated by using the formula: (Wang *et al.*, 2020).

$$Relative\ seed\ germination = \frac{Number\ of\ seeds\ germinated\ in\ the\ supernatant}{Number\ of\ seeds\ germinated\ in\ control} \times 100$$

Phylogenetic relationships of strains

To get evolutionary relationship for bacteria strains through compare the nucleotide sequence of 16s rRNA gene within program Mega 12 by using Unweighted Pair Group Method with Arithmetic mean UPGMA, depending on (Tamura *et al.*, 2021).

Scanning electron microscope (SEM)

The dried plastic pieces were assessed by scanning electron microscopy (SEM), The test performed in Alkhora laboratory in Baghdad city (Al-Salem *et al.*, 2019).

Energy dispersive X-Ray spectroscopy (EDS)

The powerful technique which study composition of nano elements and chemical properties of degraded polyethylene terephthalate, utilized in combination with scanning electron microscopy, detector of EDS placed near electron beam path. When sample bombed with a focused beam of high-energy electrons in Scanning Electron Microscope, atoms of the sample excited, transition of electron lead to releases energy in the form of X-rays, this energy is specific for each element, permit us to measure nano elements existing (Eriksen *et al.*, 2013).

Results and Discussion

Bacterial growth

After (120) days, 12 samples of our study have ability to growth and utilize LDPE and PET as sole source of carbon. Morphological and microscopic observations appeared that twelve bacterial isolates were Gram negative. Based on the capacity of degrade polyethylene terephthalate PET, four isolates from soil and two isolates from *Galleria mellonella* were identified using 16s rRNA gene and submitted to National Center for Biotechnology Information (NCBI) and each one given accession number as detailed in Table 2. The results of the active role of *Enterobacter* sp. in deterioration of polyethylene plastic are in agreement with (Bombelli *et al*., 2017; Ren *et al*., 2019). Regarding the role of *Pseudomonas* sp. in biodegradation of LDPE plastic was consistent to the results of (Gupta and Devi, 2020; Mehmood *et al*., 2023). The study conducted by Ajuzie *et al*. (2010) appeared the role of *Bordetella* sp. in bio-degradation of plastic and that agree with our result about its active role in bio-deterioration of polyethylene plastic. The results showed that LDPE degradation requires more time than PET, because the energies of the C–C and C–H bonds that present in Low density polyethylene are higher than energies of the C–O and C–N bonds which existing in PET, therefore LDPE more resistant to degradation than ester-bonded polymers in Poly ethylene terephthalate, and the combination of heteroatoms into the carbon chain makes PET more sensitive to biodeterioration for these reasons LDPE need to increase period time (Zhang and Yan, 2021).

Table 2: *Bacterial strains degraded PET and LDPE with their accession number.*

No. of bacteria	No	Bacterial strains	Accension number	Percentage of identification in NCBI
1	1	*Enterobacter kobei* ZEV4.iq	15313736	99%
2	2	*Bordetella petrii* ZE5.iq	15299810	99%
3	3	*Enterobacter cloacae* ZEV1.iq	15310482	98%
4	4	*Pseudomonas aeruginosa* ZE2.iq	15298602	99%
5	5	*Klebsiella aerogenes* ZE7.iq	15298591	99%
6	6	*Enterobacter hormaechei* ZEV5.iq	15310460	98%
7	7	*Achromobacter piechaudii*		80%
8	8	*Enterobacter cloacae*		98%
9	9	*Enterobacter* sp		84%
Bacteria isolate from *Galleria mellonella*				
11	1	*Enterobacter* sp ZE6.iq	15298586	84%
12	2	*Enterobacter cloacae*		99%
13	3	*Stenotrophomonas maltophilia* V3		99%

The highest degradation rate was obtained by *Enterobacter hormaechei* ZEV5.iq and *Enterobacter cloacae* at 56% based on weight loss of polyethylene terephthalate, while the lower rate of degradation was 34% by *Klebsiella aerogenes* ZE7.iq as showed in (Table 3).

Table 3: *Type of plastic degrading by bacteria and the percentage of weight loss.*

Bacteria	Type of plastic	Percentage of weight loss
Enterobacter hormaechei ZEV5.iq	PET	56%
Enterobacter kobei ZEV4.iq	PET	55%
Enterobacter sp.	PET	44%
Bordetella petrii ZE5.iq	PET	40%
	LDPE	
Achromobacter piechaudii	LDPE	50%
Pseudomonas aeruginosa ZE2.iq	LDPE	40%
Enterobacter cloacae ZEV1.iq	LDPE	40%
Enterobacter sp.	LDPE	38%
Klebsiella aerogenes ZE7.iq	LDPE	34%
Bacteria isolate from *Galleria mellonella*		
Enterobacter cloacae	PET	56%
Stenotrophomonas maltophilia	LDPE	45%
Enterobacter sp. ZE6.iq	PET	40%

Fourier-transform infrared spectroscopy FTIR

Changes in the structure of PET and LDPE after 120 days treatment with bacterial strains confirmed by FTIR spectroscopy. Results indicated to the change in peaks between the control and plastic treated with bacteria (Figure 1). These changes include decrease in the absorption peak in main structure region and disintegration of PET chains into smaller pieces. Appearance of new chemical bond formed at 3400 cm^{-1} and new functional hydroxyl group gave a sign to the role of bacteria and their enzymes in hydrolyzing long polymer chain. Formation of alcohol or carboxylic acid indicate hydrolysis of ester bond in polymer chain. Changes involved decrease intensity of peak at 1400 cm^{-1} indicate to depolymerized process of polyethylene occurred (Ren *et al*., 2019), that reflects

the capability of bacteria to degrade Polyethylene and use it as a source for their nutrition.

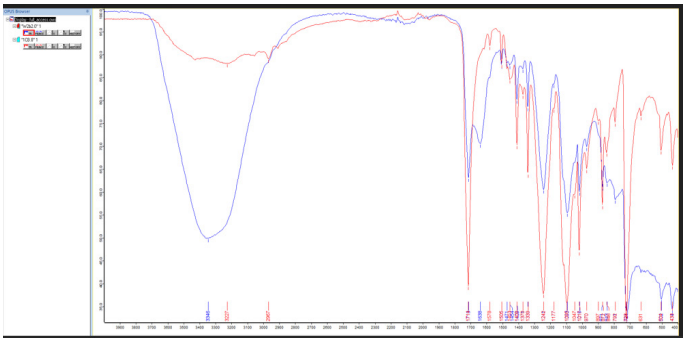

Figure 1: *The FTIR of PET polymer degrading by Enterobacter cloacae, Red color before treatment, Blue color after treatment with bacteria.*

Gas chromatography coupled with mass spectrometry (GC-MS)

Results of GC-MS demonstrated that polyethylene chains cleaved into smaller fragments during biodeterioration which conformed by forming various carboxylic acid (Jeon *et al*., 2021), (Table 4). Formation of intermediate products as fatty acids, alcohol, aldehyde, nitrile, and small alkanes compounds as a result of oxidative processes on polyethylene and that aline with which agree with the study conducted by Kumar *et al*. (2019).

Table 4: *Compounds produced through biodegradation by bacteria using GC–MS analysis.*

Bacterial strains obtained from *G. mellonella*	Type of plastic	Compounds formation
Enterobacter sp ZE6.iq	PET	Cyclobutanol, Cyclobutyl hydroxide, Cyclobutyl alcohol, Pyrrole, carbonitrile, -Amino -cyclohexyl-ethyl, -pyrano, pyran-3-carbonitrile, Cyclohexylphenylacetonitrile, Benzeneacetonitrile, cyclohexyl, Pentanenitrile, Valeronitrile, Isoamyl cyanide, Isocapronitrile, Methylpentanenitrile, Methylvaleron
Enterobacter cloacae	PET	Oxalic acid, cyclobutyl ethyl ester, Pyrrole-3-carbonitrile, thiophene-2-carboxaldehyde, Methyl-1-benzothiophene, carbaldehyde, Isobutyl nitrite, Nitrous acid, methylpropyl ester, isobutyl ester, Pentanenitrile, 4-methyl, Valeronitrile, 4-methyl, Isoamyl cyanide, Methylpentanenitrile, Methylvaleron. Formic acid, 2-methylpropyl ester, isobutyl ester, Tetryl formate, Methylpropyl formate, 1-Hexene, 5,5-dimethyl, Dimethylhexene, Fenoterol, N-trifluoroacetyl, trimethylsilyl,phenyl, trimethylsilyl.

Phylogenetic relationships of strains

The species under study clustered at each other with high similarity level as in Figure 2 *Klebsiella aerogenes* closely related to *Enterobacter* sp. and within same order Enterobacterales, clustered at similarity level 99.5%, *Pseudomonas aeruginosa* ZE2.iq and *Bordetella petrii* ZE5.iq clustered at similarity level 98.5%. While *Achromobacter piechaudii* clustered with them at similarity level 97.7% (Al-Najim and Alsammak, 2024).

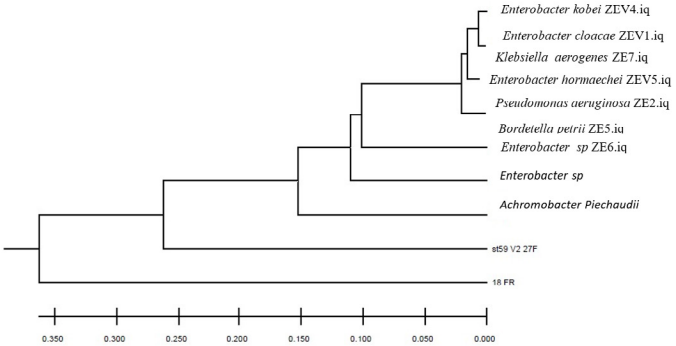

Figure 2: *Phylogenetic Tree of Bacterial strains under study within program Mega 12 by using UPGMA.*

Scanning electron microscope SEM analysis

SEM result indicated to presence cracked areas on the surface of polyethylene polymer and many holes with different sizes spread in polymer which treated with bacteria compared to control as shown in Figure 3A, B. This reflects the role of enzymes that secreted by bacteria in breaking down the bonds in polyethylene polymer and decomposing it. Using SEM with EDS indicated to presence of nano particle as in (Figure 4), due to salts of media or from decomposed plastic compounds.

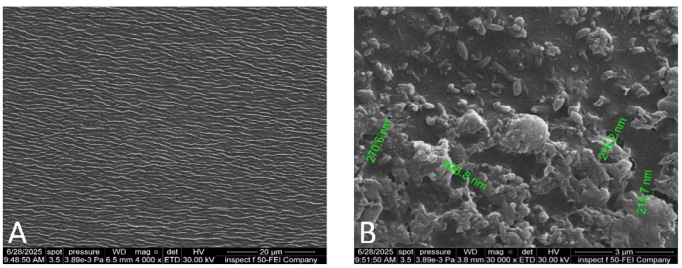

Figure 3: *A: Micrographs showing SEM examination of control plastic polymer, B: Micrographs showing SEM examination of treated plastic polymer with bacteria.*

Energy dispersive x- ray spectroscopy EDS

EDS results obtained from the plastic surface post bacterial treatment indicated to presence of chemical and structural differences consistent with biodeterioration as in (Table 5). High atomic percentage of oxygen (45.6%) were detected which signifying to

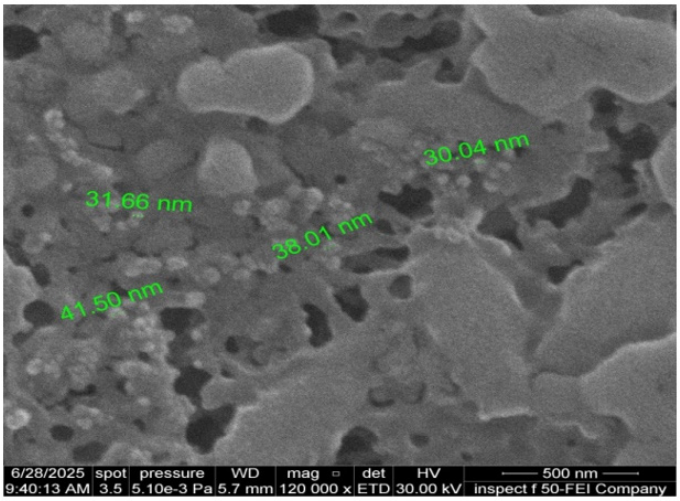

Figure 4: *Nano particles appeared through examin SEM technique.*

Table 5: *Nano elements detected through EDS technique.*

Element	Atomic %	Atomic % Error	Weight %	Weight % Error
N	11.8	0.7	7.7	0.4
O	45.6	0.5	33.9	0.3
Na	10.5	0.1	11.2	0.1
Mg	1.6	0.0	1.8	0.1
Si	17.6	0.1	23.0	0.1
S	2.3	0.0	3.5	0.1
Cl	3.0	0.0	5.0	0.1
K	4.5	0.0	8.2	0.1
Ca	2.8	0.0	5.1	0.1
Fe	0.2	0.0	0.6	0.1

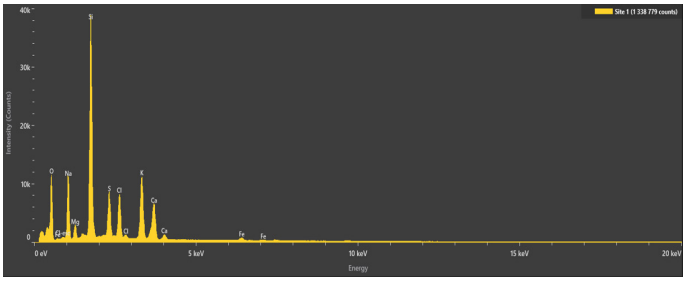

Figure 5: *Elemental mapping with EDS.*

oxidation on the surface of polymer occurs. That may due to produce oxygen-containing functional group by bacterial enzymatic activity through aerobic plastic degradation as carboxyl or hydroxyl groups where microbial action breaks down long polyethylene chains into minor oxidized fragments (Liu *et al.*, 2022). The presence of nitrogen at (11.8%) supports microbial colonization and degradation, in addition nitrogen is one of component of culture medium and metabolites of bacteria as in (Sakariyachan *et al.*, 2018). Further elements such as iron, magnesium, sulfur, chlorine, potassium, calcium, and sodium detected, which regards among components of media. The existence of silicon (14.9%) may points to the contamination through sample prepared as in (Zafar *et al.*, 2020). While lake of carbon atom means that there is no plastic nanoparticles in degrading product as explained in (Figure 5).

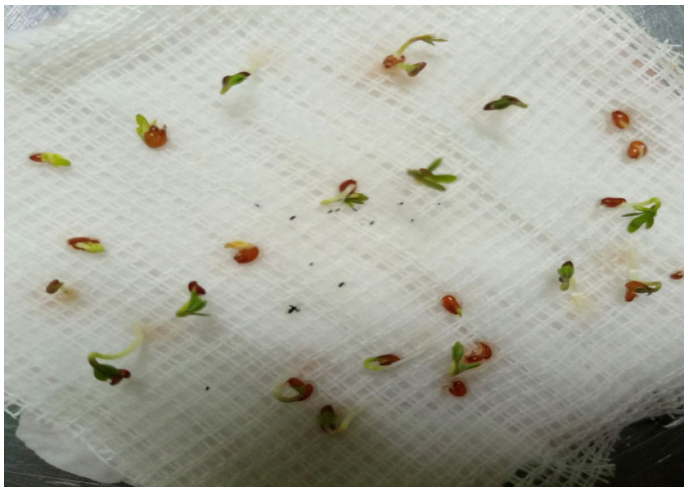

Figure 6: *Germination seeds of Lepidium sativum using bacterial plastic degradation product.*

Table 6: *Phytotoxicity of product of bacterial degradation of plastic.*

Bacterial strains	Type of plastic	Percentage of germination after 5 days
PET		
Enterobacter hormaechei ZEV5.iq	PET	90%
Enterobacter kobei ZEV4.iq	PET	86%
Enterobacter sp ZE6.iq	PET	86%
Enterobacter cloacae	PET	86%
Bordetella petrii ZE5.iq	PET	80%
Enterobacter sp	PET	80%
LDPE		
Enterobacter cloacae ZEV*1*.iq	LDPE	86%
Achromobacter piechaudii	LDPE	86%
Klebsiella aerogenes ZE7.iq	LDPE	80%
Enterobacter cloacae	LDPE	80%
Pseudomonas aeruginosa ZE2.iq	LDPE	80%
Stenotrophomonas maltophilia	LDPE	80%

Phytotoxicity

Using the end product of bio-degradation of polyethylene plastic (that treated with bacteria) showed a good germination of *Lepidium sativum* seeds as in (Figure 6). The germination rate of all seeds, treated with different end products of bacterial strains, were high ranging between 90% to 80%. particularly with end product of *Enterobacter hormaechei* ZEV5.iq (Table 6). This gives evidence

that these products are non-toxic and safety for plants and agree with study conducted by Mehmood *et al.* (2023), whom confirmed to the safety of products compound of deterioration of LDPE polymer and help in germination.

Conclusion

Bacteria distributed in the biosphere due to their metabolic capability and easily grown under a wide range of environmental conditions and produce various enzymes. These enzymes isolated from microbial sources participate in the bio-remediation of pollutants. Utilize *Galleria mellonella* to remove plastic waste consider as green and new alternative choice to get rid of pollutants that are difficult to decompose and represents a good synergistic relationship between insects and their gut microbes. Eliminating polyethylene waste using biodegradation is an effective, low- cost, and safe technology that does not lead to formation nanoparticles that is considered harmful to environment.

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Novelty Statement

Plastic-degrading Gram-negative bacteria from polluted soil environments. While previous studies have reported microbial degradation of plastics, limited attention has been given to the diversity and efficiency of Gram-negative bacterial strains in heavily contaminated soils, this research contributes to the development of eco-friendly solutions for managing plastic waste and environmental pollution.

Author's Contribution

Zhara Tareq Abdul Hameed Baqqal: Collected soil samples and performed the laboratory experiments, including the isolation and screening of plastic-degrading Gram-negative bacteria.
Essra Ghanim Hazim Alsammak: Conducted the data analysis, technical Input at every step.

Generative AI and AI-assisted technology statement

The authors declared that no generative AI and AI-assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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