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Isolation of Plastic Degrading Micro-organisms from Soil Samples Collected at Various Locations in Mumbai, India

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Abstract

Accumulation of plastics, especially Polyethylene terephthalate (PET) and Polystyrene (PS), is an ever increasing ecological threat due to its excessive usage in everyday human life. In contribution to regulate this potent ecological threat an attempt has been made to isolate plastic degrading micro-organisms from five different soil samples viz. Garden soil, Mangrove soil, Forest soil, soil near Petrol Pump, and Garbage soil. In the present study, it was found that after four months of incubation period the percentage loss in weight of PET and PS was highest in the Garden soil and Garbage soil respectively as compare to other soil samples in regards with Gram positive coccobacillus, Gram negative cocci, Gram negative rod shaped bacillus, Gram positive cocci (in clusters) in Garden soil and Gram negative cocci (in singles) in Garbage soil. In addition to this, the degradation rate of PET and PS by Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, and Aspergillus Niger was also observed separately. It was also noted that the percentage loss in weight of PS was highest by Bacillus subtilis (in Nutrient broth and Bushnell Hass broth), whereas in the case of PET, percentage loss in the weight was highest by Bacillus subtilis in Nutrient broth, Pseudomonas aeruginosa in Bushnell Hass broth, and Aspergillus niger in Rose Bengal broth.

Keywords: Polyethylene terephthalate, polystyrene, bacillus subtilis, gram positive coccobacillus, pseudomonas aeruginosa.

Introduction

Plastics are non-biodegradable, strong, durable, moisture resistant, light weight polymers of carbon along with hydrogen, nitrogen, sulphur, and other organic and inorganic elements and are manufactured from fossil fuel which is a non-renewable source[1-2]. Based on type of chemical reaction plastics are classified as "thermoplastics" and "thermoset". According to their chemical structure they are classified as polystyrene, polypropylene, low density polyethylene, high density polyethylene, polycarbonate[1]. As per a case study, plastic contribute about 8% of the total solid waste generated[3].

Styrene molecules polymerized to form long chain hydrocarbon molecule known as Polystyrene (PS) which is a synthetic, thermoplastic, non-biodegradable plastic and has preeminent features like high impact resistance, food expectance, flame retardant, fast moulding capacity[4-6]. PS in all can be moulded into different shapes to form commercial products such as disposal plastic cutlery, food containers, CD jewel, License, insulator at low temperatures, plate, Petri dishes and other laboratory containers and many commercial products[5-6]. Even for producing light grade fuel by mixing with Polypropylene and Fe_2O_3 as catalyst and PS sheets can be as component for Green Building[6-8]. PS and Expandable Polystyrene (EPS) are markedly used in packaging and construction sectors. The global demand for PS and EPS was 13 million tons by 2000 with further increase to 14.9 million tons in 2010 and now it is estimated to increase around approx 23.5 million tons by 2020[9-10]. Styrene molecules are potential human carcinogen and neurotoxin which consequently increases the danger of lukemia and lymphoma, and are also believed to affect epithelial ion channels[11]. Working staff who are exposed to styrene, xylene, toluene, methyl ketone, and many such harmful toxins which are used in styrene manufacturing units, experience lack of concentration, hearing problem, decreased colour discrimination and also some abnormalities like decrease in sperm count[12-13]. Styrofoam can also enter into food chain by aquatic and terrestrial organisms and Styrofoam debris even has potential to bind to mercury[14].

Ethylene glycol react with either terephthalic acid or dimethyl terephthalate to form bis (hydrooxyethyl) terephthalate (BHET) which further polymerizes to Polyethylene terephthalate (PET) which belongs to polyester, a synthetic polymer[15-16]. By the end of 2015, it is estimated, that the total rise in plastic consumption will boom to 18.9 million tonnes. In India, the collection rate of PET is 75% which is substantially higher than the global standard collection rate which is around 36% and also one of the major hindrances in recycling process. PET utilization in various sectors has been found to increase on an average by 11% per year which is one of the rapid growths among synthetic plastics. On an average, in Mumbai, the total utilization of PET bottles is estimated to be 25,03,334 [17]. Antimony, a metalloid

and a catalyst used in polycondensation reaction of manufacturing process, leached from PET in to bottled water, causes chronic and acute health effects such as diarrhoea, vomiting, and stomach ulcers. Along with antimony there are also some other chemicals like brominates compound that possibly can leach into water which may lead to irritation of the skin and mucus membrane. But study is still not clear whether this leaching would cause any harm to human. Other than antimony, phthalate added as a softening agent is another endocrine disruptor found to be released from PET in bottled water. This is supported by many studies which include bioassays using estrogenic sensitive snail and modified yeast having human estrogenic receptor in order to confirm its leaching and estrogencity[18-20]. Other metals like cadmium, zinc, lead, etc. which are used in plastic toys leads to various chronic and acute health hazards and minor disorders[21].

At present there are principally three ways to get rid of plastic - incineration, dumping in landfills and recycling. But all three methods are not proficient to manage the existing bulk of plastic waste generated due to its surplus demand in various sectors. So to get rid of the existing plastic waste, natural and eco-friendly methods should be used. Landfills cover large area which can otherwise be used for more productive purpose such as agricultural practices. Absence of oxygen in landfill further resists its natural degradation process. In incineration process harmful gases and greenhouse gases are released in environment. Colorants, stabilizers and other contaminates overall compels recycling process of PET ineffective[16].

There are three basic steps through which synthetic plastic can be degraded - photo oxidation, thermo oxidative degradation and biodegradation. From above three methods most acceptable method is biodegradation since it is cheap and environmentally friendly, followed by photo oxidation using UV radiations and microbes. Thermo oxidative is not preferred as it requires energy more than that is available in natural environment[18]. Degradation can be microbial or enzyme based, but in both the processes microorganisms adheres on plastic surface. In microbial degradation, those surfaces of plastic are then exposed for colonization, whereas the enzymatic degradation proceeds with hydrolysis, followed by adherence. Enzymes interact with polymer surface and break down hydrolytic bonds of the polymer to convert it into simpler form, may be a monomer, a dimer or a trimer.

Degraded plastic is further processed by microorganisms by two metabolism method- aerobic and anaerobic metabolism, which results in the formation of carbon dioxide and water as the end products in both the metabolisms. In addition to the formation of above end products, methane is also released in anaerobic metabolism[22-23].

Biodegradation of a polymer depends upon its molecular weight which determines various physical properties and its chemical composition. Degradability is inversely proportional to molecular weight[22]. Kevin O' Connor and his European colleagues found out that *Pseudomonas putida* CA3 can degrade styrene and its metabolism is triggered by styrene and styrene-degradation products[24]. Other than *P. putida* there are some micro-organisms like *Micrococcus luteus* and *Masoniella sp., Actinomycetes*[22-25]. Degradation of PET was found to be associated with *Nocardia sp.* and esterase enzyme along with some bacteria belonging to *Bacillus* species[26].

As synthetic plastic, biodegradable plastics[27] such as polycaprolactone (PCL) and polybutylene succinate (PBS) are petroleum based but they can be degraded to some extent by micro-organisms[12]. Biodegradable PET manufactured from PET waste was found to be capable of getting degraded by soil microorganisms such as *P. Putida* GO16, *P. Putida* GO19, and *P. frederiksbergensis* GO23[27]. Some are bio-based plastic such as polyhydrooxybutyrate (PHB) and polylactide (PLA) which are derived from biomass or renewable sources and hence are biodegradable[29]. Bio-based plastic and biodegradable plastic comes under bioplastic category. Bioplastic can be one of the best alternatives for synthetic plastic[22,30]. PLA and PHA are not easily recycled and require special recycling technologies and are 2-10 times more expensive even though they have relatively poor mechanical strength as compared to conventional plastics[31].

It is a need in today's world to regulate the plastic pollution. One of the solutions to degrade existing plastic, thereby reducing plastic pollution, is microbial degradation since micro-organisms are capable of utilizing organic and inorganic molecules. Soil is a major natural resource of varieties of micro-organisms. The main objective of the present study was to isolate PET and PS degrading micro-organisms from five different soil samples collected at five different locations in Mumbai city. Also, the degradation rate of *Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes*, *and Aspergillus niger* on plastic samples was observed separately by calculating percentage weight loss.

Material and Methods

Soil sample collection: Soil was dug few meters in depth and was collected in a container.

Table-1
Five different soil samples were collected from different locations, within Mumbai city

Soil Sample	Location
Garden Soil	Siddhivinayak Temple Garden
Mangrove Soil	Versova Beach
Forest	Sanjay Gandhi National Park
Soil near Petrol Pump	Worli
Garbage Soil	Prabhadevi

Preparation of Winogradsky's column: The collected soil samples were placed separately in the respective bottles (1.5 litre "Thumbs Up" bottle, top part was cut off). Equal strips of PET (bottle sample) and PS (disposable plate) were cut, weighed accurately on accurate weighing machine and were placed separately in each soil samples. Bushnell Hass Broth was poured in each column such that it formed a layer of few inches above the settled soil samples. Columns were closed by sealing bottles with the same top parts of each bottle which was cut earlier. These columns were kept for incubation at room temperature for 4 months.

Biodegradation of plastic samples after 4 month of incubation period: Sterile test tubes containing Bushnell Hass Broth were prepared. Winogradsky's columns were opened by removing sealed top of bottles. With the help of sterile gloves plastic samples from all the soil samples were removed and were further washed in sterile Bushnell Hass Broth in aseptic conditions. Plastic samples were placed on filter papers for drying and were weighed on the same weighing machine which was used earlier.

Isolation of probable plastic degrading microorganisms: These probable plastic degrading microorganisms were isolated on Sabourauds Agar medium and Nutrient Agar medium (incubated at room temperature for 24 hours). Gram staining of colonies was performed.

PET and PS degrading microorganisms: To know exactly which colonies were responsible for degradation, each colony was isolated on its own plate by standard streaking method. Few strips of PET and PS samples were cut. They were weighed accurately and placed in the appropriately labelled test tubes containing Bushnell Hass Broth. This whole set up was sterilized in an autoclave. The whole sterile set up was inoculated with isolated colonies and was incubated for a month at room temperature. Control set was maintained (no inoculation with any microbe) simultaneously. After one month in aseptic conditions, plastic samples were removed from broth and were placed on filter papers for proper drying[32-33]. Percentage loss in weight was calculated. Colonies which degraded plastic samples were indicated by weight loss. These colonies can be possibly further studied for their colony characteristics, biochemical test and their respective genetic makeup.

Check for Biodegradation of PET and PS by five different microorganisms: Small strips of PET and PS were weighed and placed aseptically in conical flasks containing 50 ml of sterile Nutrient broth, Bushnell Hass Broth, Rose Bengal Broth which were inoculated with *Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, Aspergillus niger.* This set up was then incubated at room temperature for a month. Control set was maintained. After one month, these plastic samples were then removed and washed with water and dried completely. Different flasks were maintained for each treatment[33]. They were weighed accurately and percentage loss in their weight was calculated by the formula:

$$\% \text{ Total weight loss} = \frac{W_i - W_f}{W_i} \times 100$$

Where: W_i = Initial weight (Weight of plastic before incubation), and W_f = Final weight (Weight of plastic after incubation)

Results and Discussion

The pervasive presence of microorganisms and their ability to perform various functions such as consuming oil spills, or degradation activities on waste, plays a crucial role in maintaining environmental health[34].

From table-2 and table-3, it can be summarized that the maximum percentage of biodegradation of PET was by Gram positive *coccobacillus*, Gram negative *cocci*, Gram negative *bacilli* (rod shape), Gram negative *cocci* (in clusters), Gram positive *cocci* (in clusters) from garden soil and of PS was by Gram negative *cocci* (in single) isolated from garbage soil. This Garden soil was transported from mountainous areas of New Mumbai, particularly from Panvel, and then it was provided with biofertilizer in garden for making soil more fertile for growth of different plants in the garden. Use of biofertilizer enhanced the nutritional quality of the soil, thus, giving an advantage for growth of many microorganisms. Out of which some have the ability to degrade PET and PS but the rate of degradation of PET is high, as can be seen in Table-4 and Table-5.

Garbage soil was rich in nutrients because of the waste dumped on it. So the soil was rich in microbial flora and some of them found to be plastic degrading through experimental evidence. PS was degraded much faster in garbage soil than PET as per table-6.

Significant number of researches supports such degradation ability of micro-organisms. Polystyrene can be degraded by *Pseudomonas sp*. These polystyrene samples were not given any toxic pre-treatment. Species of *Pseudomonas* were isolated from the areas which were polluted with polyolefins and then subcultured on synthetic media in the lab[35]. *A. niger and Rhodococcus fascians* biodegrade PS at a faster rate as compared to *B. subtilis, P. aeruginosa* and *Micrococcus luteus*[4]. S. Umeshwari *et al*. revealed that PET and PS foam buried in soil, cow-dung, and sewage can be degraded by fungi by cleaving bonds of PET and PS foam polymer and these decomposition can be confirmed by FTIR spectroscopy which showed stretching between the constituent bonds like C=C, C-H, OH, C-O, and C=O of polymer[36]. Studies also support the degradation of other plastics by various soil microorganisms[37-38].

Table-2
Percentage weight loss of PET (in 4 months at R.T.) in five different soil samples collected from five different locations

Sr. No.	Soil Sample	Polyethylene terephthalate		Total loss in weight (gm)	Percent weight loss
		Weight of PET before incubation (gm)	Weight of PET after incubation (gm)		
1	Forest Soil	1.416	1.383	0.033	2.330
2	Garbage soil	1.171	1.142	0.029	2.553
3	Garden soil	1.387	1.315	0.072	**5.191**
4	Mangrove Soil	1.437	1.387	0.05	3.479
5	Soil near petrol pump	1.435	1.380	0.055	3.832

Table-3
Percent weight loss of PS [in 4 months at R.T] in 5 different soil samples collected from 5 different locations

Sr. No.	Soil sample	Polystyrene		Total weight lost (gm)	Percent loss in weight
		Weight of plastic before incubation (gm)	Weight of plastic after incubation (gm)		
1	Forest Soil	0.074	0.053	0.021	28.378
2	**Garbage soil**	0.153	0.108	0.045	**29.411**
3	Garden soil	0.129	0.099	0.03	23.256
4	Mangrove Soil	0.079	0.077	0.002	2.531
5	Soil near petrol pump	0.098	0.092	0.006	6.122

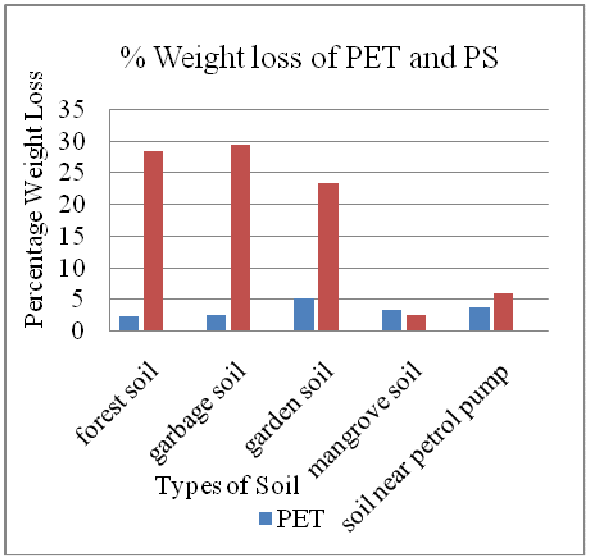

Figure-1
Graphical representation of percentage loss in weight of PS and PET in five different soil samples (Mangrove Soil, Forest Soil, soil near Petrol Pump, Garden Soil, and Garbage Soil)

Table-4
Isolation of probable PET degrading organisms from Garden soil on Nutrient agar medium

Colony no.	1	2
Colour	Yellow	White
Margin	Circular	Filamentous
Elevation	Concave	Flat
Opaque / translucent	Opaque	Opaque
Shape	Round	Irregular
Size(cm)	0.1	2.5
Consistency	Butyrous	Butyrous
Gram nature	Gram positive coccobacillus	Gram positive cocci in cluster
Initial weight (gm)	0.252	0.287
Final weight (gm)	0.252	0.250
Total loss in weight (gm)	0.252	0.037
% loss in weight	0	12.891

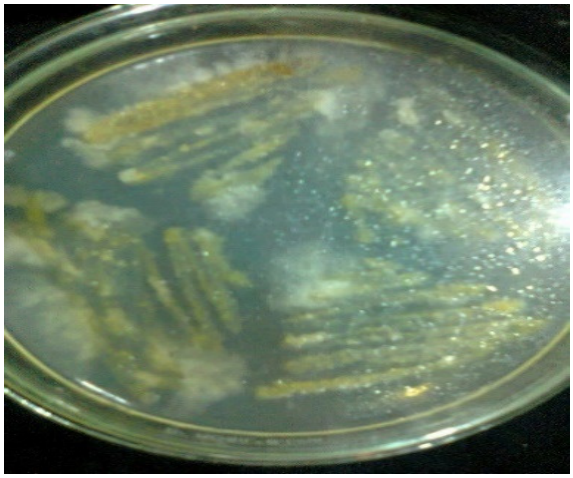

Figure-2
Isolation of probable PET degrading organisms from Garden soil on Nutrient agar medium

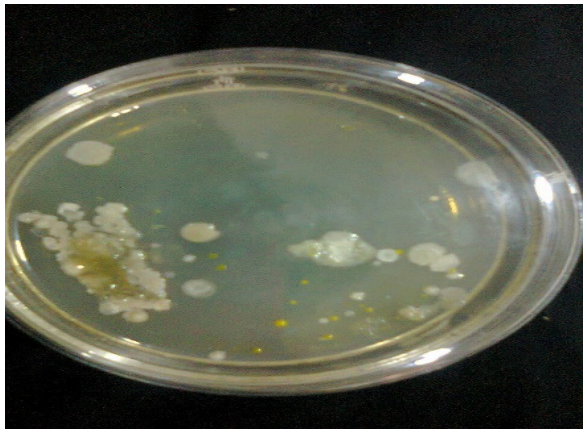

Figure-3
Isolation of probable PET degrading organisms from Garden soil on Sabouraud's agar medium

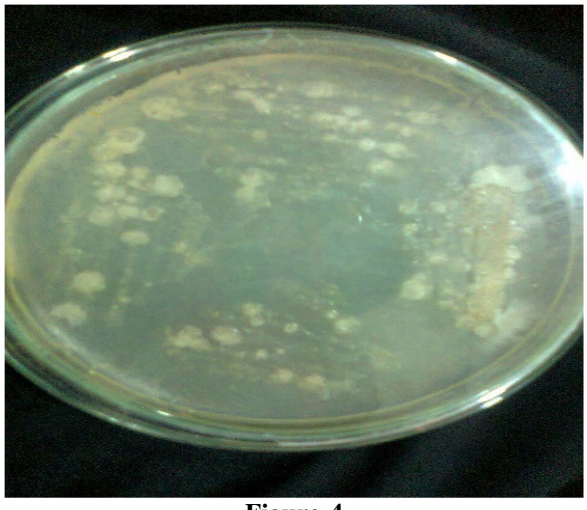

Figure-4
Isolation of probable Polystyrene degrading organisms from Garbage soil on Sabouraud's agar medium

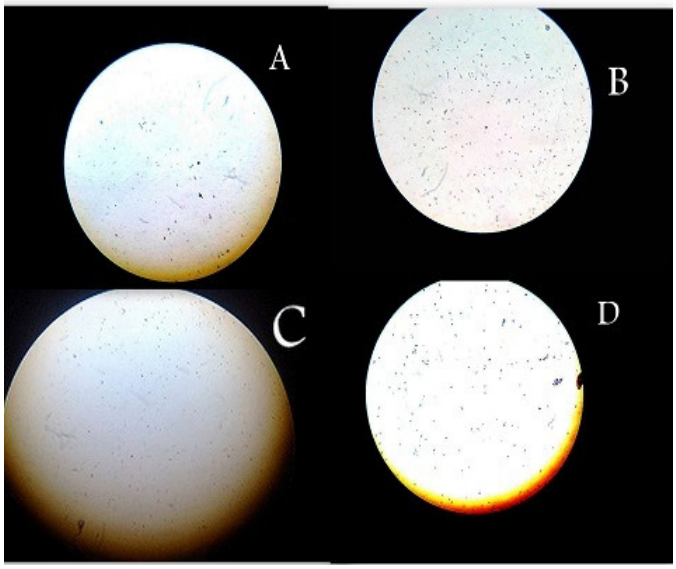

Figure-5
Gram staining results of isolates A-M1S1, B- P2N2, C-F2N1, and D- Garb1S1
[F- Forest soil, P- soil near petrol pump, Garb-Garbage soil, M-Mangrove soil, S- Sabouraud's agar medium, N-nutrient agar medium, 1- PET, 2- PS]

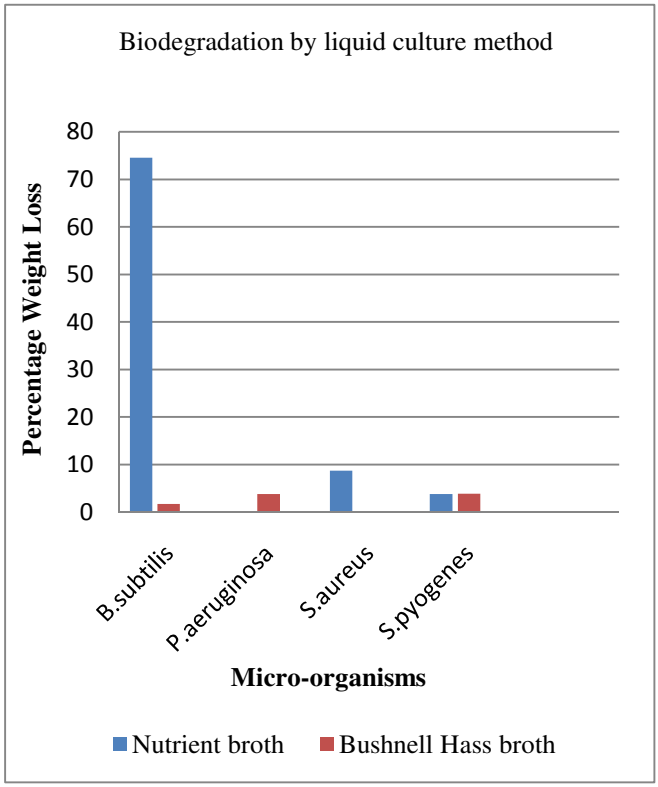

Figure-6
Percentage loss in weight of PET by *Streptococcus pyogenes*, *Bacillus subtilis*, *Pseudomonas aeruginosa, Staphylococcus aureus* in Nutrient broth and Bushnell Hass Broth

Table-5
Isolation of probable PET degrading organisms from Garden soil on Sabouraud's agar medium

Colony no.	1	2	3	4
Colour	Yellow	White	Red	Creamish white
Margin	Circular	Circular	Circular	Wavy
Elevation	Raised	Flat	Raised	Flat
Opaque / translucent	Opaque	Opaque	Opaque	Opaque
Shape	Round	Round	Round	Irregular
Size(cm)	0.1	0.3	0.1	0.8
Consistency	Butyrous	Butyrous	Butyrous	Butyrous
Gram nature	Gram positive Coccobacillus in cluster and singles	Gram negative Cocci in single	Gram negative Bacilli Rod shape	Gram negative Cocci in clusters
Weight of plastic before incubation (gm)	0.268	0.245	0.263	0.260
Weight of plastic after incubation (gm)	0.261	0.243	0.262	0.259
Total loss in weight of plastic. (gm)	0.007	0.02	0.001	0.001
% loss in weight of plastic	2.612	8.163	0.380	0.384

Table-6
Isolation of probable Polystyrene degrading organisms from Garbage soil on Sabouraud's agar medium

Colony no.	1	2
Colour	Yellow	White
Margin	Circular	Circular
Elevation	Concave	Raised
Opaque / translucent	Opaque	Opaque
Shape	Round	Round
Size(cm)	0.2	00.1
Consistency	Butyrous	Butyrous
Gram nature	Gram negative cocci in single	Gram positive coccobacillus In clusters
Weight of plastic before incubation.(gm)	0.029	0.027
Weight of plastic after incubation.(gm)	0.028	0.027
Total loss in weight of plastic.(gm)	0.001	0.000
% loss in weight of plastic	3.45	0

Table-7
PET in Nutrient Broth inoculated separately with following microorganisms

Microorganisms	**Initial weight (gm)**	**Final weight (gm)**	**Total loss in weight (gm)**	**% loss in weight**
B.subtilis	0.244	0.062	0.182	**74.59**
P.aeruginosa	0.048	0.048	0	0
S.aureus	0.08	0.073	0.007	8.75
S.pyogenes	0.052	0.050	0.002	3.846

Table-8
PET degradation in Bushnell Hass Broth inoculated separately with following microorganisms

Microorganisms	**Initial weight (gm)**	**Final weight (gm)**	**Total loss in weight (gm)**	**% loss in weight**
B.subtilis	0.057	0.056	0.001	1.754
P.aeruginosa	0.052	0.050	0.002	3.845
S.aureus	0.086	0.086	0	0
S.pyogenes	0.051	0.048	0.002	**3.922**

Table-9
PS degradation in Nutrient Broth inoculated separately with following microorganisms

Microorganisms	Initial weight (gm)	Final weight (gm)	Total loss in weight (gm)	% loss in weight
B.subtilis	0.010	0.008	0.002	**20**
P.aeruginosa	0.020	0.019	0.001	5
S.aureus	0.021	0.020	0.001	4.762
S.pyogenes	0.012	0.011	0.001	8.33

Table-10
PS degradation in Bushnell Hass Broth inoculated separately with following microorganisms

Microorganisms	Initial weight (gm)	Final weight (gm)	Total loss in weight (gm)	% loss in weight
B.subtilis	0.017	0.007	0.010	**58.823**
P.aeruginosa	0.008	0.008	0	0
S.aureus	0.016	0.010	0.006	37.5
S.pyogenes	0.009	0.008	0.001	11.11

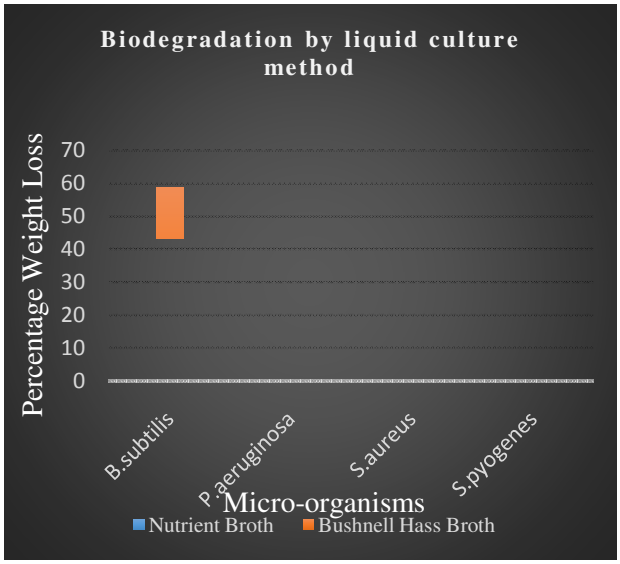

Figure-7
Percent loss in weight of PS by *Bacillus subtilis*, *P. aeruginosa*, *S. pyogenes, S. aureus* in nutrient broth and Bushnell Hass broth

The present study also deals with microbial degradation of PET and PS in liquid culture medium. Small pieces (0.5 x 1 cm) of PET and PS were inoculated in liquid culture medium (Nutrient broth, Rose Bengal broth, Bushnell Hass Broth) containing four bacterial species (*P. aeruginosa, S. aureus, S. pyogenes, B. subtilis)* and one fungal species (*A. niger*) for one month. Maximum weight loss of PET was by *Streptococcus pyogenes* and *Bacillus subtilis* in Bushnell Hass broth and Nutrient broth respectively, according to table-7 and Table-8.

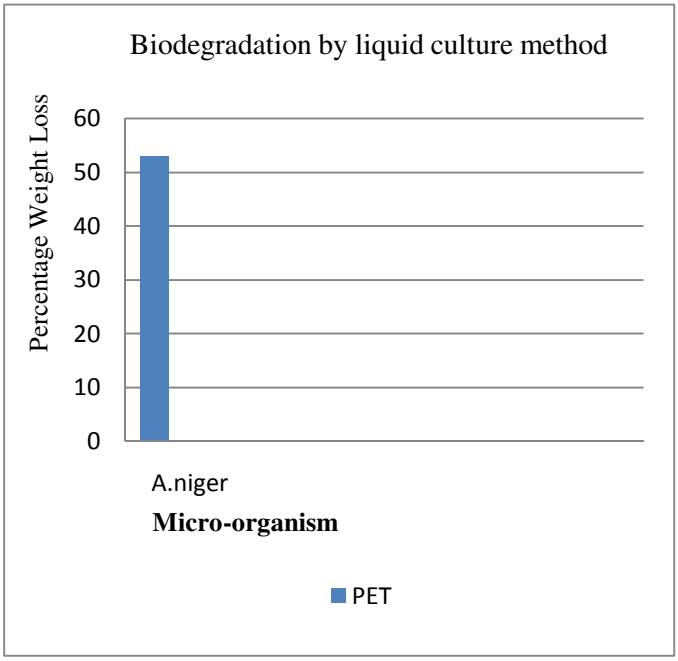

Figure-8
Percentage loss in weight of PET and PS by *A. niger* in Rose Bengal Broth

Table-11
PET and PS degradation in Rose Bengal Broth inoculated separately with following micro-organism.
Micro-organism – *A. Niger*

Plastic sample	Initial weight (gm)	Final weight (gm)	Total weight loss (gm)	% of weight loss
PET	0.085	0.040	0.045	**52.94**
PS	0.022	0.022	0	0

In nutrient broth and Bushnell Hass broth maximum weight loss of PS was by *B. subtilis*, as per *t*able-9 and table-10. In Rose Bengal broth *A. niger* degraded PET but not PS, shown in table-11. This indicates that PET and PS can be degraded if provided with suitable medium and microorganisms by liquid culture medium method. Previous studies indicate and support biodegradation of various other types of plastics by microorganisms such as *Pseudomonas sp*., *Streptococcus sp*., *Staphylococcus sp*., *Micrococcus sp*., *Moraxella sp*., including fungi like *A. niger* and *A. glaucus*[39-40].

Conclusion

After considering the above results of the present study, it is to be concluded that PET and PS can be degraded by micro-organisms (biodegradation) like *Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, and Aspergillus niger*, present in different types of soils. The methods used in the present set of experiments are cost effective, easy to perform, and environmentally friendly.

Future Scope: Similar experiments should be performed by increasing soil type variants and furthermore, genetic and biochemical studies should be carried out with potential plastic-degrading microorganisms.

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