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Microbial assisted High Impact Polystyrene (HIPS) degradation

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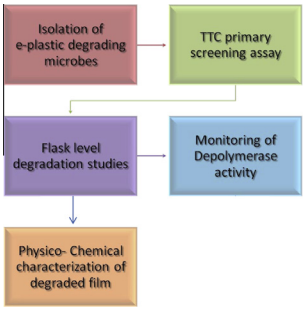
HIGHLIGHTS

- Bacterial degradation of High Impact Polystyrene (HIPS) was observed.
- Degradation with *Bacillus* spp. showed a weight loss of 23% (w/w) of HIPS film in 30 days.
- Bromine release in the form of methyl bromine from decabrominated HIPS was confirmed by NMR.
- Culture supernatant of *Pseudomonas* spp. showed 97.4% reduction in turbidity of HIPS emulsion.

GRAPHICAL ABSTRACT



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ABSTRACT

The efficacy of newly isolated *Pseudomonas* and *Bacillus* strains to degrade brominated High Impact Polystyrene (HIPS) was investigated. Viability of these cultures while using e-plastic as sole carbon source was validated through Triphenyl Tetrazolium Chloride (TTC). Four days incubation of HIPS emulsion with *Bacillus* spp. showed 94% reduction in turbidity and was 97% with *Pseudomonas* spp. Confirmation of degradation was concluded by HPLC, NMR, FTIR, TGA and weight loss analysis. NMR spectra of the degraded film revealed the formation of aliphatic carbon chain with bromine and its release. FTIR analysis of the samples showed a reduction in C—H, C=O and C=N groups. Surface changes in the brominated HIPS film was visualized through SEM analysis. Degradation with *Bacillus* spp. showed a weight loss of 23% (w/w) of HIPS film in 30 days.

1. Introduction

Plastic is becoming an inevitable material in the modern society with its wide range of application. The main constituent of e-plastic material is High Impact Polystyrene (HIPS), which is a blend of polystyrene and polybutadiene which can improve the strength of the resulting polymer (Thallada et al., 2003). Toxic metals released from e-plastic such as Lead, Mercury, Cadmium, Hexavalent chromium, Barium, Beryllium, etc., can lead to damage of central and peripheral nervous, neural and respiratory systems. Asthmatic bronchitis, DNA damage and skin warts are other side effects related to the release of e-waste into the environment. Incineration is the main technique adopted which melts away the plastic materials and burns away the non-valuable metals (Lakshmi and Nagan, 2009). Dioxides and furans, which are considered to be carcinogenic, are released into the environment in large quantities during incineration. Hence, microbial approach toward this issue can be a better option in the current situation (Yutaka et al., 2010).

Present study deals with the microbial degradation of a decabrominated HIPS and is the first report on this kind of e-plastic. The isolates showed a greater tendency to degrade the native HIPS film. The rate and gravity of degradation was studied by analyzing the structural changes in the plastic film, biofilm formation on film surface, by detecting polystyrene degrading

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intermediates in the culture medium and by looking into the enzyme profile of the cultures responsible for depolymerisation.

2. Methods

2.1. High Impact Polystyrene

HIPS with decabromodiphenyl oxide and antimony trioxide was in the form of small white opaque beads provided by CSIR-IIP and the films were made by dissolving 200 mg of beads in 10 ml of chloroform and pouring that into a wide open glass plate and were kept at room temperature for overnight in a fume hood to obtain films with consistent structure.

2.2. Medium for HIPS degradation

In this study, HIPS (20 mg/ml) was the sole carbon source and degradation of it was done in mineral medium containing, 0.01% Yeast extract; 0.02% $MgSO_4{\cdot}7H_2O$; 0.02% KH_2PO_4; 0.01% $(NH_4)_2SO_4$; 0.005% $MnSO_4$; 0.005% $ZnSO_4$; 0.016% K_2HPO_4. Cultures were also grown on Nutrient broth or agar (Peptic digest 5 g l^{-1}; Beef extract 3 g l^{-1}; NaCl 0.5 g l^{-1}; agar 0.16 g l^{-1} agar).

2.3. Culture enrichment and isolation

Soil samples were collected from plastic dump yard in Thiruvananthapuram (08°29′15″N 76°57′9″E on the west coast). Samples were collected 5 cm below the soil surface in a sterile container. Enrichment culture method was followed in which 10 gm of soil sample was transferred into a conical flask with mineral medium (95 ml), 0.85% NaCl and HIPS film as the sole carbon source (Cheng et al., 2010). Incubation was done for 14 days at 30 °C at 150 rpm. 5 ml of the culture supernatant was transferred to 45 ml fresh mineral medium with plastic. Further, plating was done on mineral medium to obtain pure cultures.

2.4. Primary screening of isolates

Organisms obtained from enrichment culture method and serial dilutions were undergone for TTC assay. Bushnell-Hass medium was used for assay (0.1% NH_4NO_3; 0.02% $MgSO_4{\cdot}7H_2O$; 0.1% K_2HPO_4; 0.01% $CaCl_2{\cdot}2H_2O$; 0.015% KCl) and HIPS film was added as the sole carbon source. In the control medium, glucose was added as the carbon source. To check the degradation, aliquot of 20 µl of TTC was added to 5 ml of culture medium. If the organisms are capable of surviving in the medium utilizing HIPS as sole carbon source, the enzymes in electron transport chain of the organisms will convert colorless TTC into red colored Triphenyl formazan.

2.5. Isolation of genomic DNA and identification of isolates

Genomic DNA was isolated from the organism using kit protocol (Sigma NA21101KT, Gen Elute™ Bacterial Genomic DNA Kits). 16SrRNA Sequencing was done using the universal forward 27F (*Escherichia coli* position 8-27, 5′-AGA GTT TGATCC TGG CTC AG-3′) and reverse 1492R (*E coli* position 1510-1492, 5′-GGC TAC CTT GTT ACGACT T-3′) 16SrRNA primers. The PCR conditions given were 37 cycles of 3 min at 94°C, 1 min at 55°C, and 1 min at 72°C (Eppendorf-epgradientMastercycler, Germany). Obtained PCR product was resolved on 1% agarose gel and purified using kit protocol (QIAGEN, QIAquick PCR Purification Kit). Sequencing (Applied Biosystem ABI 3500 Series Genetic Analyzer, USA) was done and the nucleotides were compared with NCBI data bank.

2.6. Estimation of bacterial biomass colonized on HIPS film

Estimation of the protein content on the film surface can be a good option to confirm bacterial adhesion to film. Colonized HIPS film samples were collected from the liquid culture broth at regular intervals of time and boiled in 5 ml 0.5 M NaOH for 30 min. After required time of incubation, the suspension was centrifuged and the pellets and supernatant were separated. The steps were repeated and the supernatants obtained were pooled and protein content was estimated through Bradford's assay (Sedmak and Grossberg, 1977).

2.7. SEM analysis

Selected strains were inoculated into 250 ml of mineral medium with surface sterilized HIPS film (1 × 1 cm) as sole carbon source. Incubation was done at 30 °C for 30 days at 200 rpm (Amnat et al., 2002). After incubation, HIPS film was recovered washed with 2% SDS and distilled water and air dried; the film was then undergone for SEM analysis (Carl Zeiss EVO 18, Germany) to check the surface attrition. The film was sputter coated with gold layer, in an Argon atmosphere with 25 mA and 0.3 MPa conditions before imaging.

2.8. Determination of dry weight of residual HIPS

HIPS film after incubation for 30 days at 30 °C with microorganisms was recovered and surface sterilized using ethanol and distilled water. Film was then air dried and residual weight was taken (Mor and Sivan, 2008). Residual film weight was calculated using the formula,

$$\text{Percentage weight loss} = \left[\frac{\text{Initial film weight} - \text{Final film weight}}{\text{Initial film weight}}\right] \times 100$$

2.9. FTIR spectroscopy

A Nicolet corporation, USA model: Nexus-870 FTIR was used at a frequency range of 4500–400 cm^{-1} for analysis. C—H stretch at a range of 3000 cm^{-1}, C—Br stretch at a range of 600 cm^{-1}, C—H bending at 1340 cm^{-1} were evaluated. The results obtained for degraded film was compared with control non degraded film (Allen et al., 2004).

2.10. Thermo gravimetric analysis (TGA)

Thermo gravimetric analysis of the HIPS film was done to check the weight loss of the film after and before treatment with the bacterial isolates. TA, USA model TGA Q50 was used for analysis. Temperature range was adjusted between 50 and 700 °C. Film was heated in a platinum pan and nitrogen was flushed into the pan at a rate of 50 ml min^{-1}, this was done in order to reduce the corrosion due to release of HBr, from flame retardant HIPS (Thallada et al., 2003).

2.11. NMR analysis

HIPS film was dissolved in deuterated chloroform, which was used as an internal reference and ^1H NMR was done (Bruker AVANCE II 500, Switzerland). Wavelength used was 500 MHz and the concentration of plastic film in deuterated chloroform was maintained at 0.01 gm ml^{-1} (Schlemmer et al., 2009). HIPS film after incubation with bacterial isolates was used as the test film and untreated film was used as the control.

2.12. High pressure liquid chromatographic analysis

Mineral medium with inoculated organism and surface sterilized HIPS film were incubated for 30 days at 30 °C and after that cells were removed and the collected supernatant was filtered through a 0.2 μm filter paper and the clear filtrate was subjected to HPLC analysis (Shimadzu UFLC, Japan). Phenyl acetaldehyde, Styrene oxide, 1-phenyl-1,2ethanediol, and 2-phenyl ethanol (Sigma, USA) were used as the standards. LC solution software was used for analysis. Detector used was Photo Diode Array and measurement was done at UV 210 nm. The chromatographic column used was C18 (Gemini NX 5u C18 110A, Size 150 × 4.6 mm, Particle size 5 μ), 50:50 acetonitrile and water were used as the mobile phase. Injection volume was maintained at 10 μl and flow rate was regulated at 0.6 ml min^{-1}.

2.13. Depolymerase assay

A HIPS emulsion (2 mg ml^{-1}) in 20 mM phosphate buffer with pH 6.8 was used as the substrate. For reaction, 3 ml of the substrate was added to 1 ml culture supernatant. Initial incubation was done at 50 °C for 20 min (Zhanyong et al., 2012) and second set of incubation was done at 30 °C for 4 days. Initial absorbance was taken at 650 nm before incubation and final absorbance was taken after incubation at same wavelength. Reduction in turbidity was calculated.

$$\text{Reduction in turbidity} = \left[\frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}}\right] \times 100$$

3. Results and discussion

3.1. Isolation of HIPS degrading bacteria

Serial dilution and enrichment method were carried out for isolating cultures from soil sampled collected from plastic duping area. Both bacterial isolates used in this study were responded positively to confirm their viability while using HIPS as sole carbon source.

3.2. Identification of bacterial strains

Genomic DNA was isolated from two bacterial strains selected through TTC reduction assay and by doing 16SrRNA sequencing they were identified to be a *Bacillus* spp. and other one was *Pseudomonas* spp. There are reports on degradation of Polyethylene (Prosun et al., 2013; Sharma et al., 2013), Polyhydroxyalkanoates (Volova et al., 2011; Sang et al., 2002) and High density polyethylene (Balasubramanian et al., 2010) with *Pseudomonas* spp. which were isolated from plastic dumping sites. Based on the qualitative enzyme assays, *Bacillus* spp. was found to be positive for both lipase and esterase but the *Pseudomonas* spp. was positive for esterase activity.

3.3. Total protein content estimation

Both cultures were grown on nutrient medium until it reached OD 0.6, 100 μl of the culture were transferred to mineral medium with plastic cut in uniform size (1 × 1 cm). Incubation was done and at regular intervals of time and plastic film was recovered aseptically. Organisms will get attached to the film surface since the HIPS film is hydrophobic (Hadad et al., 2005). Film recovered was boiled in NaOH and supernatant was undergone for Bradford's assay. After an initial lag phase of two days, there was a sharp increase in protein content on third day and the amount was at peak on fourth and fifth days giving the indication of cell adhesion to the film (Supplementary Fig. 1).

3.4. SEM analysis

HIPS film without any microbial treatment had a plane and smooth surface, the samples that were treated showed that organisms were found colonized near and around the incisions and formation of pits and holes indicating degradation (Supplementary Fig. 2). Such results were previously reported for other polymers such as Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (Zhanyong et al., 2012) and Poly Lactic Acid.

3.5. Determination of dry weight

Percentage weight loss of the HIPS film after incubation with the organisms showed considerable film lost. HIPS films after treatment with bacterial isolates were recovered air dried and weighed. The weight loss percentage of HIPS film obtained after treatment with *Bacillus* spp. was of significant 23.7% (w/w) and was less than 10% with *Pseudomonas* sp. No weight loss was found with the untreated film. Percentage weight loss can be correlated with SEM result and total protein content in the biofilm.

3.6. FTIR spectroscopy

Evident changes in certain peak intensities were seen on FTIR results (Supplementary Fig. 3). Rather than shifts in the spectrum, we were able to visualize a reduction in frequency. This reduction was observed at a region of wave number between 1000 and 500 cm^{-1}, which generally corresponds to C–X, where X can be any halogens. As our compound is a brominated one, it can be assumed that there is a change in bromine related portion of the HIPS. Similar reduction in peaks were observed at, 1000–1500 cm^{-1} (C═C), 2500–3500 cm^{-1} (sp^2 C—H, sp^3 C—H) (Donald et al., 2010).

3.7. Thermo gravimetric analysis

Gravimetric analysis was done to compare the weight reduction pattern of control and treated films with respect to temperature. The thermogram obtained for control had a curve which was evenly distributed and the HIPS film after biodegradation showed remarkable alterations in the thermogram (Fig. 1). Reports states that, the thermal stability of High Impact Polystyrene nanocomposites, produced by various extrusion processes can be measured by TGA analysis (Sanchez-Olivares et al., 2008).

3.8. NMR analysis

NMR analysis of the HIPS samples after and before biodegradation was done using deuterated chloroform as the internal reference. When compared to the control there were presences of few new peaks. One of the major peaks obtained was in the area between 3 and 4 ppm, which generally corresponds to —CH_2—Br— (Supplementary Fig. 4). Where as in control there were no traces of that peak in the same region. Hence, it can be concluded that due to microbial attack bromine in the form of methyl bromine is released from the HIPS. Release of bromine as such into the environment during incineration of e-waste can be a potential health risk. Hence, it will be always better to process brominated plastics in a controlled biological route.

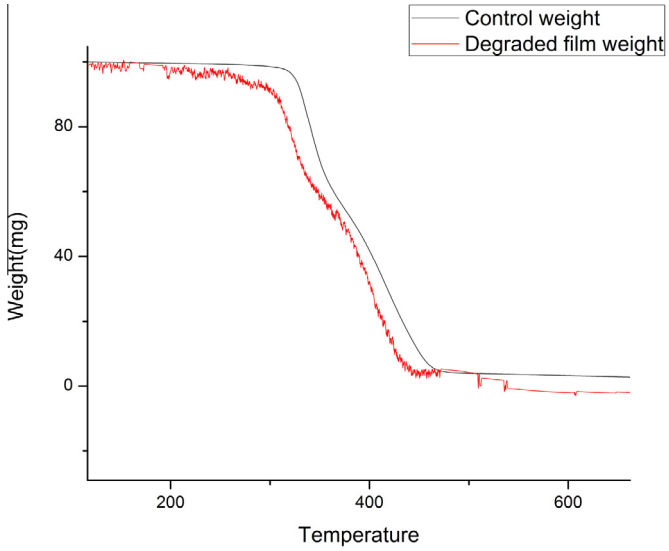

Fig. 1. Thermo gravimetric analysis of HIPS film. (a) Before degradation. (b) After degradation.

3.9. High pressure liquid chromatography

Polystyrene on microbial degradation will lead to the formation of many intermediates and some of the impending ones were found to be, Styrene oxide, Phenyl ethanol, Phenyl acetaldehyde, 1-phenyl-1,2-ethane diol (Marconi et al., 1996). The chromatogram obtained showed the presence of some of these intermediates. Culture medium treated with *Bacillus* spp. and *Pseudomonas* spp. showed the presence of Phenyl ethanol (Supplementary Figs. 5a, 5b). S. Fig. 5c shows the chromatogram of Phenyl ethanol standard represented by the peak 'a' (RT 3.9).

3.10. Depolymerase assay

When the plastic emulsion was treated with the culture supernatant of *Pseudomonas* and *Bacillus* sp. it showed turbidity clearance of 97.4% and 92.6% of the total emulsion respectively. As a control, heat treated culture filtrate was used and no turbidity clearance was observed indicating the catalytic activity of a depolymerase enzyme.

4. Conclusion

The study demonstrated the potential of the newly isolated cultures for the degradation of brominated HIPS. A feasible bioprocess using a consortium of selected microbes for treating the e plastic could be a promising and challenging work to carry out further

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.03.021.

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