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Research Article

ISOLATION AND MOLECULAR CHARACTERIZATION OF PLASTIC DEGRADING BACTERIA FROM DUMPED GARBAGE SOIL

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ABSTRACT

Objective: Plastics are polymers that are widely in application in our day-to-day life. The plastic wastes accumulation is causing threat to the environment as it causes environmental pollution and creates imbalance in the ecosystem. The natural degradation of plastic is too time consuming and the ways to do it are not highly successful. An eco-friendly approach to plastic degradation is using microbial degradation method. Microorganisms play an important role in biological decomposition of various materials in our environment. Thus, the main objective of the present study is isolation and molecular characterization of plastic degrading bacteria from dumped garbage soil.

Methods: The samples were collected from two different locations in Bengaluru and further subcultured to obtain pure culture. Gram's staining was performed to identify bacterial strains and few tests were conducted to find out the biochemical properties of the bacteria. Molecular characterization of isolated bacteria was performed using 16S rRNA method, BLAST and phylogenetic tree were constructed.

Results: The bacterial isolates were found to be, namely, *Aeromonas caviae*, *Aeromonas hydrophila*, *Salmonella enterica,* and *Pseudomonas aeruginosa*. The polythene bag biodegradation was analyzed by incubation for a period of 2 months in liquid culture method.

Conclusion: The results of the study ensure that *A. hydrophila* showed more degrading capability which is followed by *A. caviae*, whereas *Pseudomonas aeruginosa* and *S. enterica* did not contribute in much of the plastic degradation. The weight loss of polythene bag is maximum with higher incubation period rate. From the observation, we conclude that bacteria have more capability to cleave polymer.

Keywords: Biodegradation, Ecosystem, Microorganisms, Molecular characterization, Plastic.

INTRODUCTION

The word "polymer" is derived from *Greek* word, that is, *poly* means many and *mer* means unit. These repeating units combine to form a polymer. As we all know plastics are used in our day-to-day life in various applications such as in packaging of certain materials, agricultural applications, and medical purposes [1]. The usage of plastic is mainly due to their stability and durability as well as thermal properties [2]. Nearly 140 million tons of polymers are manufactured every year [3]. These plastics are of various kinds, such as polythene-terephthalate, high-density polyethylene, and polyvinyl chloride, low-density polyethylene, polypropylene, polystyrene, and others as polytetraflouroethylene and polyurethane [4]. Among the total production, 64% are polythene bags. Yearly 500 billion to 1 trillion polymers are regularly used in our daily life [5].

The accumulation of polythene bags is generating a great threat to the ecosystem as it leads to environmental pollution and causes imbalance in the ecosystem, nearly 267 species are affected in the marine life as well as terrestrial animals such as cows and other domestic animals may consume the plastic bags unknowingly and thus may get affected [6]. The degradation of polythene bags is great challenge as natural degradation is still time consuming. Hence, the alternate method is using microbial degradation which is more efficient, cheaper as it as easily found in the environment. Microbes producing extracellular enzymes have the capacity to cleave polymer into small subunits such as monomers, oligomers, and water-soluble intermediates that can assimilate into the cells of bacteria producing eco-friendly by-products such as carbon dioxide and water [7]. From the previous literature studies, *Bacillus* species showed more capable of degrading bacteria when compared to *Staphylococcus* species [8]. *Pseudomonas alcaligenes* has significant polymer degradation ability than *Desulfotomaculum nigrificans* [9].

The main aim of the work was to isolate, identify, and characterize the organisms and to check the biodegradative ability of polythene degrading bacteria from the plastic contaminated garbage soil.

METHODS

Sample collection

The plastic contaminated samples were collected from two separate regions in and around Bengaluru. One of the samples was collected from garbage soil near Vidyaranyapura and the other was collected from plastic contaminated soil near NCBS. The soil samples on collection were air dried and stored at 4°C.

Isolation of bacteria using serial dilution and spread plate technique

After the collection of bacterial samples, 1 g of each sample was dissolved in 9 ml of sterile water to make 1:10 dilutions and the same was repeated for the rest. Further, selected bacterial sample was taken up to 20 µl and was added into Petri plates containing nutrient agar media using L-shaped glass rod by spread plate technique and was incubated at room temperature for 24 h and hence the bacterial growth observed [10].

Staining

Gram's staining was done to identify the shape, size, and structure which differentiates whether the bacteria are Gram (+ve) or Gram (–ve) based on the cell wall composition.

Biochemical tests

Biochemical identification method is a standardized colorimetric identification system which utilizes supplied energy sources. The basic principle involved is by substrate utilization and change in pH. Metabolic

changes can be seen by color change on incubation in the media that is either inferred on adding the reagents or through naked eye.

Catalase test

The enzyme catalase interposes the disruption of hydrogen peroxide. As to check these activities in the bacteria, add a loop full of culture is mixed into hydrogen peroxide solution (3%) and observed for emergence of oxygen bubbles.

Citrate test

The ability of bacteria to convert citrate into oxaloacetate further metabolized into pyruvate and CO_2. Where change from green to bright blue color indicates increase in pH level (above 7.6) of the media. If bacteria cannot use citrate, there would be lack of bacterial growth.

Indole test

Indole test determines the ability of bacteria to break amino acid tryptophan to indole compound. The production of the indole was tested using minor drops of Kovac's reagent after 48 h of growth in tryptone broth, appearance of red color in the media indicates the presence of indole.

Motility test

The motility test was done to observe the motile nature of the organism by stabbing method (to stab in the media using the inoculating loop containing microbial culture) in semi-solid agar media, which is incubated at 37°C for 2 days. Turbidity was observed around the stab line indicates that a bacterium is motile.

Methyl red test

Methyl red test was performed by injecting bacterial isolates in MR media at 35°C for 48 h by adding few drops of methyl red solution to the media representing red color in the presence of mixed acid fermentation products in glucose media. Fermentation products are produced when bacteria consumes glucose.

Voges–Proskauer (VP) test

VP test was performed by inoculating bacterial isolates in the VP medium and incubating at 35°C for 2 days by addition of alpha-naphthol and potassium hydroxide. Red color formation indicates the production of acetone from glucose which is positive.

DNA isolation using silica column-based technique

DNA isolation is carried using kit method to obtain purified form of DNA from samples. The bacterial cell culture requires only liquid broth containing essential nutrients at optimum concentration. For the growth and division of bacterial cells, they just require a complex medium like lactose broth. Later, the cells are separated by centrifugation and further resuspended in 1% of the initial culture. A 750 µl of homogenizing ×1 buffer is added to dissolve DNA, 750 µl of lysis buffer is added to lyse the cell, and isopropanol was added for DNA precipitation followed by wash buffer and elution buffer. The DNA thus obtained was quantified.

Primers

The primers used are forward and reverse primers which vary in length of 30–60 nucleotides. Primers restrict the DNA sequence that has to be amplified. As the length of primers needs to be short, this determines the start and end of the region to be amplified. Here, we have used these primers for amplifying the DNA between positions 27 (5'AGAGTTTGATCCTGGCTCAG-3') and 1492 (5'-TACGGTACCTTGTTACGACTT-3') of bacterial 16S rRNA genes under the polymerase chain reaction (PCR) conditions as shown in Table1

PCR

PCR is a popular and scientific method used in molecular biology to obtain millions to billions of copies of a specifically targeted DNA sample, which is carried out under specific temperature conditions following three steps, namely, denaturation, annealing, and extension, the reactions depend on specific time required for various samples of DNA obtained from each and every individual species [11]. Table 2 shows the PCR program set for the bacterial species.

Gel purification and Sanger sequencing

Obtained bands were cut and eluted with 20 µl gel and this purified gene product is taken further for sequencing. Sanger sequencing is the most commonly used method of DNA sequencing which is carried out depending on the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during an *in vitro* DNA replication. This method was developed by Kahl in 1977 [12]. Sanger sequencing can be carried out manually or, more commonly, in an automated fashion through sequencing machine which follows mainly three steps: PCR with fluorescent chain terminating ddNTPs; size separation by capillary gel electrophoresis and laser detection by sequencing machine. For a single reaction, one-sided primer, template DNA, and BDT which contains fluorescent BigDye terminator, dNTPs, ddNTPs, and Taq polymerase were added. PCR conditions used were initial denaturation 95°C for 2 min followed by final denaturation 95°C for 30 s and annealing 50°C for 30 s. The reaction was terminated at 60°C for 4 min for 30 cycles. Followed by ethanol wash and drying of PCR product, it was subjected further for capillary electrophoresis. The plate was linked to sequencer (Genetic analyzer 3130XL) and the obtained .ab1 files were further used for data analysis.

Data analysis

".ab1" files were opened in a software called Finch TV to observe the peaks obtained in electropherogram. These files were converted to ".fsta" and ".pdf" formats using software sequence scanner. The obtained FASTA file was analyzed for the presence of nucleotide pattern and performed blast for further analysis. From the blast, the unknown sequence was selected based on the percentage similarity and e-value. The hits showing maximum percentage and minimum e-value was confirmed.

Phylogeny

Obtained DNA sequences were aligned with nearby hits from BLAST in Clustal omega to construct tree. Phylogenetic tree was constructed to find the useful evolutionary perspectives of existing organisms.

Degradation of polythene bag

Nutrient broth was prepared and autoclaved at 121°C for 15 min and 250 ml was measured and poured into conical flasks. Further, 0.2 g of plastic pieces were weighed and added into conical flask containing nutrient broth. In addition, a loopfull of bacterial isolates was aseptically transferred into

Table 1: The compositions of polymerase chain reaction mixture

S. No.	Components	Concentrations	Volume taken
1	Forward primer (27F)	10 pM	1 µl
2	Reverse primer (1492R)	10 pM	1 µl
3	10 Mm dNTPs	10 µM	1 µl
4	×10 polymerase chain reaction buffer	2x	2.00 µl
5	25 Mm $Mgcl_2$	2.5 mM	0.50 µl
6	Template DNA	25–40 ng	2 µl
7	Taq polymerase	5 U	0.50 µl
8	Nuclease-free water	Make up to 25 µl	4.51 µl

Table 2: Polymerase chain reaction program used for bacterial species

Initial denaturation	95°C	5 min	1 cycle
Final denaturation	95°C	30 s	35 cycles
Annealing	55°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	10 min	1 cycle

the culture. Observation was recorded for a period of 30 and 60 days to measure the degrading capability of bacteria as shown in Table 5 [9].

RESULTS

Spread plate technique

The serial diluted inoculums were spread aseptically and incubated at 37°C for 24 h on nutrient agar plate. A day later, the bacterial growth was observed and colonies were obtained. They were found to be morphologically small, smooth with defined margins. The Grams' nature and biochemical properties of bacteria obtained are as tabulated in Tables 3 and 4.

PCR and sequencing

The genomic DNA isolated from the bacterial colonies and was electrophoresed to quantify the approximate concentration in comparison to standard DNA ladder. After PCR amplification, the desired amplicons of size 1.2 bp of 16S rRNA gene were obtained for all the samples. The PCR product bands were cut and gel purified to get rid of the salts and smears present. The details of all above are shown in Fig. 1. The purified samples were further subjected to DNA sequencing by Sanger sequencing method.

Data analysis

Sequencing result was obtained in the form of electropherogram in software called Finch TV, where the peaks of the graph represent the obtained DNA sequences (Fig. 2). The file format obtained was .ab1 file which was convertedto.pdf format to get overall view of sequence (Fig. 3). For further classification, the FASTA sequence obtained was used to perform BLAST analysis to find out the close relatedness between the organisms. Phylogenetic tree was constructed using Clustal Omega to find out the evolutionary relationship of the organisms based on 16S rRNA gene sequence (Fig. 4). Further, this procedure was carried out for rest of the collected samples.

Table 3: Gram's staining results

Sample	Shape	Color	Characteristics	Bacterial isolates
S_3	Rod	Pink	Gram –ve	*Aeromonas caviae*
S_6	Rod	Pink	Gram –ve	*Aeromonas hydrophila*
P_2	Rod	Red	Gram –ve	*Pseudomonas aeruginosa*
P_4	Rod	Pink	Gram –ve	*Salmonella enterica*

DISCUSSION

The present study deals with the isolation, identification, molecular characterization, and ability of plastic degrading bacteria isolated from dumped garbage soil. The samples were subcultured from the collected samples the bacterial isolates (S_3, S_6, P_2, and P_4). Pure cultures were recovered from serial dilution followed by spread plate technique. Through Gram's staining technique, S_3, S_6, P_2, and P_4 organisms where found to be rod shaped. The isolates obtained were subjected to standard biochemical tests (catalase test, citrate test, motility test, indole test, and MR-VP test). Phylogenetic tree was constructed on amplification by 16S rRNA method. The bacterial isolates were found to be, namely, *Aeromonas caviae*, *A. hydrophila*, *Salmonella enterica,* and *Pseudomonas aeruginosa. A. hydrophila*, *S. enterica,* and *Pseudomonas aeruginosa* isolates showed Gram negative having pink and red color colonies. The biodegradation of polythene bag was analyzed using plastic pieces which were aseptically transferred into the liquid nutrient broth containing bacteria and it was observed for 2 months for weight reduction (Fig. 5). From the above studies, *A. hydrophila* showed more degrading capability. This bacterium was able to degrade up to 48.29% at 60 days (Table 5). Hence, as incubation period increases, polythene bag weight decreases periodically. From literature survey, based on 30 days, we interpreted that *A. hydrophila* (32.68%) showed more degradation when compared to *P. alcaligenes* (20.1%) and *D. nigrificans* (16.2%) [13].

Table 4: Biochemical properties of bacteria

S. No.	Catalase	Citrate	Indole	Motility	MR test	VP test	Bacterial isolates
S_3	+	+	–	+	–	+	*Aeromonas caviae*
S_6	+	+	–	+	–	+	*Aeromonas hydrophila*
P_2	+	+	–	+	–	+	*Pseudomonas aeruginosa*
P_4	+	+	–	+	–	+	*Salmonella enterica*

Table 5: Biodegradation of polythene bag by various bacterial isolates

S. No.	Days of treatment	Initial weight of polythene bag (g)	Final weight of polythene bag (g)	% of weight loss	Species name
S_3	30	0.205	0.169	17.56	*Aeromonas caviae*
	60	0.205	0.125	39.02	
S_6	30	0.205	0.138	32.68	*Aeromonas hydrophila*
	60	0.205	0.106	48.29	
P_2	30	0.205	0.149	27.31	*Pseudomonas aeruginosa*
	60	0.205	0.128	37.56	
P_4	30	0.205	0.192	6.34	*Salmonella enterica*
	60	0.205	0.152	25.85	

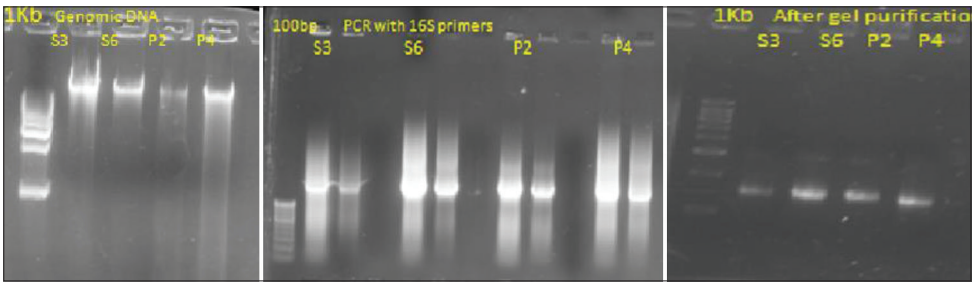

Fig. 1: Genomic DNA isolated; amplified 16s rRNA gene and gel purified product of four different bacteria

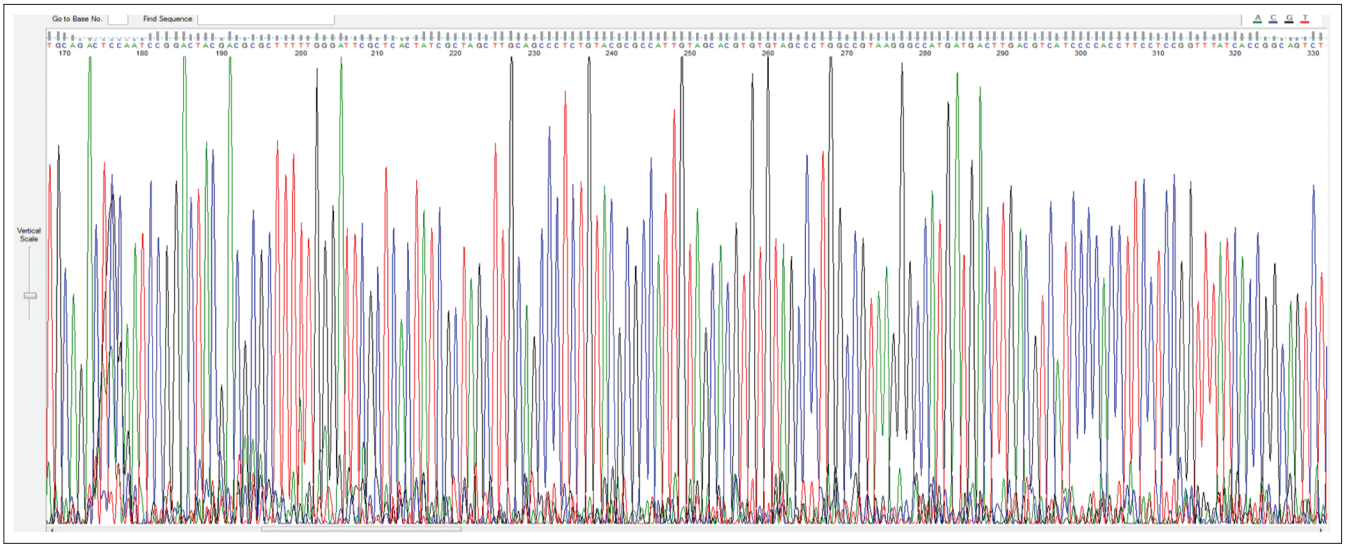

Fig. 2: Electropherogram of S_6 sample opened in Finch TV

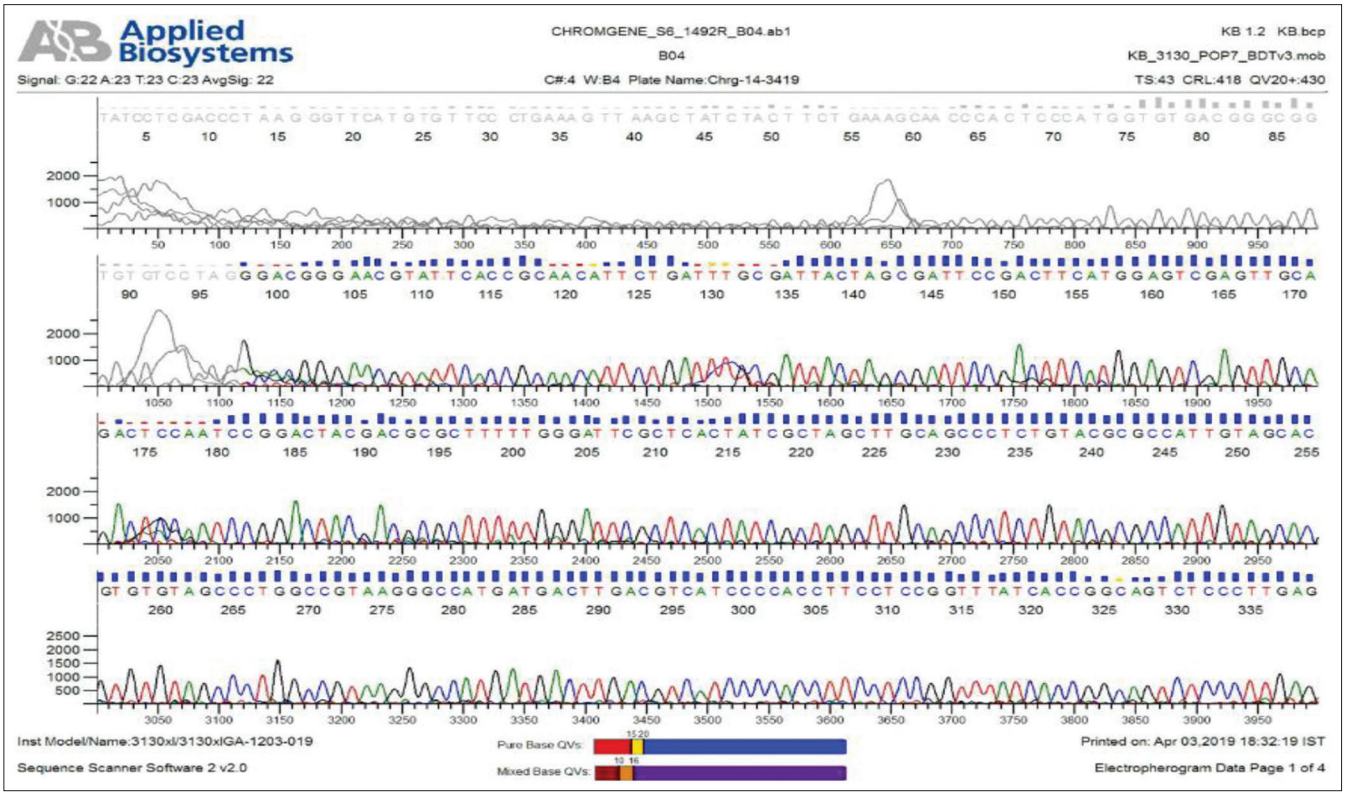

Fig. 3: Pdf file showing S_6 electropherogram

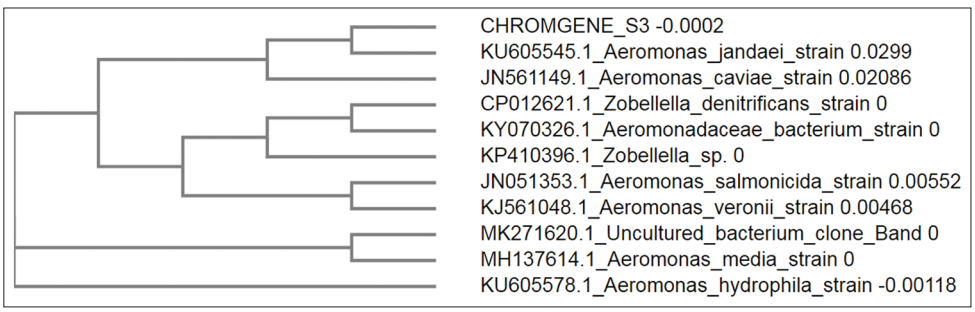

Fig. 4: Phylogenetic tree obtained for S_6 sample

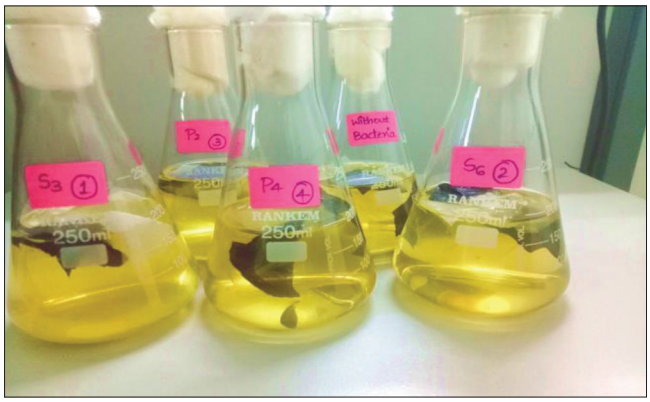

Fig. 5: Biodegradation of polythene bag by various bacterial isolates

A similar study previously conducted by Usha *et al.* included biodegradation of polythene bag and plastic cups which were analysed by 2, 4, and 6 month of incubation in liquid culture method respectively. The microbial species associated with the polythene materials were identified as *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., *Aspergillus nidulans, Aspergillus flavus,* and *Streptomyces* sp. Their results concluded that, among the bacteria *Pseudomonas* sp., degrade faster compared to *Streptomyces* species [14]. This work revealed that *Streptomyces* species possess greater potential to degrade polythene and plastics when compare with other bacteria and fungi which was partially proved in our study. In another study, microbes were isolated from garbage soils of Andhra Pradesh and Telangana. Those microbes were screened by clear zone technique using polythene powder to confirm the degradation activity. To check the efficiency of the biodegradation, weight method was applied under laboratory conditions for 2, 4, and 6 months. Moreover, their results confirmed that *Streptomyces* sp. have highest plastic degradation capacity which is followed by *Pseudomonas* sp. A fungal species *Aspergillus niger* was also found to be effective. Thus, this study revealed that *Streptomyces* sp. plays the most effective role in degrading polythene powder and polymer granules [15]. Research done by Gauri *et al.* stated that out of 15 bacteria that were recovered from different areas three showed the positive results and identified to be *Staphylococcus* sp., *Pseudomonas* sp., and *Bacillus* sp. They concluded that *Bacillus* sp. may act as solution for the problem caused by polythene in nature [8]. Hence, from this study, it can be speculated that microbes have enough potential to degrade plastic with due course of time.

CONCLUSION

The microbial strains from the collected sample were successfully isolated with potential to degrade the synthetic polymer polyethylene. The bacteria were identified to be *A. caviae, A. hydrophila, S. enterica,* and *P. aeruginosa*; these findings have important application in solving plastic waste problem. This suggests that bacteria possess plastic degrading potential and plastic waste problem can be efficiently managed with these organisms. Degradation capacity will obviously show better results in *in vitro* condition compared to natural environment. Research toward engineering the microbes at genetic level to increase its plastic degrading capability should be the priority in future days.

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