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RESEARCH ARTICLE

BIO-DEGRADATION OF POLYETHYLENE BY *Bacillus horikoshii* ISOLATED FROM DUMPYARD SOIL

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ABSTRACT

The present study deals with the isolation, identification and degradative ability of plastic degrading microorganisms from soil. Different types of changes are produced by the microorganism during morphological and biochemical analysis. The biodegradation of polythene bag was analyzed two weeks of incubation in liquid culture method. The microbial species found associated with the degrading materials were identified as two Gram positive bacteria's. The microbial species associated with the polythene materials were identified as *Bacillus sp* and *Bacillus horikoshii*. The efficacy of microbes in the degradation of polyethylene were analyzed in liquid (shaker) culture method, among the bacteria *Bacillus horikoshii* degrades polyethylene more in two weeks (29% weight loss/ two weeks) period compared to others and lowest degradation rate was observed in case of *Bacillus sp* (14% weight loss/ two weeks). This work reveals that *Bacillus horikoshii* possess greater potential to degrade plastics when compared with other bacteria.

Key words: Biodegradation, polyethylene, *Bacillus horikoshii*

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INTRODUCTION

During the last two decades the development of science and technology has led to the production of a number of synthetic polymers worldwide. The polymers are chains of monomers linked together by chemical bonds. The natural polymers such as lignin, starch, chitin, etc., are present in the environment naturally. Currently, synthetic polymers are used in numerous industries, of which packaging application covers 30% of plastic use throughout the world (Shah *et al.,* 2008b; Dey *et al.,* 2012; Kumar *et al.,* 2011). In the nineteenth and twentieth century's plastic played a revolutionary role in the packaging industries. Subsequently, the introduction of carrying bags made of polyethylene assumed a remarkable role in transportation (Nerland *et al.,* 2014). The low cost and high durability of synthetic polymers made it as a significant packaging material but it now have been emerged as a challenge for solid waste management, and it is a major source of pollution (Song *et al.,* 2009; Dey *et al.,* 2012). These synthetic compounds have developed in to an annoyance by contaminating the natural resources like water quality and soil fertility (Bhatnagar and Kumari 2013; Ojo 2007; Arutchelvi *et al.,* 2008).

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Plastic materials have become flexible, economical and dependable substitutes for traditionally used metal, leather and wood materials in the past 5 decades because of their durability, elasticity and physical properties (Sivan 2011; Singh and Sharma 2008). Permanence and unwanted accumulation of synthetic polymers are major extortions to the environment. On the other hand the only way of waste management is by biodegradation were the organic substances are broken down by living organisms. (Shah *et al.,* 2008b; Ojo 2007; Ali *et al.,* 2014).

Distribution of different types of polymers

Polymers are made up from non-renewable as well as renewable feedstock. These polymers are well known for their diverse applications in industries, domestic appliances, transportation, construction, shelters, storage and packaging practices. Such polymers are differentiated according to their chemical nature, structural arrangement, physical properties and applications (Shah *et al.,* 2008b; Dey *et al.,* 2012; Kumar *et al.,* 2011; Smith 2005).

Natural polymers

Natural polymers are found abundantly in nature in the forms of biopolymers and dry material of plants (Leschine 1995). The constitution of the plant cell wall differs with the

composition of the lignocellulosic biomass (cellulose, hemicellulose and lignin), which provides strength (Premraj and Doble 2005). Lignocelluloses play a critical role in developing plant biomass, in which cellulose, hemicellulose and lignin are the major building blocks of the natural polymer (Perez *et al.,* 2002).

Synthetic polymers

Plastics are manmade compounds that consist of a long chain of polymeric molecules and unusual bonds, with excessive molecular mass and halogen substitutions. Nowadays plastic manufacturing involves different inorganic and organic materials, including carbon, hydrogen chloride, oxygen, nitrogen, coal and natural gases (Shah *et al.,* 2008b). The most widely used polymers contributing to plastic waste are low-density polyethylene (LDPE), high-density polyethylene (HDPE), polyvinyl chloride, polystyrene and polypropylene with 23, 17.3, 10.7,12.3and 18.5%, respectively, and the remaining 9.7% of other types of polymer (Puri *et al.,* 2013). The polymer production in 2012 was estimated as polyethylene 30% (LLDPE and LDPE 18%, HDPE 12%), polypropylene 19%, polyvinyl chloride 11%, polystyrene 7%, polyethylene terephthalate 7% and polyurethanes 7% worldwide (Nerland *et al.,* 2014). Microorganisms can degrade plastic over 90 genera, from bacteria and fungi, among them; *Bacillus megaterium, Pseudomonas sp., Azotobacter, Ralstonia eutropha, Halomonas sp., etc.* (Chee *et al.,* 2010). Plastic degradation by microbes due to the activity of certain enzymes that cause cleavage of the polymer chains into monomers and oligomers. Plastic that has been enzymatically broken down further absorbed by the microbial cells to be metabolized. Aerobic metabolism produces carbon dioxide and water. Instead of anaerobic metabolism produces carbon dioxide, water, and methane as end products (Usha *et al.,* 2011). The persistence of this study was to isolate bacterial microorganisms from dumped soil area and screening of the potential plastic degrading bacteria and identifying the high potential microorganisms that degrade the plastics.

MATERIALS AND METHODS

Sample collection

Soil sample was collected from the plastics dumped soil of Municipal waste dump yard, vellalore, Coimbatore, Tamil Nadu, India and brought to the laboratory, preserved under laboratory conditions for further use.

Isolation of microorganisms

One gram of soil sample was transferred into a conical flask having 99ml of sterile distilled water. The mixture was shaken and serially diluted from 10^{-1} to 10^{-9}. Dilution 10^{-6} in bacteria. Then prepare the sterile nutrient agar plates. Then take 0.1 ml of sample present on the surface of agar was spread evenly by using L-rod plates were incubated at 37^{o}C for 24 hrs to get the isolated colonies.

Screening of polyethylene degrading microorganisms

The isolated microorganisms were inoculated in mineral salt medium supplemented low density polyethylene powder (LDPE sheets were cut into bits and immersed in 20 ml of xylene. It was boiled for 15mins as xylene dissolves the LDPE film and the residue was crushed while it was warm by using band gloves. The LDPE powder so obtained was washed with ethanol to remove residual xylene and allowed to evaporate to remove ethanol. The powder was dried in hot air oven at 60ºC over night) as a sole source of carbon at a final concentration of 0.1% (w/v) and incubated in rotary shaker at room temperature for 24 hours. After incubation, the turbidity of the medium was checked for growth (Ponniah Saminathan *et al.,* 2014).

Pure culture technique

To study the characteristics of single species. That particular species must be separated from all other species by pure cultures. This pure culture was obtained by streak plate technique. The microbial mixture was transferred into the edge of the agar plate with the help of the inoculation loop and then streaked. After the plates were incubated at 37^{o} C for 24 hrs.

Bacterial identification

The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterizations. The parameters investigated included colonial morphology, gram's reaction, endospore formation and specific biochemical tests.

Colony morphology

This was done to determine the morphology of selected strains on the basis of shape, size and colour.

Gram staining

Sterile glass slide was taken, heat-fixed smear of sample was flooded with crystal violet for 1min and the slide was washed with water then flooded with mordant: Gram's iodine for then 1 minute, then decolorizing agent is added and kept for 10-15 seconds; Slide was washed and flooded with counter stain safranin for 30 seconds.

In-dole test

Peptone broth was Prepared and dispense into sterile test tubes (5 ml), loopful of culture was inoculated into the peptone broth and incubated for 24-48 hrs at 37^{o}C, after incubation 0.2ml of kovac's reagent is added and a red colour ring formation was observed.

MR and VP teat

MR-VP broth was Prepared and dispense into sterile glass tubes, loopful of culture was inoculated into glass tube and incubated for 24-48 hrs at 37^{o}C, after incubation 0.6ml of barrit's reagent A and 0.2 ml of barrit's reagent B was added, test tube was shaked well and allowed to stand for 15mins and a pink to red colour was observed.

Catalase test

The cultures were transferred to clean glass slide over that few drops of hydrogen peroxide was added and observed for bubbles.

Oxidase test

The cultures were transferred to oxidase disc on clean glass slide and observed for the colour change.

Citrate utilisation test

Simmon' s citrate agar slant was prepared and dispensed into sterilised test tubes (5 ml) and kept it into slating position for solidification, by using sterile inoculation techniques culture was inoculated and incubated at 37°C for 24-48 hrs, after incubation growth of the organism on the surface of the slant and blue colouration of the slant was observed.

2.5.8. Nitrate reduction test

Nitrate broth was prepared and dispensed into sterile test tubes, loop full of culture was inoculated into the nitrate broth and incubated for 24-48 hrs at 37°C. after incubation 5 drops of sulfanilic acid and then alpha-naphthylamine was added and a red colour was observed.

Carbohydrate fermentation test

Fermentation broth was prepared and desired sugars (glucose) were added and isolates were inoculated and Starch hydrolysis test. Starch agar plates were prepared and test organisms is inoculated by single line streak method, plates are incubated in an inverted position for 24-48 hrs at 37°C, after incubation plates are flooded with gram' s iodine and clear areas of starch hydrolysis was observed.

Endospore formation test

Smear of the organism was prepared and stained with malachite green and the slide was heated for 3-5 minutes and malachite green was poured frequently to keep it moist, slide was drained and 0.5% of safranin, red colour vegetative cells and green colour endospores were observed.

Microbial Degradation Polyethylene of in Laboratory condition

Initially weighed strips of 3×3-cm size of 51 micron polythene were aseptically transferred to the conical flask containing 50 ml of nutrient broth medium and inoculated with bacteria (0.5 ml). Control was maintained with plastic discs in the microbe-free medium. Different flasks will be kept in a shaker for two weeks respectively. After the respective duration of shaking, the polythene strips were collected, washed thoroughly using distilled water, shade-dried and then go to further studies (Determination of weight loss, FTIR analysis, XRD analysis, Scanning electron microscopy analysis).

Fourier Transform Infrared (FTIR) and Attenuated Total Reflectance (ATR) spectroscopy

FTIR analysis is a useful tool to determine the formation of new or disappearance of functional groups. So degradation products, chemical moieties incorporated into the polymer molecules such as branches, co-monomers, unsaturation and presence of additives such as antioxidants can be determined by this technique. Fourier transform-attenuated total reflectance (FT-ATR) infrared spectroscopic studies were carried out on plastic samples using a Shimadzu in the horizontal ATR mode, using a zinc-selenide crystal. A total of 3 scans were taken (omar saad jumaah., 2017).

Scanning Electron Microscopy Analysis: The surface morphological changes in degradation of polythene were analyzed by Scanning electron microscopy (SEM).

XRD Analysis of polyethelene

Crystallographic structural analysis was analyzed by XRD peak height method of raw and pretreated samples using XRD-6000 Schimadzu diffractometer. Diffraction patterns were recorded by using Cu–Ka radiation at 40 kv and 30 mA and grade range between 10^0 and 30^0 with a step size of 0.03^0. Degradation polyethylene crystallinity was calculated using the pragmatic equation.

$$Crl=[(l_{020} - l_{am}) / l_{020}] \text{ X100}$$

The bacterial strains isolated with the ability to degrade and performed on the basis of macroscopic and microscopic examination and biochemical test. The bacterial isolates were identified macroscopically by examining colony characteristics, pigment, size shape, margin, and microscopic examination including, grams staining to study the staining behavior, shape, and cell arrangement and granulation, spore staining was also per performed biochemical test. A total of two bacteria's were isolated from dumped soil of Municipal Corporation of vellalore, Coimbatore, Tamil Nadu. These two bacteria's were purified in order to tilt to the next test and screened for plastic degradation by incubation for two weeks in an incubator shaker at 100 rpm agitation in a 37^0C temperature conditions. The bacteria which were identified from the above biochemical tests are *Bacillus sp* and *Bacillus horikoshii* by the MALDI-TOF method.

Table 1. Colony morphology of the bacterial strain on the basis of serial dilution

Colony characteristics and Morphological Tests		
Test	Isolate 1	Isolate 2
Configuration	Circular	Circular
Margin	Entire	Entire
Elevation	Convex	Raised
Size	Moderate	Large
Surface	Smooth	Smooth
Pigment	White	Pale yellow
Opacity	Opaque	Opaque
Grams reaction	Positive	Positive
Arrangement	Single	Single
Spore(s)	Positive	Positive
Physiological Test		
Growth at temperature	37^0C	37^0C
Growth at Ph	pH 7.0	pH 7.0
Biochemical Test		
Indole Test	Positive	Positive
Methyl Red Test	Negative	Negative
Voges Proskauer Test	Negative	Negative
Citrate Utilization Test	Positive	Positive
Nitrate Reduction Test	Positive	Positive
Catalase Test	Positive	Positive
Oxidase Test	Negative	Negative
Starch Hydrolysis Test	Positive	Positive
Carbohydrate Fermentation Test	Acid/Gas	Acid/Gas

Fig. 1. Colony morphology of the strains on the basis of serial dilution

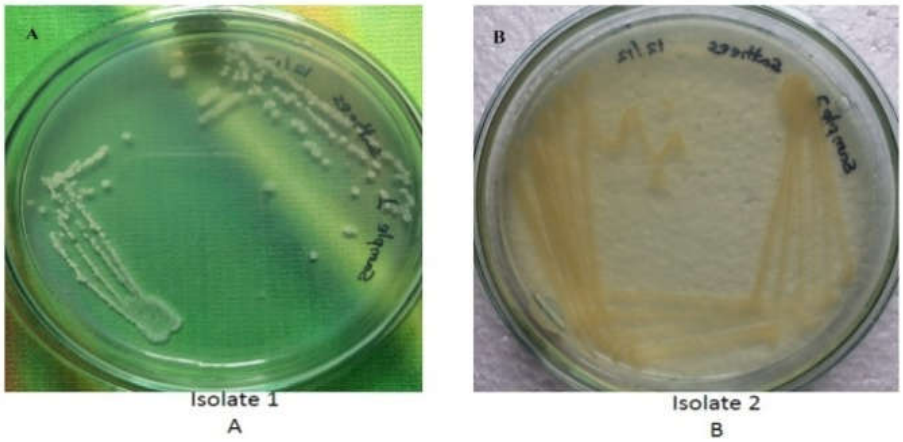

Fig. 3. Pure Culture Technique

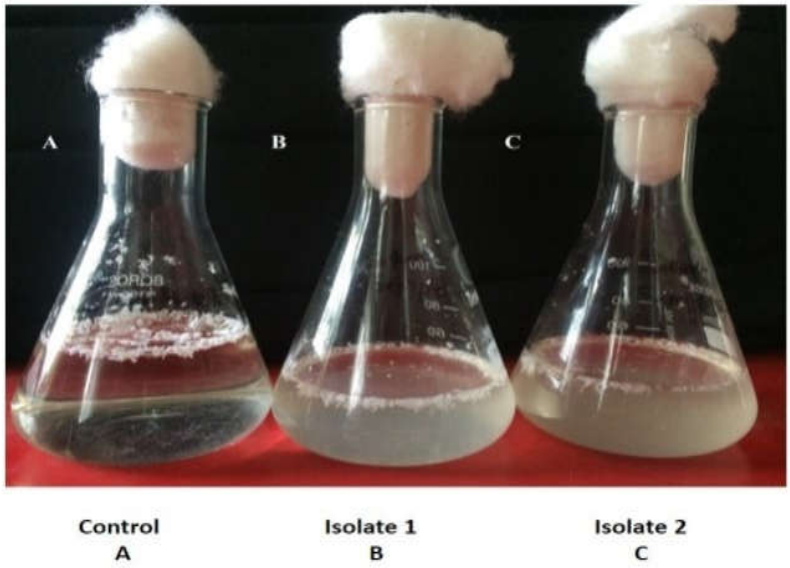

Fig 4. Screening of polythene degrading bacteria's

Table 2. OD value 620nm

Hrs	Control	Lsolate1	Isolate2
24	0.059	.359	0.504
48	0.059	0.611	1.751
72	0.061	0.853	4.000
96	0.063	1.377	4.120

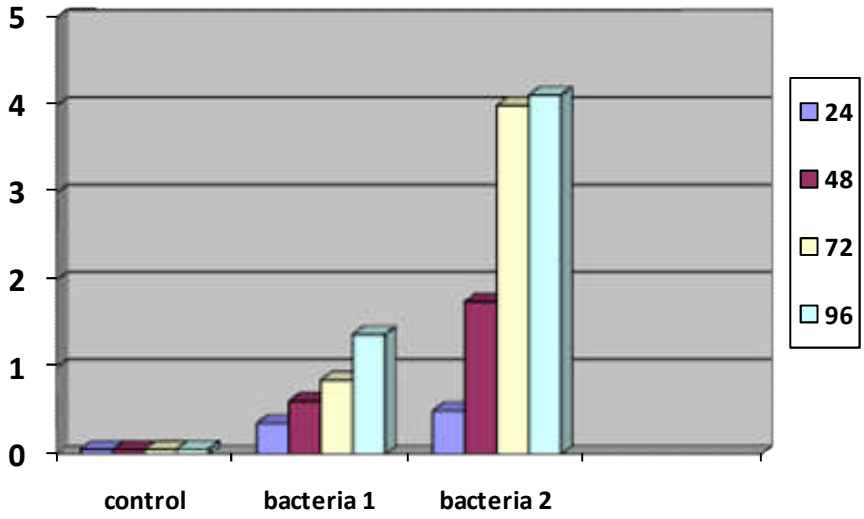

Fig. 5. OD VALUE 620nm

Table . No 3.Probable Identification of Isolates

S.NO	Isolates	Identified Isolate
1	Isolate 1	*Bacillus sp*
2	Isolate 2	*Bacillus horikoshii*

Table No 4. Result of degradation of polythene sample by bacteria

Isolates No.	Name of Bacteria	Initial weight (mg)	Final weight (mg)	Difference of Weight	Weight loss/2weeks (%)
1	*Bacillus sp*	100	98.6	1.4	14
2	*Bacillus horikoshii*	100	97.1	2.9	29

Fig. 6. Microbial degradation of polyethylene under laboratory conditions

Degradation of Polyethylene Waste was carried out. Weigh polyethylene (initial weight) and then washed with sterile distilled water and sprayed with 70% alcohol. Polythene is inserted into the Nutrient broth as much as 50 ml aseptically. So as much as two loops inoculated bacterial isolates to the media. Then incubated in an incubator shaker at 37^0C temperature, with agitation of 100 rpm for a two weeks. Polyethylene plastic that has been incubated for a two weeks, washed with sterile distilled water and then sprayed with alcohol dried aired then weighed (final weight).

Determination of the percentage of degradation of polyethylene plastic by bacteria by using following formula:

$$\% \text{ Degradation} = \frac{\text{Final weight}}{\text{Initial weight}} \times 100\%$$

Microbial degradation of polyethylene under laboratory conditions

To assess this, the pre-weighed discs of 3-cm diameter prepared from 51 micron polythene bags were aseptically transferred to the conical flask containing 50 ml of culture broth medium, inoculated with different bacterial species separately. Control was maintained with plastic discs in the microbe-free medium. Two flasks were maintained for each treatment and left in a shaker (fig .6). After two weeks of shaking, the polythene discs were collected, washed thoroughly using distilled water, shade-dried and then weighed for final weight. From the data collected, weight loss of the polythene bag was calculated.

The species tested were *Bacillus sp* and *Bacillus horikoshii*. The *Bacillus horikoshii* were found most active in degrading 29% in two weeks period. FTIR result confirmed polyethylene degradation. Following are the figures showing FTIR control, bacteria isolate1 (*Bacillus sp*) degrading polyethylene and bacteria isolate2 (*Bacillus horikoshii*) degrading polyethylene. FTIR spectra of polyethylene material. (Fig.7) shows the FTIR spectra of polyethylene material after degradation by isolated bacterial strain. The overlapping spectra in the expanded form are also provided for comparison, which clearly depict the changes observed. *Bacillus horikoshii* degrades polyethylene more than of *Bacillus sp*. *Bacillus sp* has less capacity to degrade polyethylene as compared to *Bacillus horikoshii*. The XRD data illustrates further significant differences in the patterns in degradation of polyethylene (Fig .8). The XRD data also confirmed the polyethylene degradation. *Bacillus horikoshii* degrades polyethylene more than of *Bacillus sp*. *Bacillus sp* has less capacity to degrade polyethylene as compared to *Bacillus horikoshii*. We identified *Bacillus horikoshii* has high capacity to degrade polyethylene as compared to *Bacillus sp* by weight loss, FTIR analysis and XRD analysis.so directly we go SEM analysis to control and isolated *Bacillus horikoshii*. (Fig 3.9) showed morphological changes when observed through SEM. Formation of surface changes, disruption of polyethylene structure confirmed high degradation capacity of *Bacillus horikoshii*.

DISSCUSSION

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. It is clear that most recalcitrant polymers can be degraded to some extent in the appropriate environment at the right concentration. The results revealed that the bacillus bacterial species showed a significant effect in biodegradation of polyethylene. On the other hand the analytical tools like FTIR , XRD and SEM were done to study the efficacy of the bacterial isolates were they are genetically identified as *Bacillus horikoshii.* This strain *Bacillus horikoshii* showed a better result when comparing to the *Bacillus sp*. FTIR spectroscopy is used as analytical technique in many biodegradation studies (Kiatkamjornwong *et al.,* 1999; Klrbas *et al.,* 1999; Arboleda *et al.,* 2004; Drímal *et al.,* 2007). It is a useful tool to determine the formation of new or disappearance of functional groups. Control spectra of polymer film (not treated with microbes) displayed a number of peaks reflecting the complex nature of the LDPE (Fig.7). There was a variation in the intensity of bands in different regions when test samples (after incubation with microbes, BSM-1 and BSM-2) were analyzed (Fig .7). For control spectrum, the characteristic absorption bands were assigned at 719 cm^{-1} (C–H bend-mono), 1,472 cm^{-1} (C=C stretch), 2,660 cm^{-1} (CHO stretch), and 2,919, 2,850 cm^{-1} (both due to C–H stretch). Significant and similar changes were found for both microbial strains. The peak at 2,660 cm^{-1} corresponds to CHO stretching vibration that has been disappeared in case of BSM-1 and 2 while new band has been observed at 939 cm^{-1} (O–H bend) which supports the depolymerization activity of the microbial isolates. The strong absorption peaks at 719 and 1,472 cm^{-1} became weaker after microbial treatment. In addition, the intensity of those peaks reduced more in case of BSM-2 than BSM-1 whereas peaks at 2,919 and 2,850 cm^{-1} became sharper in the treated sample than the control one, here also the same microbial activity pattern was seen. The change in the peak values of almost all functional groups supporting the conformational change on polymer surface.

The XRD data illustrates further significant differences in the patterns of control film and microbial treated film were the control film showed a crystallinity index of 50.43 % and that of biodegraded polyethylene is 20.49 %. Similar observation has been reported by (Sowmya *et al.,* 2014). The XRD data also confirmed the polymer degradation using *Bacillus horikoshii.* The SEM analysis of control sample has showed an appearance of smooth surface having no pits, cracks or any particles attached on the surface (Fig .9A). In the case of polyethylene film treated with the *Bacillus horikoshii*, it was found that several cracks on the surface developed after 15 days of treatment. Simultaneously, microbes were also noticed on the film surface indicate its strong adhering capabilities as well as LDPE utilization capacities (Fig .9B). The film treated with the bacterial isolate *Bacillus horikoshii*, found to have bacterial attachment on higher rate as compared to BSM-1. Clear mark of degradation can be seen at places where initially microbes were attached along with the pockets and pits around (Fig 9). For both the strains, at different places on the surface several colonies forming biofilm can be observed. The study revealed that initial microbial treatment of the polyethylene films ensured initiation of degradation. Microbial degradation lead to introduction of oxygen in the polymer matrix, to form oxygen containing compounds which were made available for utilization by the bacteria. *Bacillu*s sp., and *Bacillus horikoshii* grew better on abiotically weathered Polyehylene films. On the basis of this study it can be concluded that *Bacillus horikoshii* indigenous to the dump yard soil have potential for use in biodegrade 16ation of polyethylene.

Conclusion

The abiotic bacteria *Bacillus horikoshii* degrades polyethylene more than that of other bacteria. *Bacillus horikoshii* has higher capacity to degrade plastic as compared to other bacteria and to the credit it is the first reported polythene degradation using *Bacillus horikoshii.* The isolated microbes were native to the site of polyethylene dump yard and shown some degradability in natural conditions, yet they also exhibited biodegradation in laboratory conditions on synthetic media.

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