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Full Length Research Paper

Brevibacillus parabrevis, Acinetobacter baumannii and Pseudomonas citronellolis - Potential candidates for biodegradation of low density polyethylene (LDPE)

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Low density polyethylene is one of the polymers that is up till date nearly impossible to be degraded safely. Biodegradation is the safest method of breakdown that possibly leaves behind less toxic residue and shows potentials of bio-geo chemical cycling of the substrate. Considerable amount of work has been carried out in this area, but most of them are associated with blended low density polyethylene (LDPE). Previous reports also rely on host consortia for degradation. Various techniques have been implicated in designing this kind of study. BATH test, to evaluate the hydrophobicity of the isolates was performed apart from the calculation of generation time of the isolates in LDPE incorporated growth medium. Biofilm formation was also quantified by using protein estimation technique. Our findings corroborated previous findings in most of the techniques, but in Sturm test, a technique to evaluate concentration of carbon dioxide, a final product of biodegradation of LDPE, we have taken dissolved CO_2 (that is, CO_2 present in the soluble form in the growth medium, apart from the gaseous CO_2 collected in the KOH tube) also in consideration which is not reported by any of the previous workers. The current article investigated the biodegradation ability of bacteria isolated from a municipal landfill area near Pallikaranai, Chennai, South India. The bacteria were subjected to growth in a medium containing LDPE as the sole carbon source with and without a nitrogen source. Four bacterial species were isolated. According to the *16S rRNA* gene sequences they were identified as *Brevibacillus parabrevis* (PL-1)*, Acinetobacter baumannii* (PL-2, PL-3) and *Pseudomonas citronellolis* (PL-4). Bacterial adhesion to hydrocarbon (BATH test) was done to determine the bacterial hydrophobicity. Bacterial biomass was quantified to estimate the population density of the biofilm. This work clearly identifies with our objective to find a right microbe to degrade the resistant LDPE by giving out promising results from the present study.

Key words: Biodegradation, low density polyethylene (LDPE), bacterial adhesion to hydrocarbon (BATH test), sturm test, *Pseudomonas*, *Acinetobacter, Brevibacillus.*

INTRODUCTION

Plastics are polymers that consist of monomers linked together by chemical bonds (Shah et al., 2009). The polymers include, polyethylene, polypropylene, polystyrene, polyurethane, nylon etc., Polyethylene is a thermoplastic polymer produced by combining monomers of ethylene. Low density polyethylene is a thermoplastic made from petroleum (Shah et al., 2009). Plastic bags do bring a lot of convenience to people's life, but at the same time, it also generates long term harms. When the plastic products mix in the soil and accumulate continuously, they affect the absorption of nutrients and water by the crops, thus reducing their output. The littering also reduces rate of rainwater percolation. Biodegradation is a process where complex organic molecule breaks into

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simple molecules as a result of the action of micro-organisms like bacteria, fungi, or algae. Biodegradation of the materials has to be scientifically measurable. Since most biodegradation produces CO_2 as a by-product, usually this is measured by the amount of CO_2 produced after degradation of carbon containing compounds. The following strategies are used to assess and monitor the biodegradation of the polymer:

1. Accumulation of biomass (experimentally determine the growth rate of micro organisms with the polymer as the sole carbon source).
2. Oxygen uptake rate.
3. CO_2 evolution rate.
4. Surface changes.
5. Changes in the mechanical and physical properties of the polymer.

Biodegradation of polyethylene has been studied extensively earlier (Pometto et al., 1992) but the results were based on PE blend with starch (Pometto et al., 1993; Breslin, 1993).

Fourst et al. (1997) have reported the biodegradation of LDPE/cellulose blends by cellulose blends by common fungi. El-shafei et al. (1998) have reported the biodegradation of disposable polyethylene by fungi and *Streptomyces* sp. Yamada-Onodera et al. (2001) have reported the degradability of polyethylene by *Penicillium simplicissimum.* Kathiresan (2003) has reported isolating fungi from the mangrove soil which has the potential to degrade polyethylene materials. Watanabe et al., (2009) isolated and identified three types of low density polyethylene (LDPE) degrading microbes *Bacillus circulans*, *Bacillus brevies* and *Bacillus sphaecicus* by soil burial method. Sindhuja et al. (2011) have reported degradation of LDPE by marine fungi.

Biodegradation of polyethylene as revealed by structural changes have been studied by many workers using Scanning Electron Microscopy (SEM). (Raghavan et al.,1992; Weiland et al.,1995; Bonhomme et al. 2003; Gilan et al., 2004; Aamer, 2007; Zahra et al., 2010; Mumtaz et al., 2010; Pramila and Vijaya, 2011) The previous findings do have some bearing about microbial remediation strategies but the onus lies on the time taken for the degradation. The work has proceeded towards isolating microbial species that can degrade LDPE in lesser time. Reports of Sturm test done in this study reveals some amount of evidence in this line.

MATERIALS AND METHODS

Preparation of LDPE powder

LDPE sheets were cut into small bits and immersed in xylene and boiled for 15 min. Xylene dissolved the LDPE and the residue was crushed while it was warm by hand with help of gloves. The LDPE powder so obtained was washed with ethanol to remove residual xylene and allowed to evaporate (approximately 2 to 3 h) to remove ethanol. The powder was dried in hot air oven at 60°C overnight. The LDPE powder was stored in closed containers in room temperature.

Isolation of microorganisms

Soil sample was collected from the municipal solid waste landfill area, Pallikaranai, Chennai Tamil Nadu, South India. To isolate bacteria, 5 g of soil sample and 500 mg LDPE powder was added in 100 ml sterilized synthetic medium (SM) and incubated at 37°C for 24 h. The organisms grew by utilizing LDPE as carbon source. Bacteria isolated were obtained as pure cultures.

Determination of growth rate of bacteria colonized on LDPE

250 mg of LDPE powder was added to overnight culture inoculated into 50 ml SM and shaken. OD values were taken every 30 min at 500 nm using UV-VIS spectrophotometer (Model SL-159). From the values obtained for period of 5 h, generation time was calculated. SM without culture served as a control.

Determination of bacterial hydrophobicity

Bacterial cell surface hydrophobicity was determined by the BATH test (Bacterial adhesion to hydrocarbon). 24 h culture (5 ml) in nutrient broth was centrifuged at 10,000 rpm for 15 min and washed twice with Phosphate-Urea-Magnesium (PUM) buffer. Supernatant was discarded and the pellet was re-suspended in PUM buffer. Absorbance of the suspension was measured at 400 nm using UV-VIS spectrophotometer (Model SL-159). 0.2 ml of hexadecane was added to the suspension and shaken for 20 min. Test tubes were kept undisturbed for 5 min, which resulted in separation of two phases into organic and aqueous. Absorbance of aqueous layer was measured at 400 nm using UV-VIS spectrophotometer (Model SL-159). Culture free buffer served as the blank.

Estimation of bacterial biomass colonizing the LDPE

LDPE films of similar size and weight were used for the colonizing studies. The LDPE sheets were disinfected with 70% ethanol for 30 min and transferred to sterile distilled water for 10 min. The disinfected LDPE sheets were used for further studies. Biofilm formed on the polyethylene surface was quantified by protein estimation.

Culture of 24 h was inoculated in 100 ml SM. 3 pieces of disinfected LDPE sheets were transferred to the medium. The conical flasks were kept over magnetic stirrer at 37°C. One polyethylene film was taken out after every 2 days, boiled with 5 ml of 0.5 mol^{-1} NaOH and the suspension was centrifuged at 10,000 rpm for 15 min. Supernatant was kept aside and the pellet was subjected to the same procedure. The two supernatants were combined and the protein concentration was determined according to Lowry et al. (1951).

Quantification of biofilm

Bacterial culture of 24 h was inoculated in 100 ml SM. 4 pieces of disinfected LDPE sheets were transferred to the medium and incubated at 37°C. After 2 days, one LDPE film was taken out. 10 ml of 95% ethanol was added to the LDPE sheet and shaken vigorously for 10 min to remove biofilm from the sheets. Absorbance was taken at 540 nm using the UV-VIS spectrophotometer. 95% ethanol served as blank.

Table 1. Generation time of the bacterial isolates

Name of the isolate	Generation time in min
PL- 1	167
PL- 2	79
PL- 3	80
PL- 4	28

CO_2 evolution test (Sturm test)

Capacity autoclavable plastic containers of 100 ml were used for the study. A separate set up was kept for control that was maintained un-inoculated. Briefly, sterile CO_2 free air was passed into the container having inoculated synthetic medium, supplemented with LDPE powder. After the stipulated time [48 h for bacteria], potassium hydroxide (KOH) solution [1 M] that had trapped the CO_2 liberated by the inoculants, [after utilization of LDPE the sole carbon source] was gravimetrically quantified for test as well as control. The dissolved carbon dioxide present in the medium was also estimated using titration method. Briefly, sample (25 ml) was taken in a conical flask and 0.05 ml of 0.1 N sodium thiosulphate solution was added. After the addition of 2 drops of methyl orange indicator, this solution was titrated against 0.02 N sodium hydroxide solution. End point was the change in color from orange red to yellow. Following this, two drops of phenolphthalein indicator was added and titration continued till a pink color developed. Volumes of the titrant used were noted and the amount of CO_2 calculated using the formula:

$$\frac{A \times B \times 50 \times 1000}{V}$$

Where,
A = ml of NaOH titrant
B = normality of NaOH
V = ml of the sample

Identification of bacteria

To perform the morphological and biochemical studies on the isolated bacteria for identification, the cultures were streaked onto nutrient agar (NA) plates and Hi Media Biochemical Kit for identification of Gram negative bacteria K22. For the morphological identification following parameters were taken into consideration:

1. Gram character.
2. Motility.
3. Basic biochemical utilization tests like Catalase and Oxidase.
4. Other biochemical tests like carbon, amino acids, nitrate, other enzyme production etc.

16S rRNA gene sequencing

Genomic DNA was extracted by boiling-lysis method and stored at – 80°C until PCR assay. Amplification of *16S rRNA* was carried out using previous described primers

Forward – CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG, Reverse -CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC) (Weisburg et al., 1991).

PCR was carried out in a 50 µl reaction volume which contained 5 µl of the template, 10 pM of each primer (Sigma Aldrich), 0.25 mM of each dNTP (Bangalore Genei, India), 2U of *Taq* polymerase (Bangalore Genei, India).The thermocycling conditions included, an initial denaturation step at 94°C, 3 min, followed by 35 cycles of denaturation at 94°C, 30 s, annealing at 65°C, 30 s and extension at 72°C, 1 min and a final extension at 72°C, 7 min.

The amplified PCR products were resolved in 1% agarose gel in TBE. The amplicons were visualized in a UV transilluminator and gel documentation was carried out (BioRad, Hercules, CA). DNA sequencing was performed to identity the amplified PCR products using applied biosystem (ABI) 3130 Genetic Analyser with ABI PRISM BigDye Terminators V 3.1. The gene sequences were compared with sequences in the GenBank database by using BLAST program. The sequences were deposited in the GenBank database.

RESULTS

Bacterial generation time

Experiments performed to calculate generation time (Table 1) of each of the bacterial isolates in SM (synthetic medium) with LDPE as a carbon source revealed that isolate PL-4 had doubling time of 28 min, culture PL–2 and PL–3 had a generation time of 79 min and 80 min respectively, followed by PL-1 that showed a doubling time of 167 min.

BATH test

BATH test (Table 2) of the bacterial culture revealed some level of hydrophobicity in PL-2 and PL-3 followed by PL -1 and PL-4

Bacterial biomass colonized on LDPE

Bacterial colonization was measured as extractable protein of the polyethylene. Isolate PL-1 showed considerable increase in protein concentration than PL-2 & PL-3 and PL-4 (Table 3)

Bio-film quantification

The bacterial isolates PL-1, PL-2 and PL-3 rose during 2nd day and remained stable for 2 additional days,

Table 2. BATH test.

Name of the isolate	OD value before adding hexadecane	OD value after adding hexadecane
PL- 1	0.432	0.28
PL- 2	0.86	0.35
PL- 3	0.87	0.35
PL- 4	0.40	0.380

Table 3. Measurement of bacterial colonization (490 nm).

Name of the isolate	Protein concentration (µg mg^{-1})	
	After 2 days	After 6 days
PL- 1	15	3
PL- 2	17	19
PL- 3	17	20
PL- 4	3	9

Table 4. Quantification of bacterial bio-film.

Name of the isolate	OD value after 2 days	OD value after 4 days	OD value after 6 days
PL- 1	0.020	0.023	0.008
PL- 2	0.008	0.010	0.006
PL- 3	0.008	0.010	0.005
PL- 4	0.055	0.008	0.017

Table 5. Sturm test for bacteria (48 h).

Name of the Isolate	Amount of CO_2 (g l^{-1})
PL- 1	0. 7042
PL- 2	1. 0603
PL- 3	1. 0604
PL- 4	0. 5706

followed by a gradual decrease in the 6th day whereas PL-4 showed a gradual decrease from the 4th day onwards (Table 4)

Sturm test

The value for CO_2 evolution from the degradation of LDPE sample by bacterial isolates (Table 5) shows that PL-2 and PL-3 degraded LDPE with a higher efficiency than the other isolates.

Identification of bacterial strains

Based on the *16S rRNA* gene sequencing, the bacterial isolates were identified as *Brevibacillus parabrevis* (PL-1), *Acinetobacter baumannii* (PL-2), *Acinetobacter baumannii* (PL-3) and *Pseudomonas citronellolis* (PL-4).

The DNA sequences of these bacterial isolates were deposited in the GenBank- Genetic sequence database at the National Center for Biotechnical Information (NCBI) under the following accession numbers JQ304812 (strain PL1), JQ304813 (strain PL2), JQ294033 (strain PL3), JQ304811 (strain PL4).

DISCUSSION

The list of pollutants which pose environmental problems and health hazard and are tough for biodegradation, is a long one. Present study ealt with the isolation of

polyethylene degrading micro organisms from the municipal landfill soil.

Low density polyethylene films were used in this study. The micro organisms with ability to degrade LDPE were isolated in synthetic medium supplemented with LDPE powder and these micro organisms used for degradation study. Several methods were employed to monitor the biodegradation of polyethylene.

Rosenberg et al. (1980) have described BATH test to estimate the bacterial cell surface hydrophobicity that can be directly related to the ability to form an effective biofilm over any hydrophobic surfaces. This test was followed by Hadad et al. (2005) where the results showed lower reduction in turbidity of the bacterial suspension. Our results of BATH test revealed some levels of decrease in hydrophobicity of strains PL-2, PL-3 and PL-1 followed by PL-4.

Estimating bacterial biomass density in the biofilm is a difficult task since the bacterial cells are strongly attached to the polyethylene surface. We had followed the technique of (Hadad et al., 2005) and the results proved that the bacterial biomass of isolate PL-4 increased from 2^{nd} day to 6^{th} day of incubation followed by PL-1 and PL-2 and PL-3.

We had followed the technique of O'Toole et al (2000) to measure the absorbance of the biofilm extracted with ethanol. There was a mild increase in the OD value of the isolates PL-1, PL–2 and PL-3 from 2^{nd} to 4^{th} day but decreased in 6^{th} day whereas PL-4 isolate showed a poor formation of biofilm, but interestingly, PL-4 isolate showed a doubling time of about 28 min, indicating the fact that biofilm formation cannot be relied upon totally for deciding the rate of degradation as also observed by Hadad et al. (2005).

Sturm test is commonly employed for evaluation of the biodegradability of polymer materials. We used a Modified Sturm test for the measurement of CO_2 both in gaseous and dissolved form in SM. Incidentally, all previous reports did not take into account the dissolved CO_2 during Sturm Test evaluation (Shah et al., 2007; Muhammad et al., 2009; Shah et al., 2009)

The CO_2 evolution test gave a valid data about the degradation rate by the bacterial isolates. CO_2 evolved by isolate PL-2 and PL-3 was measured to be 1.0603 g l^{-1} followed by isolate PL-1 that evolved 0.7042 g l^{-1} and isolate PL-4 which gave out 0.5706 g l^{-1} of measurable CO_2. Shah et al. (2007) performed this test with a consortium of bacterial and fungal isolates and he reported a CO_2 concentration of 1.85 g l^{-1} for 4 weeks. Studies done by Muhammad Ishtiaq Ali et al. (2009) reported a concentration of about 10 g l^{-1} of CO_2 after a period of 30 days. Shah et al. (2009) also reported CO_2 concentration of about 1.85 g l^{-1} after a 30 day period of growth of *Fusarium* sp. on LDPE films. On comparison with the above reports, our studies have revealed a much higher concentration of CO_2 for our isolates and this shows that there are potential abilities in these microbes that could be tapped to draw out a biodegradation strategy.

Our Sturm Test study is unique in the fact that we have worked with individual organisms rather than consortia as reported by many workers.

Conclusion

LDPE can be biodegradable if the right microorganism is isolated. We have proved that the hydrophobic LDPE film can act as a substratum for some groups of micro-organisms which formed a biofilm on the LDPE film. The isolates also grew on minimal medium containing only LDPE in the powdered form as the carbon source even without any nitrogen source. We have also proved that the LDPE can be totally degraded into carbon dioxide which brings us closer to the fulfillment of the objective of isolating a microorganism that can completely degrade the recalcitrant polyethylene if the right conditions are provided.

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