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Isolation, characterization of Microplastic degrading bacterial strains isolated from Plastic dumping site in Panchkula, Haryana India.

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Abstract
Microplastics are plastic particles with diameters less than 5 mm, which have become the widespread environmental contaminants within both water and soil, causing risks such as physical harm, reduced nutritional quality, and increased pathogen exposure. The primary aim of the present study is the isolation and characterization of plastic dumping site-associated bacterial strains in their ability to degrade various types of microplastics. Based on biochemical and molecular techniques, it was found that two bacterial strains isolated from a dumping site in Panchkula Haryana, India, showed high enzymatic activity against polyethylene. Our findings under two types of controlled conditions show that, bacterial strains degraded the microplastic 15% and 10% by two isolates respectively whereas reduced the weight of polyethene film by 20% and 14% by two isolates respectively. In addition to plastic degrading experimentation, Biochemical tests was also performed from which Sample [A] was identified as *Acinetobacter johnsonii*, making microbial remediation strategies a good approach.
Keywords: Microplastic, Identification, Isolation, Polyethene

I. INTRODUCTION

Microplastics are the plastics of size less than 5 mm. They are non-metallic, present in water and soil, leading to physical harm, reduced nutritional quality, and increased risk of pathogen exposure [1]. Exposure to microplastics may result in toxicity by inducing oxidative stress, inflammatory damage, and enhanced absorption or movement within the body, potentially causing metabolic imbalances, neurotoxic effects, and a heightened risk of cancer [2]. These studies suggest that microplastics are primarily composed of polymers such as polyethylene, polypropylene, and polystyrene, and are commonly found in forms like fibers, fragments, and spheres. Micro-plastics can also be categorized based on their sources into primary sources such as industrial uses and secondary sources from the degradation of larger plastics [3].
Around 1,500 tons of microplastics from personal care products bypass wastewater treatment facilities annually, entering aquatic ecosystems and exacerbating microplastic pollution. By 2030, the volume of microplastics in certain oceanic regions is expected to double [4]. Microplastic contamination in seafood is prevalent, with annual human intake potentially reaching up to 55,000 particles, emphasizing the urgency of mitigating human exposure through seafood consumption [5].
Currently there are mainly three means of disposing of plastic incineration, land filling and recycling. But all three processes are not efficient enough to cope with the prevailing volume of plastic waste produced because of its excess demand in various fields. Therefore, to dispose of the prevailing plastic waste, natural and green means should be adopted. Landfills occupy huge land area which can be otherwise utilized for more productive use like agricultural activity. Lack of oxygen in landfill also prevents its natural decomposition process. In incineration process toxic gases and greenhouse gases are emitted into environment. Colorants, stabilizers and other impurities in total forces recycling process of PET useless [6].
microbial approaches are vital for degrading synthetic polymers such as polyethylene, PET, and polystyrene into less harmful substances, offering effective solutions to combat plastic pollution and promote environmental sustainability. Studies suggest that the degradation of common plastics like polyethylene, polypropylene, polystyrene, and polyethylene terephthalate can take decades to centuries, with factors such as environmental conditions, plastic type, and microbial activity influencing the rate of degradation. Few researches show that bacterial species such as pseudomonas, bacillus are effectively degrading plastics [7].
Other microplastic degradation methods are also explored in many studies. Photolytic degradation using nitrogen doped TiO_2, Catalytic methods including carbon catalytic processes and Advanced oxidation processes can also be used to degrade microplastic [8-10]. Despite the advancement in degradation techniques, current methods are still insufficient to degrade microplastics efficiently [11].
This research aims to isolate and characterize the bacterial strains from soil samples collected from local plastic dumping site to evaluate their efficacy in degrading microplastics. The study involves Biochemical characterization and identification of bacterial

isolates. it also investigates the biodegradation efficacy of these strains on different types of microplastics, such as polyethylene (PE), polypropylene (PP), under laboratory conditions to understand degradation mechanisms and metabolic processes, this research aims to promote sustainable plastic waste management through microbial bioremediation methods.

II. MATERIALS AND METHODS

[A] Soil samples

Soil was collected from a garbage dumping site in Sector 23, Panchkula, Haryana as shown in fig. 1. in India. The sample was collected from the superficial layer by digging 10-12 cm deep. Sample was instantly transferred to sealed plastic bags after collection and stored at 4 ºC for further processes.

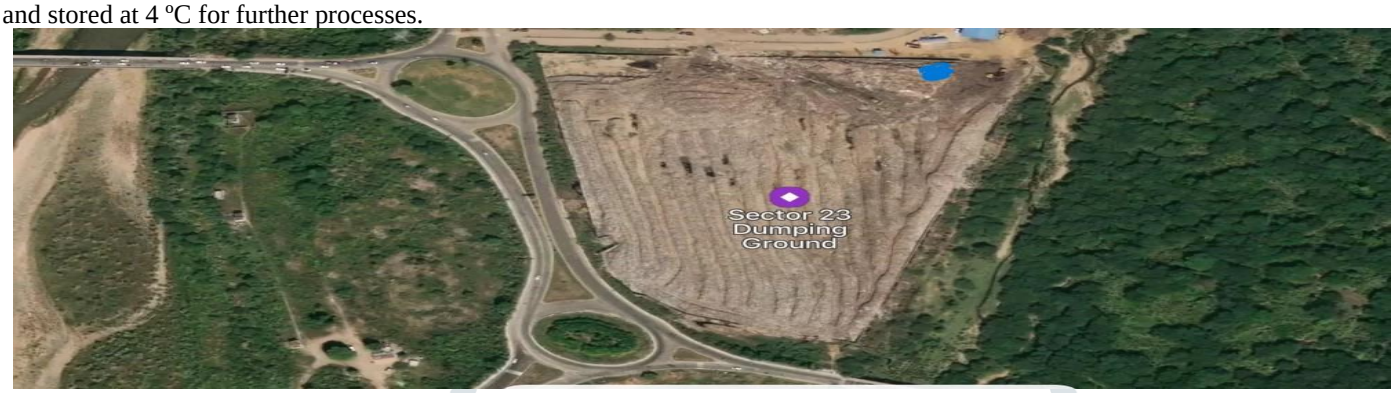

Fig. 1. Satellite view of plastic dumping site in Panchkula with geographical Latitudinal and longitudinal coordinates 30.68058° or 30° 40' 50" north and 76.88205° or 76° 52' 55" east respectively. Mark (blue) shows the sample collection site.

[B] Bacterial isolation

Isolation of bacteria was done through standard procedure by serial dilution, 10^{-5} and 10^{-4} dilutions was poured on Nutrient agar plates. The plates were stored in incubator at 37° for 24 hours. Pure culture was obtained by streaking one colony from spread plate.

[C] Biochemical Tests

Gram staining: A clean, grease-free slide was prepared, and a bacterial culture smear was made on it using a sterile loop. The smear was air-dried and heat-fixed before being treated with the following staining reagents. Crystal violet was applied to the smear for 1 minute, then rinsed with distilled water. Gram's iodine was added for 1 minute, followed by a wash with alcohol. Finally, the slide was counterstained with safranin for 30 seconds and rinsed again with distilled water. The slide was observed under microscope [12].

Sugar Test: Monosaccharide is monomeric sugar that is easily fermented by various bacteria. In a test medium containing glucose, the fermentation process leads to the production of acids and sometimes gases, A lowering the pH of the medium. This is indicated by a color change in the pH indicator. Prepared the fermentation broth with each specific sugar (dextrose, mannitol, maltose, sucrose) as the sole carbohydrate source. Add the appropriate concentration of phenol red as the pH indicator (0.02%). Using a sterile inoculating loop or needle, transfer a small amount of the bacterial culture into each test tube containing the sugar-based medium. Place the test tubes in an incubator at 37°C for 24-48 hours, depending on the organism being tested. After the incubation period, A color change in the phenol red indicator from red to yellow indicates acid production due to sugar fermentation. If the pH remains unchanged, no fermentation has occurred.

Catalase test: The catalase test was performed to detect the presence of catalase enzyme by inoculating a loopful of culture into tubes containing 3% of hydrogen peroxide solution. Positive result was indicated by formation of effervescence or appearance of bubbles due to the breaking down of hydrogen peroxide to O_2 and H_2O [13].

Indole test: The indole test is a biochemical test for the detection of indole, a compound produced by bacteria that contain the enzyme tryptophanase. Tryptophanase catalyzes the hydrolysis of the amino acid tryptophan into indole, pyruvic acid, and ammonia. Then, the indole is detected by its reactivity towards a specific regent, which produces a characteristic red or pink color, usually using Kovac's reagent or Diazotized p-aminobenzene sulfonic acid. This test is useful for the distinction between bacterial species according to their ability to metabolize tryptophan.

Motility test: The motility test was conducted to assess the movement of the microorganism. About 5ml of nutrient broth was inoculated with the isolated microorganism and incubated overnight for growth. A drop of the nutrient broth was then placed on a grooved slide using the hanging drop method and examined under phase contrast microscopy.

Citrate test: This assay evaluates the capability of bacteria to metabolize citrate into oxaloacetate, utilizing citrate as the sole carbon source. A positive outcome is indicated by bacterial growth and a bright blue coloration of the medium.

Urease Test: The urease test assesses the ability of bacteria to hydrolyze urea into ammonia and carbon dioxide using the enzyme urease. A positive result is observed when the medium turns bright pink due to an increase in pH. It involves inoculating bacteria into a urea-containing medium with a pH indicator. Hydrolysis of urea produces ammonia, raising the pH and turning the medium bright pink, indicating positivity.

MR-VP test: The MR-VP test separates organism based on the pathway they use for glucose fermentation. Turn red (positive), shifted from neutral to positive, MR test means mixed-acid fermentation (acids drop pH) The Voges-Proskauer (VP) test detects the production of acetoin, a neutral end product of fermentation. If acetoin is present (positive), the addition of reagents will produce a red color. These tests focus on distinguishing bacterial species based on their unique metabolic pathways and are frequently employed in differentiating Enterobacteriaceae.

Ability to grow on plastic containing media: Bacterial growth was assessed on a plastic-containing nutrient agar medium (NAM) to evaluate their ability to thrive in a plastic-enriched environment. Standard NAM preparation techniques were followed, with 10% finely chopped polyethylene incorporated as the sole carbon source. Bacteria was spread over the medium and kept at 37 ºC overnight and observed after 24 hours.

Antibiotic Susceptibility Test: The purpose of the antibiotic susceptibility test is to determine the sensitivity of different bacterial strains towards different antibiotics Two NAM plates were made and 20µl of bacterial culture was spreaded on each plate. Four wells were made in each plate for four antibiotics Amoxicillin, Penicillin, Cephloxin and Levoquinn. 10µl of each antibiotic is put in each well. plates were incubated at 37ºC for 24 hours. The purpose of the antibiotic susceptibility test is to determine the sensitivity of different bacterial strains respond to different antibiotics. Plates are incubated to promote the growth of bacteria and the diffusion of antibiotics. Clear regions surrounding the discs are known as zones of inhibition. To evaluate bacterial sensitivity or resistance to particular antibiotics, the size of these zones is assessed.

Interaction with other micro-organisms: Four fresh agar plates were prepared, two inoculated with Pseudomonas suspensions and two with Salmonella suspensions. Sterile cork borers were used to create three agar wells on each plate. One well per plate was filled with distilled water, while the other two were filled with pure culture solutions grown overnight in nutrient broth. The solutions were added separately to the plates containing Pseudomonas and yeast cultures. Plates were incubated at 37°C for 24 hours and examined for results.

[D] Identification of Bacterial Strains

One bacterial colony was isolated from each spread plate and then pure culture was made using standard procedure. DNA was isolated from the bacterial culture and then run on agarose gel. The bands were visualised under UV illuminator. The culture giving good results was sent for identification using 16s RNA sequencing. Similarity search was done using BLAST from which most similar strain was identified.

[E] Microbial Degradation of Microplastics and Plastic

Polythene film degradation

Polyethene strips (4 cm^2) were made in the laboratory. Pour plate technique was used for inoculation of cell suspension in the agar medium. Cell suspension of the culture was added to sterile petri-plate followed by addition of warm NA media. The plate was swirled and the culture was mixed homogenously. The Polyethene strips were then aseptically placed on each plate containing the two bacterial samples. The plates were then sealed with polythene bags to avoid desiccation and were kept at 30° for 30 days [14]. The plates were periodically sub cultured and the film was observed for any sign of microbial growth.

Weight Loss determination

A mixed bacterial culture was cultivated in a basal medium consisting of 2.34 g/L K_2HPO_4, 1.33 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.5 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, and 1 mL of trace element solution per litre of distilled water. The trace element solution contained 21.8 mg/L $CoCl_2 \cdot 6H_2O$, 21.6 mg/L $NiCl_2 \cdot 6H_2O$, 24.6 mg/L $CuSO_4 \cdot 5H_2O$, 1.62 g/L $FeCl_3 \cdot 6H_2O$, 0.78 g/L $CaCl_2$, and 14.7 mg/L $MnCl_2 \cdot 4H_2O$ [15-16]. Ten percent of the bacterial strains were inoculated into Erlenmeyer flasks containing 100 mL of basal medium supplemented with 40 mg of sterilized polyethylene (PE) microplastics. Culture was left at 37ºC for 30 days. Weight loss was determined after 30 days using following formula:

(Initial weight – final weight) / initial weight * 100

III. RESULTS AND DISCUSSION

TABLE I. SOIL SAMPLE CHARACTERSITICS

CHARACTERS	OBSERVATIONS
Location	Dumping ground, Panchkula
Weight	100gm
Ph	6.2
Nature	With Poly-ethene and pebbles
Temperature	29ºC
Colour	Brown

TABLE II. COLONY MORPHOLOGY

DILUTION FACTOR	MORPHOLOGY	GRAMSTAINING
10^{-4}	White, opaque, round colonies on NAM. Coccus shaped and Pink under microscope	Gram negative
10^{-5}	White, opaque colonies on NAM, Coccus shaped and Voilet under microscope.	Gram positive

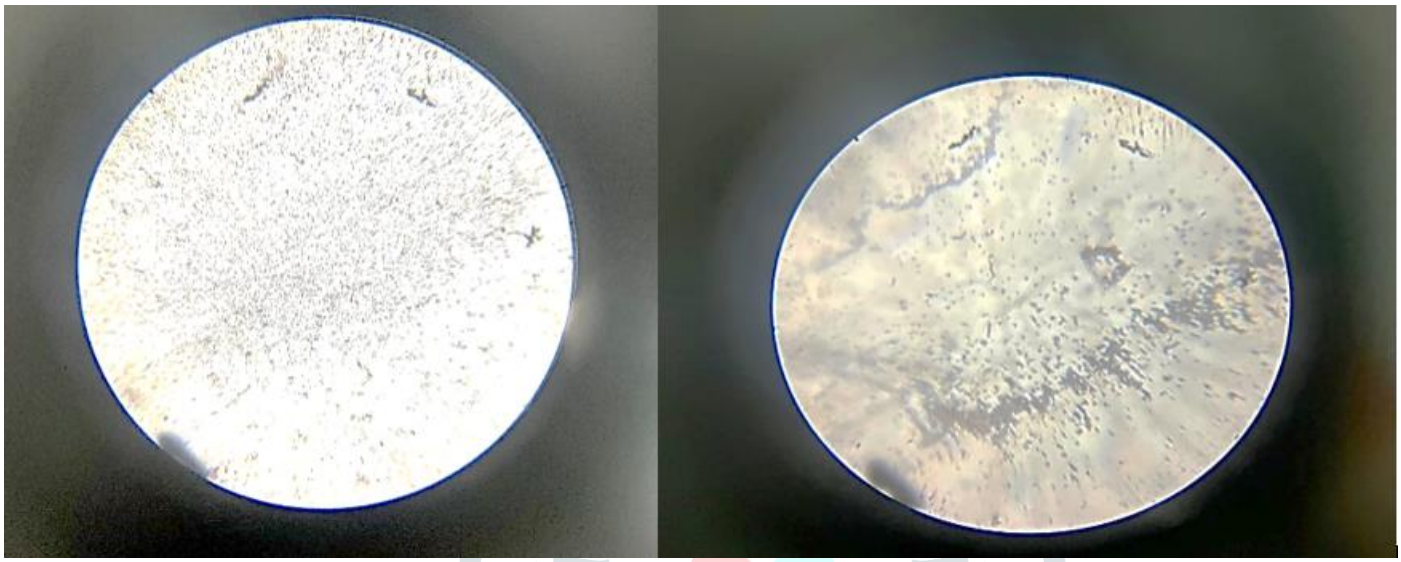

Fig. 2. (a) Gram staining of strains 10^{-4} (Gram negative) and 1(b) Gram staining of 10^{-5} (Gram Positive).

TABLE III. RESULTS FOR BIOCHEMICAL TEST

BIOCHEMICAL TEST	10^{-4}	10^{-5}
Catalase test	Positive	Positive
Urease test	Positive	Positive
Indole test	Negative	Negative
Gram staining	Negative	Positive
Citrate test	Negative	Negative
Dextrose test	Negative	Negative
Mannitol test	Negative	Negative
Lactose test	Positive	Positive
Motility test	Negative	Negative
MR-VP test	Positive	Positive

All the biochemical test identifies the 10^{-4} strain as *Acenetobacter johnsonii* and 10^{-5} as *Kocuria palustris*.

[A] Identification of Bacteria

To prove the biochemical testing best performing bacterial strain (10^{-4}) identification 16S rRNA sequencing was done, after isolation of DNA samples were for 16s rRNA sequencing. Sequence similarity search was performed by using BLAST. Phylogenetic tree was made and results proved the same, confirming the presence of the targeted microbial species. Further analysis revealed insights into their genetic diversity and potential functional roles within the ecosystem.

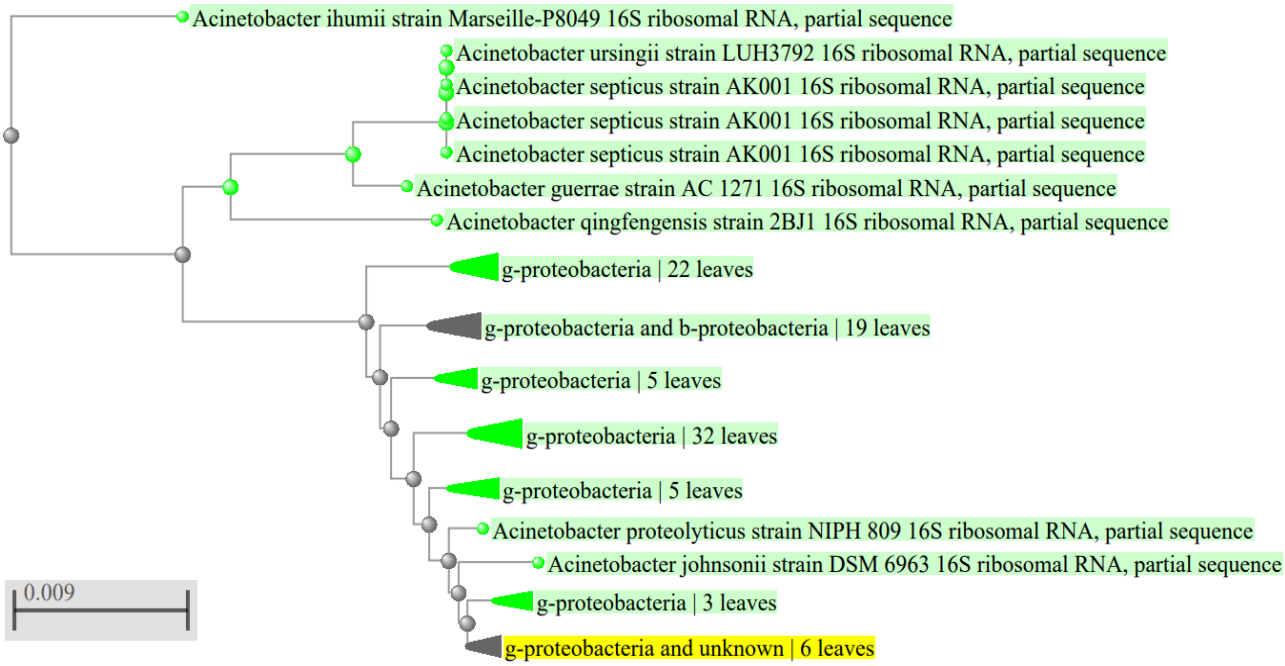

Fig. 3. Phylogenetic tree showing bacteria is closely related to *Acenetobacter sp.*

[B] Results for Growth of Bacteria on Plastic Containing Medium

The microbial population in disposal sites with significant plastic pollution shows the capacity to use both natural and artificial materials as a source of carbon hence, possess the ability to degrade the plastic to some extend [17] Strains 10^{-4} and 10^{-5} proved to have the ability to grow in the plastic-containing medium, although at a reduced growth rate, with colonies visible after incubation for 48 hours as shown in fig 4. This finding indicates that the bacteria are able to use plastic microparticles as a good carbon source. The growth also indicates the ability of the bacteria to grow at low Supplementation levels, as peptone was not added into the medium. The growth under such conditions indicates metabolic flexibility, which can be proved crucial during the plastic degradation process. This finding adds to the existing knowledge of microbial plastic biodegradation and possible applications of bacteria in environmental bioremediation.

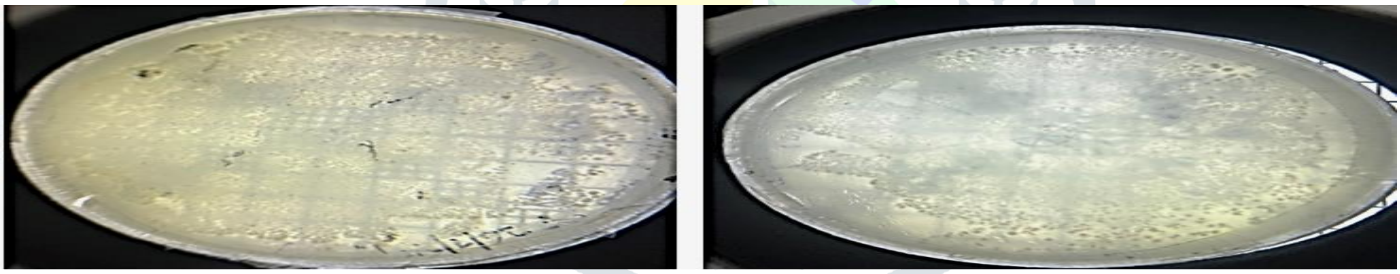

Fig. 4. (a) Growth of bacteria 10^{-4} and 10^{-5} in plastic containing media after 48 h incubation respectively.

[C]Antibiotic Susceptibility Test

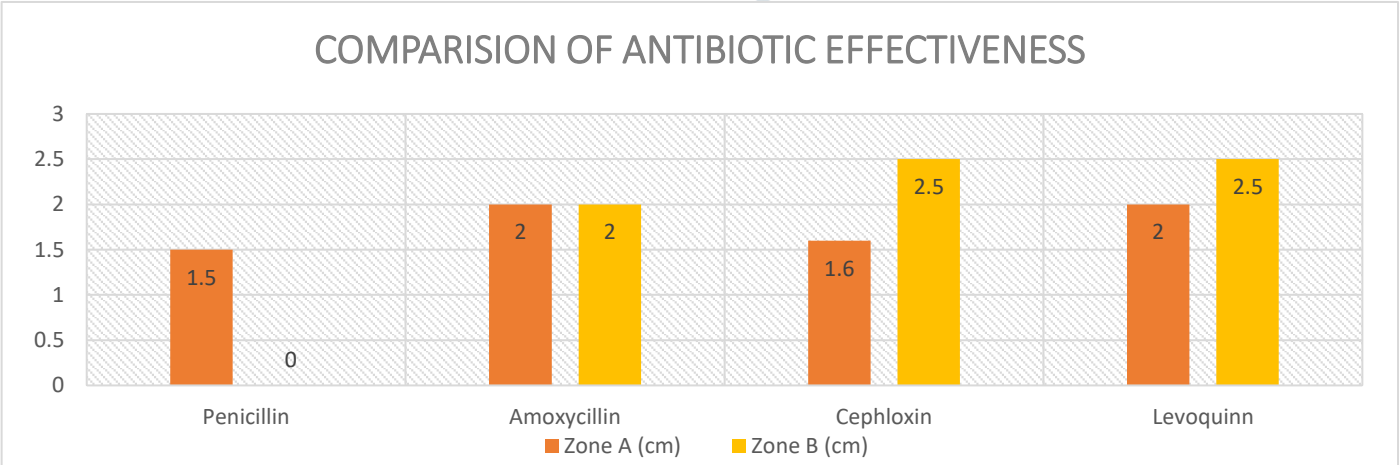

Fig. 5. Comparative table of inhibition zone shown by different antibiotics in two sample [A] for 10^{-4} and [B] for 10^{-5} 2.5 cm zone means that antibiotic has resisted the growth of bacteria completely and 0cm means that Bacteria resisted the antibiotic completely.

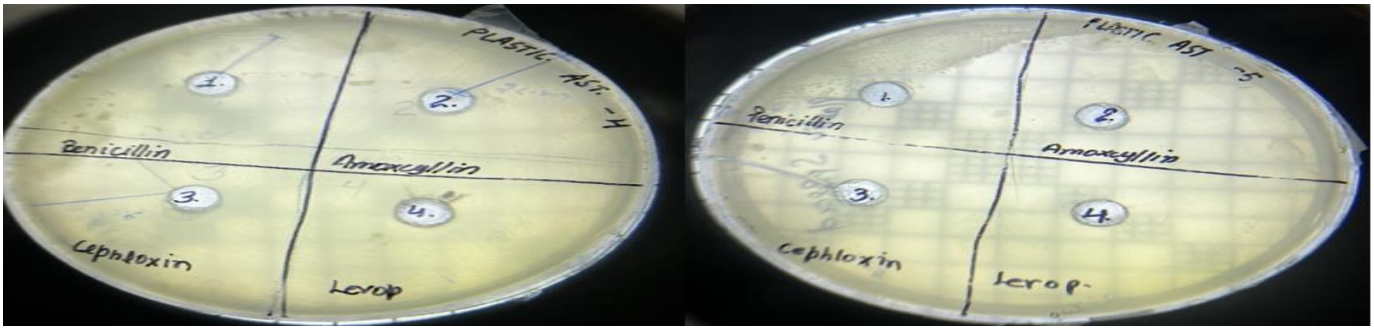

Fig 6. (a) Results showing Antibiotic resistance in strain 10^{-4} and 5(b) 10^{-5} strain respectively.

Strain 10^{-4} and strain 10^{-5} have varying resistance to the antibiotics used as shown in fig. 5. and fig 6. which indicated possible genetic or metabolic differences in their antibiotic susceptibility profiles. No inhibition zone for Penicillin for strain 10^{-5} (0 cm) refers to complete resistance. However, strain 10^{-4} has a minimal inhibition zone (1.5 cm), referring to partial sensitivity. This finding indicates the likelihood of β-lactamase activity in strain 10^{-5}, which would lead to breakdown of Penicillin. The two strains have moderate sensitivity to Amoxicillin and Cephalosporins (Cephlaxin) with inhibition zones ranging from 1.6 cm to 2.5 cm. This implies that while resistance mechanisms of β-lactam may be present, they are not strong enough to inhibit such action of the antibiotics. Among the antibiotics under test, Levofloxacin had the greatest inhibition zone (2.5 cm) for both strains, which shows its efficacy as a drug. This implies that the strains do not have strong mechanisms of resistance towards fluoroquinolones. This also suggests that the bacterial isolates having microplastic degrading capacity have some resistant genes due to environmental exposure.

[D] Interaction with Other Bacteria Strains

A zone of inhibition was found when bacteria is grown with *Salmonella typhi* to check the interaction whereas both the strains grow with *Pseudomonas sp*. Other observations is shown in Table 4.

TABLE IV. SHOWING INTERACTION OF SAMPLE STRAINS WITH TWO OTHER BACTERIAL SPECIES.

SAMPLE	OBSERVATION
Pseudomonas sp. + Sample (10^{-4})	Growth of Pseudomonas around the bacterial suspension and growth of both bacteria can be seen.
Pseudomonas sp. + Sample (10^{-5})	Growth of Pseudomonas found less around the place where bacterial suspension was poured.
Salmonella sp. + Sample (10^{-4})	Widely dispersed colonies were observed.
Salmonella sp. + Sample (10^{-5})	Clear zone of inhibition near bacterial suspension was observed signifying the antagonist negative relationship between two bacteria

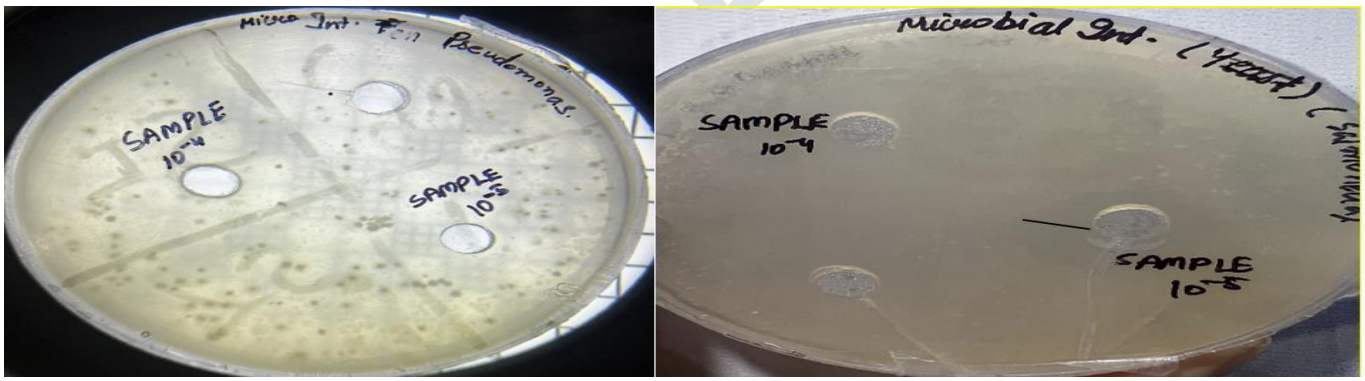

Fig. 7. Interaction of bacterial strains 10^{-4} and 10^{-5} with two other bacteria *Pseudomonas sp*. and *Salmonella typhi* respectively.

TABLE V. RESULTS FOR PLASTIC DEGRADATION

S.no	STRAIN	MICROPLASTIC				POLYTHENE FILM			
		In. wt.	Final Wt.	Diff.	Loss percentage	Initial Wt.	Final Wt.	Diff.	Loss percentage
1.	10^{-4}	40mg	34	6mg	15%	55 mg	44	11	20%
2.	10^{-5}	40mg	36	4mg	10%	55 mg	47	8	14%
3.	Blank	40mg	40	-	-	55 mg	55	-	-

Fig. 8(a). Polyethene Biofilm formation on 10^{-4} strain, incircle show polyethene degradation by bacteria after 10 days of incubation. 8(b). Polyethene Biofilm formation on 10^{-5} strain after 10 days of incubation.

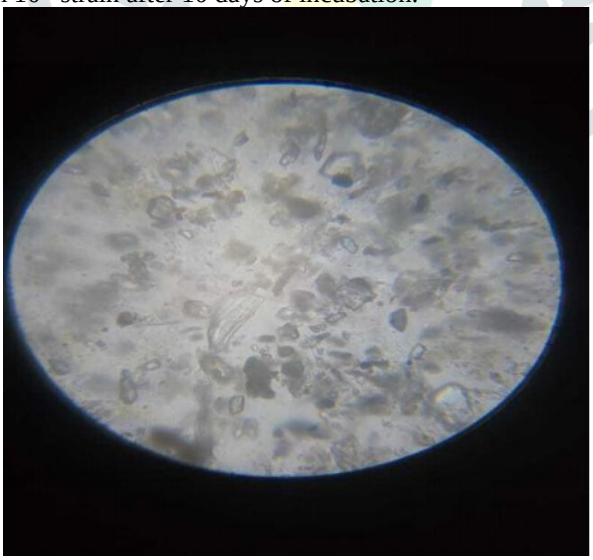

Fig. 9. Plastic degradation by Bacteria under 40x microscope showing plastic fragmentation and degradation.

Discussion

The findings from the experiment setup after a month of incubation are listed in Table The bacterial sample were continuously shaken throughout the incubation period [18]. In order to prevent saturation, the culture medium weas also routinely replaced. Visible

difference in microplastic sample was also observed as in fig 8. When observed under microscope it was noticed that the plastic is more fragmented hence, slowly degrading as shown in fig. 9. Based on the weight difference between the treated and control strips at the end of the incubation time, it was concluded that the bacterial isolates had the ability to break down the plastic strips. Microplastics sample weight was reduced 15% and 10% by 10^{-4} identified as *Acinetobacter johnsonii* and 10^{-5} respectively as shown in Table 5. Also, the weight of Polyethene strip was reduced 20% and 14% by 10^{-4} identified as *Acinetobacter johnsonii* and 10^{-5} respectively. Similar results were also found in recent research studies [19].

Microplastics have a strong carbon backbone and hence they are resistant to conventional natural biodegradation methods [20] Photocatalytic degradation of pre-treated PET microplastics using spherical and nanosheet BiOCl photocatalysts is 8.8 and 6.9 times more efficient than untreated microplastics [21]. Reactive oxygen species, facilitate the breakdown of microplastics into smaller, less harmful components, but even these methods are not cost and time efficient and hence not feasible [22-24]. Hence, biodegradation of microplastic using microbes can be optimize to find a feasible process for micro-plastic degradation.

IV. CONCLUSION

The current study presents concrete facts regarding the capability of bacteria collected from a plastic dumping ground to break down microplastics and their potential use in bioremediation. The discovery of numerous plastic co-kinetic bacterial strains presents a promising opportunity for reducing environmental microplastic pollution. Given that the potential of such microbes is well established, further research is required to improve the state for degradation and optimize biotechnological potential. Based on the study we can conclude that the bacteria isolated from soil sample collected from Dumping ground in Panchkula has microplastic degrading capacity. Microplastic were degraded on using biofilm by 20% and 14% from two isolates respectively. Microplastics were degraded using MSM medium by 15% and 10% from two isolates respectively. One of the isolates was identified as *Acenetobacter johnsonii* Microplastic pollution requires an interdisciplinary approach comprising microbial bioremediation among other regulatory approaches to develop sustainable and effective means for plastic waste accumulation minimization.

V. ABBREVIATION

PET: Polyethylene Tetraphthalate
NAM: Nutrient Agar Media
MR-VP: Methyl Red- Voges-Proskauer

VI. CONFLICT OF INTEREST

There was no conflict of Interest

VII. AUTHOR'S CONTRIBUTION

Experimentation is done by Devyani Gautam. Manuscript preparation is done by Devyani Gautam and Joshika Sharma. Proof reading is done by Dr. Puneet. Infrastructure is provided by Harpreet Kaur.

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