Plastic-Degrading Bacteria From Municipal Wastewater

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Abstract- ***The accumulation of plastic wastes in the environment are creating continuous an ever increasing ecological threat. Biodegradable plastics are considered as environment friendly, they have an increasing range of potential uses and are driven by the growing use of plastics in packaging. In this study, the biodegradation of polythene bag was analyzed 1 month of incubation in liquid culture medium. Bacterial counts in the degrading materials were recorded up to*** 0.0268×10^9 ***per gram for total heterotrophic bacteria. The bacterial strains found associated with the degrading materials were identified as two Gram positive and five Gram negative bacteria. The bacterial strains associated with the polythene materials were identified as Bacillus subtilis, Bacillus amylolyticus, Arthobacter defluvii. The efficacy of bacteria in the degradation of plastics were analyzed in liquid (shaker) culture method, among the bacteria Bacillus amylolyticus degrades plastic more in 1 month (31% weight loss/month) period compared to others and lowest degradation rate was observed in case of Bacillus subtilis (21% weight loss/month). This work reveals that Bacillus amylolyticus possess greater potential to degrade plastics than other bacteria.***

Key words: Biodegradation, plastics, bacterial counts, total heterotrophic bacteria.

INTRODUCTION

Plastics are defined as the polymers (solid materials) which on heating become mobile and can be cast into molds. They are nonmetallic moldable compounds and the materials that are made from them can be pushed into any desired shape and sizes (saymour, 1989). Commonly plastics are used in many purposes including packaging, disposable diaper backing, agricultural films and fishing nets. Plastics and their use has become a part in all sectors of economy. Infrastructure such as agriculture, telecommunication, building and construction, consumer goods, packaging, health and medical are all high growth areas that ensures present demand for plastics. Plastic is the mother industry to hundreds of components and products that are manufactured and used in our daily life like automobiles parts, electrical goods, plastic furniture, defense materials, agriculture pipes, packages and sanitary wares, pipes and fittings, tiles and flooring, artificial leathers, bottles and jars, PVC shoes and sleepers hundreds of household items.

Plastics are used in packaging of products such as food, pharmaceuticals, cosmetics, detergents and chemicals. Approximately 30% of plastics are used worldwide for packaging applications and the most widely used plastics used for packaging are polyethylene (LDPE, MDPE, HDPE, LLDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyurethane (PUR), polybutylene terephthalate (PBT), nylons. At present the industry is split into organized and unorganized sectors. The organized sector produce quality products whereas unorganized sector is not capable of producing quality products, it produces low quality, cheap products through excessive use of plastic scrap.

Almost invariably, organic polymers mainly comprise plastics. The majority of these polymers are based on chains of carbon atoms alone or with sulfur, oxygen or nitrogen as well. The backbone is the part of the chain on the main "path" linking a large number of repeat units together. In order To customize the properties of a plastic, different molecular groups "hang" from the backbone (usually they are "hung" as part of the monomers before linking monomers together to form the polymer chain). This property of the polymer by repeating unit's molecular structure has allowed plastics to become an indispensable part of the twenty-first century world. Plastics are usually classified by their chemical structure of the polymer's backbone and side chains. Important groups in these classifications include acrylics, silicones, polyesters, polyurethanes, halogenated plastics. Plastics can be classified by the chemical process that is used in their synthesis. There are two types of plastics: thermoplastics and thermosetting polymers. Thermoplastics are plastics that do not undergo chemical change in their composition when heated and can be molded again and again. Thermosets are assumed to have infinite molecular weight. These chains are made of many repeating molecular units, known as repeating units, derived from monomers; each polymer chain will have several thousand repeating units. Thermosets can melt and can be molded into various

shapes. After they are solidified, they remain solid. In the thermosetting process, a chemical reaction occurs which is irreversible. Vulcanization of rubber is a thermosetting process. The polyisoprene is a tacky, slightly runny material, before heating with sulfur, but after vulcanization the product is rigid and non-tacky.

Many bacteria and archaea synthesize biodegradable plastics which are a group of biopolymers. Polyhydroxyalkanoates (PHA) are a good alternative to petrochemical plastics among the various biodegradable polymers because they are biodegradable, eco-friendly and biocompatible. Non petroleum based biological polyesters are considered to be one of the most important next-generation polymers in the future in light of limiting natural resources. The properties of PHA are also similar to those of polyethylene (PE) and polypropylene (PP) (Kim and Lenz, 2001; Rehm, 2003). Many micro-organisms accumulate PHA as intracellular energy and storage of carbon inclusions when the carbon is in excess to the other nutrients such as nitrogen, sulfur, phosphorus and oxygen (Madison and Huisman, 1999; Reddy et al., 2003). PHA is produced by almost 250 organisms to be known, but only a few species can produce PHA at a high concentration e.g. Alcaligenes latus (Yamane et al., 1996), Pseudomonas oleovorans (Brandl et al., 1988), Cupriavidus necator (formerly Ralstonia eutropha) (Kim et al., 1994). Classification of PHA can be done into different types according to the number of repeating units in the polymers. Short-chain-length PHA (scl-PHA) is the polymer that contain monomers of C3 to C5 hydroxyl fatty acids e.g. polyhydroxybutyrate (PHB) and hydroxyvalerate (PHV). Similarly, the polymers composed of C6 to C16 hydroxyl fatty acids or aliphatic carbon sources are termed as medium- chain -length PHA (mcl-PHA) (Kim and Lenz, 2001; Sudesh et al., 2000). Heavy metals and antibiotics contaminate the environment from natural sources or directly and indirectly due to human activities and anthropogenic sources (Ware et al., 2006). The general use of antibiotics has been increased in many activities leaded by man, as agriculture, hospitals, animal husbandry, industry and prophylaxis. Heavy contamination from environment in the fermenter affects the production of PHA for industrial scale. Different scientists prefer to exploit the strains which are resistant to some antibiotics for controlling contamination (de Lima et al., 1999). Any physical or chemical change in polymer as a result of environmental factors such as light, heat, moisture, chemical conditions and biological activity is termed as degradation of plastic. Biodegradable polymers are designed to degrade upon disposal by the action of living organisms. Microbial degradation of plastics is caused by enzymatic activities that lead to a chain cleavage of the polymer into monomers. Microorganisms utilize polythene film as a sole source of carbon resulting in partial degradation of plastics. They colonize on the surface of the polyethylene films forming a biofilm. Cell surface hydrophobicity of these organisms was found to be an important factor in the formation of biofilm on the polythene surface, which consequently enhances biodegradation of the polymers. Once the organisms get attached to the surface, starts growing by using the polymer as the carbon source. In the primary degradation, the main chain cleaves leading to the formation of low-molecular weight fragments (oligomers), dimers or monomers. The degradation is due to the extra cellular enzyme secreted by the organism. These low molecular weight compounds are further utilized by the microbes as carbon and energy sources. The resultant breakdown fragments must be completely used by the microorganisms, otherwise there is the potential for environmental and health consequences. Thus, the purpose of this study was to isolate bacteria from municipal wastewater, screening of the potential polyethylene degrading bacteria and identifying the high potential microorganism that degrade the plastics.

MATERIALS AND METHODS

Sample Collection and Isolation

Plastic sample was collected from municipal wastewater near railway pulliya, MNNIT Campus. After the collection of plastic sample, these were taken and 1gm of this sample was cut into pieces and added to 9 ml of sterile water to make 1:10 dilution, adding 1ml of the 1:10 dilution of 9ml of sterile water makes a 1:100 dilution and so on.

Total heterotrophic count: C.F.U. /g= Number of colonies/ inoculum size (ml) X dilution factor

Identification

Identification of the isolates were performed according to their morphological, cultural and biochemical characteristics by following Bergey's Mannual of Systematic Bacteriology (Kandler and Weiss, 1986). All the isolates were subjected to Gram staining and specific biochemical tests.

Morphological-

Gram Staining Method

A clean grease free slide was taken and a smear of the bacterial culture was made on it with a sterile loop. The smear was air-dried and then heat fixed. Then it was subjected to the following staining reagents: (i) flooded with Crystal violet for 1 min. followed by washing with running distilled water. (ii) Again, flooded with Gram's

Iodine for 1 min. followed by washing with running distilled water. (iii)Then the slide was flooded with Gram's Decolourizer for 30 seconds. (iv)After that the slide was counter stained with Safranin for 30 seconds, followed by washing with running distilled water. (v) The slide was air dried and cell morphology was checked under microscope. Colony morphology was done to determine the morphology of selected strains on the basis of shape, size and color.

Biochemical Tests:

Biochemical identification of the isolated strains was done by using Biochemical identification kit (Hibacillus identification kit, HIMEDIA) and some manual biochemical methods. Biochemical Identification test kit is a standardized colorimetric identification system utilizing conventional biochemical tests and carbohydrate utilization tests. The test is based on the principle of change in pH and substrate utilization. Organisms undergo metabolic changes on incubation which are indicated by a colour change in the media that is either interpreted visually or after addition of a reagent.

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 test was indicated by formation of effervescence or appearance of of bubbles, due to the breaking down of hydrogen peroxide to O2 and H2O.

Oxidase Test:

The oxidase test was done with the help of commercially available disc coated with a dye N- tetramethyl paraphenylene diamine dihydrochloride (Himedia), to detect the presence of cytochrome 'c' oxidase which is responsible for the oxidation of the dye. Rubbing a small quantity of bacterial culture by means of a sterile toothpick on the disc causes formation of purple colour within 10-30 sec indicating positive reaction whereas no colour change indicates a negative reaction.

Mannitol Test:

This experiment is generally performed to determine whether the bacteria are capable of fermenting mannitol sugar or not. Whenever organisms ferment mannitol agar, the pH of media becomes acidic due to production of acids. The fermentation of the media starts red to yellow which shows positive test result.

Motility Test:

The motility test was done to determine the motility of the organism. Bacterial cultures were stabbed into the motility test medium (Himedia) and were incubated at 37 C for 48 hrs. Turbitity and observation of growth besides the stab line indicated a positive reaction whereas clear visibility with growth indicated a negative reaction.

Malonate Utilisation:

Malonate utilization test was performed to observe the utilization of malonate present in the malonate test medium (Himedia). Malonate test medium contains Bromothymol blue as indicator. Sodium malonate is the carbon source and ammonium sulphate is the nitrogen source. Organisms, which are able to utilise malonate, release sodium dioxide. The resulting alkaline conditions cause the indicator to change from light green to blue. Colour of the medium changes from light green to blue if the test is positive. Medium remains in light green colour if the test is negative.

Nitrate Reduction Test:

This test was done to test if microorganisms are able to convert nitrate to nitrite or not by adding 1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl-Napthylanine reagent to the kit medium. Immediate development of pinkish red colour there on addition of reagent indicates positive reaction. Negative reaction could be observed if there is no change in the colour.

Citrate Utilisation Test:

This test determines the ability of bacteria to convert citrate (an intermediate of the Kreb's cycle) into oxaloacetate (another intermediate of the Kreb's cycle). Citrate is the only carbon source available to the bacteria in this media. If bacteria cannot use citrate, it will not grow. Positive result is seen if the bacteria grow and the media turns into bright blue colour as a result of an increase in the pH of the media.

Gas Production From Glucose:

Gas production from glucose was assessed by inoculating the isolated strains in MRS broth containing glucose containing Durham tube in inverted condition and incubated at 37oC for 48-72 hrs. The upward movement of inverted Durham tube indicates positive reaction (gas production).

Microbial Degradation of Plastics in Laboratory Condition

Determination of Weight Loss:

Pre-weighed discs of 1-cm diameter prepared from polythene bags were aseptically transferred to the conical flask containing 50 ml of culture broth medium, inoculated with different bacterial 22 strains. Control was maintained with plastic discs in the microbe-free medium. Different flasks were maintained for each treatment and left in a shaker. After one month of shaking, the plastic discs were collected, washed thoroughly using distilled water, shade-dried and then weighed for final weight. From the data collected, weight loss of the plastics was calculated.

RESULTS AND DISCUSSION

This study has covered the major concerns about the natural and synthetic polymers, their types, uses and degradability also it has looked at the disposal methods and the standards used in assessing polymer degradation. Another area examined has been the biodegradation of plastics by the liquid culture method (Albertsson et al., 1980). It is clear that most recalcitrant polymers can be degraded to some extent in the appropriate environment at the right concentration.

The present study deals with the isolation, identification and degradable ability of plastic degrading microorganisms from municipal wastewater. Different types of changes are produced by the microorganism during morphological and biochemical analysis. Synthetic plastic sample was collected from municipal wastewater was used in this study.

Table no. 1: Colony morphology of the bacterial strain on the basis of serial dilution.

Dilution no.	S. No.	Colony morphology	Code
10^{-1}	1	Large round white	PLRW
	2	Small round yellow	PSRY
	3	Small round white	PSRW
	4	Large irregular white	PLIW
10^{-2}	1	Large round pale yellow	PLRP
	2	Small round yellow	PSRY
	3	Small round transparent	PSRT
	4	Large irregular white	PLIW
10^{-3}	1	Large round white	PLRW
	2	Small irregular yellow	PSIW
10^{-4}	1	Large irregular white	PLIW

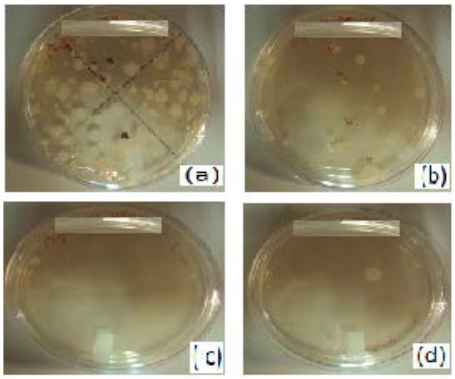

Fig 1: colony morphology of the strains on the basis of serial dilution

(a):10-1, (b):10-2, (c):10-3, (d):10-4

This plastic was used to study their biodegradation by microorganisms isolated from them. Microbial degradation of a solid polymer like polyethylene requires the formation of a biofilm on the polymer surface to enable the microbes to efficiently utilize the non-soluble substrates by enzymatic degradation activities. Developments of multicellular bacterial communities known as biofilm which are attached to the surface of synthetic wastes have been found to be powerful degrading agents in nature (Albertsson et al., 1990). When the total biodegradation process of any organic substrate is considered the formation of microbial colony is critical to the initiation of biodegradation.

Table no.2: The bacterial strains identity on the basis of gram staining.

Bacterial Isolates	Shape	Color	Characteristics
PLRW	Rods in chain	Purple	Gram +ve, bacillus
PSRY	Coccus in chain	Pink	Gram –ve, coccus
PSRW	Coccus in chain	Pink	Gram –ve, coccus
PLIW	Rods in chain	Purple	Gram +ve, bacillus
PLRP	Rods in chain	Pink	Gram –ve, bacillus
PSRT	Rods in chain	Pink	Gram –ve, bacillus
PSIY	Rods in chain	Pink	Gram –ve, bacillus

The table contains the bacterial strains which are gram +ve & Gram –ve. Bacterial strains like 1 and 4 were found to be Gram +ve & strain 2,3,5,6 and 7 were Gram –ve.

Bacterial strains like 1,4,5,6 abd 7 were bacillus & strains 2 and 3 were coccus. The code stands for the morpholohical characteristics of the bacterial strain: LRW- Large Round White, SRY- Small Round Yellow, SRW- Small Round White, LIW- Large Irregular White, LRP- Large Round Pale, SRT- Small Round Transparent, SIY- Small Irregular Yellow.

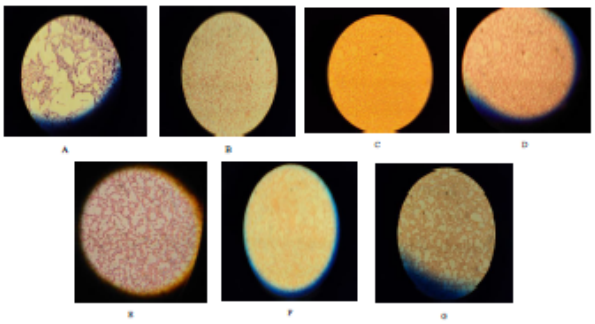

Fig 2: Gram staining of seven selected strains (A-G) on the basis of colony morphology.

Thus, the duration of the microbial colonization is an important factor that effects total degradation period.

Table no 3: Total heterotrophic bacterial count

Dilution	Number of colonies	Inoculums size (in mL)	CFU/g
10^{-3}	268	0.1	0.0268×10^{9}

Microbial counts in the degrading materials were recorded up to 0.0268×10^{9} per gram for total heterotrophic bacteria. The microbial species found associated with the degrading materials were identified as two Gram positive and five Gram negative bacteria.

Table no. 4: Result Of Biochemical Test

S. n.	Catalase test	Oxidase test	Mannitol test	Motility test	Citrate utilisation test	Nitrate reduction test	Malonate utilisation test	Gas production from glucose
1	+	+	+	Non-motile	+	–	–	
2	+	+	+	Non-motile	–	–	+	+
3	+	+	+	Non-motile	+	–	–	+
4	+	+	+	Non-motile	–	+	–	+
5	+	+	+	Non-motile	–	–	–	–
6	+	+	–	Non-motile	–	+	+	–
7	+	+	+	Non-motile	–	–	–	–

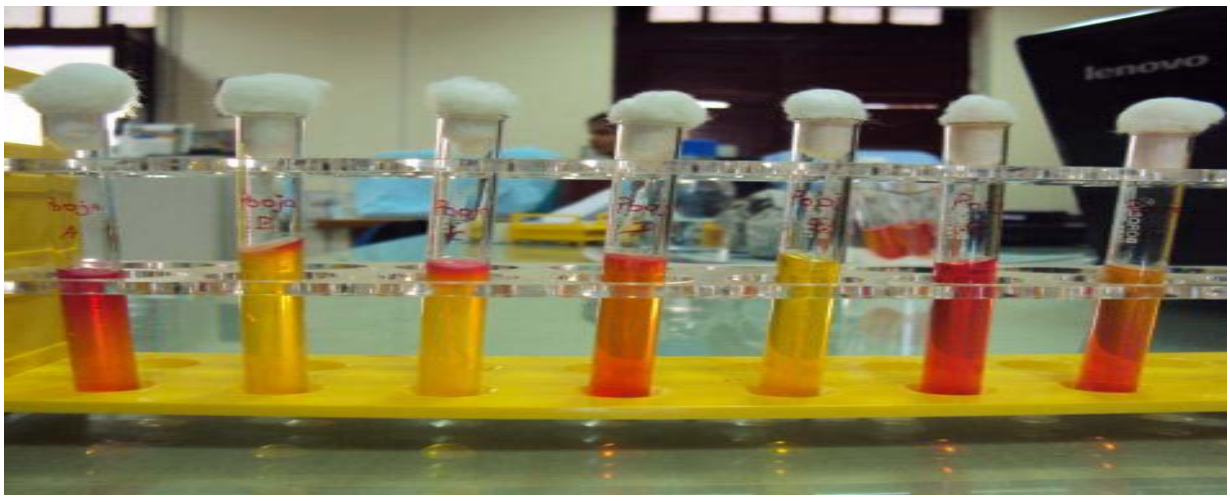

Fig 3: Mannitol-Motility test.

Fig 4: Citrate utilization test.

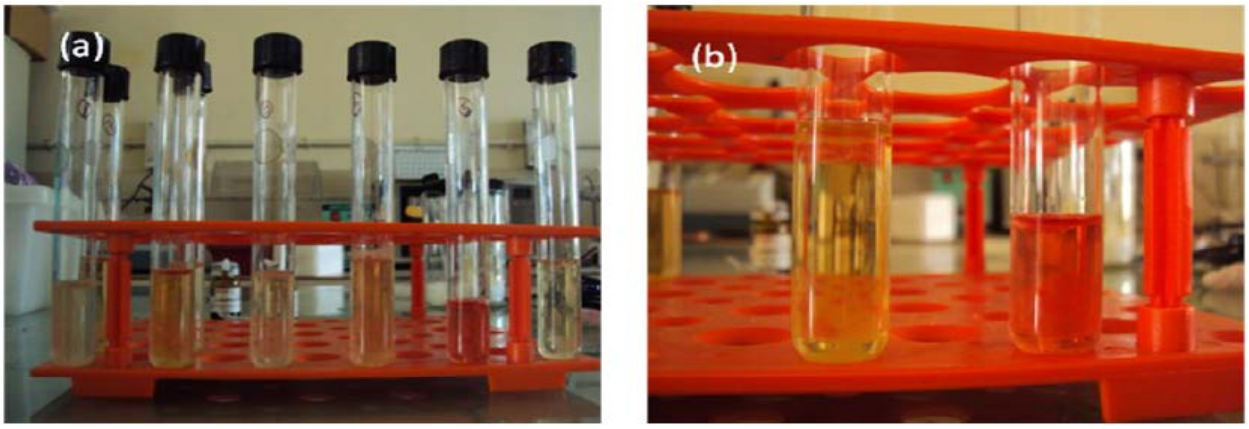

Fig 5 (a & b): Nitrate reduction test.

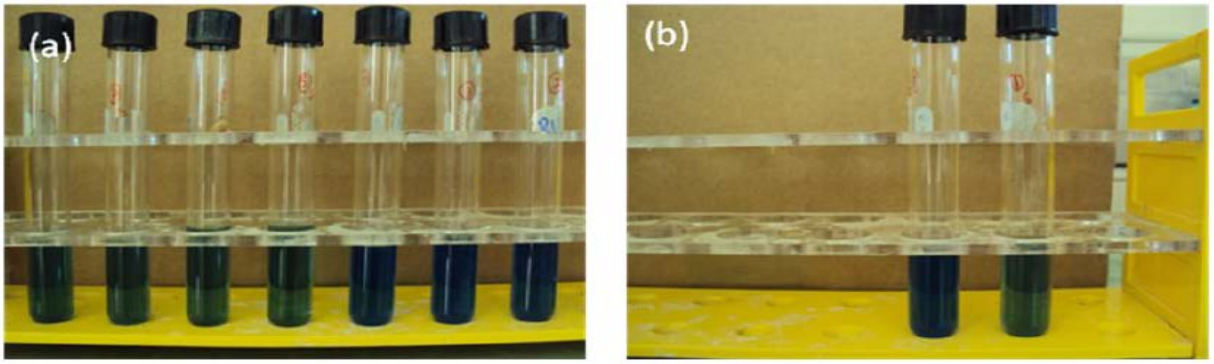

Fig 6 (a & b): Malonate utilization test.

Table no. 5: Degradation of plastic sample by bacteria after 1 month.

Strain no.	Initial wt. (mg)	Final wt. (mg)	Difference	Weight loss/month (in %)
1	50	39.5	10.5	21
2	50	34.5	15.5	31
3	50	38	12	24
4	50	37	13	26
5	50	39	11	22
6	50	37	13	26
7	50	38	12	24

Biochemical tests shows, catalase and oxidase test result of all the strains were found to be positive. Mannitol test of the strains were also found positive excluding strain no.6, Motility test shows all the strains are non-motile. Citrate test of strain no 1 and 4 were found positive and rest of them showed a negative result. Nitrate reduction test of strain no.4 was found positive and rest of them showed a negative result. Malonate test shows only strains 4 and 6 gave a positive result. The test named gas production from glucose shows strains 2, 3 and 4 showed a positive result.

In the present study pieces of plastics were inoculated in the liquid culture medium containing bacterial isolates and kept for 1 month to observe the percentage of weight loss by bacteria.

The result shows the degradable ability of the microorganisms after one month of incubation. The percentage of weight loss due to degradation was found more by Bacillus amyloliticus. This shows it has the greater potential of degradation compared to other bacteria.

Fig 7: Gas production from glucose.

PIBWIN (Probabilistic identification of bacteria) programme provides probabilistic identification of unknown bacterial isolates against identification matrices of known strains. This programme has following three major functions: (1) it identifies an unknown isolate. (2) it selects additional tests in order to distinguish between possible strains if identification is not achieved. (3) it has storage and retrieval of results.
The program makes use of Excel (2007) files to store identification matrices. The program is designed to use probabilistic identification matrices that have either published in the literature or created by the user.

The bacteria which are identified from the above biochemical tests are Bacillus Subtilis (strain-1), Bacillus Amylolyticus (strain-2) and Arthobacter defluvii (strain-3) by the software PIBWIN (Probabilistic identification of bacteria). These three bacterial strains were also found on the basis of common morphological characteristics.

CONCLUSION

The bacteria were identified to be Bacillus Subtilis, Bacillus Amylolyticus and Arthobacter defluvii. Bacillus amylolyticus degrades plastic more than other bacteria. Bacillus subtilis has less capacity to degrade plastic as compared to other bacteria. The isolated microbes were native to the site of polyethylene disposal and shown some degradability in natural conditions, yet they also exhibited biodegradation in laboratory conditions on synthetic media. Therefore, the current study reveals that Bacillus amylolyticus were found to be efficient bacteria for bioremediation of plastic material.

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