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ORIGINAL ARTICLE

High-density polyethylene (HDPE)-degrading potential bacteria from marine ecosystem of Gulf of Mannar, India

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biodegradation, biofilm, biomass, high-density polyethylene, marine ecosystem.

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Abstract

Aims: Assessment of high-density polyethylene (HDPE)-degrading bacteria isolated from plastic waste dumpsites of Gulf of Mannar.

Methods and Results: Rationally, 15 bacteria (GMB1-GMB15) were isolated by enrichment technique. GMB5 and GMB7 were selected for further studies based on their efficiency to degrade the HDPE and identified as *Arthrobacter* sp. and *Pseudomonas* sp., respectively. Assessed weight loss of HDPE after 30 days of incubation was nearly 12% for *Arthrobacter* sp. and 15% for *Pseudomonas* sp. The bacterial adhesion to hydrocarbon (BATH) assay showed that the cell surface hydrophobicity of *Pseudomonas* sp. was higher than *Arthrobacter* sp. Both fluorescein diacetate hydrolysis and protein content of the biofilm were used to test the viability and protein density of the biomass. Acute peak elevation was observed between 2 and 5 days of inoculation for both bacteria. Fourier transform infrared (FT-IR) spectrum showed that keto carbonyl bond index (KCBI), Ester carbonyl bond index (ECBI) and Vinyl bond index (VBI) were increased indicating changes in functional group(s) and/or side chain modification confirming the biodegradation.

Conclusion: The results pose us to suggest that both *Pseudomonas* sp. and *Arthrobacter* sp. were proven efficient to degrade HDPE, albeit the former was more efficacious, yet the ability of latter cannot be neglected.

Significance and Impact of the Study: Recent alarm on ecological threats to marine system is dumping plastic waste in the marine ecosystem and coastal arena by anthropogenic activity. In maintenance phase of the plastic-derived polyethylene waste, the microbial degradation plays a major role; the information accomplished in this work will be the initiating point for the degradation of polyethylene by indigenous bacterial population in the marine ecosystem and provides a novel eco-friendly solution in eco-management.

Introduction

During the past three decades, plastic materials have increasingly used in food, clothing, shelter, transportation, construction, medical and recreation industries. A very general estimate of worldwide plastic waste generation is about 57 million tons annually. Particularly in India, more than 59 000 and 61 000 tons of plastic wastes have found their way in to India in the years 1999 and 2000, respectively (Statistics of Foreign Trade of India, March 2000 and March 2001, DGFT, GoI). These drastic rises in the use of plastic materials have not been accompanied by a corresponding development of procedures for the safe disposal or degradation of these materials. Synthetic plastics accumulate at a rate of 25 million tons per year in the terrestrial and marine coastal environment.

However, polyethylene represents up to 64% of the synthetic plastics produced, and they are mainly used for manufacturing plastic bags, bottles, disposable containers, which are discarded within a short time (Byuntae *et al.* 1991). High-density polyethylene (HDPE) is a polyethylene thermoplastic, prepared by a catalytic process, containing carbon and hydrogen as backbone elements and has little branching, giving it stronger intermolecular forces and tensile strength (4550 psi approx.) greater than low-density polyethylene (LDPE). Generally, it was believed that higher the density greater the stability because of shortened bond length and tight packaging. Physically, HDPE is harder, more opaque and can withstand somewhat higher temperatures (120°C). HDPE is also used in many products and packaging such as carry bags, milk jugs, detergent bottles, margarine tubs, garbage containers and water pipes. Thus, they have wide industrial and day-to-day application. The extensive usage of HDPE poses severe environmental threats to terrestrial and marine ecosystem, as they are hardly degradable and voluminously dumped after usage.

The Gulf of Mannar, a marine national park on the south-eastern tip of India is biologically diverse; these areas are at risk of pollution caused by synthetic polymer wastes primarily because of high rate of anthropogenic activities. The polyethylene from plastic bags could sometimes cause blockage in intestine of fish, birds and marine mammals as plastic bags are often mistaken as food by marine mammals and they choke to death (Spear *et al.* 1995; Secchi and Zurzur 1999). Hence, it is apposite to find a suitable remedial process, which is also eco-friendly. Microbial degradation of solid polymers such as HDPE requires the formation of a biofilm on the surface of the polymer, which enable the microorganisms to degrade the nonsoluble substrate efficiently.

On this regards, no records describe the clear evidence of biodegradation process of polyethylene (Potts 1978). Only partial degradation was observed in polyethylene film that had been buried in soil for as long as 32 years (Otake *et al.* 1995). ^{14}C-labelled polyethylene subjected to 26 days of artificial UV irradiation before buried in to soil evolved <0·5% carbon dioxide (CO_2) by weight after 10 years. In another report, without prior UV treatment, <0·2% CO_2 was produced (Albertsson and Karlsson 1990).

Most of the studies on the biodegradation of polyethylene are based on the biotic environment, but some studies have used axenic bacterial strains amended with polyethylene. This study is aimed to isolate the HDPE-degrading bacteria from plastic waste dumped arena in Gulf of Mannar region, India and described the HDPE degradation efficacy with the results of FT-IR spectra.

Materials and methods

Substrate

Commercially available HDPE materials were used as substrate in this study, which is the grade of environmental pollution rather than pure polyethylene to access the direct impact on environment and application-oriented solution, because the composition of commercially available HDPE varies from pure polyethylene by the addition of additives like antioxidants and colourant.

Source of bacteria

Partially degraded polyethylene along with soil samples adhering and adjacent to it was collected from 15 plastic waste dumped sites in the Gulf of Mannar region, India.

Isolation of HDPE-degrading bacteria

Soil samples, 10 g each, were inoculated in 100 ml of synthetic media (SM) containing (per litre of distilled water): g l^{-1}: NH_4NO_3, 1·0; $MgSO_4{\cdot}7H_2O$, 0·2; K_2HPO_4, 1·0; $CaCl_2{\cdot}2H_2O$, 0·1; KCl, 0·15; yeast extract, 0·1 (Difco) and micronutrients for 1·0 mg l^{-1} of each of the following: $FeSO_4{\cdot}6H_2O$, $ZnSO_4{\cdot}7H_2O$ and $MnSO_4$. In addition, 300 mg of partially degraded polyethylene material was added as substrate collected from same place to the SM. The samples were incubated for 12 weeks at 30°C. After 12 weeks of incubation, the mixture of bacteria was purified by spread plate technique, and subsequent purified cultures were maintained on slant nutrient agar (Difco). Further, the HDPE-degrading bacteria was screened.

Screening of HDPE-degrading bacteria

Isolated individual bacterial colonies were tested for HDPE-degrading efficiency according to Gilan *et al.* (2004). Briefly, flasks containing 50 ml of SM were amended with preweighed HDPE and isolated bacteria. Further, the experiments were performed with or without 0·05% (v/v) mineral oil (light white oil, d = 0·84 g l^{-1}; Difco) in the medium to colonize bacteria on HDPE surface. The dry weight of the HDPE was determined after incubated at 30°C for 30 days after which, the polyethylene samples were removed, washed, dried at 60°C and weighed. Furthermore, the degradation was confirmed with the aid of FT-IR, and the biofilm formation on HDPE surface was determined using the same.

Determination of dry weight of residual HDPE

To facilitate accurate measurement of the dry weight of residual HDPE, the bacterial biofilm was washed off from the HDPE surface with 2% (v/v) aqueous sodium dodecyl sulfate (SDS) solution for 4 h and further washed with distilled water (Gilan *et al.* 2004). The residual HDPE containing mineral oil was treated with chloroform prior to SDS wash to remove mineral oil. The washed HDPE was placed on a filter paper and dried overnight at 60°C before weighing. The weight loss was calculated using the following formulae (Konduri *et al.* 2010): Percentage of weight loss = [(Final weight − Initial weight)/Original weight] × 100.

Evaluation of bacterial hydrophobicity

The bacterial adhesion to hydrocarbon (BATH) assay was performed in nutrient broth medium containing isolated bacteria with phosphate urea magnesium sulfate (PUM) buffer. The bacterial cells were resuspended in PUM to an optical density at 400 nm value of 1·0–1·2. Aliquots (1·2 ml each) of this suspension were transferred to a set of test tubes to which were added increasing volumes (range 0–0·2 ml) of hexadecane. The test tubes were shaken for 10 min and allowed to stand for 2 min. The OD_{400} of the aqueous suspension was measured. Cell-free buffer served as the blank (Rosenberg *et al.* 1980).

Viability of the bacterial biofilm

Viability of the bacterial biofilm was determined by the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Carlsbad, CA, USA), according to the manufacturer's instructions. Live cells (green in colour) can be differentiated from dead cells, which emit red colour.

After 30 days, HDPE containing biofilms were removed from medium, washed in sterile water and further viewed under an epifluorescent microscope equipped with a fluorescein isothiocyanate filter (Chavant *et al.* 2002). The viability and activity of the bacterial biofilm was determined indirectly by measuring the hydrolysis of fluorescein diacetate (FDA) to fluorescein according to Schnurer and Rosswall (1982). Samples without FDA served as blanks, and a sample of HDPE from a sterile SM medium served as a control.

Quantitative estimation of bacterial biomass of biofilm

The bacterial biofilm was strongly attached to the HDPE surface; hence, it was difficult to estimate the population density by standard techniques, such as direct cell counting or plating. Therefore, the population density of the biofilm on the HDPE surface was estimated by determination of protein concentration. Pieces taken from HDPE colonized in SM or SM plus 0·05% (v/v) mineral oil were washed gently in water and then boiled for 30 min in 5 ml of 0·5 mol l^{-1} NaOH. The suspension was centrifuged, collected supernatant and the pellet were subjected to the same procedure once again. The collected supernatants were combined; the protein concentration was determined in each supernatant according to Sedmak and Grossberg (1977) and calculated the mean value.

FT-IR analysis

A Perkin Elemer, USA, Model: Spectrum RX1 was used at a resolution of 2 cm^{-1}, in the frequency range of 4000–400 cm^{-1}. Relative absorbance intensities of the ester carbonyl bond at 1740 cm^{-1}, keto carbonyl bond at 1715 cm^{-1}, terminal double bond (vinyl) bond at 1650 cm^{-1} and internal double bond at 908 cm^{-1} to that of the methylene bond at 1465 cm^{-1} were evaluated using the following formula (Albertsson *et al.* 1987): Keto carbonyl bond index (KCBI) = I_{1715}/I_{1465}; Ester carbonyl bond index (ECBI) = I_{1740}/I_{1465}; Vinyl bond index (VBI) = I_{1650}/I_{1465}; Internal double bond index (IDBI) = I_{908}/I_{1465}. The percentage crystallinity of the polymer was measured based on the method suggested by Zerbi *et al.* (1989) and calculated by following formulae: % of Crystallinity = $100-[(1 - (I_a/1{\cdot}233I_b)/1 + (I_a/I_b)) \times 100]$.

Statistical analysis

All experiments were replicated five times, and the results were expressed as mean ± standard deviation.

Results

HDPE-degrading competent fifteen bacterial (GMB1-GMB15) strains were isolated by enrichment method. These bacterial strains were named with numbers as GMB1-15 (Gulf of Mannar Bacteria). Of which, two were capable of HDPE degradation using HDPE as sole carbon source, namely GMB5 and GMB7 were screened for degradation analysis based on their biofilm formation ability on HDPE and weight loss of HDPE after incubation. GMB5 and GMB7 bacterial strains were identified as *Arthrobacter* sp. and *Pseudomonas* sp., respectively. These bacterial strains were inoculated separately in to SM containing HDPE as the sole carbon source, both of them colonized on the HDPE surface within 5 days. After 30 days of incubation, the HDPE was subjected to weight loss analysis. The HDPE were degraded, and percentage weight loss was calculated as 12·23 ± 0·6 and 15·18 ± 0·7

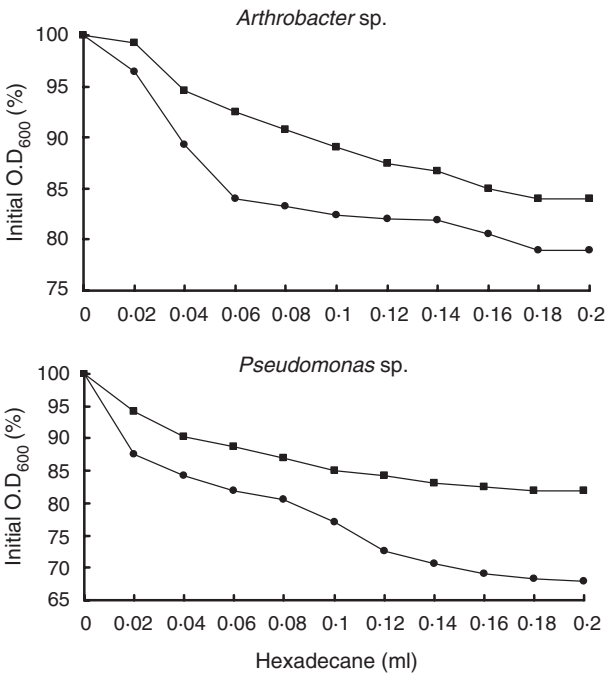

Figure 1 Hydrophobicity of bacterial isolates determined by bacterial adhesion to hydrocarbon test. Aliquots of logarithmic and stationary cell suspensions were added with increasing concentrations of hexadecane. Hydrophobic cells transfer from aqueous phase to the hexadecane is reflected as a decrease in the turbidity (O.D. at 600 nm) of the bacterial suspension (■ Logarithmic cells; ● Stationary cells).

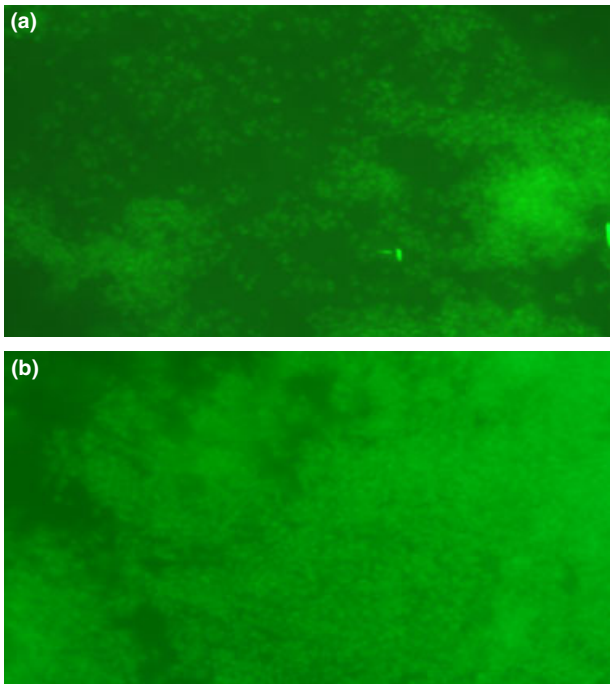

Figure 2 Live and dead cells attached on high-density polyethylene surfaces after 30 days of incubation (a) *Arthrobacter* sp., (b) *Pseudomonas* sp. Green and red colour indicates live and bacterial cells, respectively.

of its weight by *Arthrobacter* sp. and *Pseudomonas* sp., respectively and normalized against control medium without microbial inoculation.

The BATH assay showed higher hydrophobicity for *Pseudomonas* sp. when compared to *Arthrobacter* sp. (Fig. 1). For *Pseudomonas* sp., the adhesion of bacteria cells to hexadecane was evident even at the lowest concentration of the hydrocarbon, resulting in the reduction of more than 25% turbidity in the cultures. Cells of logarithmic phase in both cultures were more hydrophobic than stationary phase. The viable bacteria on HDPE surface after incubation with both strains was determined separately. In both cultures, more living bacterial cells (green spots) were observed on biofilm of the HDPE surface (Fig. 2a,b). The FDA hydrolysis by extracellular esterases and protein content in the biofilm were analysed for *Arthrobacter* sp. and *Pseudomonas* sp., as an indirect method to measure the biofilm viability and metabolic activity. Both cultures showed efficient hydrolysis of FDA in 2–5 days of incubation with steep raise in peak (Fig. 3) and after fifth day, the efficacy was slightly inconsistent with increasing time, yet *Pseudomonas* sp. followed minimal variation in terms of efficacy (Fig. 3). The results of protein content of the biofilm of both bacterial strains followed a pattern similar to that of the viability of the biofilm measured by the FDA assay of *Arthrobacter* sp. and *Pseudomonas* sp. The results of FDA hydrolyzing activity showed that both bacterial strains of biofilm population were viable and very active in metabolism.

On overall, both bacterial strains were effective in colonizing on the HDPE surface. This may explain the sustained biodegradation of polyethylene by weight loss. Further, the degradation was confirmed by FT-IR. Interestingly, examined HDPE degradation by FT-IR analysis showed changes in functional group(s) and/or side chain modification because of microbial activities. Monitoring the formation or disappearance of acids (1715 cm^{-1}), ketones (1740 cm^{-1}) and double bonds (1640 and 721 cm^{-1}) were analysed using the FT-IR to explain the mechanism of the biodegradation process (biotic or abiotic). KCBI, ECBI, VBI and IDBI were also calculated (Fig. 4). The KCBI, ECBI and VBI were increased; however, IDBI was increased in *Arthrobacter* sp. and decreased in *Pseudomonas* sp. when compared to control after incubation.

Discussion

The present findings demonstrate that *Arthrobacter* sp. and *Pseudomonas* sp. isolated from plastic waste

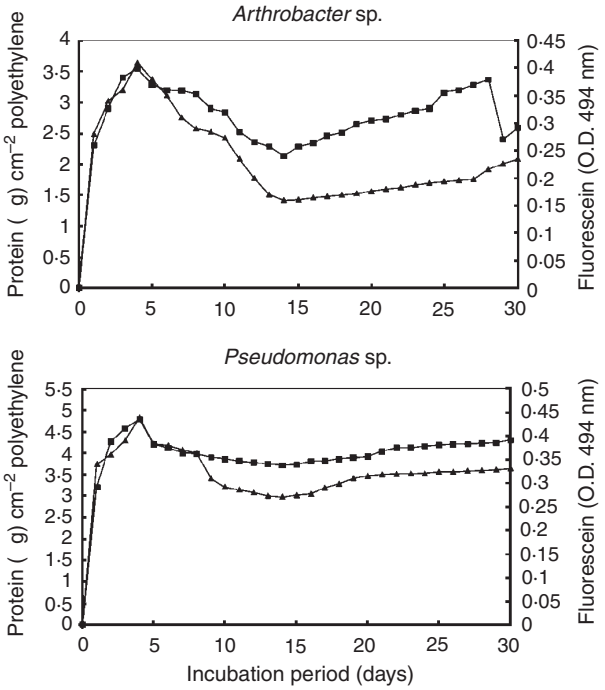

Figure 3 Protein content and hydrolysis of fluorescein diacetate (FDA) by extracellular esterases in the biofilm of *Arthrobacter* sp. and *Pseudomonas* sp. on the surface of high-density polyethylene (■ protein; ▲ FDA).

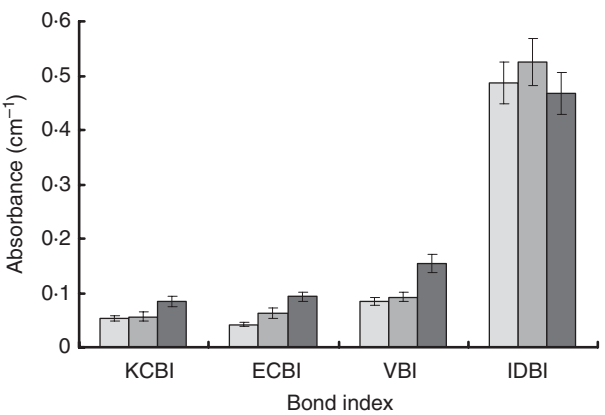

Figure 4 Fourier transform infrared of high-density polyethylene exposed to *Arthrobacter* sp. and *Pseudomonas* sp. for one month (KCBI – Keto carbonyl bond index; ECBI – Ester carbonyl bond index; VBI – Vinyl bond index; IDBI – Internal double bond index). (□) Control; (▒) *Arthrobacter sp.*; and (■) *Pseudomonas sp.*

dumpsites along the coastal side of Gulf of Mannar were capable of utilizing HDPE as a sole carbon source. The maximal biodegradation of the polyethylene was observed during 30 days of incubation with both the aforementioned bacterial strains. *Arthrobacter* sp. degraded the polyethylene up to 12·23% of HDPE weight loss without any prior oxidation by thermal, acids and/or UV irradiation. This shows better degrading ability than the previously reported work by Albertsson *et al.* (1998) in which they have documented the degradation of thermooxidized LDPE only after 3·5 years of incubation with *Arthrobacter paraffineus,* whereas the identified strain was able to degrade HDPE in 30 days. The biodegradation of HDPE level is higher in present study compared to the values ranging from 3·5 to 8·4% of previous reports of polyethylene incubated in the soil for 10 years (Potts 1978; Albertsson and Karlsson 1990; Yabannavar and Bartha 1994). The synthetic polyethylene degradation was very slow process according to Otake *et al.* (1995), ten years is relatively considered as short period, whereas the degradation of the same was achieved in 30 days with *Arthrobacter* sp. and *Pseudomonas* sp. in the present study. Kathiresan (2003) has isolated the *Pseudomonas* sp. from indigenous place (Mangrove forest) and tested the biodegradation ability with polyethylene, the isolated *Pseudomonas* sp. results shows similar to our study, and we were able to achieve 15% of HDPE weight loss in laboratory conditions. Karlsson and Albertsson (1998) have studied the degradation of ^{14}C-labelled LDPE in soil over a 15-year period and found that the total weight loss during degradation was 16% and in addition to H_2O and CO_2, short hydrocarbons, alcohols, organic acids, ketones and aldehydes were detected in the products (Carlsson and Wiles 1969a,b; Albertsson and Karlsson 1990).

In the present study, HDPE was added with or without mineral oil showed the same level of degradation in 30 days as those observed after 4 years of incubation in a biotic environment by Albertsson and Karlsson (1993), and no effect on overall biodegradation by mineral oil was observed. However, the mineral oil plays a functional role in providing bacterial adherence to the surface of HDPE by enhancing biofilm formation.

The biofilm formation ability of bacterium on HDPE surface was attributed to the hydrophobicity of its cell surface (Gilan *et al.* 2004). Further, addition of a small amount (0·05%) of mineral oil to the culture medium increased both biofilm formation and the subsequent biodegradation of the polyethylene (Gilan *et al.* 2004), presumably by increasing the hydrophobic interactions between the bacterial biofilm and the polymer. Albeit the statement is logical, no such observations were made in our study except facilitating the adherence property.

FDA hydrolysis by extracellular esterases and protein assay proved to be efficient tools for analysing the state of colonization and biofilm formation on the HDPE (Fig. 2). FDA hydrolysis and protein assay were provided strong evidence of rapid colonization of both *Arthrobacter* sp. and *Pseudomonas* sp. on the surface of the polyethylene during first 2 days of incubation. After fifth day,

decrease in biomass density was observed. It showed that the rapid early colonization of the polyethylene was because of utilization of the mineral oil adhering to the surface of the HDPE as a carbon source. Hence, this may be hypothesized that low-density bacterial cells with low growth rate were able to utilize polyethylene as a carbon source.

The formation and disappearance of ester, keto, vinyl and internal double bond index were clearly showed by FT-IR spectra, which help in the confirmation of HDPE biodegradation. The biodegradation of polyethylene was originally initiated by the abiotic process. Oxidation of polymer chain occurred because of the dissolved oxygen or that which is present in the ambient leading to the formation of carbonyl groups. Subsequently, carbonyl groups will form carboxylic groups and enter into β–oxidation process. As a result, CO_2 and H_2O are formed after total degradation *via* citric acid cycle by micro-organisms (Albertsson *et al.* 1987). The amount of carbonyl residues in the polyethylene increased after incubation with both bacterial strains, which was considered predominantly because of enzymatic activities. The KCBI, ECBI, VBI and IDBI were increased after treated with *Arthrobacter* sp. However, when incubated with *Pseudomonas* sp. KCBI, ECBI and VBI were increased, whereas IDBI (internal double bond index) was decreased on comparison against the control. Albertsson *et al.* (1987) reported the formation of terminal double bond when exposed to a biotic environment, the same was observed for terminal double bonds after incubation with both the bacterial strains. This could have resulted from Norrish mechanism of degradation (Albertsson *et al.* 1987) or through the formation of esters. In our cases, the KBCI and EBCI were increased because of biotic factors (microbial enzymatic activity), Keto and ester carbonyl have been reported as major products in the presence of enzyme oxidoreductase (Karlsson and Albertsson 1998). However, in control, there was no change in carbonyl bond index (keto and ester). The formation of double bonds in the polymer chain occurred may be because of the Norrish type II reaction proposed earlier (Albertsson *et al.* 1987). In the present study, fraction of carbonyl (keto and ester) and double bonds (-CH=CH-) was higher than that of the terminal/vinyl double bond (-CH=CH_2). The percentage of crystallinity of HDPE decreased up to 7% after incubated with both the bacterial strains. On the grounds, we were able to augment the validation of proposed hypothesis stating the degradation is more effectual using the selected strains considering the weight loss and biofilm formation. Furthermore, the FT-IR data strongly suggests the participation of biotic factors, which was the epicentre of our investigation. Based on our studies, among Gulf of Mannar isolated strains of *Pseudomonas* sp. showed high degradation efficiency to HDPE by the measures of weight loss, carbonyl (keto and ester) index, double-bond (internal and vinyl/terminal) index, hydrophobicity, viability of bacterial biofilm and bacterial biomass.

Conclusion

The biodegradation of HDPE by two marine bacteria, namely *Arthrobacter* sp. and *Pseudomonas* sp., is reported here under *in vitro* condition in the synthetic medium. The degradation of HDPE by *Pseudomonas* sp. was faster than *Arthrobacter* sp., and the viability of the bacterial biofilm observed under epifluorescent microscope showed that the live bacterial cells were green colour and showed high metabolic activity. Both *Arthrobacter* sp. and *Pseudomonas* sp. showed decrease in hydrophobicity of HDPE and crystallinity percentage after 30 days of incubation. Based on the results of biofilm formation, weight loss and FT-ID data, we could able to interpret that *Pseudomonas* sp. is a better degrader of HDPE. Between the two bacterial strains selected for the study, *Pseudomonas* sp. efficiently degrade HDPE in 30 days without any prior treatment, yet we have not studied the long-term effect that would be laid as a good rationale for future studies. Therefore, we could able to recommend the exploitation of HDPE biodegrading property of *Pseudomonas* sp. in coastal regions exposed to environmental hazards posed by plastic waste.

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