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RESEARCH ARTICLE

DEGRADATION OF LOW DENSITY POLYTHENE BY *ACHROMOBACTER DENITRIFICANS* STRAIN S1, A NOVEL MARINE ISOLATE

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ABSTRACT

The present article reveals the biodegradation of low density polythene by bacterial strain isolated from marine water near Visakhapatnam. The degradation ability of the bacterial strain was evaluated by performing polythene zone of clearance method. The bacterial strain was identified and confirmed, by 16S rRNA gene sequencing and phylogenetic analysis, as *Achromobacter denitrificans* strain S1 (MTCC 12203). Lipid profiling was done by FAME analysis using GCMS and ESI-MS. Then polythene degradation ability of the strain was evaluated by measuring the dry weight loss in low density polythene (LDPE) films (20 and 40micron thickness). The LDPE degradation was further confirmed by tensile testing, NMR, XRD, thermo gravimetric, carbon analysis, and GCMS analysis.

INTRODUCTION

Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compounds [Alexander and San Diego 1994]. Indeed, biodegradation is the process by which organic substances are broken down into smaller compounds by living microbial organisms [Marinescu *et al*., 2009]. When biodegradation is complete, the process is called "mineralization". However, in most cases the term biodegradation is generally used to describe almost any biologically mediated change in a substrate [Bennet *et al*., 2002]. So, the process of biodegradation requires an understanding of the microorganisms that make the process work. The microbial organisms transform the substance through metabolic or enzymatic processes. It is based on two processes: growth and co-metabolism. In growth, an organic pollutant is used as sole source of carbon and energy. This process results in a complete degradation (mineralization) of organic pollutants. Co-metabolism is defined as the metabolism of an organic compound in the presence of a growth substrate that is used as the primary carbon and energy source [Fritsche and Hofrichter 2008]. Several microorganisms, including fungi, bacteria and yeasts are involved in biodegradation process. Algae and protozoa reports are scanty regarding their involvement in biodegradation [Das and Chandran 2011]. Biodegradation processes vary greatly, but frequently the final product of the degradation is carbon dioxide [Pramila *et al*., 2012]. Organic material can be degraded aerobically, with oxygen, or anaerobically, without oxygen [Fritsche and Hofrichter 2005, Mrozik *et al*., 2003].

Polyethylene (PE) bags have been used for over 5 decades for a variety of storage and packaging applications. One million tons of plastic bag waste was generated per year [Miller 2008]. The primary challenge in disposing these bags after use is, its lack of degradation of the polymer. Thus, plastic bags are one of the largest contributors to the material waste produced in the United States. Only a fraction of the plastic bags are recycled or reused, resulting in a significant contribution to landfills as well as a large deficit of plastic waste. Out of 30.05 million tons of plastic waste generated, only 2.12 million tons was recovered in India [US-Environment Protection Agency (EPA) 2001]. In 2008, about 55 percent of the total MSW (Municipal Solid Waste) was directly transported into landfills [US-EPA 2008]. Additionally, resins and colors added into PE have only made the material more difficult to recycle [Koutny *et al*., 2006]. This is especially the case when plastic manufacturers blend multiple resins.

There are many reports on the degradation of environmental pollutants by different bacteria. Several bacteria are even known to feed exclusively on hydrocarbons [Yakimov *et al*., 2007]. Bacteria with the ability to degrade hydrocarbons are named hydrocarbon-degrading bacteria. Biodegradation of

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hydrocarbons can occur under aerobic and anaerobic conditions, it is the case for the nitrate reducing bacterial strains *Pseudomonas* sp. and *Brevibacillus* sp. isolated from petroleum contaminated soil [Grishchenkov *et al*., 2000]. However, data presented by Wiedemeier *et al*., [1995] suggest that the anaerobic biodegradation may be much more important. Twenty five genera of hydrocarbon degrading bacteria were isolated from marine environment [Floodgate 1984]. Furthermore, among 80 bacterial strains isolated by Kafilzadeh *et al*., [2011] belonged to 10 genera as follows: *Bacillus, Corynebacterium, Staphylococcus, Streptococcus, Shigella, Alcaligenes (Achromobacter), Acinetobacter, Escherichia, Klebsiella* and *Enterobacter*.

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 low density polyethylene from marine water source. The bacterium was identified by molecular approach. The biodegradation rate was evaluated by dry weight loss measurement and various analytical techniques like tensile testing, NMR, XRD, thermo gravimetric, carbon analysis, and GCMS analysis.

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 other chemicals were from E-Merck (Germany).

Screening of Polythene degrading bacterial isolates from Marine water sample

Marine water samples were collected near Visakhapatnam coastal area. Then standard dilution plating technique on Tryptone soy and Zobella Marine agar (Hi media) at 30°C was performed. Then yellow colonies were picked up, purified by re streaking and further confirmation was done by growing them on polythene (PE) Minimal salt medium (PE powder from Sigma chemicals) for observation of zone of clearance around the colonies. Then the isolates were maintained on the same medium for future applications.

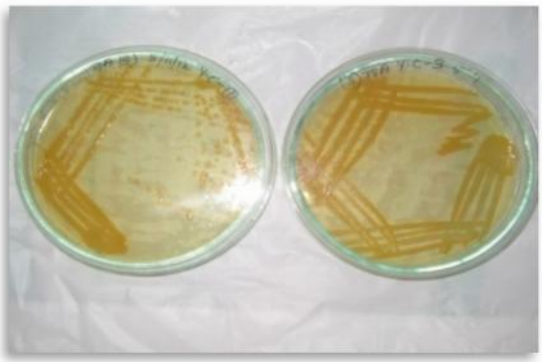

Figure 1 Yellow colonies of strain S1

Biodegradation Testing

Polythene bag films were subjected to UV treatment for 70hrs and then thermal ageing in the oven for 3 days at 70°C. Then PE films 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.

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 films 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 the 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°C at 180rpm shaking condition.

Determination of dry weight of the residual PE

After degradation, PE films 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 overnight at 60°C before weighing.

Molecular Characterisation and Identification of potent strain

DNA extraction from pure culture and PCR amplification

Genomic DNA was extracted from 1ml of bacterial culture; the culture was pelleted by centrifuging at 12,000rpm for 2 min. The pellet was treated with lysis solution and proteinase k and incubated at 60°C for 30min. Nucleic acids were precipitated with isopropanol, followed by the ethanol treatment and centrifuged at 10,000 rpm for 10mins. The pellet was washed with 1 ml of 70% (v/v) ethanol solution and dissolved in 0.1 ml of Tris HCl and EDTA (TE) buffer. The purity and quantity of DNA was examined by UV absorption spectrum and agarose gel electrophoresis. 16S rRNA gene PCR amplification was performed with the help of suitable primers. The following PCR conditions were followed (35 cycles of 3min at 94°C , 1min at 50°C, 2 min at 72°C and 2min at 72°C) and performed in a thermal cycler (Gradient Master cycler, Eppendorff, USA). The amplified products were subsequently subjected to gel electrophoresis (Banglore Genei, India), stained with ethidium bromide and documented by gel documentation system.

Sequencing of 16S rRNA gene and phylogenetic analysis

The purified PCR product was sequenced by dideoxy chain termination method using ABI Prism Big Dye Terminator Cycle Sequencing Ready reaction kit as directed in the manufacturer protocol. Sequence reactions were electrophoresed and analysed by ABI Prism 3100 genetic analyser (Applied Bio systems, USA). The sequences were analysed using the CHECK CHIMERA and the SIMILARITY RANK programs of the Ribosomal Database project [Stephen *et al*., 1990]. The BLAST analysis was carried out (National Centre for Biotechnology information) to determine the closest bacterial sequences; the closest bacterial sequences were aligned using the Clustal W program [Shingler *et al*., 1996].

Phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbour–joining method [Saitou and Nei 1987]. The confirmed sequences were deposited in Gen bank for public access with accession number KT22273.

The nucleotide sequencing was thus, performed by Applied Bio System Automatic Sequencer Inc., USA. Deoxy ribonucleic acid base Sequence Assembler v. 1.0 was used to assemble both the forward and reverse sequence file [Anuraj *et al*., 2012; Shah *et al*., 2013]. The 16S rRNA gene sequences were aligned and sequence similarity was assessed.

Lipid analysis of potential bacterial strain

Electron Spray Ionization ion trap Mass Spectroscopy (ESI-MS)

To the cell paste (after centrifugation), water was added to be 95% water content. One volume of the suspension was homogenized with three volumes of methanol-chloroform (2: 1, v/v) in a Waring blender for two minutes. Following the addition of one volume of chloroform, the mixture was homogenized for 30 seconds, and then rehomogenized for 30 seconds after the addition of one volume of distilled water. The final mixture consisting of homogenate and a two phase solvent was centrifuged. The liquid phase was decanted into a separating funnel and the chloroform phase was collected. The cell residue was homogenized with one volume of chloroform-methanol (2: 1, v/v) and the homogenate was centrifuged again three times. The combined chloroform extracts were evaporated to dryness in a rotary evaporator under 35°C in *vacuo*. The residue, dissolved into a small volume of chloroform, containing the total lipids, was dissolved into a small volume of chloroform and stored at -20°C until ESI-MS analysis was done using Thermo LCQ deca ESI-MS, USA**.**

FAME analysis by GCMS

The lipid samples were analysed on GC MS (HP 5890 Series II Plus system, Hewlett-Packard, Palo Alto, CA) using 60m DB-23 column with I.D. 0.25mm and 0.25µm film thickness. Helium was used as carrier gas with 1mL/min constant flow compensation, split ratio is 20:1, injection temperature of 300ºC and temperature range from 125 to 240ºC at 3º/min. Total run time was 40 min, with acquisition from 8 to 40 min. Gas Chromatography Mass Spectroscopic analysis 10% of samples were measured with GC-MS (Varian CP-3800 GC equipped with a Varian Saturn 2200 MS) to confirm peaks. The mass range is 50-400 m/z, scan time 0.5 sec/scan and emission current of 10uA, Acquisition is from 8 to 40 min.

Confirmation of polyethylene degradation

Polyethylene degradation was confirmed by tensile testing, NMR, thermo gravimetric, XRD, total carbon and GCMS nalysis.

Tensile testing/ Mechanical properties (macroscopic)

Changes in mechanical properties, i.e. tensile strength, elongation at break and modulus, were studied according to ASTM D 882–02, in a Universal Testing Machine (Shimadzu Autograph AG-I Series) (Polish standard). Six samples of each specimen were strained at a rate of 50mm/min and average values of tensile strength, elastic modulus and elongation at break were determined. The length between the jaws at the start of each test was fixed as 40mm. The tests were undertaken in an air conditioned environment at 21ºC and a relative humidity of 65%.

NMR analysis

^{13}C NMR spectra were recorded on a Jeol JNM FX- 100FT-NMR Spectrophotometer operating at 25MHz. For Analysis, films were dissolved in 1,2,4-trichlorobenzene in 10mm tubes at 120°C and a few drops of di methyl sulphoxide (DMSO) were added as an internal lock. Hexamethyl disiloxane (HMDS) was used as chemical shift reference. Spectra were recorded in complete decoupling mode under the following conditions: pulse interval, 1s; pulse delay, 5s; spectral width 500Hz; number of accumulations, 1500-2000; number of data points per spectrum 8000.

Thermo gravimetric analysis (TGA)

Thermo gravimetric analysis of LDPE strips were done for thermal characterization prior to biodegradation and after biodegradation. The thermo gravimetric (TG) analysis of the samples was carried out on a TGA 607 (Leco Analyzer, U.S.A) thermal analyzer under nitrogen atmosphere. Polythene sample weights varied from 5 to 15mg. The thermo grams were recorded for the range from room temperature to 800°C at a heating rate of 20ºC/min. The nitrogen gas flow was 50-70cm^3/min. The maximum degradation temperature, the onset degradation temperature and the percentage residual weight were found out [Satlewal *et al*., 2008]. The weight loss data were recorded as a function of time and temperature using special software in computer.

XRD analysis

The X-ray diffraction patterns of the films were measured with an X-ray diffractometer (D5000, Siemens Diffractometer/ Jeol – JDX 830) which is operated fully automatically using Cu Kα radiation (λ = 1.5418 A°). The scattered radiation was registered in the angular interval (2⊖) from 2°to 40°. A current of 30mA and a voltage of 40 kV were used. All diffraction patterns were examined at room temperature and under constant operating conditions. The solvent cast films were cut into a size of about 15 mm"#"15 mm and the X-ray diffractograms were recorded in the range ca 5–40° with a step size of 0.04, and a time per step of 1 second.

Carbon analysis using C- analyser/ Ultimate Elemental analyser

Total carbon was analysed by placing approximately 0.1g of dried, ground and homogenized sample into a clean, carbon-free combustion boat. Each sample boat was treated with phosphoric acid drop by drop until the sample stops “bubbling” and the sample was completely moist with acid. The sample was placed into an oven set at 40°C for 24 hours and then transferred to an oven set at 105°C. Once the sample was dry,

the boat was placed on the auto sampler rack assembly and loaded onto the LECO carbon analyser.

Reactive intermediates observation by GCMS analysis

After 2 months of incubation period, the bacterial pellet on LDPE strips centrifuged and was removed by filtration, and the filtrates were extracted with distilled ether. The degraded products can be identified by GC–MS analysis of the samples. The plastic films weighing 0.1g were cut into pieces and mixed with 10ml chloroform. The mixture was ultrasonicated for 2 hr at 55°C. The extract was evaporated at room temperature and it was mixed with 2ml of chloroform before filtering using 0.2ml PTFE syringe filter. This filtered sample was analysed in GC coupled with MS using helium as carrier gas. The GC was equipped with HP5 column, HP 5MS or DB 5MS (30 m 9 0.25 mm ID, film thickness 0.25 l) of medium polarity. The oven temperature was programmed for 40°C for 3 min to 280°C at 10°C/min, and then held for 4 min at 280°C. The degraded products of PE were determined by Gas chromatography-mass spectrometer(JEOL GCMATE II GC-MASS SPECTROMETER) using HP5 column, helium gas, was programmed to raise the oven temperature from 70°C to 200°C(maximum temperature-250°C at 15°C/min, Injection liquid 1microliter). Mass spectrometer consists of tungsten filament as electron source which works with 70eV, a double focusing analyser and photo multiplier tube as detector with resolution of maximum 5000. Using Per Fluoro Kerosene (PFK) as standard, mass spectrometer was calibrated [Wen chai *et al*., 2008].

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

RESULTS

Sampling and Screening

From marine water samples (collected from Visakhapatnam coastal area) many yellow colonies were formed by standard dilution plating technique on Zobella Marine agar at 30°C. Fifty yellow colonies (Figure 1 and 2) were selected and checked with Polythene (PE) Zone of clearance test. From the selected fifty yellow colonies, fifteen colonies (M1- M3, M10, B1, B7, B8, A4, A9, A10, S1, S2, S4, S5, T1-T4, T7, and T9)

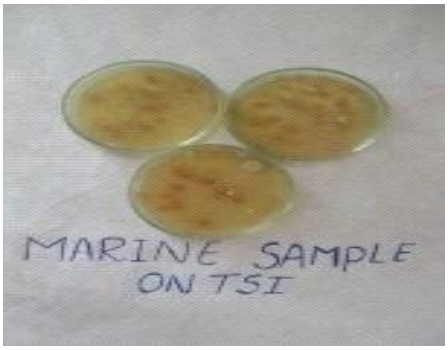

Figure 1 Agar plate with yellow colonies

had shown clear prominent zones (Figure 3) around the wells on PE emulsified agar. The zone of clearance diameters were calculated and recorded (Table 1), which indicates the efficiency of bacterial colonies to degrade LDPE. Among all, S1 strain had shown maximum (4.5cm) zone of clearance on PE emulsified agar.

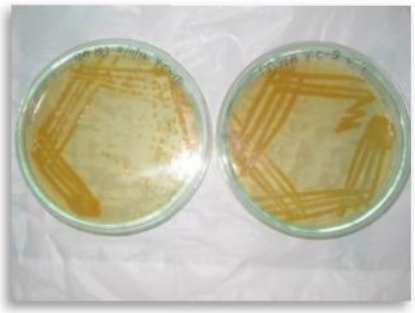

Figure 2 Yellow colonies

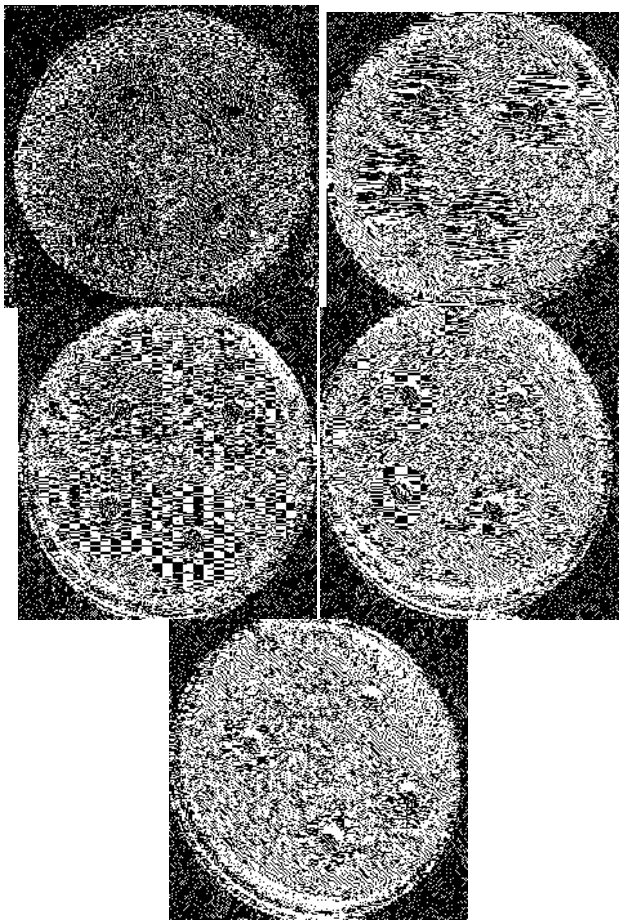

Figure 3 Zone of clearance of PE

Determination of dry weight of the residual PE

S1 strain degraded 40 micron PE to 11% and 20 micron PE to 6% in **fifteen days** of incubation; in **one month** incubation 40 micron PE degraded to 24% and 20 micron PE was degraded to 14% (Table 2). And in **forty five days** of incubation 40 micron PE was degraded to 37% and 20 micron PE was degraded to 20%; in **two months** of incubation 40 micron PE was degraded to 40% and 20 micron PE was degraded to 26% (Table 3). The results showed greater degradation ability of S1 strain towards both types of PE films.

Table 1 Growth on PE and Zone of clearance of isolated bacterial colonies

S.no	Colony name	Well Diameter (cm) (r)	Zone Diameter (cm) (R)	Zone of clearance (cm) (R-r)
1	M1	1	3.8	2.8
2	M2	1	4.3	3.3
3	M3	1	3.1	2.1
4	M4	1	-	-
5	M5	1	-	-
6	M6	1	-	-
7	M7	1	-	-
8	M8	1	1.8	0.8
9	M9	1	-	-
10	M10	1	1.2	0.2
11	B1	1	4.2	3.2
12	B2	1	-	-
13	B3	1	2.1	1.1
14	B4	1	2.0	1.0
15	B5	1	-	-
16	B6	1	2.5	1.5
17	B7	1	3.6	2.6
18	B8	1	-	-
19	B9	1	-	-
20	B10	1	-	-
21	A1	1	1.7	0.7
22	A2	1	1.2	0.2
23	A3	1	3.0	2.0
24	A4	1	3.9	2.9
25	A5	1	-	-
26	A6	1	-	-
27	A7	1	-	-
28	A8	1	1.2	0.2
29	A9	1	3.2	2.2
30	A10	1	1.9	0.9
31	**S1**	**1**	**5.5**	**4.5**
32	S2	1	4.8	3.8
33	S3	1	-	-
34	S4	1	3.1	2.1
35	S5	1	1.9	0.9
36	S6	1	-	-
37	S7	1	-	-
38	S8	1	2.7	1.7
39	S9	1	1.8	0.8
40	S10	1	-	-
41	T1	1	3.9	2.9
42	T2	1	4.5	3.5
43	T3	1	5.0	4.0
44	T4	1	3.0	2.0
45	T5	1	-	-
46	T6	1	-	-
47	T7	1	-	-
48	T8	1	-	-
49	T9	1	3.9	2.9
50	T10	1	2.2	1.2

Molecular Characterisation and Identification of potent strain

16S rRNA gene sequencing of Strain S1

The strain S1 was further confirmed by 16S rRNA gene sequencing. Based on DNA extracts of strain S1, 16S rRNA gene (1404bp) was amplified by PCR using 35 cycles. The bacterial 16S rRNA gene sequences were aligned with Blast search of NCBI databases. The sequence aligned has 100% similarity with *Achromobacter denitrificans;* 99% with *Achromobacter xylosoxidans, A. ruhlandii, A.anxifer, A.dolens, A.inslitus, A.aegrifaciens, A.pulmonis, A.animicus, A.marplantensis, A.spanius, A.spiritinus, A.mucicolens*; 98% similarity with *A.piechaudii, Bordetella hinzii, B.avium, B.parpertusis,* and *B.bronchiseptica* .

Phylogenetic study of Strain S1

For the 16S rRNA gene sequence, seventeen highly homologous sequences were identified by Blastin results and were downloaded and phylogenetic tree was constructed with neighbour joining method (Figure 4). The nucleotide sequences of these seventeen bacterial strains were submitted to the Gene bank with accession numbers AY170848, ADM501000149, EU150134, HE613447, HE613446, HE613448, AY170847, HF586507, CP006958, HE798552, HF586509, HF586508, Y14907, AB010840, KC986352, NR025669, and AY131212. The isolate was identified, as *Achromobacter denitrificans* strain S1 (MTCC 12203) by the molecular analysis with accession number KT22273.

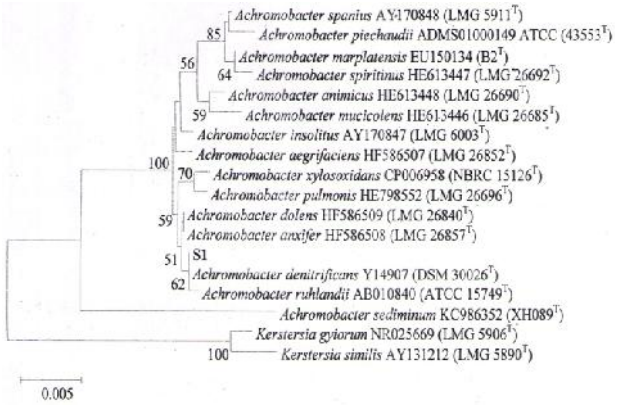

Figure 4 Phylogenetic tree of strain S1

Table 2 Biodegradation ability of S1 strain

		Fifteen days			One month		
	Weight of PE(g)	Dry weight after Degradation (g) *	Weight loss (g)	Percentage of Dry weight loss	Dry weight after Degradation (g) *	Weight loss (g)	Percentage of Dry weight loss
S1 40	0.1	0.089±0.003	0.011	11	0.076±0.001	0.024	24
S1 20	0.1	0.096±0.001	0.004	6	0.086±0.003	0.014	14

Table 3 Biodegradation ability of S1 strain

		Forty five days			Two months of Incubation		
	Weight of PE(g)	Dry weight after Degradation (g) *	Weight loss (g)	Percentage of Dry weight loss	Dry weight after Degradation (g) *	Weight loss (g)	Percentage of Dry weight loss
S1 40	0.1	0.063±0.003	0.037	37	0.06±0.002	0.04	40
S1 20	0.1	0.08±0.002	0.02	20	0.074±0.002	0.026	26

Significant reduction ($P < 0.05$) in dry weight loss was demonstrated by films treated with the bacterium after incubation.

Table 4 FAME analysis of strain S1by GCMS

RT	Response	Ar/Hr	RFact	ECL	Peak Name	Perecent	Comment1	Comment2
0.7361	9.944E+8	0.013	----	6.7130	SOLVENT PEAK	----	<min rt	
1.1371	392	0.010	1.143	9.6079	10.0 iso	0.08	ECL deviates 0.008	Reference -0.016
1.2038	764	0.014	----	10.0693		-----		
1.3036	1025	0.013	1.079	10.6265	11.00 iso	0.19	ECL deviates 0.008	Reference 0.003
1.3623	778	0.010	1.061	10.9544	Sum In Feature 2	0.14	ECL deviates 0.002	Unknown 10.9525
1.5071	3274	0.009	1.030	11.6219	12.0 iso	0.59	ECL deviates 0.001	Reference -0.002
1.5407	521	0.011	----	11.7749		----		
1.5909	6569	0.009	1.015	12.0031	12.0	1.17	ECL deviates 0.003	Reference 0.001
1.6433	758	0.010	----	12.2081		----		
1.7185	626	0.010	----	12.5025	unknown 12.502	----	ECL deviates 0.000	
1.7497	20582	0.008	0.993	12.6244	13.0 iso	3.58	ECL deviates 0.001	Reference 0.000
1.7728	3751	0.009	0.990	12.7149	13.0 anteiso	0.65	ECL deviates 0.001	Reference 0.000
1.8457	778	0.011	0.982	13.0003	13.0	0.13	ECL deviates 0.000	Reference -0.001
1.9033	4669	0.009	0.977	13.2029	12.0 2OH	0.80	ECL deviates -0.001	
1.9827	1184	0.009	0.971	13.4824	12.0 3OH	0.20	ECL deviates -0.001	
2.0237	9659		0.968	13.6270	14:0 iso	1.64	ECL deviates -0.001	Reference -0.001
2.1293	20654	0.009	0.961	13.9991	14:0	3.48	ECL deviates -0.001	Reference -0.001
2.2329	849	0.009	----	14.3417		-----		
2.2851	1129	0.010	----	14.5146	unknown 14.502	-----	ECL deviates -0.001	
2.3198	50056	0.009	0.952	14.6293	15.0 iso	8.36	ECL deviates -0.003	Reference -0.003
2.3476	11919	0.009	0.951	14.7214	15.0 anteiso	1.99	ECL deviates -0.004	Reference -0.004
2.3929	616	0.011	0.950	14.8713	15.0 w6c	0.10	ECL deviates -0.004	
2.4071	742	0.010	0.949	14.9186	15.0 w5c	0.12	ECL deviates -0.007	
2.4315	3285	0.009	----	14.9991	15.0	-----	ECL deviates -0.001	
2.4865	531	0.012	----	15.1752		-----		
2.5598	1413	0.009	0.946	15.4101	16:1 w7c alcochol	0.23	ECL deviates -0.004	
2.5917	21643	0.010	0.946	15.5123	Sum In Feature 2	3.59	ECL deviates -0.003	14:0 30H/16:1 iso1
2.6293	13320	0.009	0.945	15.6325	16:0 iso	2.21	ECL deviates -0.001	Reference -0.002
2.6929	113279	0.010	0.945	15.8364	Sum In Feature 3	18.76	ECL deviates -0.004	16”1 w7c/16:1 w6c
2.7444	127863	0.009	0.944	16.0012	16:0	21.17	ECL deviates 0.001	Reference -0.001
2.7734	3767	0.015	----	16.0935		-----		
2.8236	495	0.010	0.945	16.2534	15:0 2OH	0.08	ECL deviates -0.002	
2.8533	1318	0.012	0.945	16.3479		-----		
2.8733	3496	0.009	0.945	16.4117	17:1 iso w10c	0.58	ECL deviates -0.002	
2.8956	6446	0.011	0.945	16.4829	17:1 iso w5c	1.07	ECL deviates 0.000	
2.9226	1717	0.010	0.945	16.5688	17:1 antciso A	0.28	ECL deviates -0.002	
2.9433	17933	0.010	0.945	16.6347	17:0 iso	2.97	ECL deviates -0.002	Reference -0.006
2.9733	4860	0.009	0.945	16.7304	17:0 ante iso	0.81	ECL deviates -0.003	Reference -0.006
2.9995	5124	0.011	0.946	16.8138	17:1 w8c	0.85	ECL deviates -0.001	
3.0303	12241	0.009	0.946	16.9119	17:0 cyclo	2.03	ECL deviates -0.003	
3.0586	4574	0.009	0.946	17.0020	17:0	0.76	ECL deviates 0.002	Reference -0.002
3.1410	783	0.009	0.948	17.2660	16:0 2OH	0.13	ECL deviates 0.000	
3.2180	739	0.008	----	17.5124		-----		
3.2291	1095	0.009	0.950	17.5481	16:0 3 OH	0.18	ECL deviates 0.000	
3.2459	2449	0.011	0.950	17.6018	18:3 W6C (6,9,12)	0.41	ECL deviates 0.002	
3.3061	77054	0.010	0.951	17.7946	18:1 W9C	13.00	ECL deviates 0.001	
3.3217	23115	0.009	0.952	17.8446	Sum In Feature 8	3.86	ECL deviates -0.003	18:1 w7c
3.3713	19403	0.010	0.953	18.0035	18:0	3.24	ECL deviates 0.003	Reference -0.004
3.4306	1861	0.026	0.953	18.1977	17:0 iso 3OH	-----	>max ar/ht	
3.4741	779	0.016	----	18.3400		-----		
3.4825	461	0.011	----	18.3676		-----		
3.5145	3171	0.011	----	18.4725		-----		
3.5342	4407	0.010	----	18.5370		-----		
3.5739	5105	0.022	----	18.6668		-----		
3.6557	646	0.012	0.962	18.9350	19:0 cyclo w8c	0.11	ECL deviates 0.003	
3.6741	1265	0.010	0.963	18.9954	19:0	0.21	ECL deviates -0.005	
3.7217	1359	0.017	0.965	19.1541	18:1 2OH	0.23	ECL deviates 0.008	
4.1665	1235	0.011	----	20.6267		----	>max rt	

Lipid analysis of potential bacterial strain

Electron Spray Ionization ion trap Mass Spectroscopy (ESI-MS)

The ESI-MS analysis of the aerobe S1 revealed the presence of different unique polar lipids, predominantly alanyl-, lysyl-, and glucosyl-phosphatidylglycerols and cardiolipins (Figure 5). Negative-ion ESI–MS yielded over 200 peaks attributed to the degraded PE films.

The major peaks of m/z (mass-to charge) values of 200–300 are mostly attributable to carboxylate anions. The strain showed major anion peaks with m/z 239 (C15: 1), m/z 253 (C16: 1), m/z 255 (C16: 0), m/z 279 (C18: 2), m/z 281 (C18: 1), m/z 283 (C18: 0), m/z 293 (C19: 2) and m/z 297 (C19: 0). Strain S1 had major anions of m/z 265 (C17: 2) and m/z 269 (C17: 0), The major peaks of m/z values between 600 and 800 are mostly attributed to the expected presence of polar lipid anions. Strain S1 displayed major phospholipid peaks with m/z 714, PE (34: 2), together with its first isotope peak at m/z 715 and m/z 742, PE (36: 2) and its first isotope peak at m/z 743. The other peaks

were putatively assigned the following identities: m/z 758, PE (37: 1), m/z 786, PE (39: 1) and m/z 614, PE (27: 2). However, strain S1 had additional major peaks of m/z 700, PE (33: 2) and m/z 728, PE (35: 2). Strain had an extra major peak with m/z 455, PE (32: 2). Negative-ion ESI–MS of the PE fraction observed from bacterial strain showed that electrospray mass spectra were highly reproducible for repeated continuous cultures.

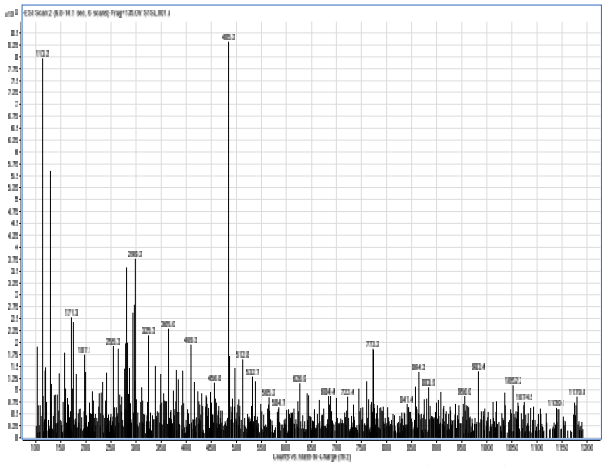

Figure 5 ESI-MS Negative Ionization Spectrum of lipids of S1 strain

Five classes of unidentified polar lipids were detected in the strain. The lipid composition consisted of 22.5% 'of non-conjugated lipids and 77.5% of conjugated lipids. The major lipids were phosphatidylethanolamine (35.5%), phosphatidic acid (20.1%), phosphatidylglycerol (10.9%), and wax (8.0%). Lyso-phosphatidylethanolamine (3.0%), Iyso-phosphatidylglycerol (2.9%), hydrocarbon (2.3%) and coenzyme Q (2.0%) were present, but in small proportion.

FAME analysis of Strain S1

It provides a means for the determination of fatty acids (FA) in tissues and lipids. This is accomplished by solvent extraction driven by high temperature and pressure in an inert atmosphere of nitrogen. The analytes were derivatized into free fatty acids (FA) by a base catalysed reaction and esterified to form fatty acid methyl esters (FAMEs) in an acid catalysed reaction. As shown in the Table 4, S1 strain contains 16”1 w7c/16:1 w6c 21.17 % and 18:76% W9C 13%. Lipid analysis results showed different compositions of fatty acids and other lipids of bacterial strain S1 indicating the Gram negative cell wall nature of this strain.

Confirmation of polyethylene degradation
Tensile testing/ Mechanical properties (macroscopic)

The tensile strength, percentage elongation at break and modulus of elasticity measures the stress at fracture of the specimen and the extension of the material under load respectively. Sixty days incubated PE films with *A.denitrificans* strain S1 had shown the following results of mechanical properties as noted in the Table 5.

- 60% reduction in 40 micron PE tensile strength compared with the untreated 40micron.
- 42.5% reduction in 20 micron PE tensile strength.
- 46.5% reduction in percentage elongation for 40 micron PE.
- 31.5% reduction in percentage elongation for 20 micron PE.
- 52.5% reduction in modulus of elasticity for 40 micron PE.
- 28.8% reduction in modulus of elasticity for 20 micron PE.

Table 5 Mechanical properties of treated PE after two months of incubation with S1 strain

PE material	Tensile Strength (MPa)	% Elongation at break	Modulus (MPa)
Untreated 20 micron	11.8±0.002	299±0.002	128.35±0.002
Untreated 40 micron	17.9±0.002	490±0.002	371.27±0.002
S1 treated 20 micron	6.785±0.002	204.815±0.002	98.3852±0.002
S1 treated 40 micron	7.16±0.002	262.15±0.002	176.353±0.002

NMR Analysis

Micro structural (chemical) changes in the PE were detected when the biodegraded films were characterized by ^{13}C NMR. The NMR spectral assignments of these films were shown in Figures 6a and 6b. The intensities of peaks corresponding to short chain branching (34.7ppm) decreased considerably in treated polythene film.

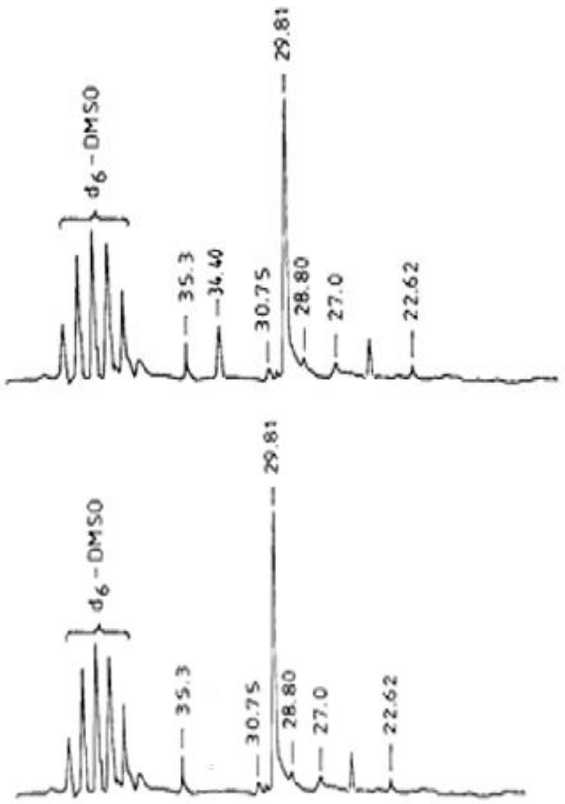

Figure 6a and **b** ^{13}C NMR spectrum of untreated and treated PE

Thermo gravimetric analysis (TGA)

Thermal profile of LDPE films was checked before and after degradation assay to know the influence of implicating bacterial degradation. The thermal profile of undegraded LDPE shows weight loss percent of 99, 98, 98, 87 and 25, one step steep degradation between 450-500°C (Figure 7a), whereas the thremogravimetric analysis of *A. denitrificans* strain S1 degraded 20 micron LDPE films, causing weight loss percent of 99, 76, 54, 34 and 35, respectively and degraded 40 micron LDPE films causing weight loss percent of 99, 51, 39, 21 and 19 respectively between 200-500°C (Figures 7b, and 7c).

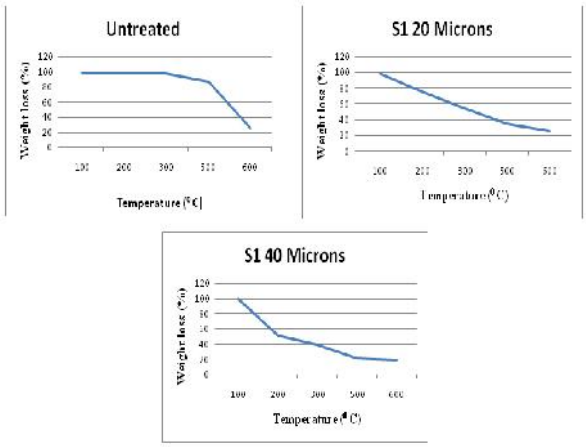

Figure 7 TGA of a) untreated, b) 20 and c) 40microns PE

XRD analysis

The XRD spectrum of the LDPE films was shown in Figure8, b efore and after 60 days of incubation in the presence and absen ce of the selected bacterium. As depicted in this figure, the XRD spectrum shows distinguished peaks at 21.4 and 23.5 of the angular position 2⊖. The intensity of the peaks of untreated films is higher than that of treated one. This difference clearly demonstrated the decrease in the degree of polyethylene crystalinity. The intensity of the peaks was significantly decreased after 60 days of incubation in the presence of the selected bacterium, *A.denitrificans* strain S1.

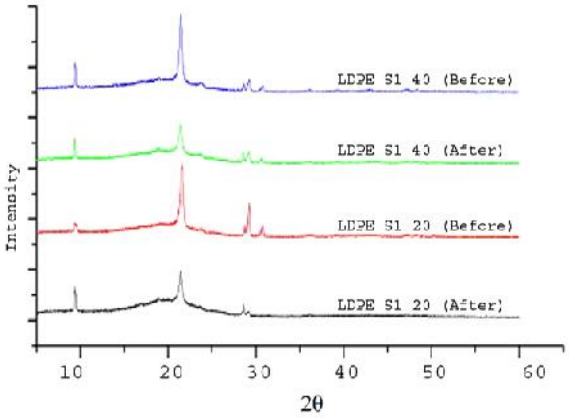

Figure 8 XRD Spectrum of untreated and treated LDPE

Carbon analysis

Total carbon was analysed by LECO CR-412 Carbon Analyser for the treated and untreated polythene samples after thirty and sixty days of incubation. Polythene samples treated with *A.denitrificans* strain S1 were analysed. As shown in the Table 6, carbon percentage was clearly reduced after degradation process. For untreated 20 micron PE, carbon percentage was 93% and for 40 micron it was PE 91.6%: one month treated 20micron PE carbon percentage was reduced to 91.7%; for one month treated 40 micron PE carbon percentage was reduced to 90.1%: after two months incubation of 20 micron PE carbon percentage was further reduced to 86.6% whereas 40 micron carbon percentage was reduced to 65.1% (Figure 9).

Table 6 Total Carbon percentages of degraded PE by incubating with S1 strain

Type of PE	Untreated LDPE C%	S1 Treated LDPE C% 30 days	S1 Treated LDPE C% 60 days
20 micron	93	91.7	86.6
40 micron	91.6	90.1	65.1

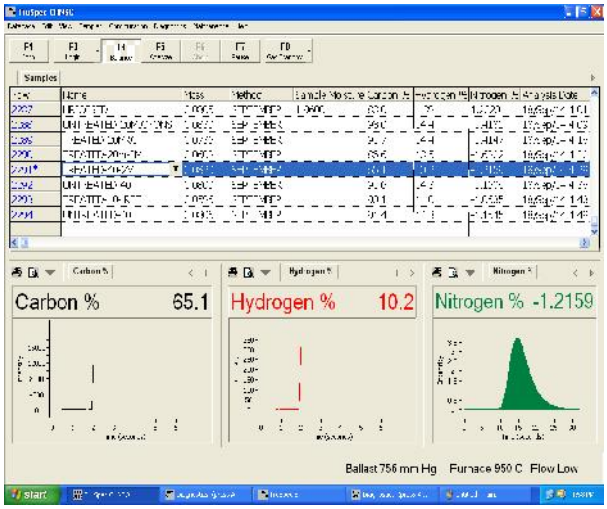

Figure 9 Carbon Analysis of untreated and treated LDPE

Reactive intermediates observation by GCMS analysis

A wide variety of both volatile and semi volatile compounds were eluted by gas chromatography. Analysis using GC-MS was performed for samples incubated with *A. denitrificans* strain S1. The degradation products after exposure to S1 strain include long chain fatty acids, esters, hydrocarbons, oxygenated chemical compounds predominantly containing aldehydes, ketones, esters and ether groups, unsaturated fatty acids and certain unknown compounds.

Most of the chemical compounds were eluted and detected in initial stage of the experiments (i.e. minutes). The intermediate products included were alkanes (such as Octadecane, Tetracosane, Pentacosane, Octane, 1-Tetradecene, 1-Eicosanol and Hexacosane), fatty acids (such as Hexadecanoic acid and Octanoic acid), ester group of alcohol and other compounds. Compounds of low molecular weight such as benzene, trichloroethylene were eluted first whereas compounds having high molecular weight unsaturated fatty acids were eluted later (Table 7). The dissociation rate of S1 treated PE was found to be higher than the untreated PE. In twenty micron treated LDPE some compounds like Octane, 1-Eicoanol, and 1-Tetradecene have not been eluted indicating more degradation of forty micron LDPE films with *A. denitrificans* strain S1 (Figures 10 and 11).

In the present study, the degraded products in the culture supernatant extracted with distilled ether were determined by GC-MS analysis. Thus compounds like Octadecadienoic acid, Octadecatrienoic acid, Benzene dicarboxylic acid, Cyclo propanebutanoic acid were also found to be produced by the PE degrading cultures (Table 7).

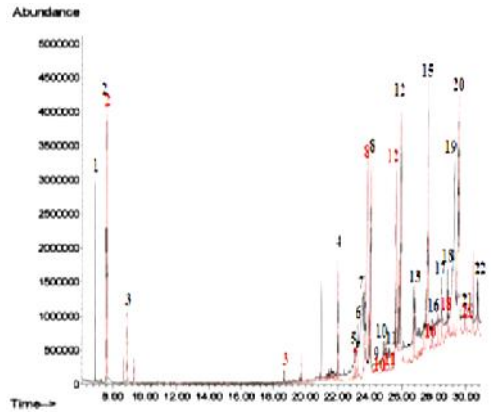

Figure 10 GC–MS chromatogram showing the degradative products after incubation with *A. denitrificans* strain S1 with 20 micron LDPE compared to untreated LDPE

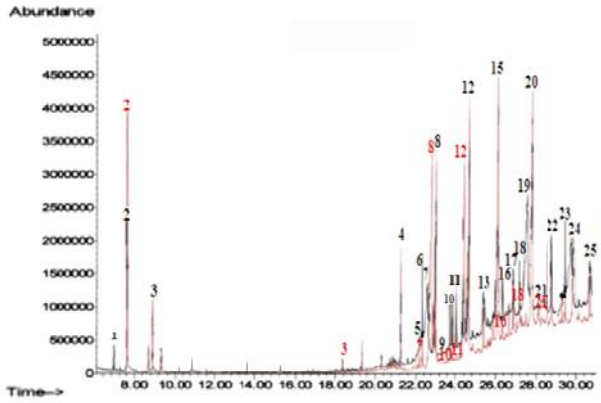

Figure 11 GC–MS chromatogram showing the degradative products after incubation with *A. denitrificans* strain S1 with 40 micron LDPE compared to the untreated LDPE

Table 7 Total compounds eluted for degraded PE

Peak	Compound Detected	Peak	Compound Detected
1	Benzene, methyl-	12	Docosane
2	Tetrachloroethylene	13	3-Chloropropionic acid,
	heptadecyl ester		
3	Benzene, 1,3-dimethyl	14	Tricosane
4	Octadecane	15	Octadecanoic acid, butyl
5 7, 9-	Di-tert-butyl-1-oxaspiro(4,5)	16	ester
	deca-6,9-diene-2,8-dione	17	1-Nonadecene
6	Unknown	18	Tetracosane
7	Hexadecanoic acid	19	Pentacosane
8	Hexadecanoic acid, ethyl ester	20	1,2-Benxenedicarboxylic acid,
	diisoostyl	21	
9	Eicosane	22	Octadecatrienoic acid
10	Octadenoic acid	24	Hexacosane
11	Undecane		Cyclo propane
23	Octane		butanoic acid
25	1 Eicosanol		1-Tetradecene

DISCUSSION

From marine water samples (collected from Visakhapatnam coastal area) PE degrading bacteria were screened, fifteen colonies (M1- M3, M10, B1, B7, B8, A4, A9, A10, S1, S2, S4, S5, T1-T4, T7, and T9) had shown clear prominent zones around the wells on PE emulsified agar.S1 strain had shown maximum (4.5cm) zone of clearance on PE emulsified agar. The result is in agreement with the previous studies, where *Pseudomonas chlororaphis* was grown in broth medium containing polyurethane for 4 days. Polyurethane zone of hydrolysis around the colonies was represented by Howard *et al*., (2001). Isolation of Poly lactic acid degrading thermophiles from the compost sample was reported. The isolates forming clear zone around the colonies were isolated by Prema and Uma Maheswari Devi (2012). The Poly Chloro Lactic acid-degradation ability of the isolates was detected by the presence of a clear zone around the colonies on agar plates containing emulsified PCL [Te-Kuan 2013].

The bacterial isolate was identified, as *Achromobacter denitrificans* strain S1 (MTCC 12203) by molecular analysis. Belhaj *et al.*, [2002] isolated and identified, by using same analysis, *P. aeruginosa* from the soil which had been contaminated with hydrocarbons. Kloos *et al.*, [2006] performed the PCR for the total DNA extraction and sequencing for bacteria from the soil sample at Bavaria, Germany. High density polyethylene (HDPE) film 0.1µm thickness degradation was done using soil burial method for 3 months with isolated microbes like *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus oryzae* and identified by molecular level (16S rDNA sequencing) [Konduri *et al*., 2010]. Molecular level (16S rRNA gene sequencing) was used for identification of *Bacillus cereus*, *B. megaterium*, *B. subtilis* and *Brevibacillus borstelensis* which were used in biodegradation of photo-degraded mulching films based on polyethylenes and stearates of calcium and iron as pro-oxidant additives [Abrusci *et al*., 2011].

Mass spectrometric studies indicated the lipid profile of the S1 strain using Mass Spectroscopy. Kunimoto *et al*., [1975] reported the determination of phospholipids of two *Achromobacter* sp. isolated from the Atlantic salmon. Oliver and Colwell [1973] examined systematically the phospholipid composition of 16 strains, isolated from marine origins, consisting of genus *Vibrio*, *Agrobacterium*, *Achromobacter*, *Spirillum*, *Pseudomonus*, *Photobacterium*, *Arthrobacter* and unidentified genera. Phosphatidyl ethanolamine is the most abundant component attaining 35.5% of the total lipids. Oliver and Colwell [1973] had already shown a similar result for the amount of phosphatidyl ethanolamine of *Achromobacter aquamarinus*.

Previous studies on the degradation capacity of bacterial and fungal consortium under natural conditions with 12.5% weight loss in 2 months was reported [Kathiresan 2003] which was lower than the present work. Scientists from the Central Salt and Marine Chemicals Research Institute, Bhavnagar, have discovered three species of marine bacteria which have demonstrated their ability to degrade polyethylene [Harshvardhan and Jha 2013]. Three out of the sixty marine bacterial species – *Kocuria palustris*, *Bacillus pumilus* and *Bacillus subtilis* demonstrated their ability to help to degrade polyethylene. The loss in dry weight of polyethylene films used was found to be 1%, 1.5% and 1.75% after 30 days of incubation with the *K.palustris*, *B.pumilus* and *B.subtilis* isolates respectively, indicating although *B.subtilis* to be the most efficient among the three bacteria, degradation ability was very low in comparison to the *Achromobacter denitrificans* strain S1.

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

isolated. After 30 days of incubation, degradation was nearly12% with *Arthrobacter* sp. and 15% with *Pseudomonas* sp. [Balasubramanian *et al*., 2010], whereas *A. denitrificans* strain S1 has shown 24% degradation after 30 days of incubation.

The drop in elongation and other mechanical properties of the PE films become more drastic as the period of incubation with S1 strain is increased. Similar reduction in the percentage elongation of the LDPE film after thermal oxidation was reported by Jakubowicz *et al*., [2011]. S everal researchers [Greco and Maffezzoli 2008; Breuer *et al*., 1997; Joseph and Thomas 2003] also reported similar trends on mechanical properties of PE strips and other polymers. In addition, Orhan and Buyukungor [2000], Jakubowicz *et al*., [2011] and Nowak *et al*., [2011] reported a reduction in the percentage elongation of polyethylene films after the biodegradation process.

The intensities of peaks by NMR analysis corresponding to short chain branching (34.7ppm) decreased considerably in treated polythene film. Cheng [1986] reported the similar findings of chemical changes in biodegraded PE.

The thremogravimetric profile of *A. denitrificans* strain S1 treated LDPE films are in line with research conducted by Satlewal *et al.*, [2008] according to the documented thermal profile, the implication of consortium has shown more than one step degradative mechanism with respect to control i.e. undegraded LDPE and HDPE. With respect to this, in the present study the degradation profile had shown occurrence of similar deformities in the polymer structure, resulting in degradation.

The crystalinity of PE films decreased significantly as indicated by XRD analysis findings, which are in agreement with the previous reports of crystalinity. The crystal sizes of treated PE films decreased during the process with the selected bacteria [Rehim *et al*., 2004]. Thermally treated low density polyethylene biodegradation by using *Penicillium pinophilum* and *Aspergillus niger* after 31 months was studied. Maximum (5%) reduction in crystallinity by *Aspergillus niger*, 11.07% reduction in crystalline thickness by *Pencillium pinophilum*, were reported using X-ray diffraction (XRD) [Volke *et al*., 2002].

Less percentages of total carbon of treated PE films were in accordance with previous results observed in degradation of Poly Lactic Acid film with *Geobacillus thermocatenulatus* [Tomita *et al*., 2003] and *Amycolatopsis mediterrani* ATCC27643 [Tokiwa and Calabia 2006].

In the present study, the degraded products in the culture supernatant extracted with distilled ether were determined by GC-MS analysis. This was in line with the previous reports by Andersson *et al*., [2002]. A large number of different aldehydes, ketones and carboxylic acids were identified in smoke generated on film extrusion of LDPE in an extrusion coating process.

From the present study it can be concluded that bacterial isolate was able to grow on minimal medium with LDPE as a sole carbon source. Hydrophobic nature of LDPE film acts as substratum for bacterium which colonizes the surface of the LDPE films. Confirmatory tests indicate positive degradability for the polyethylene and these tests have given fulfillment to the objective of this study.

Plans to employ selected bacterial strain in the biodegradation of polyethylene waste often focus on compostation processes. It appears that *Achromobacter denitrificans* strain S1 is most suitable candidate for this purpose, as *A.denitrificans* strain S1 is mesophilic, capable of growing and degrading polyethylene at 30°C.

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