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Isolation of microorganisms from petroleum contaminated soil and its effect on degradation of polythene bag and plastic cup.

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ABSTRACT

Plastic waste accumulation is still an increasing problem all over the world. In this view, the biodegradation of polythene bag and plastic cup (both in native and in-vitro conditions) by various group of microorganisms isolated from petroleum contaminated soil is investigated. Five bacterial strains, five fungal strains and five Actinomycetes strains were isolated and identified biochemically. In native conditions, at the end of 6th month, the degradation percentage of plastic was noted as 2.37±0.25% in petroleum contaminated soil and in normal soil as 1.70±0.20% and polythene samples got degraded up to 4.60±0.34% in petroleum contaminated soil and 3.33±0.20% in normal soil. In in-vitro condition, among the five bacterial organisms, *Pseudomonas aeruginosa* showed highest degradation percentage of both plastic cup (1.34%) and polythene (26.7%). Among the five fungal organisms, *Aspergillus niger* showed highest degradation percentage of both plastic cup (8.13%) and polythene (32.4%). Among the five Actinomycete sp, Actinomycete sp.1 showed highest degradation percentage of both plastic cup (11.3%) and polythene (34.9%). On comparing to all organisms, Actinomycete strain 1 showed highest degradation percentage. The 16S rRNA gene sequencing revealed it as *Streptomyces clavuligerus* MTCC 7037. It may be concluded from this work that the petroleum contaminated soil sample is a good source of microbes capable of degrading plastic cup and polythene bag.

Keywords: Biodegradation, petroleum contaminated soil, polythene bag, plastic cup, gene sequencing.

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INTRODUCTION

The worldwide annual production of synthetic polymers has reached about 14 billion metric tons[1]. During the past 3-decades, plastic materials have been increasingly used in food clothing, shelter, transportation and construction, medical, and recreation industries. Plastics are advantageous as they are strong, light-weighted, and durable. However, they are disadvantageous as they are resistant to biodegradation, leading to pollution, harmful to the natural environment. A worldwide increase in the use of these materials has generated issue of solid waste disposal[2]. Millions of tons of solid waste are disposed off annually in the world and a large proportion consists of plastics[3]. Plastic is used very commonly in the world because they are cheap, easy to make and they will last long as well. These useful qualities make plastic a real menace to the environment. As it is so cheap that people discards it soon especially carries bags and disposable bottles. As these materials are long lasting and difficult to decompose, it persists in the earth for many centuries resulting in enormous environment pollution. Plastic wastes clog the drains and thus hit especially urban sewage systems. The plastic wastes being dumped into rivers, streams and seas contaminate the water, soil, marine life and also the very air we breathe. Choked drains provide excellent breeding grounds for disease-causing mosquitoes besides causing flooding during the monsoons. The polythene could sometimes cause blockage in intestine of fish, birds and marine mammals[4]. The successful production and marketing of biodegradable plastics will help alleviate the problem of environmental pollution. In the past 10 years, several biodegradable plastics have been introduced into the market. However, none of them is efficiently biodegradable in landfills. For this reason, none of the products has gained widespread use[5]. Hence, there is an urgent need to develop efficient microorganisms and their products to solve this global issue[6]. Plastic degradation by microorganisms has become the main area of research and now advanced studies are being carried out. Studies have been made in this regard for the past 5 decades and successfully many degrading organisms have been identified and isolated. Enzymes degrading plastics have been studied from these microorganisms and being investigated for their practical applications to reduce the environmental pollution due to plastic accumulation[7]. Biodegradation of plastics has been studied extensively earlier by many scholars and its importance has been highlighted[8-16]. Polythene and plastic are the two polymers with wide ranging applications. They are recalcitrant and hence remain inert to degradation and deterioration leading to their accumulation in the environment, and therefore creating serious environmental problems. In this context, an attempt was made to study the biodegradation of

polythene and plastic strips inside the laboratory (under controlled condition) and outside the laboratory (under natural condition) with the help of microbial tools. In view of this, the present investigation was carried out to identify various microorganisms from petroleum contaminated soil to degrade plastic and to reduce the environmental pollution. Since petroleum and natural gas is being the raw materials used for the production of most plastics, microorganisms isolated from petroleum contaminated soil may have a greater ability to degrade plastic because they adapt to the environment and start utilizing the plastic as carbon source for their survival[17].

MATERIALS AND METHODS

Microorganisms from petroleum contaminated soil sample

Petroleum contaminated soil sample was collected from the automobile shop at Gowrivakkam, Chennai. They were transported to the laboratory and stored at 4^{o}C. The collected soil samples were mixed thoroughly and passed through a 2 mm sieve to remove gravel and debris. Serial dilution was carried out and the colonies of Bacteria, Fungi and Actinomycete were isolated from the respective dilutions using respective media (Bacteria –Nutrient Agar, Fungi- Sabouraud Dextrose Agar, Actinomycete- Actinomycete isolation Agar). The well isolated bacterial colonies were identified based on their colony morphology, Gram'staining, motility and biochemical tests using Bergey's Manual of Determinative Bacteriology[18]. The well isolated fungal colonies were identified based on the macroscopic (colony morphology, Colour and Texture of the colony) and microscopic (Hyphae and Spore morphology) observations. For the identification of yeast, Gram's staining, carbohydrate assimilation test, carbohydrate fermentation test and Germ tube tests were performed. Based on the macroscopic (Colony morphology, Colour and Texture of the colony) and microscopic observations the Actinomycetes species also confirmed. The microbial colonies were also enumerated at their respective standard dilutions.

Degradation in native conditions (Kathiresan[6])

Strips of polythene bag and plastic cup of one gram in weight were buried at a depth of 5 cm in the area where the petroleum contaminated soil was collected and in also normal soil. The materials were allowed to degrade naturally for 6 months. The samples were collected at the intervals of 2 months using sterile forceps and they were aseptically brought to the laboratory, washed thoroughly with distilled water and shade dried for 24 hours. After, which the weight loss of the samples were measured and noted. The degradation percentage of plastic cup and polythene bag were calculated and tabulated.

Degradation in in-vitro condition using isolated organisms (Kathiresan[6])

50ml of the liquid media was prepared and inoculated with the isolated microorganisms. Triplicates were kept for each organism. After 24 hours of incubation, sterilized plastic and polythene discs were added separately and kept for 30 days incubation in shaker. The broth used for Bacterial degradation is Nutrient broth, fungal degradation is Sabourauds Dextrose broth and Actinomycete degradation is Actinomycete Isolation Broth. After 30 days, the plastic and polythene strips were removed from the flask using sterilized forceps and washed thoroughly with distilled water, shade dried for 24 hours. The weight of the plastic and polythene strips were measured. The degradation percentage of plastic and polythene strips by each microorganism were calculated. Degradation percentage of plastic and polythene strips was calculated by using the following formula and also compared.

Degradation percentage = Weight loss x 100/ Original weight

DNA extraction and 16S rRNA Sequencing

Genomic DNA was extracted from the organism that showed highest percentage of degradation of polythene and plastic cup. The method described by Ausubel *et al*[19] was slightly modified and used for genomic DNA isolation. The purity of DNA solutions was checked spectrophotometrically at 260 and 280 nm, and the quantities of DNA were measured between 260 and 280 nm. The 16S ribosomal RNA gene was amplified by using the PCR method with Taq DNA polymerase and primers F (5'AGAGTTTGA TCCTGGCTCAG 3') and R (5'ACGGCTACCTTGTTACGACTT 3'). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94^{o} C for 5 min followed by 30 cycles at 94 ^{o} C for 45 sec, primer annealing at 42 ^{o} C for 1 min and primer elongation at 72 ^{o} C for 40 sec. At the end of the cycling, the reaction mixture was held at 72 ^{o} C for 10 min[20]. PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The PCR product obtained was sequenced by an automated Sequencer (ABI PRISM ® 377 DNA Sequencer (Applied Biosystems)). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species contained in genomic database banks, using the NCBI BLAST available at http://www. ncbinlm-nih.gov/.

RESULTS AND DISCUSSIONS

Microorganisms

Environmental pollution is an increasing problem at present and plastics play a major role in polluting the environment. The present study is aimed at the elimination of plastics from the

environment by microbial degradation that leaves no harmful residues. Since petroleum and natural gas is being the raw materials used for the production of most plastics, microorganisms isolated from petroleum contaminated soil may have a greater ability to degrade plastic because they adapt to the environment and start utilizing the plastic as carbon source for their survival[17]. Atiq *et al.*, [21] isolated six bacterial strains from soil buried expanded polystyrene films showing adherence and growth with the polystyrene as a sole carbon source. *Brevibacillus borstelensis* and *Rhodococcus ruber* have been shown to degrade the CH_2 backbone and use polyethylene as its sole carbon source due to the hydrophobic nature of the cell membranes[22]. Nayak and Tiwari[16] aimed to isolate various plastic and polythene degrading microorganisms from the soil. An attempt has been made to cover the mechanism of biodegradation, the various bacterial and fungal organisms that have been reported for the same, method adopted for the studies and different characterization techniques followed to measure the extent of degradation.

In the Present investigation, a total of 15 isolates were isolated from the soil samples. Among them, five were found to be bacterial strains, five were found to be fungal strains and five were found to be actinomycetes strains. They were designated as B1, B2, B3, B4 and B5 (bacterial strains), F1, F2, F3, F4 and F5 (fungal strains) and A1, A2, A3, A4, A5 (actinomycetes strains) respectively. The bacterial strains were identified as B1- *Staphylococcus aureus*, B2- *Bacillus megaterium*, B3- *Micrococcus luteus*, B4- *Serratia marsescens* and B5- *Pseudomonas aeruginosa*. The four fungal molds were identified as F1-*Aspergillus niger*, F2*Mucor sp*, F3-*Aspergillus fumigatus*, and F4 – *Fusarium oxysporum* and the yeast F5 was identified as *Candida albicans.* The actinomycetes colonies initially resembled that of bacteria and after 14 days of further incubation at 30^{o}C, the colonies became powdery in appearance with each colony having different pigmentation. Microscopic appearance in slide culture technique revealed filamentous structures with various types of spore arrangement. Thus the five strains were confirmed to be Actinomycetes species A1, A2, A3, A4 and A5. These microbial colonies were also enumerated using Quebec Colony Counter and the results were tabulated (Table-1).

Table 1. Enumeration of microorganisms in petroleum contaminated soil

Organism	Number of colonies (cfu/mg)
Bacteria	$57 \pm 1 \times 10^{7}$
Fungi	$15 \pm 2 \times 10^{3}$
Actinomycete	$6 \pm 2 \times 10^{5}$

(Mean value of the Triplicates ± SD)

Kathiresan,[6] reported that the microbial species of mangrove soils degrading polythene bags were *Bacillus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Diplococcus* sp., and *Micrococcus* sp. (belong to Gram- positive bacteria); *Moraxella* sp. and *Pseudomonas* sp. (belong to Gram-negative bacteria); and, *Aspergillus niger, A. ornatus, A. cremeus, A. flavus, A. candidus, A. ochraceus, A. nidulans,* and *A. glaucus* (belonging to fungi). Similar types of organisms were reported to be associated with polythene degradation by Vijaya and Mallikarjuna Reddy[11], and Orhan *et al.*, [23]. Similar report was given by various authors stating that *Pseudomonas aeruginosa, Aspergillus fumigatus*, have shown an ability to degrade polyethylene [24, 25]. Kathiresan[6] reported that the microbial species from mangrove soil recorded in degrading plastic bags were *Staphylococcus* sp., *Streptococcus* sp., *Micrococcus* sp., *Moraxella* sp., *Pseudomonas* sp., *Aspergillus niger and A. glaucus.* Khan *et al.*, [26] also reported that *Bacillus lentus, B. circulans, B. badius, B. laterosporus, B. larvae, B. thuringiensis* and *Micrococcus sedentarius* was associated with plastic floppy disc detoriation.

Degradation in native conditions

In both normal and petroleum contaminated soil, there was no weight loss of plastic cup up to 4 months. Gradual degradation was observed after 4th month and at the end of 6th month. The degradation percentage of plastic was noted as 2.37±0.25% in petroleum contaminated soil and in normal soil as 1.70±0.20%. On the other hand, polythene did not show any degradation up to 2 months in petroleum contaminated soil and up to 4 months in normal soil. The degradation percentage in petroleum contaminated soil after 4 months was noted as 1.83±0.04%. At the end of 6th month, 4.60±0.34% of polythene was degraded in petroleum contaminated soil and 3.33±0.20% in normal soil (Table-2 a & b).

Table 2. Degradation percentage of plastic samples in native conditions

Degradation percentage for plastic cup

Duration in months	Normal soil	Petroleum contaminated soil
2	0	0
4	0	0
6	1.70±0.2	2.37±0.25

(Mean value of the Triplicates ± SD)

Degradation percentage for polythene bag

Duration in months	Normal soil	Petroleum contaminated soil
2	0	0
4	0	1.83±0.04
6	3.33±0.20	4.60±0.34

(Mean value of the Triplicates ± SD)

Similar study was reported by Kathiresan[6], where the plastic cups and polythene bags were buried at a depth of 5 cm in the mangrove soil under two zones, colonized by *Rhizophora sp.* and or *Avicennia sp*., along the Vellar estuary (11°29' N; 79°46' E; southeast coast of India). He reported that in both *Rhizophora* zone and *Avicenia* zone, the polythene was not degraded up to 4 months and gradually it started to degrade after 4th month. In contrast, plastic did not show any degradation for 6 months in both the zones. Labuzek *et al*., [27] also reported that after 8 months of soil burial, the degradation percentage of LDPE polyethylene were only 0.5% which is much less than the present report. Suseela and Kiran Toppo[28] also reported that partial decomposition/degradation of polythene due to a consortium of aquatic microbial activity during investigations of oligotrophic water bodies of Uttar Pradesh. Vijaya and Mallikarjuna Reddy [11] also reported that, composting of polythene films for 4 months did not show any degradation. After 4 months of composting in municipal solid wastes, the loss in weight was 2.9-4.5% for HDPE films and 10.5-11.6% for LDPE films at the end of 12 months.

Degradation in invitro conditions

In invitro conditions, the highest degradation percentage was found in A1 strain of actinomycete that showed 11.3% of plastic and 34.9% of polythene degradation. In fungal strains, *Aspergillus niger* showed highest degradation of 8.13% for plastic and 32.4% for polythene and in Bacteria, *Pseudomonas aeruginosa* showed 1.34% of plastic and 26.7 % of polythene degradation (Figure 1, 2 and 3). Kumar *et al.*, [29] reported that the degradation of commercial polythene carry bags made of high density polyethylene (HDPE) and low density polyethylene (LDPE) by *Staphylococcus, Micrococcus, Listeria* and *Vibrio* isolated from mangrove soil was showed only 5 % degradation over a period of 8 weeks. Labuzek *et al*., [27] also reported that 84 days exposure of LDPE polyethylene films to *P.aeruginosa* showed degradation of 0.43%. On comparing the present investigations with the study of Kathiresan [6], the degradation percentage of both bacterial and fungal organism from mangrove soil were much less when compared with the microorganisms isolated from petroleum contaminated soil. On comparing the degradation percentage of plastic and polythene by all organisms, the Actinomycete strain 1 showed the highest degradation percentage and was given for gene sequencing and compared in BLAST for the strain identification. The 16S rRNA gene was sequenced and the (1454 bp) analysis clearly demonstrated that the Actinomycete strain 1 was a member of the genus *Streptomyces* and exhibited maximum similarity with the 16S rRNA sequence of *Streptomyces clavuligerus* MTCC 7037 (93% sequence similarity). Khan *et al.*, [26] also reported that, detoriating plastic floppy disc shows a diversity of *Streptomyces species*. Based on the appearance of isolates, the major

isolates belong to the *Streptomyces* genus (91.9%) and *Micromonospora* genus (8.1%). Hoang, [30] also reported that PHB-degraders are widely distributed among the families of *Pseudonocardiacece* and related genera, including *Micromonosporaceae, Thermonosporaceae, Streptosporangiaceae* and *Streptomycetaceae*.

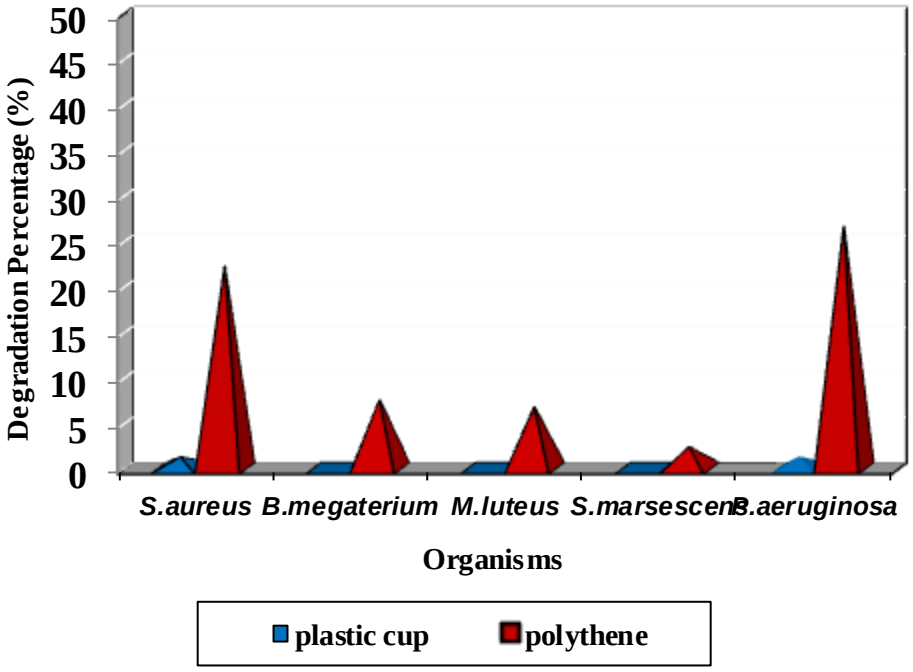

Figure 1. Percentage of degradation by bacteria

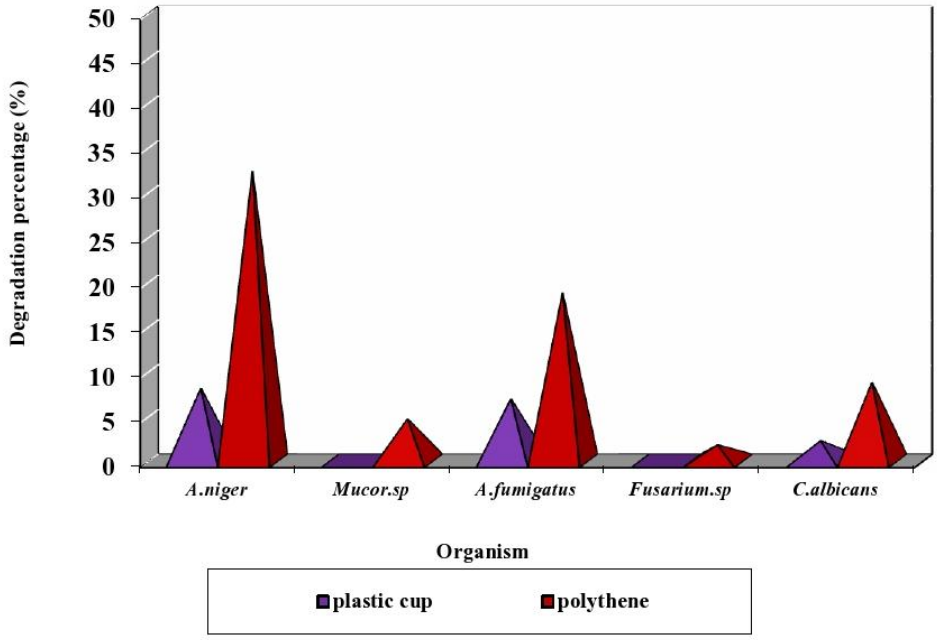

Figure 2. Percentage of degradation by fungi

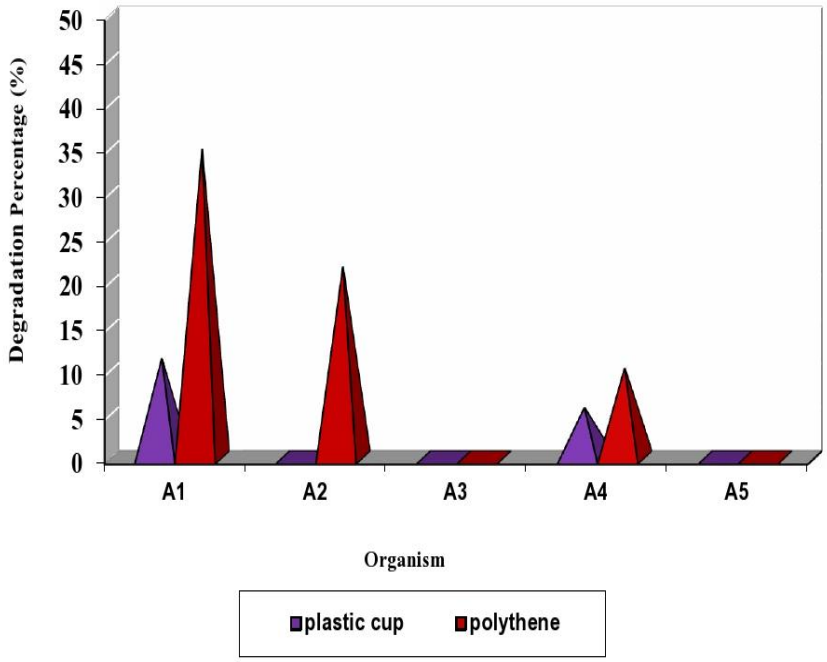

Figure 3. Percentage of degradation by Actinomycetes

CONCLUSION

Petroleum contaminated soil samples have microorganisms that are capable of degrading polymers and hydrocarbons, since most of the plastics are made of petroleum and natural gas, these organisms would aid in the degradation in a less time when compared with other organisms isolated from other areas. Thus the time consumption for this process would be less. However, further molecular level research has will give a clear picture of the enzyme system in these microorganisms that are involved in the process.

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