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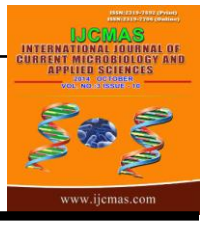

Original Research Article

Isolation of polythene degrading bacteria from marine waters of Viskhapatnam, India

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A B S T R A C T

Keywords

SM-Synthetic Medium, PE-Polyethylene, Bath-Bacterial Adhesion to Hydrocarbon

The present article reveals the biodegradation of low density and High density polythene by bacterial strains isolated from marine waters near Appikonda, Bheemili, Sagarnagar of Bay of Bengal, and Visakhapatnam. The degrading ability of the bacterial strains was evaluated by performing colonization studies, Agar over layer method, The Bacterial Adhesion to Hydrocarbon (BATH) assay and Salt Aggregation Test (SAT). Colonization studies on both LDPE films was performed over a period of one month by measuring the Bacterial biomass in biofilms colonizing the polythene surface using protein estimation method. Polythene degradation by the bacterial strains was further evaluated by measuring the dry weight loss in polythene. Degradation was confirmed by SEM and FTIR analysis. These strains were characterized morphologically and biochemically.

Introduction

Polythene bags bring a lot of convenience to people's life but at the same time they also bring long term harms. Polyethylene is a polymer made up of long chain monomers of ethylene. The worldwide utility of polyethylene is expanding at a rate of 12% per annum and approximately 140 million tonnes of synthetic polymers are produced worldwide each year (Shimao, 2001). With such huge amount of polyethylene getting accumulated in the environment, their disposal evokes a big ecological issue. It takes thousand years for their degradation.

A review has been published on backbone hydrolysis of polyesters, polyanhydrides, polyamides, polycarbonates, polyurethanes, polyureas and polyacetals. (Pierre and Chiellini 1987). The degradation of polythene begins with the attachment of microbes to its surface. Various bacteria (*Streptomyces viridosporus* T7A, *Streptomyces badius* 252, and *Streptomyces setonii* 75Vi2) (Pometto *et al.*, 1992) and wood degrading fungi produced some extracellular enzymes which lead to degradation of polythene (Iiyoshi *et al.*, 1998). The by-products of the polythene varied depending upon the conditions of degradation. Under aerobic conditions, CO_2,

water and microbial biomass are the final degradation products whereas in case of anaerobic/ methanogenic condition CO_2, water, methane and microbial biomass are the end products and under sulfidogenic condition H and H_2O and microbial biomass are reported to be the end products (Arutchelvi *et al*., 2008).

Plastics are advantageous as they are strong, light weight and durable. But, lack of degradability and the closing of landfill sites, as well as growing water and land pollution problems have led to concern about plastics. With the excessive use of plastics and increasing pressure being placed on capacities available for plastic waste disposal, the need for biodegradable plastics and biodegradation of plastic has assumed increasing importance in the last few years. Biodegradation is necessary for water soluble or water immiscible polymers, because they eventually enter water streams which can neither be recycled nor incinerated (Shah *et al.,* 2008). The polyethylene is the most commonly found solid waste that has been recently recognized as a major threat to marine life. The polyethylene could sometimes cause blockage in intestine of fish, birds and marine mammals (Spear *et al*., 1995 and Seechi *et al*., 1999). The degradation of polyethylene can occur by different molecular mechanisms such as chemical, thermal, photo and biodegradation (Gu 2003). Biodegradability is evaluated by weight loss, tensile strength loss, changes in percent elongation and changes in polyethylene molecular weight distribution. Physicochemical distribution is initiated by treatment with acid at 70°C and UV irradiation of the polyethylene film. These pre-treatment favours the microbial degradation of polyethylene.

The PE solid waste related problems pose threat to mega cities including Visakhapatnam. So, an attempt has been made to isolate the potent bacterium that degrades polyethylene from marine water source.

Materials and Methods

The polythene films used in this work were obtained from local markets (Radha polybags and Sri Shyam Plasto products Hyderabad) where they were sold as 20 and 40 micron thick carrier bags. The Nutrient medium materials were obtained from Hi-media (India) and inorganic salts and chemicals from E-Merck (Germany).

Screening of Polythene degrading bacterial isolates from Marine water sample

Marine water samples were taken from Bay of Bengal Appikonda, Bheemili, Sagarnagar near Visakhapatnam coastal area. Then standard dilution plating technique on Zobella Marine agar and Tryptone soy agar (TSA) (Hi media) at 30^0 C was performed. Then orange and yellow pigmented colonies have formed. Only creamish yellow colonies were picked up, purified by re streaking and further confirmation was done by growing them on PE Minimal salt medium (PE powder from Sigma chemicals). Then the isolates were maintained on same medium for future applications. Morphological and Biochemical characterisations of the bacterial strains were done according Bergy's Manual of Determinative Bacteriology Volume II. (Brenner *et al.,* 2004)

Biodegradation Testing

Polythene bags were subjected to UV treatment for 70hrs and then thermal ageing in the oven for 3 days at 70^0 C. Then PE

strips were disinfected with 70% ethanol and Benzene for 30mins to remove any plasticizers, colouring agents and air dried for 15mins in laminar air flow chamber under blower.

Preparation of Polymer Over layer

The Polymer over layer method used here is a modification of the ASTM-D-2676T (Annual book of ASTM standards). A glass petri dish was covered with an PE film of 40 and 20-micron thickness and was then autoclaved and enriched nutrient media comprising of 5% tryptone, 5%NaCl and 1% yeast extract with 2% agar was carefully poured by lifting the polymer film with a sterile glass rod and gently laying it back on the solidifying agar and covering it with another autoclaved petri dish. The whole process was done in a laminar flow to maintain sterility. Bacterial culture (50µl) was then spread upon the PE films and incubated at 30^0 C for two to three days in an inverted condition.

Preparation of Polythene based liquid culture

Synthetic nutrient medium composed of 1g NH_4NO_3, 1g K_2HPO_4, 0.15g KCl, 0.2g, $MgSO_4$, and 0.1g yeast extract, 0.1g $CaCl_2$, 1mg $MnSO_4$, 1mg $FeSO_4$, and 1mg $ZnSO_4$ Dist water 1ltr was prepared. Mineral oil (0.05%) was added to increase the colonization. It was supplemented with 0.1g pre treated PE strips as sole carbon source. Overnight liquid culture of bacterial strains in peptone water was centrifuged at 5000rpm for 5min to remove the nutrient medium. The centrifuged cells resuspended in same volume of sterile water, was then added in the ratio of 1:10 (v/v) in the PE based liquid culture and incubated at 30^0 C at 180rpm shaking condition.

Evaluation of viability of bacterial culture from incubated flasks

After 15 days interval of incubation at 30^0 C, 50 µl of the culture was withdrawn and spread on an enriched agar medium plate for overnight at 30^0 C, after 18hr the plates were observed for colony formation of bacteria, the purity of the culture was ascertained through biochemical testing characteristic morphology.

Quantitative estimation of bacterial biomass in PE biofilms

The population density of the biofilm on the polythene was estimated by determination of protein concentration by boiling for 30min in 15ml of 0.5N NaOH. Biofilms centrifuged, protein concentration of the supernatant was determined according to Lowry method. (Lowry *et al*., 1951)

Evaluation of bacterial hydrophobicity

a. **BATH assay:** Bacterial cell-surface hydrophobicity was estimated by the bacterial adhesion to hydrocarbon (BATH) test (Rosenberg *et al.* 1980). Bacteria were cultured in NB medium until the mid-exponential phase, centrifuged and washed twice with phosphate–urea–magnesium (PUM) buffer containing (g l^{-1}): K_2HPO_4, 17; KH_2PO_4, 7·26; urea, 1·8 and $MgSO_4 \cdot 7H_2O$, 0·2. The washed cells were resuspended in PUM buffer until it reaches an O.D. value of 1·0–1·2 at 400 nm. Aliquots (1·2 ml each) of this suspension were transferred to a set of test tubes, to which increasing volumes (ranging: 0–0·2 ml) of hexadecane were added. The test tubes were shaken for 10 min and then allowed to stand for 2 min to facilitate phase separation. The turbidity of the aqueous suspensions was measured at 400 nm. Cell-free buffer served as the blank.

b. SAT assay: (Lindahl *et al.,* 1981) Bacterial suspensions (10µl) were mixed with equal volumes of ammonium sulphate solutions of various molarities (0.2M to 4M) on a glass slide and observed for aggregation for 1 min at room temperature. The highest dilution of ammonium sulphate (final concentration) giving visible aggregation was scored as a numerical value for bacterial surface hydrophobicity, the SAT value.

Determination of dry weight of the residual PE

After degradation for 1 month PE strips were washed with chloroform to remove mineral oil. Bacterial biofilm was washed off PE surface with 0.2% aqueous Sodium Dodecyl sulphate (SDS) solution for 4 hrs then with dist water, placed on a filter paper and dried over night at 60^0 C before weighing.

Confirmation of polyethylene degradation

Polyethylene degradation was confirmed by using Scanning Electron Microscopy (SEM) and Fourier Transform Infrared (FTIR) Spectroscopy.

Observation of PE strips using SEM

The samples were mounted on the Aluminium stubs by silver paint. Gold coating was carried out in vacuum by evaporation in order to make the samples conducting. Micro structural examination was conducted in the Leo 440i scanning electron microscope. The images of the test samples were compared with the original untreated control samples.

Characterization of the PE film through Fourier transform infra-red spectroscopy (FT-IR) analysis

The micro destruction of the small samples is widely analyzed by an important tool such as Fourier Transform Infrared spectroscopy (FT-IR), and due to the recent up-gradation of this instrument the map of the identified compounds on the surface of the sample can be documented via collection of large number of FT-IR spectra (Prati *et al.,* 2010). The Infrared absorption (IR) spectrum of degraded PE was recorded with a Perkine Elmer Model ATR FT-IR spectrophotometer. Washed PE strips were scanned between 500 and 4000 cm^{-1} with air as reference.

Statistical analysis: All the experiments were repeated thrice and standard deviation was calibrated.

Results and Discussion

Screening of Polythene degrading bacterial isolates from Marine water samples

Marine water samples were taken from Bay of Bengal near Visakhapatnam coastal area. Then standard dilution plating technique on Zobella Marine agar and Tryptone soy agar (TSA) (Hi media) at 30^0C was performed. Orange and yellow pigmented colonies were formed. Yellow colonies (Fig. 3.1) were picked up, purified by restreaking and further confirmation was done by growing on PE Minimal salt medium. Ten colonies with degradation zone were selected for further biodegradation testing. They were characterized by morphological and biochemical testing (Table 3.1).

Biodegradation Testing
Preparation of Polymer Over layer

Bacterial isolates showed clear growth on 20 and 40 micron PE films (Fig. 3.2), indicating that they were able to colonize the PE efficiently. Out of ten isolates, three were

found to be best in colonization of PE. Three bacterial strains (S1, S2 and T3) with clear growth were selected for further degradation study.

Evaluation of viability of bacterial culture from incubated flasks

After 15 days of incubation at 30^0 C, in liquid culture flasks, isolates purity was confirmed by Gram staining (Fig. 3.3to 3.5) and biochemical tests.

Quantitative estimation of bacterial biomass in PE biofilms

Total protein concentration for S1 strain on 40 and 20 micron PE was found to be 92 and 120µg, for S2 strain 62 and 82 µg and for T3 strain 72 and 98 µg respectively. This indicates that biofilm formation was high for S1 than T3 and S2 (Table 3.2).

Evaluation of bacterial hydrophobicity

a. **BATH assay:** S1 strain showed 11% O.D whereas S2 and T3 showed 38% and 33% O.D indicates that lesser the O.D more will be the adhesion to hexadecane hydrocarbon. This indicates that S1 strain is having more adhesion capacity to hydrocarbon than T3 and S2 strains (Table 3.3). It is based on the affinity of bacterial cells for an organic hydrocarbon such as hexadecane. The more hydrophobic the bacterial cells, the greater their affinity for the hydrocarbon, resulting in transfer of cells from the aqueous suspension to the organic phase and a consequent reduction in the turbidity of the culture. This assay confirmed the polymer adhesion capacity of the strains.

b. SAT assay: S1strain showed maximum aggregation from 2M to 4M Ammonium sulphate whereas S2 and T3 strains showed maximum aggregation with only 4M concentration of Ammonium Sulphate. (Table 3.4) This tendency of hydrophobic bacterial cells to clump at relatively low ionic strength compared with bacteria having a more hydrophilic cell surface was used to develop a simple and accurate test to quantitate bacterial surface hydrophobicity, the SAT method. This indicates that S1 strain is more hydrophobic than S2 and T3 strain.

Determination of dry weight of the residual PE

The degradation PE has been shown by the growth of the bacterial strain in Synthetic medium where PE was the only carbon source supported not only survival but also growth of the organism as evident from Dry weight loss in the degraded PE. S1 strain degraded 40 micron PE to 24% and 20 micron PE to 20% and S2 strain degraded both 40 and 20 micron PE to 10 and 2% respectively, where as T3 degraded 40 micron PE to 11% and 20 micron PE to 14% only in one month incubation. The results showed greater degradation ability of S1 strain towards both types of PE films. (Table 3.5)

Confirmation of polyethylene degradation

Observation of PE strips using SEM

Autoclaved (Fig. 3.6), treated polyethylene (Fig. 3.7) showed morphological changes when observed through SEM. Formation of holes, disruption of polyethylene structure confirmed high degradation capacity of bacterial strains S1, and T3 than S2.

FTIR Analysis of biodegraded 20 and 40 microns PE

After bacterial treatment the same polymer film was washed, dried and subjected to FT-IR spectroscopy. FTIR analysis also indicated that S1 strain has greater intensity

Peaks than S2 strain (Figs. 3.8 to 4.3). The main band of 2940-2915 cm^{-1} was indicative of the C-H stretch and 1472 cm^{-1} of CH_2 asymmetric bending.. From the IR spectroscopy it can be stated that the bacterial degradation led to a substantial increase in the C-H stretch band of the polyethylene at (2940-2915 cm^{-1}).

Bacterial isolate S1, is Gram-negative, rod-shaped with rounded poles. Whereas, isolates S2 and T3 are gram positive rod shaped bacteria. On TSA and minimal salt agar, they form creamish-yellow pigmented colonies around 0.2mm in diameter, circular, slightly convex and opaque, smooth after 2 days of incubation at 30°C.

Isolates were found to grow on PE and colonized PE surface and formed massive biofilm on it: a process that seemed to be a prerequisite for biodegradation. Indeed, cell-surface hydrophobicity tests confirmed the hydrophobic nature of the strains. S1 showed greater hydrophobicity than S2 and T3 strains. It may be hypothesized that biodegradation of PE by these strains is mediated by the adherence of cells to the polythene surface and was detected by protein concentration on biofilm formed.

The maximium 61.0% (*Microbacterium paraoxydans*) and 50.5% (*Pseudomonas aeruginosa* and *Brevibacillus borstelensis*) (Hadad *et al.*,2005) of polythene degradation in terms of Fourier Transform Infrared coupled Attenuated Total Reflectance (FTIR-ATR) was reported (Rajandas *et al*., 2012) within two months previously.

But in terms of weight loss, the degradation of polythene was recorded as 47.2% after 3 months of incubation with the *A. oryzae* (Konduri *et al.,* 2011) followed by 50% weight loss of the polythene strips using fungus, *Phanerochaete chrysosporium* after 8 months of regular shaking with pH 4.00 at room temperature (Aswale 2010). But due to biodegradation, weight loss of the polythene is not always reported. Some workers reported gain in the polythene weight after cultivation of the microbes on the polythene, incubated at regular shaking for one month at 30^0C. Only three out of ten microbes led to weight loss. The maximum weight gain (2.02%) was reported with *Streptomyces humidus*. The possible reason for gaining of the polythene weight after the cultivation of the microbes on the strips is accumulation of cell mass on the polythene surface (El-Shafei *et al.,* 1998). In case of *in vivo* study after 32 years of polythene dumping in the soil only partial degradation was reported (Otake *et al.,* 1995).

Strains S1, T3 and S2 were identified and were found to be capable of utilizing commercial irradiated polyethylene as the sole carbon source. During 1-month incubation with *these strains*, the maximal biodegradation was observed for 40 micron PE film with S1 (24%) and for 20micron degradation is 14% whereas the biodegradation with S2 strain only 10%. For 20 micron PE film maximum degradation was observed with S2 strain (2%), whereas T3 degraded 40 micron PE to 11% and 20 micron PE to 14% only in one month of incubation. These results were higher than *Arthrobacter* sp. of marine origin (Balasubramanian *et al*., 2010).

This biodegradation level is higher than the values (3·5 to 8·4%) reported for polyethylene incubated in soil for 10 years (Albertsson and Karlsson 1990). These low rates were in agreement with the argument of Otake *et al., (1995*) that 10 years is a relatively short period for the biodegradation of synthetic polymers such as polyethylene.

Fig 3.1 Yellow colonies on TSA

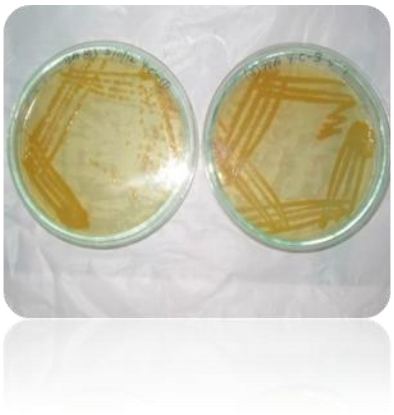


Fig 3.2 Polymer over layer method

Fig 3.3 Gram -ve rods of S1

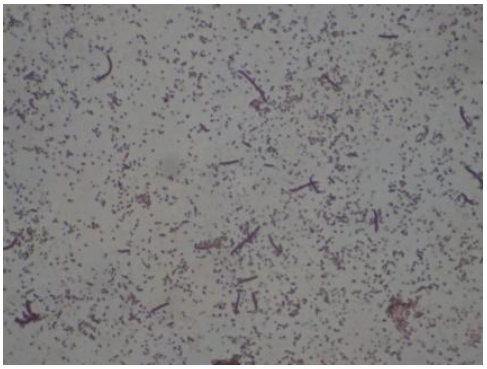


Fig 3.4 Gram +ve rods of S2

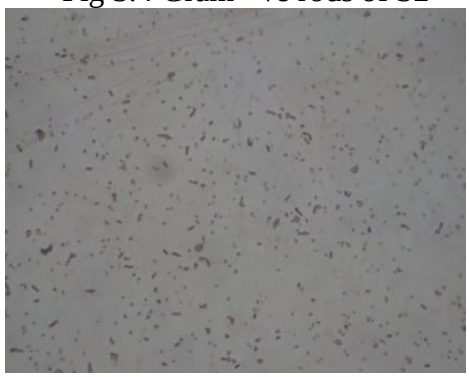


Fig 3.5 Gram +ve rods of T3

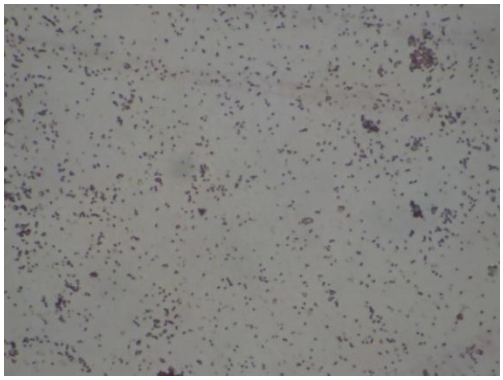


Table 3.1 Biochemical characterization of isolates

Characteristic	**S1**	**S2**	**T3**
Color	LY	Y	LY
Motility	+	+	-
Gram reaction	- rods	+ rods	+ rods
Biochemical characteristics:			
Catalase	+	+	+
Oxidase	+	+	-
Gelatin Liquefaction	-	+	+
Amylase	+	+	+
DNase	-	+	+
Aesculin hydrolysis	-	+	+
Tween80 hydrolysis	+	+	+
Urease	-	+	+
H_2S production	-	-	-
Indole	+	-	-
Methyl red	-	+	+
Vogus Proskauer	-	-	+
Citrate	-	-	-
Casein Hydrolysis	+	+	+
Nitrate test	+	-	+
Acid produced from:			
Glucose	+	+	+
Maltose	+	+	-
Arabinose	-	-	-
Galactose	-	-	-
Lactose	-	-	-
Mannose	+	+	-

Y- Yellow; +, positive; -, negative;

Quantitative estimation of bacterial biomass in PE biofilms

Table 3.2 Measurement of protein biofilm concentration

Isolate	Protein concentration (μgmg^{-1})
S1 40	92 ±1.5275
S1 20	120 ±1.633
S2 40	62 ±1.633
S2 20	82±1.5275
T3 20	79±1.633
T3 40	98±1.5275

Evaluation of bacterial hydrophobicity

Table 3.3 BATH Assay

S.no	Vol of the Culture (ml)	Vol of the Hexadecane (ml)	S1 OD_{400}	S1 % of initial OD	S2 OD_{400}	S2 % of initial OD	T3 OD_{400}	T3% of initialOD
1	1.2	0	1.12	100	1.289	100	1.24	100
2	1.2	0.04	0.52	41	0.97	88	0.89	71
3	1.2	0.08	0.29	22	0.89	55	0.85	68
4	1.2	0.16	0.21	16	0.7	53	0.75	60
5	1.2	0.2	0.14	11	0.88	38	0.77	33

Table 3.4 SAT Assay

$(NH_4)_2SO_4$	S1	S2	T3
0	0	0	0
0.2 M	0	0	0
0.6 M	0	0	0
1.0 M	0	0	0
1.6 M	1	0	0
2.0 M	1	0	1
2.5 M	2	1	1
3.0 M	2	2	1
3.5M	4	2	2
4.0 M	4	3	3

Biodegradation testing

Table 3.5 Biodegradation ability of bacterial isolates in one month incubation

Isolate	Initial Dry weight of PE(g)	Dry weight after Degradation (g) *	Weight loss (g)	Percentage of Dry weight loss
S1 40	0.1	0.76	0.24±0.01	24%
S1 20	0.1	0.86	0.14±0.03	14%
S2 40	0.1	0.90	0.10±0.005	10%
S2 20	0.1	0.88	0.12±0.02	12%
T3 40	0.1	0.8	0.2±0.02	20%
T3 20	0.1	0.99	0.11±0.02	11%

± = Standard Deviation. * = Mean

SEM Analysis of degraded 20 and 40 microns PE

Fig.3.6. SEM photographs of control/ untreated and autoclaved polyethylene

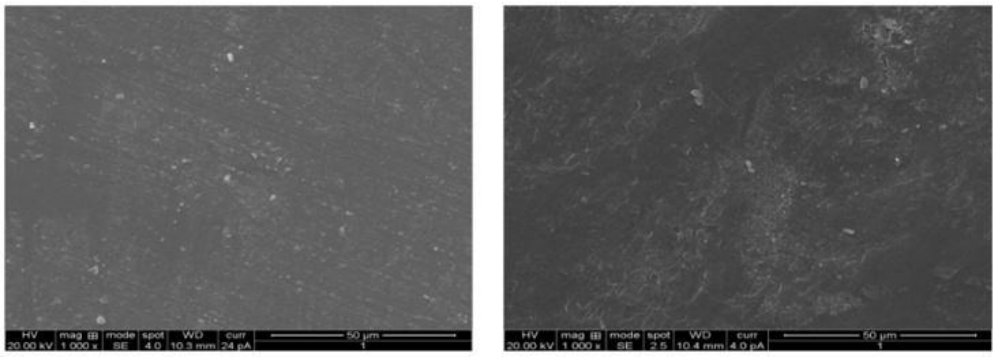
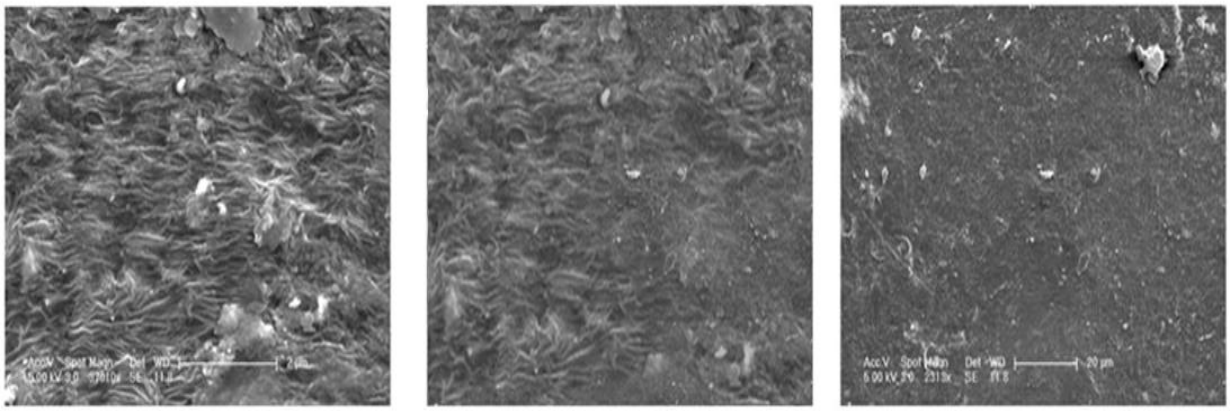


Fig. 3.7 SEM photographs of S1, T3 and S2 Treated polyethylene



FTIR Analysis of S1and S2 degraded 20 and 40 microns PE

Fig. 3.8 FTIR of S1 strain treated 20micron PE

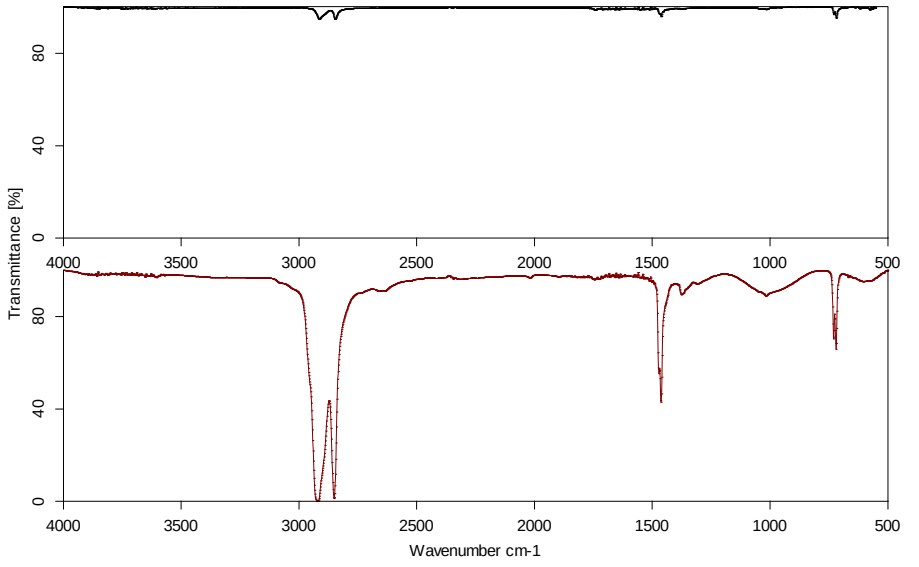


Fig.3.9 FTIR of S1 strain treated 40micron PE

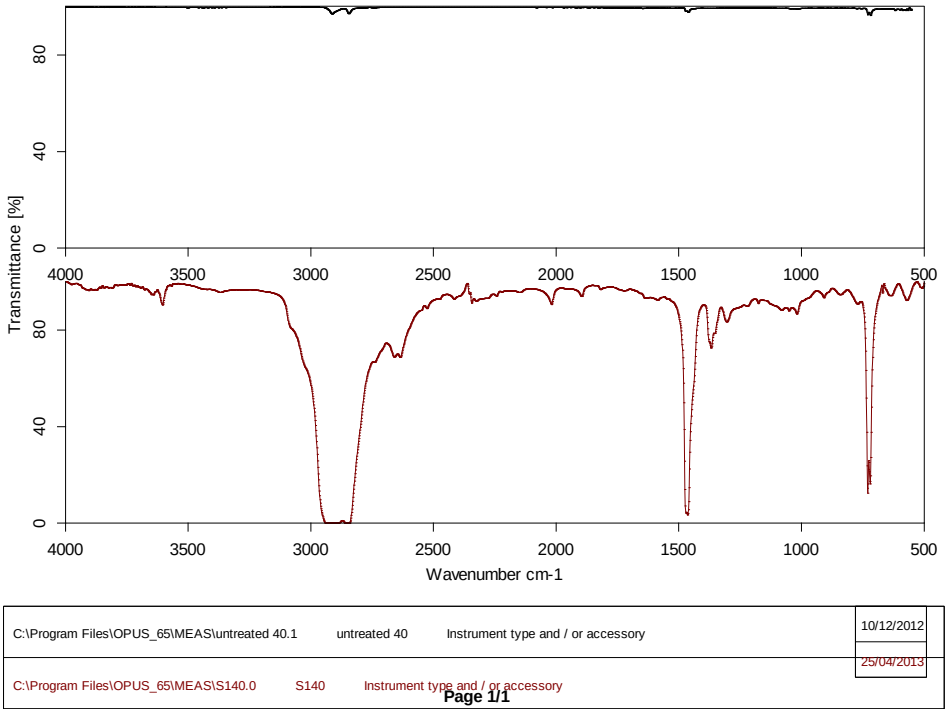


Fig. 4.0 FTIR of S2 strain treated 20micron PE

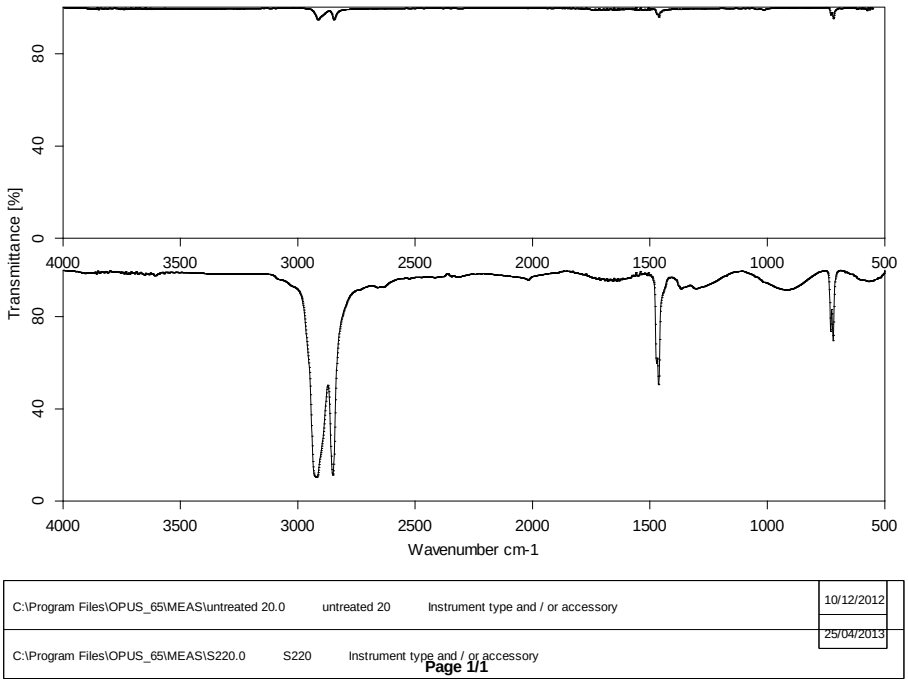
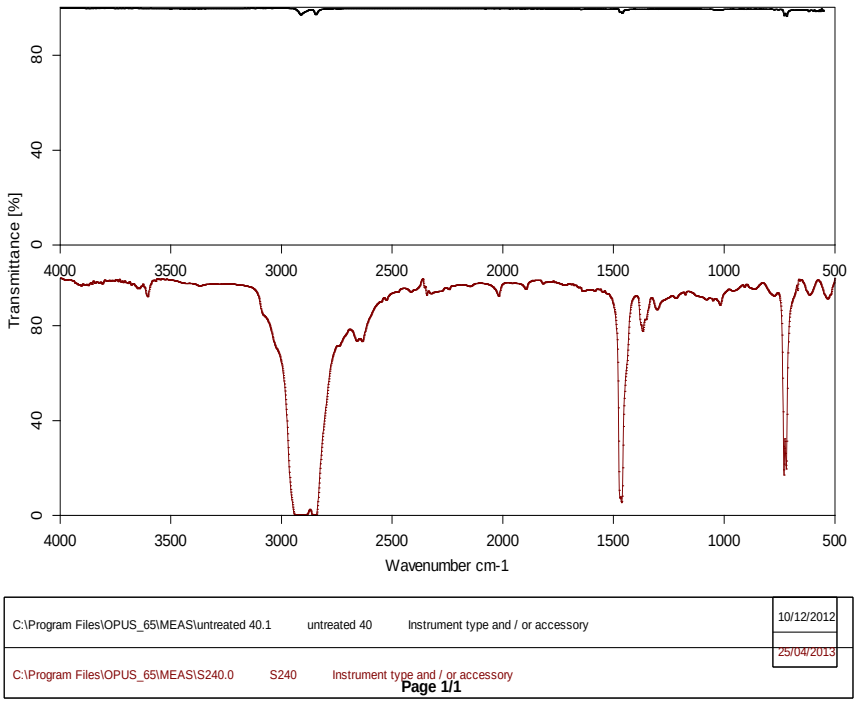


Fig.4.1 FTIR of S2 strain treated 40micron



PE

Fig.4.3 FTIR of T3 strain treated 20micron PE

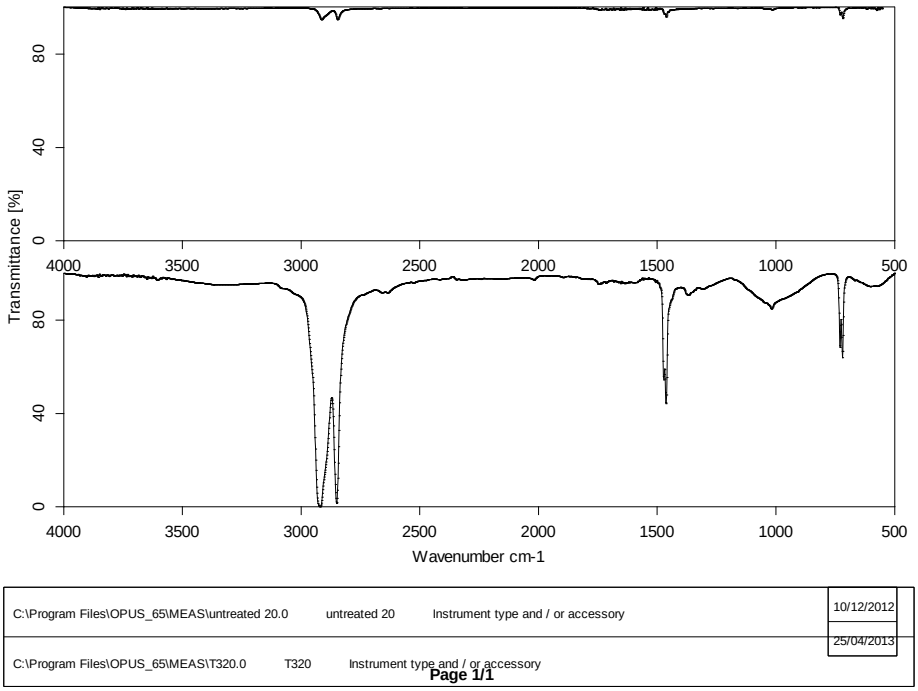
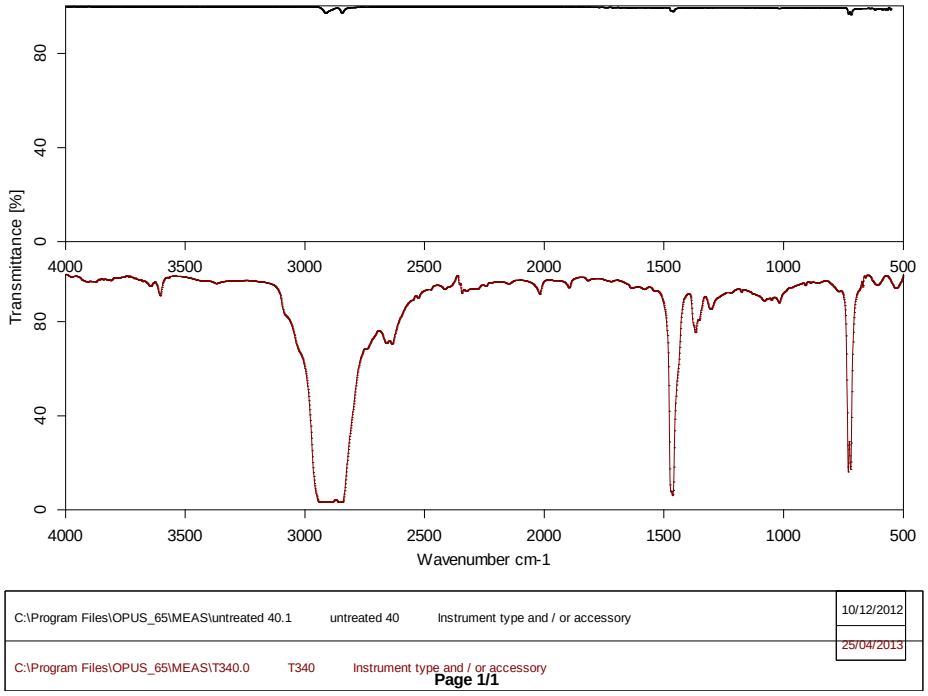


Fig. 4.2 FTIR of T3 strain treated 40micron PE



It is widely accepted that the short fragments formed in UV.-irradiated polyethylene are readily utilized by various microorganisms. For example, incubation of polyethylene with *Arthrobacter paraffineus* resulted in a small increase in the average molecular weight of the polyethylene, apparently because of the consumption of the low molecular weight fragments by the bacteria (Albertsson *et al., 1998).*

It has been reported that isolation of a strain of *Rhodococcus ruber* colonized polyethylene surface formed a massive biofilm on it: a process that seemed to be a prerequisite for biodegradation (Gilan *et al.,* 2004). Similarly, S1 and T3 strains are strong biofilm producers. The high degree of biofilm production of *S1 strain* is probably because of the hydrophobic nature of its cell surface. Indeed, two cell-surface hydrophobicity tests confirmed the hydrophobic nature of *these strains*.

However, the fact that the dry weight of polyethylene incubated with S1 was highly reduced indicates that S1 strain, unlike most other tested microorganisms, was capable of degrading the low density PE fragments efficiently. This is supported by the degradation level of polyethylene films during incubation for 30 days with *S1 strain*. Moreover, the biodegradation of irradiated polyethylene without humidity by S1 strain indicates that it is capable of degrading unmodified polyethylene.

Commercially available extruded PE can be well degraded by the S1 strain. The degradation PE has been shown by the growth of the bacterial strain in synthetic medium where PE was the only carbon source supported not only survival but also growth of the organism as evident by FTIR analysis, which indicates that S1 has greater intensity Peaks. The main band of 2940-2915 cm^{-1} was indicative of the C-H stretch and 1472 cm^{-1} of CH_2 asymmetric bending and CH_2 rocking at 1718 cm^{-1}.

After bacterial treatment the same polymer film was washed, cleaned, dried and subjected to FT-IR spectroscopy. From the IR spectroscopy it can be stated that the bacterial degradation leads to a substantial increase in the C-H stretch band of the polyethylene at (2940-2915 cm^{-1}). The increase in carbonyl absorption band at 1750 cm^{-1} region was primarily due to the formation of carbonyl bond through oxidation of the polyethylene moieties during the UV heat treatment. The bacterial action may cause a decrease in the carbonyl absorption band (Roy *et al*., 2008).

Plans to employ the selected bacteria in the biodegradation of polyethylene waste often focus on soil compostation processes. It appears that *S1 strain* is a suitable candidate for this purpose, as like other PE degrading bacteria, capable of growing and degrading polyethylene at 30°C. This study indicates the first inference, to the best knowledge of the authors, about marine bacteria in degradation of PE after UV and heat treatment alone without humidity.

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