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Connecting *agrobacterium tumefaciens* for bio-degradation of low-density polyethylene: opportunities and challenges ahead

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ABSTRACT

LDPE contributes significantly to global plastic waste accumulation, necessitating effective bio-degradation strategies. Its persistence in ecosystems raises concerns, prompting the need for innovative solutions. Microbial degradation of environmental pollutants is a widely accepted phenomenon, driven by the decomposer nature of naturally occurring microorganisms. This study aimed to explore Agrobacterium tumefaciens for bio-degradation of low-density polyethylene (LDPE). Indigenous bacteria capable of LDPE degradation were isolated from a solid waste dumping site in Durg district and screened for their bio-degradation potential using PEG assays. The research highlights that the study site sheltered a well-known genetic engineer Agrobacterium tumefaciens capable of degrading LDPE effectively. Molecular characterization supported the identification of potential isolate as Agrobacterium tumefaciens, providing authentic nomenclature for the microbial agent involved in LDPE degradation. The degradation process was monitored through various methods, including weight loss measurements and pH changes at regular intervals, which confirmed the ongoing bio-degradation of LDPE. Furthermore, Fourier Transform Infrared Spectroscopy (FTIR) analyses revealed chemical changes in the pollutant, leading to the formation of simpler compounds that could potentially be utilized or removed by other environmental organisms. Additionally, Scanning Electron Microscopy (SEM) studies provided crucial visual evidence of structural alterations on the LDPE surface due to microbial activity. SEM images depicted the formation of pits, cracks, and other signs of surface degradation, illustrating the physical changes occurring during the bio-degradation process. These findings emphasize the significant potential of indigenous bacterial strains in contributing to the remediation of LDPE pollution through microbial degradation processes.

Key words: Low density polyethylene, zone of clearance, weight loss measurements, FTIR, SEM, *Agrobacterium tumefaciens*.

INTRODUCTION

Plastic production globally amounts to approximately 368 million tons annually and is anticipated to double over the next two decades (Nakei *et al.,* 2022). The term 'plastic' derives from the Greek word 'Plastikos,' meaning capable of being shaped or molded into various forms (Zeenat *et al.,* 2021). Commonly used plastics include polyethylene (both low density and high density), polypropylene, polystyrene, polyvinyl chloride, polybutyrene, teraphthalate, and nylon (Sharma *et al.,* 2014). Out of these, Polyethylene, valued for its versatility, availability, lightweight, durability, flexibility, and cost-effectiveness, finds extensive application in everyday products such as plastic bags, bottles, food packaging, agricultural materials, electronics, and automotive components (Duddu *et al.,* 2015 and Veethavya *et al.,* 2016). Microplastics are toxic,long lasting,capable of accumulating in organisms,and resistant to breakingdown,while metallic contamination is recognized as a pollutant (Kumar *et al.,* 2024). Its molecular structure, predominantly composed of ethylene monomers with a high molecular weight and hydrophobic backbone rich in C-C and C-H bonds, renders low density polyethylene non-biodegradable (Gupta *et al.,* 2019). This non-biodegradability poses a significant environmental challenge, contributing to persistent accumulation in landfills, oceans, and natural habitats worldwide. Improper disposal of polyethylene threatens wildlife through ingestion, potentially causing severe health consequences and mortality, with implications for human health as well (Duddu *et al.,* 2015). Bacterial degradation of polyethylene refers to the enzymatic breakdown of this polymer by certain bacteria,

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offering a promising avenue for addressing its environmental impact."

The aim of this study is to explore the potential of *Agrobacterium tumefaciens*, for its efficient polyethylene degradation capabilities. This research seeks to contribute to the development of environmentally friendly waste management practices by exploring the opportunities and challenges ahead in harnessing microbial bio-degradation processes for polyethylene.

MATERIALS AND METHODS

ORIGIN OF THE BACTERIA

The soil samples were collected twice a year from the Maroda dumping site in Durg district, Chhattisgarh. Meanwhile, low-density polyethylene films with a density ranging from 0.910 to 0.940 g/cm³ were sourced from local markets in Durg. Before initiating the bio-degradation process, the polyethylene films were meticulously prepared: cut into small 2 × 2 cm pieces, washed with 2% sodium dodecyl sulfate solution, thoroughly rinsed with distilled water, and sterilized using 70% ethanol as per established protocols suggested by Priyanka *et al.,* 2011 and Gajendiran *et al.,* 2016.

The next step involved isolating bacteria capable of degrading low-density polyethylene. This was achieved using a synthetic media formulation comprising NH4NO3 (1.0 g), MgSO4·7H_2O (0.2 g), K_2HPO_4 (1.0 g), $CaCl_2$·2H_2O (0.1 g), KCl (0.15 g), yeast extract (0.1 g), $FeSO_4$·6H_2O (1.0 mg), ZnSO4·7H_2O (1.0 mg), $MnSO_4$ (1.0 mg), Tween 60 or 80 (0.01-0.50% v/v), and agar. The isolation process involved serial dilution and the spread plate method, followed by incubation at 37°C for 24 hours. The screening of polyethylene degrading bacteria involved employing the zone of clearance method within minimal media supplemented with ammonium and potassium salts. This method facilitated the detection of clear zones around bacterial colonies, indicative of polyethylene utilization as the primary carbon source. Confirmation of activity was achieved through staining with Coommassie Blue, highlighting areas where bacteria actively degraded the polyethylene substrate (Singh *et al.,* 2016; Gupta *et al.,* 2017).

Following screening, the bacterial isolates underwent comprehensive characterization. Initial identification relied on morphological criteria observed through Gram staining. Subsequent biochemical analyses included the IMViC (Indole, Methyl Red, Voges-Proskauer, Citrate) tests to assess metabolic pathways, carbohydrate fermentation tests to evaluate substrate utilization, and enzymatic assays such as gelatin and starch hydrolysis. Additionally, the isolates were tested for catalase activity and urease production, providing a detailed biochemical profile essential for species identification and further studies (Singh *et al.,* 2016; Ariba Begam *et al.,* 2015).

MOLECULAR IDENTIFICATION

The isolated bacteria were molecularly identified through analysis of the 16S ribosomal RNA gene. This molecular identification method confirmed the taxonomy and phylogenetic placement of the bacteria (Gajendiran *et al.,* 2017).

BIODEGRADATION STUDIES

The isolated bacteria were inoculated into mineral salt media composed of 1.0 g NH_4NO_3, 0.2 g $MgSO_4$·7H_2O, 1.0 g K_2HPO_4, 0.1 g $CaCl_2$·2H_2O, 0.15 g KCl, and 1.0 mg each of $FeSO_4$·6H_2O, $ZnSO_4$·7H_2O, and $MnSO_4$. The media contained low density polyethylene film (2 × 2 cm), which was aseptically transferred and incubated in a rotary flask shaker at 120 rpm for 60 days (Ibiene *et al.,* 2013).

Weight Loss Measurement

After 60 days of incubation, the polyethylene film was retrieved and sequentially washed with 2% (w/v) sodium dodecyl sulfate, distilled water, and sterilized with 70% ethanol. Subsequently, the film was air-dried and placed in a hot air oven at 60°C overnight. The extent of degradation in terms of percentage weight loss of the polyethylene film was determined using the formula suggested by Nademo *et al.,* 2023.

$$Weight\ loss\ \% = \frac{\text{initialweight} - \text{finalweight}}{\text{initialweight}} \times 100$$

pH Changes

The study monitored pH changes to assess the metabolic activity of the microbial strain and observe evidence of polyethylene degradation. pH measurements of the medium were taken at 10-day intervals, maintaining an initial pH of 7.0 throughout the experiment (Duddu *et al.,* 2015).

Fourier Transform Infrared (FTIR) Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy was applied to analyze changes in the chemical structure of the low-density polyethylene film due to bio-degradation (Mandal *et al.,* 2015)

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was employed to examine structural alterations in the low-density polyethylene film due to bacterial activity. Post 60 days of incubation in synthetic media, the polyethylene film was extracted from the conical flask, washed with 2% (w/v) sodium dodecyl sulfate, followed by distilled water and 70% ethanol. Subsequently, the film was air-dried and subjected to SEM analysis (Nademo *et al.,* 2023).

STATISTICAL ANALYSIS

All the experiments were performed in triplicate. Standard deviation and standard error was calculated for each experiment by using MS office excel program.

RESULTS AND DISCUSSION

Screening and characterization

Agrobacterium tumefaciens isolated from the dumping site in Durg district exhibited significant capabilities in degrading low-density polyethylene (LDPE). Clear zones around bacterial colonies indicated effective utilization of polyethylene as a sole carbon source, confirming its bio-degradation potential. Morphological characterization through Gram staining identified *Agrobacterium sp.* as gram-negative rods. Biochemical tests including IMViC (Indole, Methyl Red, Voges-Proskauer, Citrate), carbohydrate fermentation (glucose, lactose, sucrose), gelatin hydrolysis, starch hydrolysis, catalase, and urease production tests further characterized its metabolic profile (Table 1). Positive results in specific biochemical assays affirmed the species' ability to metabolize various substrates and produce essential enzymes conducive to polyethylene degradation.

Table 1: Morphological and Biochemical characteristics

1	2	3	4	5	6	7	8	9	10	Probable Identification
BS9	Gram –ve, Rod shaped	-ve	+ ve/ +ve	+ve	-ve	+ve	-ve	-ve	+ve	*Agrobacterium sp.*

1.Isolate code; 2. Gram Character; 3. Indole Test; 4. MR/ VP; 5. Citrate; 6. Starch Hydrolysis; 7. Catalase; 8. Urease; 9. Gelatin degradation; 10. Carbohydrate fermentation

Molecular indentification

Molecular identification via 16S ribosomal RNA gene sequencing confirmed the taxonomy of *Agrobacterium sp*. It showed highest similarity of 98.79 % with *Agrobacterium tumefaciens* strain IAM 12048 16S ribosomal RNA small sub-unit ribosomal RNA gene, partial sequence with accession number NR_041396.1 (Fig. 1). The sequencing results aligned with known genetic databases, validating its phylogenetic placement and supporting its role as a potent agent in polyethylene degradation.

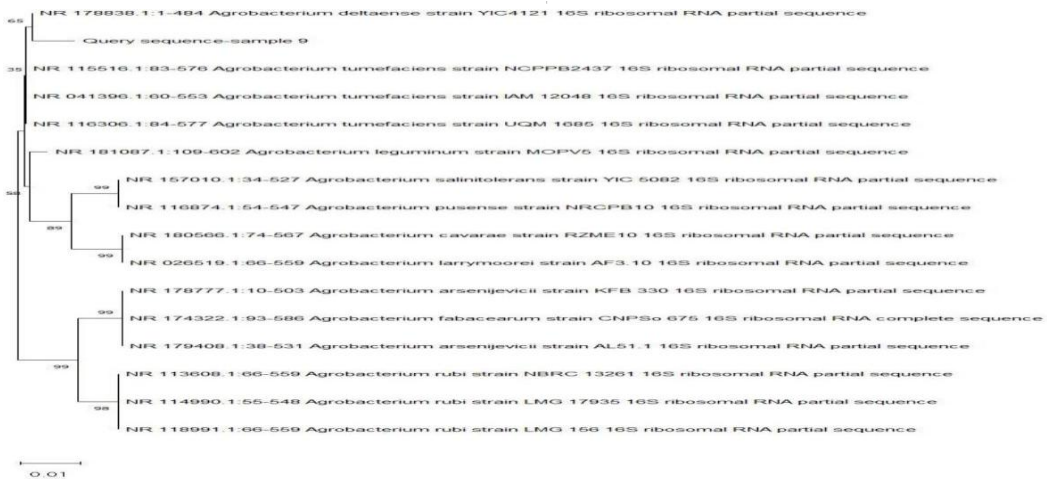


Fig. 1: Phylogenetic analysis of *Agrobacterium tumefaciens* strain

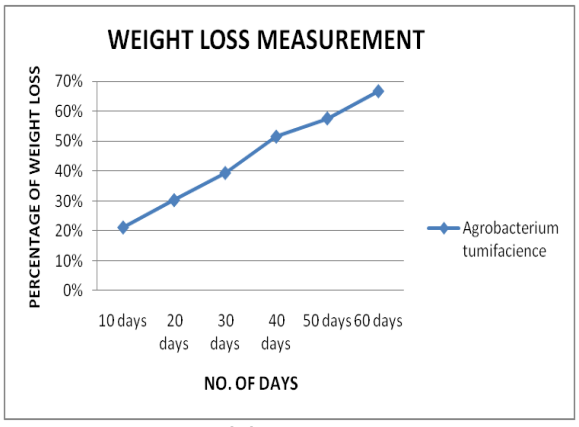
BIODEGRADATION STUDIES

Weight Loss Measurement

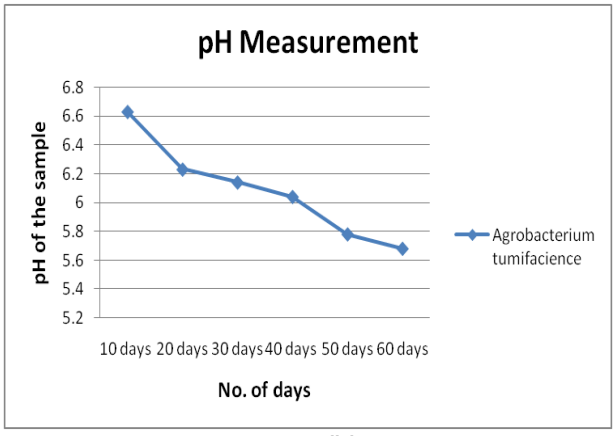
Pure culture of *Agrobacterium tumefaciens* was inoculated into mineral salt media containing LDPE films (2 × 2 cm) and incubated for 60 days in a rotary flask shaker. The weight loss of polyethylene films assessed using gravimetric analysis indicated substantial degradation efficiency {Table 2; Fig. 2 (a)}. The degradation of LDPE was observed to progressively increase over the incubation period, with up to 66.66 % of the polyethylene degraded within 60 days. Gupta *et al.,* 2010 reported the presence of Agrobacterium species in the old polyethylene waste which are capable of degrading polyethylene..

Table 2: % Weight loss at the interval of 10 days

Initial Weight (mg)	%Weight Loss during incubation (Days)					
	10	20	30	40	50	60
0.011	21.21	30.3	39.39	51.51	57.57	66.66



(a)



(b)

Fig. 2: (a) Percentage of weight loss at the interval of 10 days. (b) pH measurement of the sample at the interval of 10 days

pH Changes

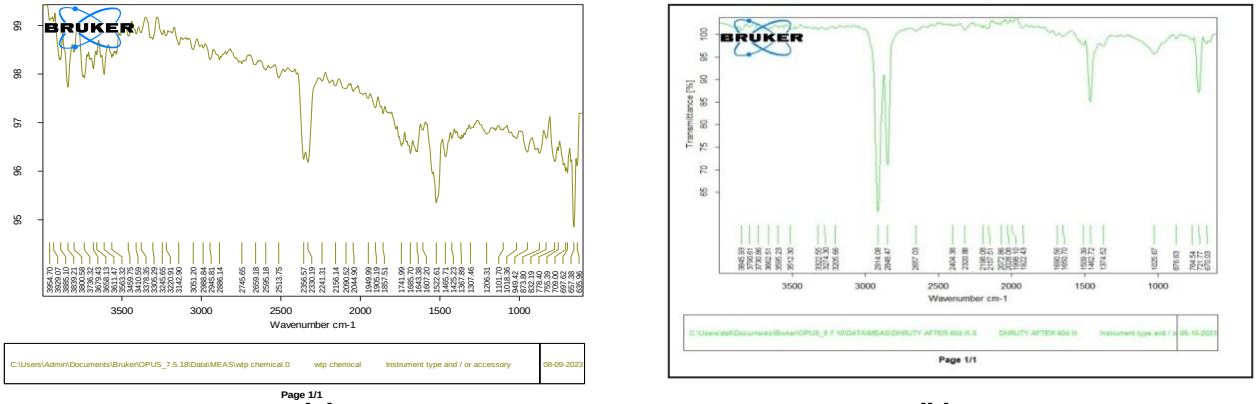
The pH was monitored every 10 days {Table 3; Fig. 2(b)}, revealing a decrease in pH indicative of acid production and confirming polyethylene degradation. The metabolism of polyethylene releases acidic by-products, leading to a decrease in pH over the time.

Table 3: pH changes at the interval of 10 days

Initial pH	pH during incubation (Days)					
	10	20	30	40	50	60
7.0	6.63	6.23	6.14	6.04	5.78	5.68

FTIR Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy revealed distinct chemical changes in the polyethylene structure post-incubation, supporting enzymatic breakdown. It was observed that the polyethylene film undergoes degradation, transforming its chemical structure from repeating ethylene monomers composed of carbon and hydrogen atoms to include COOH, -CH3, -NH2, and C-Cl bonds, as depicted in Fig. 3.



(a) **(b)**

Fig. 3: FTIR spectrum of bio-degradation of low-density polyethylene film after 60 days of incubation (a) control (b) FTIR spectrum of *Agrobacterium tumefaciens*

Polyethylene is predominantly composed of repeated ethylene monomers linked together through carbon-carbon (C-C) and carbon-hydrogen (C-H) bonds. These bonds form a long-chain polymer structure characterized by its high molecular weight and hydrophobic properties. The molecular weight of polyethylene can vary widely, influencing its physical properties such as strength, flexibility, and resistance to chemical and environmental degradation. Its hydrophobic nature contributes to its durability and inertness, making it highly resistant to moisture, acids, alkalies, and other chemical agents.

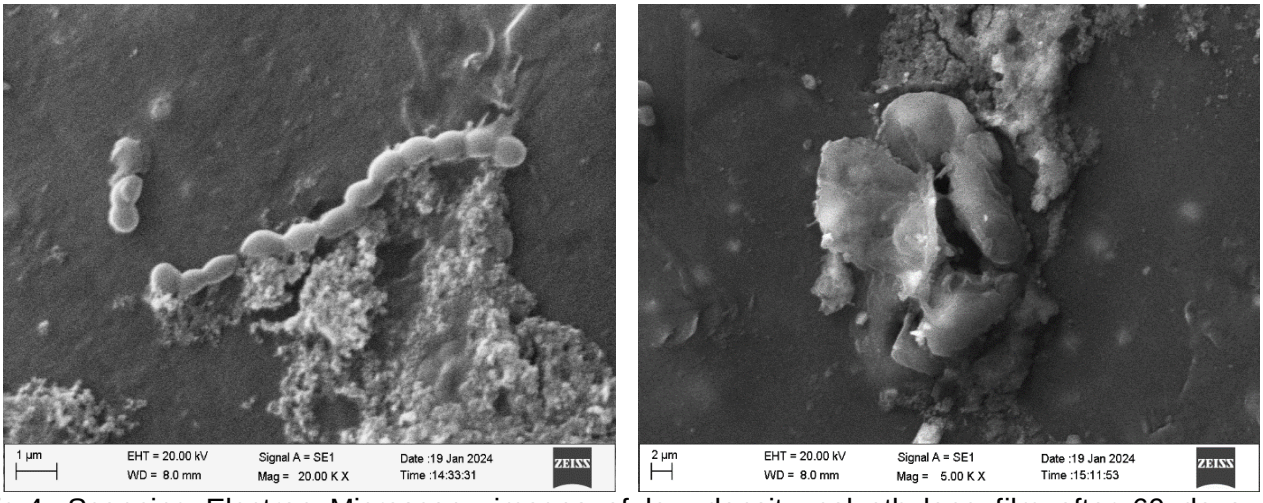


Fig.4: Scanning Electron Microscopy images of low density polyethylene film after 60 days of incubation

3.3.4 SEM Analysis

Scanning Electron Microscopy (SEM) revealed structural changes on the polyethylene surface, offering visual evidence of bacterial activity and the progression of degradation (Fig. 4).

CONCLUSION

Agrobacterium tumefaciens emerges as a promising candidate for environmentally friendly management of polyethylene waste, demonstrating robust bio-degradation capabilities. This bacterium is known for its natural ability to transfer DNA between itself and plants, causing tumors in plants. However, it has also shown potential in biotechnological applications, including bio-degradation. LDPE is a common type of plastic used in packaging and products due to its low density and flexibility. However, its persistence in the environment poses significant environmental challenges.

Supported by comprehensive screening, morphological and biochemical characterization, and molecular identification, the study employed diverse analytical methods, including weight loss measurements and pH changes, which provided quantitative evidence of the bio-degradation process over time. Fourier Transform Infrared Spectroscopy (FTIR) analysis offered insights into chemical transformations within the polyethylene, indicating breakdown into simpler compounds. Furthermore, Scanning Electron Microscopy (SEM) provided visual evidence of surface alterations and microbial activity.

The results highlighting both the opportunities and challenges involved in this promising area of research. Exploring Agrobacterium tumefaciens for bio-degradation of LDPE presents several opportunities including, utilizing the bacterium's enzymatic capabilities to break down LDPE into simpler compounds, potentially developing eco-friendly solutions for plastic waste management and contributing to sustainable practices in waste disposal and environmental conservation.

Despite the potential, there are several challenges to address such as, understanding the mechanisms of bio-degradation by Agrobacterium tumefaciens, optimizing conditions for efficient degradation, scaling up bio-degradation processes to industrial levels and assessing the environmental impact of breakdown products.

Exploring additional research avenues aimed at optimizing the efficiency and reliability of LDPE bio-degradation through microbial consortia could lead to promising advancements in tackling LDPE waste.

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