Recent Innovations in Chemical Engineering, 2020, *13*, 29-40

RESEARCH ARTICLE

Ecofriendly Degradation of Polyethylene Plastics Using Oil Degrading Microbes

Liny Padmanabhan[1,*], Shreya Varghese[2], Raj Kumar Patil[2], H.M. Rajath[2], R.K. Krishnasree[2] and M. Ismail Shareef[1]

[1]*Acharya Institute of Technology, Soladevanahalli, Bangalore, India;* [2]*Shridevi Institute of Engineering & Technology, Tumkur, India*

ARTICLE HISTORY

Received: September 26, 2018
Revised: June 24, 2019
Accepted: July 13, 2019

DOI: 10.2174/2405520412666190725114137

Abstract: ***Background & Objective*:** Plastics are strong, light weight and durable due to which it has wide applications. Degradation of plastics is difficult due to their xenobiotic origin and recalcitrant nature. Hence, accumulation of plastics in the environment is posing an increasing ecological threat.

***Methods*:** Various methods are preferred for the reduction of plastics in the environment, of which degradation by chemical and biological means are considered to be more effective. In the biodegradation of plastics, micro organisms play a pivotal role. In the present work, microbial species are isolated from different sources such as cooking oil, grease and petroleum products. Two bacterial species such as *Sphingomonas, Pseudomonas aeruginosa* and three fungal species such as *Aspergillus niger, Aspergillus flavus* and one unidentified fungal species were isolated from the sources were used for the degradation of polyethylene plastic samples (black and white).

***Results*:** *Sphingomonas* indicated 56% (black) and 31% (white) degradation of polyethylene plastic. Unidentified fungal species also indicated 64% (black) and 45% (white) degradation of polyethylene plastic. During the degradation, pH altered from 7 to 8. SEM analysis indicated the presence of appreciable surface erosions, fading, cracks and extensive roughening of the surface with pit formation.

***Conclusion*:** Sequence analysis of *Sphinogomonas* species was done in comparison with the similar known bacterial species and the phylogenetic tree was generated based on the sequence analysis.

Keywords: Biodegradation, polyethylene, *Aspergillus niger*, *Aspergillus flavus*, *Pseudomonas aeruginus*, SEM analysis, sequence.

1. INTRODUCTION

Plastics are synthetic long chain polymeric units [1] and are used as a substitute for the natural material. The stability and the durability of the plastics have been continuously increased with time and are known to be resistant to microbial degradation [2]. Every year the demand of the plastic is increasing at a high rate of 12% due to their improved physical and chemical properties such as strength, lightness, resistance to water and most waterborne microorganisms over the other packaging materials such as paper and other cellulose-based products.

*Address correspondence to this author at the Acharya Institute of Technology, Soladevanahalli, Bangalore, India; Tel: 9901890513; E-mail: liny@acharya.ac.in

2405-5212/20 $65.00+.00

Plastic production by synthetic method is one of the fastest growing fields of global industry. The plastic waste will be dangerous for the environment. Abiotic degradation of conventional plastic caused by UV radiation, oxygen, temperature, and physical stress degrade large plastic slowly. Biodegradation leads to the changes in the structure of the chemicals in the environment [3, 4].

Bacteria and fungi are considered for the degradation of natural and synthetic plastics [5]. Degradation of plastics depends on the soil conditions, type of microbes and pretreatment processes and also the polymer properties such as molecular weight, functional groups, crystalline, orientation, morphological properties, mobility and plasticizers or additives due to which biodegradation of plastic varies and the microbes have their own optimal growth conditions in the soil [6].

Plastics form the potential substrates for heterotrophic microorganisms [7-9]. During the process of degradation, the polymers are broken into monomers and they are biodegraded within the microbial cells. The oxidation or hydrolysis by enzyme to create functional groups that improve the hydrophylicity of a polymer is the primary mechanism for the degradation of high molecular weight polymer.

As the micro organisms are able to degrade organic and inorganic material, there is an increase interest to study the ability of microbes to degrade plastics. Thus the area of research for biodegradation of plastics by microbes should further be explored to gain new insight in the mechanism of action of microbial degradation of plastics. Role of chemical and physical properties of plastics has an important role in the degradation process. Factors involved in the degradation of polymers include its morphology, melting temperature, degree of crystallinity and molecular weight. Also if the polymers possess side chains, they are difficult to degrade when compared to polymers that do not possess side chains [10].

In the present work, different sources such as cooking oil, grease and petroleum products were used for the isolation of plastic degrading microbes. These plastic degrading microbes were identified and the biodegradation ability of these organisms was studied.

2. MATERIALS AND METHODS

2.1. Sample Collection

Two polyethylene plastic samples (black and white) were collected from the dumped soil Tumakuru, Karnataka, India.

2.2. Chemical-alkali Treatment of Polyethylene

Plastic samples were cut into smaller units of equal sizes and transferred into a fresh solution containing 18ml Teen, 10ml bleach and 225ml of distilled water and the contents were stirred for 30-60min. Bleach consists of 5g of sodium chloride (NaCl), 5g of sodium hydroxide (NaOH) and 10ml of glacial acetic acid (CH_3COOH). The strips were transferred into the beaker containing distilled water and stirred for two hours. They are aseptically transferred into 70% v/v ethanol solution for 30min. The polyethylene strips were transferred to petri plate and were inoculated at 45°-50°C overnight.

2.3. Isolation of Microbes for Degradation

2.3.1. Collection of Sample

Microbial samples were collected from different sources such as cooking oil, grease and petroleum products for isolation of microbes.

2.3.2. Serial Dilution

1.0g of each samples were added to 9ml of sterile water to make 1:10 dilution, adding 1ml of the 1:10 dilution with 9ml of sterile water makes 1:100 dilution and so on. After serial dilution, the sample were inoculated in the special media consisting of (K_2HPO_4-1.0g, KH_2PO_4-0.2g, NaCl-1.0g, $CaCl_2$. $2H_2O$-0.002g, $NH_4(SO_4)_2$-1.0g, $MgSO_4.7H_2O$-0.5g, $CuSO_4.5H_2O$- 0.001g, $ZnSO_4.H_2O$- 0.001g, $MnSO_4$. H_2O-0.001g, $Fe_2(SO_4)_3.6H_2O$-0.001g). Microbes were allowed to grow for 70 days.

2.3.3. Selection of Microorganisms

The microbes grown in the special media were selected based on the multiplication and subcultured in five different Petri dishes. The selected microbes consist of two fungal species and three bacterial species.

2.3.4. Total Heterotrophic Count

The plates with the selected microbes with 30-400 colonies were counted and the total plate count

was expressed as the number of colony forming units per gram (CFU/g) of soil.

C.F.U./g = Number of colonies/ inoculum size (ml) X dilution factor

The selected microbes were identified according to their morphological, cultural and biochemical characteristics by following Bergey's Manual of Systematic Bacteriology [11]. All the isolates were subjected to Gram staining and specific biochemical tests**.**

2.4. Characterization of Microbes

Morphologically different colonies were selected and aseptically transferred into special media slants for further characterization. The selected microbes were chosen for the characterization and identified by Gram staining method and colony morphology based on microscopic observations.

2.4.1. Biochemical Tests

Biochemical identification of the isolated strains were done by using a Biochemical identification kit (Hibacillus identification kit, Hi-Media) and standard manual biochemical methods. Catalase, Nitrate reduction and Citrate utilization test, Gas production from glucose and starch hydrolysis were checked.

2.5. Microbial Degradation of Plastics in Laboratory Conditions

2.5.1. Determination of Weight Loss

The plastic samples cut into smaller units were aseptically transferred into the conical flask containing 50ml of culture broth media which were inoculated with different microbial species. In the control, plastic samples were added in the microbial free medium. All the conical flasks were then subjected to shaking. At the end of one month, plastic samples were drawn from the flask containing media, it was then washed thoroughly using distilled water, dried and then final weight was checked

From the data collected, weight loss of the plastics was calculated by the formulae:

$$Percentage\ degradation = \frac{(Initial\ weight - Final\ weight)}{Initial\ weight} X100$$

2.6. pH Analysis

pH of the media was checked initially and after 35 days of the degradation change in the pH was noted.

2.7. Scanning Electron Microscope (SEM) Analysis

Degraded plastic strips were subjected to a Scanning Electron Microscope (SEM) analysis to determine the surface degradation of plastics. The microbes play a significant role in biological decomposition of materials, including synthetic polymers in natural environments.

2.8. Bacterial Identification- 16 S rRNA Sequencing

2.8.1. DNA Extraction

Using InstaGeneTM Matrix Genomic DNA isolation kit Bacterial Genomic DNA was isolated. An isolated bacterial colony was picked and suspend in 1ml of sterile water in a microfuge tube. It was centrifuged for one minute at a range of 10000 to 12000 RPM to remove the supernatant, to which 200 µl of the Insta Gene matrix was added to the pellet and incubated at 56°C for 15 minutes. It was subjected to vortexing at high speed for 10s and the tube was placed in a 100°C in heat block or boiling water bath for 8min. The contents were subjected to vortexing at high speed for 10 seconds and spin at 10,000-12,000 RPM for 2 minutes. Finally, from the supernatant, 20µl of the supernatant was used per 50 µl PCR reaction.

2.8.2. PCR Protocol

Using 16S rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler.

2.8.3. Primer Details

1µl of template DNA was added in 20 µl of PCR solution. 27F/1492R primers used for bacteria, and then the PCR reaction performed with below conditions: initial denaturation at 94^{o}C for 2 min followed by thirty five amplification cycles at 94^{o}C for 45s, 55^{o}C for 60s, and 72^{o}C for 60 s and the final extension at 72^{o}C for 10 min. In case of bacteria, DNA fragments get amplified by about 1,400bp. Also positive control (*E. coli* genomic DNA) and a negative control are included in the PCR study.

2.8.4. Purification of PCR Products

Using Montage PCR clean up kit (Millipore), unincorporated PCR primers and dNTP's were removed from PCR products. By using 518F/800R primers, PCR product was sequenced. ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems) was used for sequencing reactions.

2.8.5. Sequencing Protocol

16s rRNA universal primers were used for Single-pass sequencing on each template. The ethanol precipitation protocol was considered for purification of fluorescent-labeled fragments from the unincorporated terminators. It was resuspended in distilled water, which was then subjected to electrophoresis using ABI 3730xl sequencer (Applied Biosystems). Sequence data of primers were aligned and analyzed for identifying the sample.

2.8.6. Bioinformatics Protocol

The 16s rRNA sequence was a blast using NCBI blast similarity search tool. The phylogeny analysis of the sample sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

The program MUSCLE 3.7 was used for multiple alignments of sequences [12]. The resulting aligned sequences were cured using the program Gblocks 0.91b, which eliminates poorly aligned positions and divergent regions (removes alignment noise). Finally, for making phylogeny analysis, PhyML 3.0 aLRT program was used and HKY85 as substitution model [13].

3. RESULTS AND DISCUSSION

3.1. Selection of Microbes

The sample was collected from different sources such as cooking oil, grease and petroleum products. Fig. (**1**). indicates the growth of microbes on the culture media. The microbes which were selected for the plastic degradation based on their plastic degrading ability were given below in Table **1**. Four types of fungal and bacterial species were identified individually for the study.

3.2. Identification of Microbes

Fungal and bacterial species were selected based on their morphological studies and biochemical tests (Table **2**).

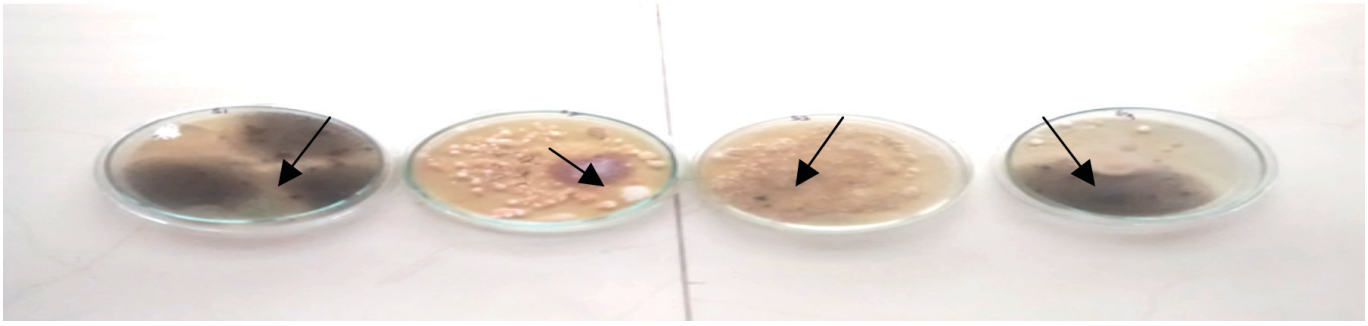


Fig. (1). Selection of microbes from various sources. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 1. Growth of four fungal and bacterial species on the media containing cooking oil, grease and petroleum products.

Samples	Fungal Species				Bacterial Species			
	F1	F2	F3	F4	B1	B2	B3	B4
Cooking oil	+	-	-	-	-	-	-	-
Grease products	-	-	+	-	-	+	-	-
Petroleum products	-	-	-	-	-	-	+	+

Table 2. Identified the bacterial species based on biochemical tests and morphological studies.

Bacterial Species	Gram Staining	Tests						
		Catalase	Nitrate Reduction	Citrate Utilization	Gas Production	Starch Hydrolysis	Shape	Color
Sphingomonas	+	+	+	-	+	-	Cocci	Cream/yellow
Pseudomonas aeruginosa	-	+	+	+	_	_	Rods	Blue

Fig. (2). Biodegraded plastic samples after 35days of incubation.

Fungal species were also identified based on colony and morphological studies, such as *Aspergillus niger, Aspergillus flavus* which are in rod and conidia forms. Along with that unknown fungal species were also found.

These microbial species were selected for the degradation of plastic samples, the plastic samples were inoculated into the conical flask containing the culture media and was subjected to incubation for one month under the laboratory conditions.

After 35 days of incubation, plastic strips inoculated with three different fungal species and two different bacterial species in shaker incubator were found to be degraded. The analysis was based on weight loss and SEM analysis (Fig. **2**).

3.2.1. Determination of Weight Loss

Initially weighed plastic strips are incubated with microbial species. After 35 days of incubation in shaker incubator the plastic strips were collected and final weight is calculated along with the percentage of degradation. Weight loss was checked in duplicates (Table **3**).

Sphingomonas species indicated 56% degradation with respect to black colored polyethylene plastic and 31% respect to white colored polyethylene plastic samples compared to that of *Pseudomonas aeruginosus* which indicated 42% and 24%. The study also indicated that the unidentified fungal species also gave a better result of degradation by 64% and 45%.

3.2.2. pH Analysis

pH of the media containing plastic and microbes are checked initially and after 35 days of incubation in shaker incubator.

The initial pH of the microbial species was 7.0 and the final pH was 8.0 for *Sphingomonas,* 7.5 for *Psuedomonas aeruginosa,* 8.0 for *Aspergillus niger* and *Aspergillus flavus,* pH of 8.5 for unknown fungi after 35 days. Change in the pH indicated the degradation of plastic. The pH of the media increased due to the course of incubation, this may be due to the release of the enzymes and increase of carbon dioxide during the course of the degradation process.

Table 3. Weight loss of degraded plastic strips.

Microbial Species	Final Weight of Plastic		Initial Weight of Plastic		% Degradation of Plastic	
	Black	White	Black	White	Black	White
Sphingomonas species	0.016	0.032	0.025	0.042	56%	31%
Pseudomonas aeruginosa	0.019	0.029	0.027	0.036	42%	24%
Aspergilus niger	0.013	0.023	0.018	0.029	38%	26%
Aspergillus flavus	0.011	0.018	0.014	0.021	27%	16%
Unknown	0.014	0.020	0.023	0.029	64%	45%

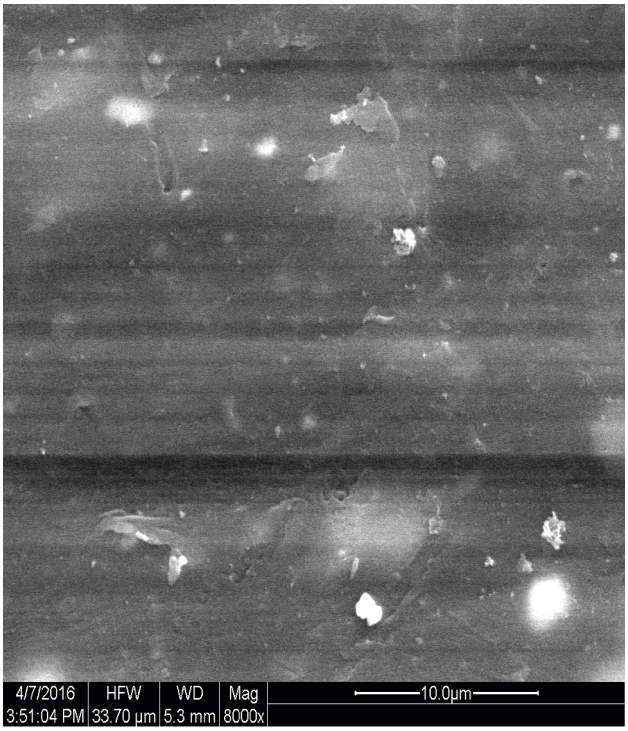


Fig. (3). SEM analysis showing non degraded Black plastic incubated as control.

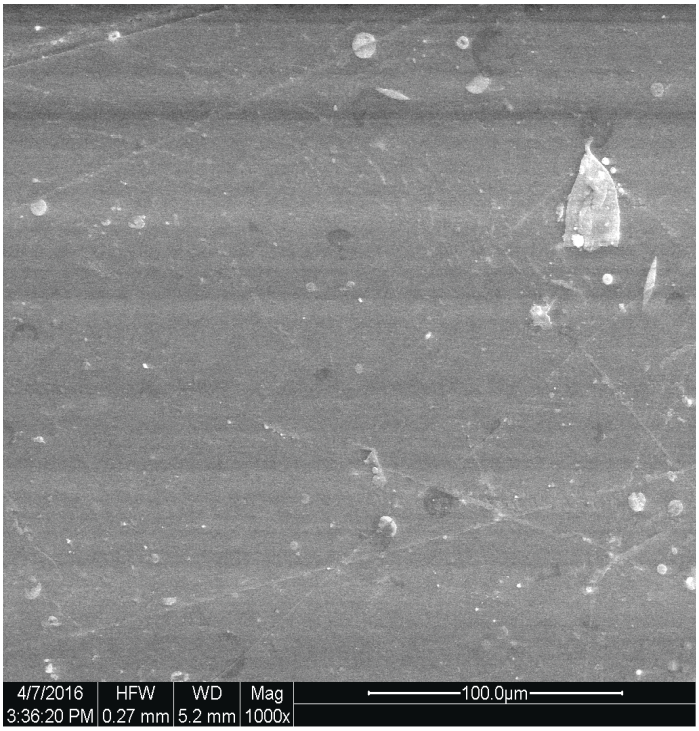


Fig. (4). SEM analysis showing non degraded white plastic incubated as control.

3.2.3. SEM Analysis

The polyethylene plastic sample both control and degraded plastics were subjected to SEM analysis to determine the surface degradation of plastics.

SEM analysis was carried out at different magnifications; SEM study reveals that the polyethylene plastic treated with microbial species showed appreciable surface erosions, folding and cracks. Extensive roughening of the surface with pit formation was observed (Figs. **3-8**). But the control which is untreated polyethylene plastics did not indicate such changes, it displayed a normal surface topography the surface degraded SEM analysis result is given in the below.

Surface degradation of polyethylene plastic also positively indicated that the degradation was more pronounced with respect to Sphingomonas species and unknown fungi.

3.3. Sequence Analysis

Sequence analysis of bacteria which gave the highest degradation rate and are compared with other

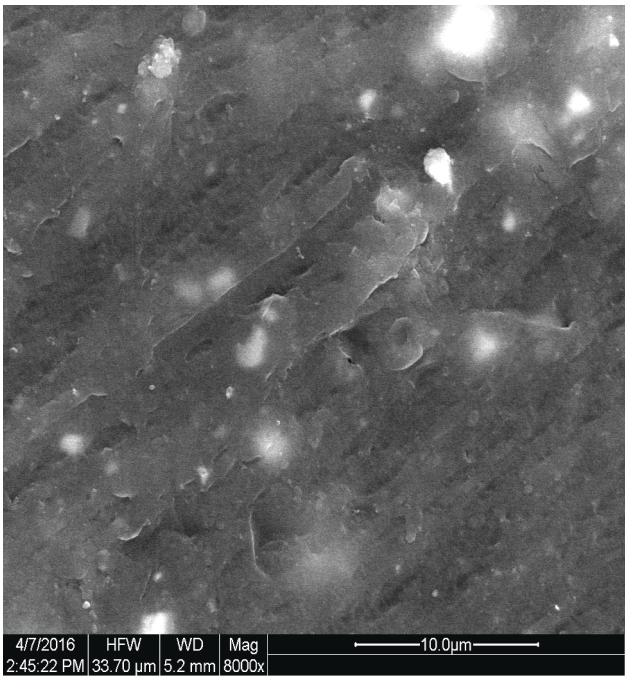


Fig. (5). SEM analysis showing surface degradation of black plastic incubated with bacteria *Sphingomonas* species.

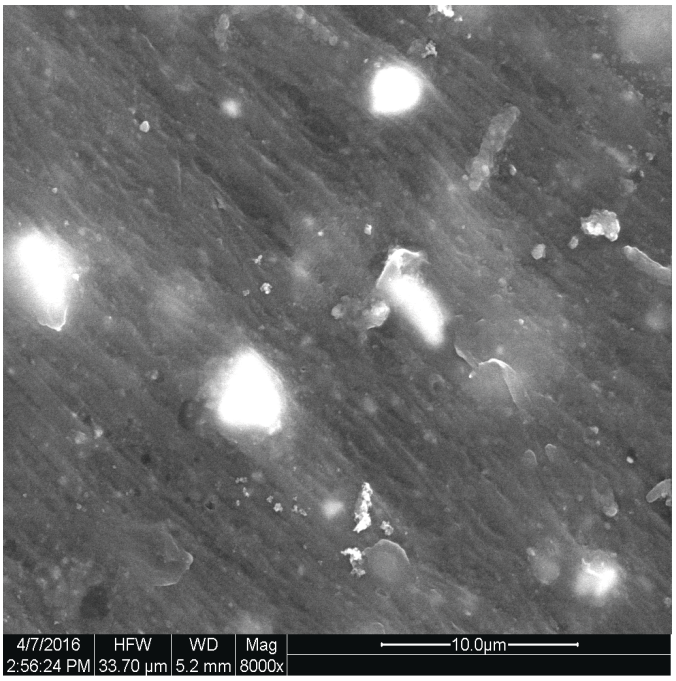


Fig. (6). SEM analysis showing surface degradation of black plastic incubated with unknown fungi.

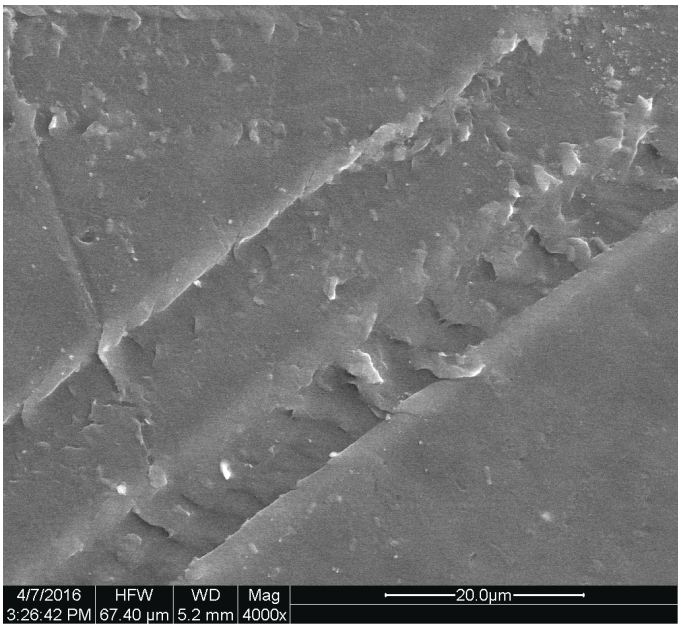
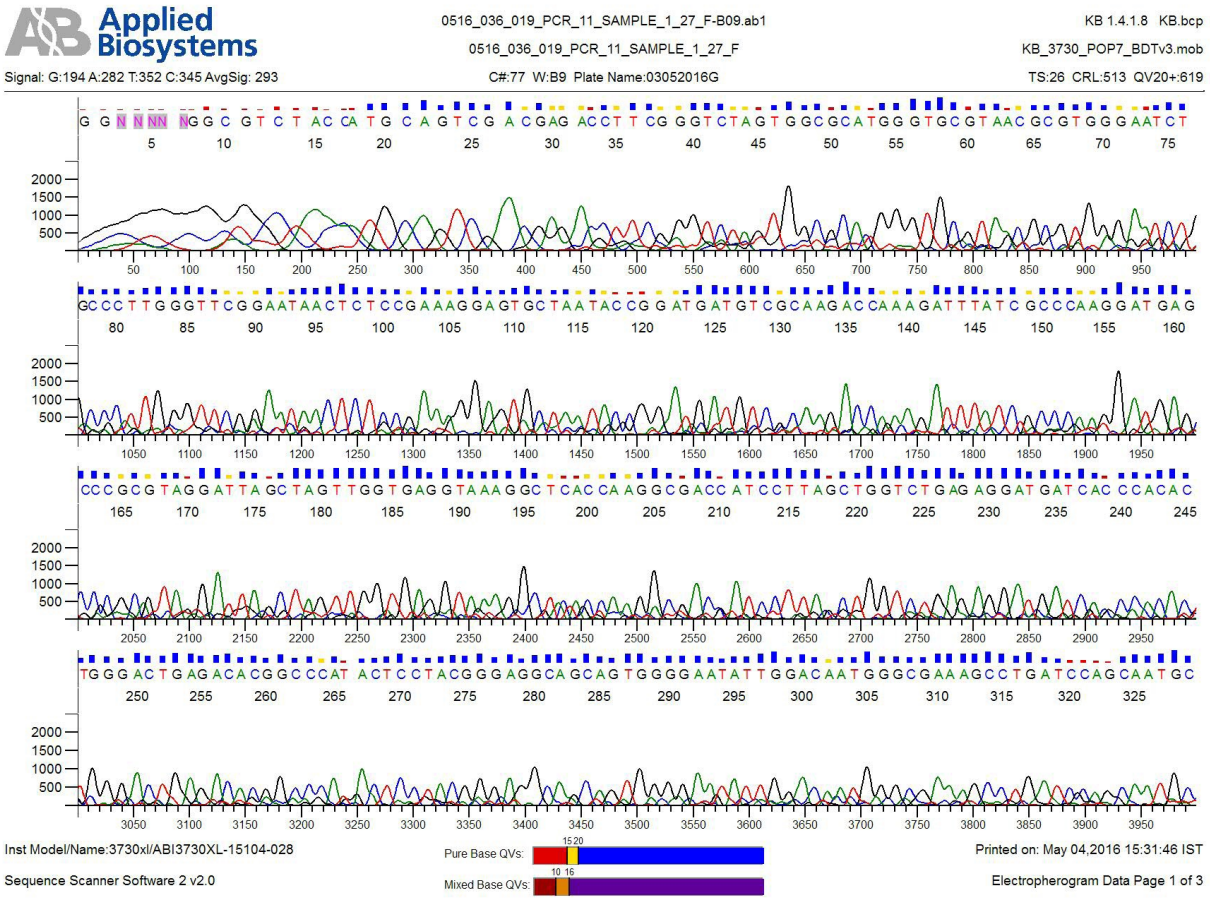


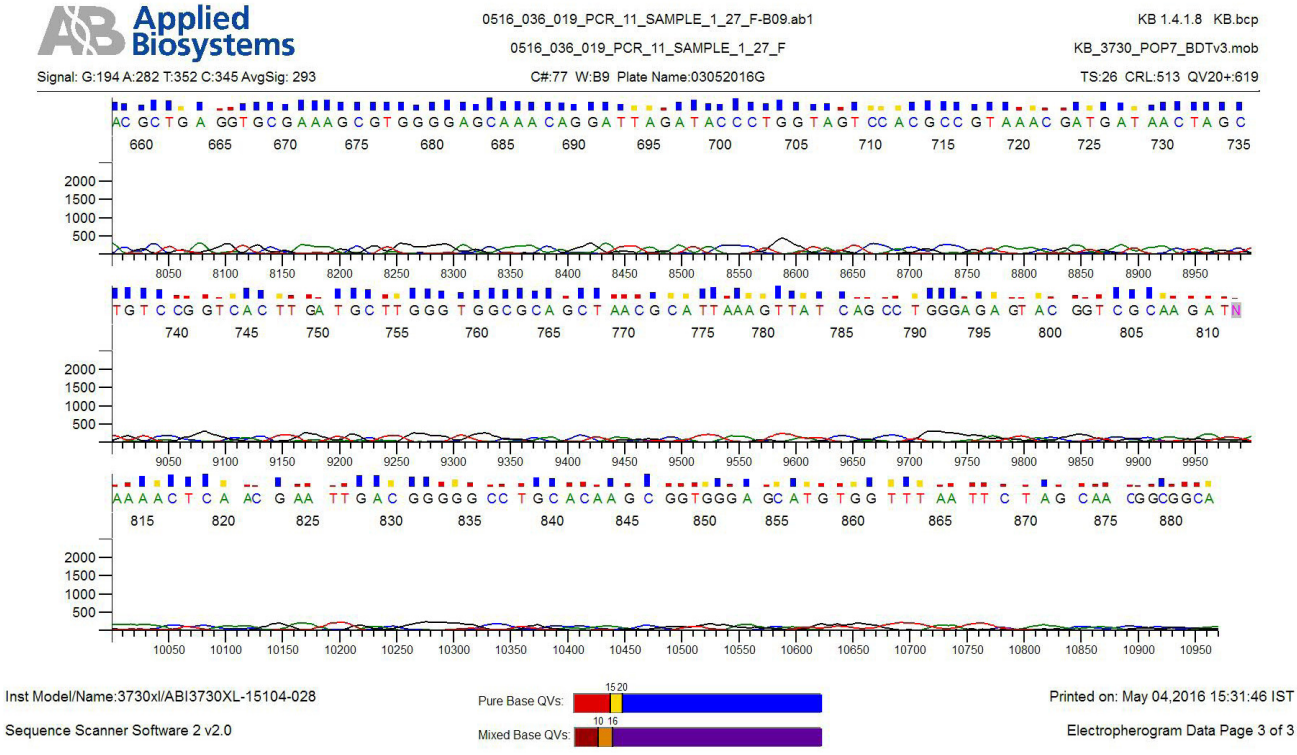
Fig. (7). SEM analysis showing surface degradation of white plastic incubated with *Sphingomomas* species.

Fig. (8). SEM analysis showing surface degradation of white plastic incubated with unknown fungi.

known bacterial species. Bacteria have shown a greater similarity to other oil and plastic degrading bacteria. The sequence analysis result is given in Fig. (**9**).

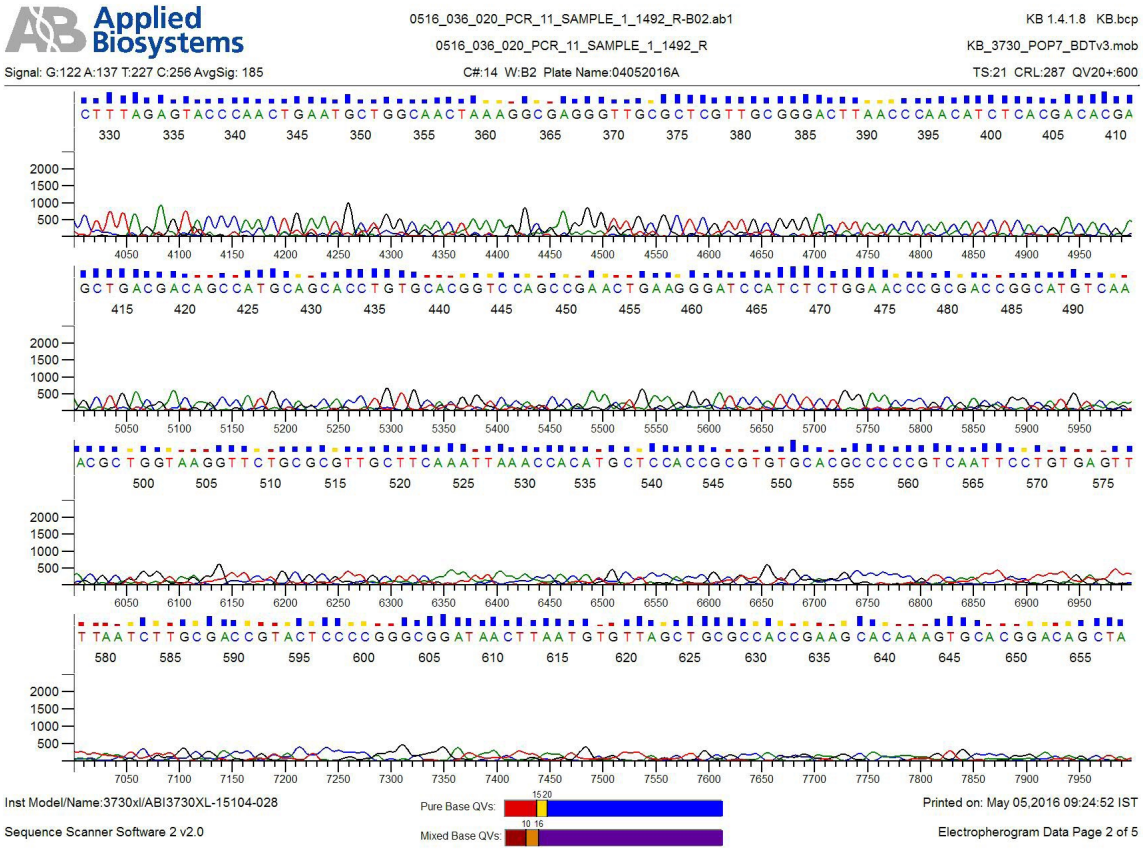


(a)

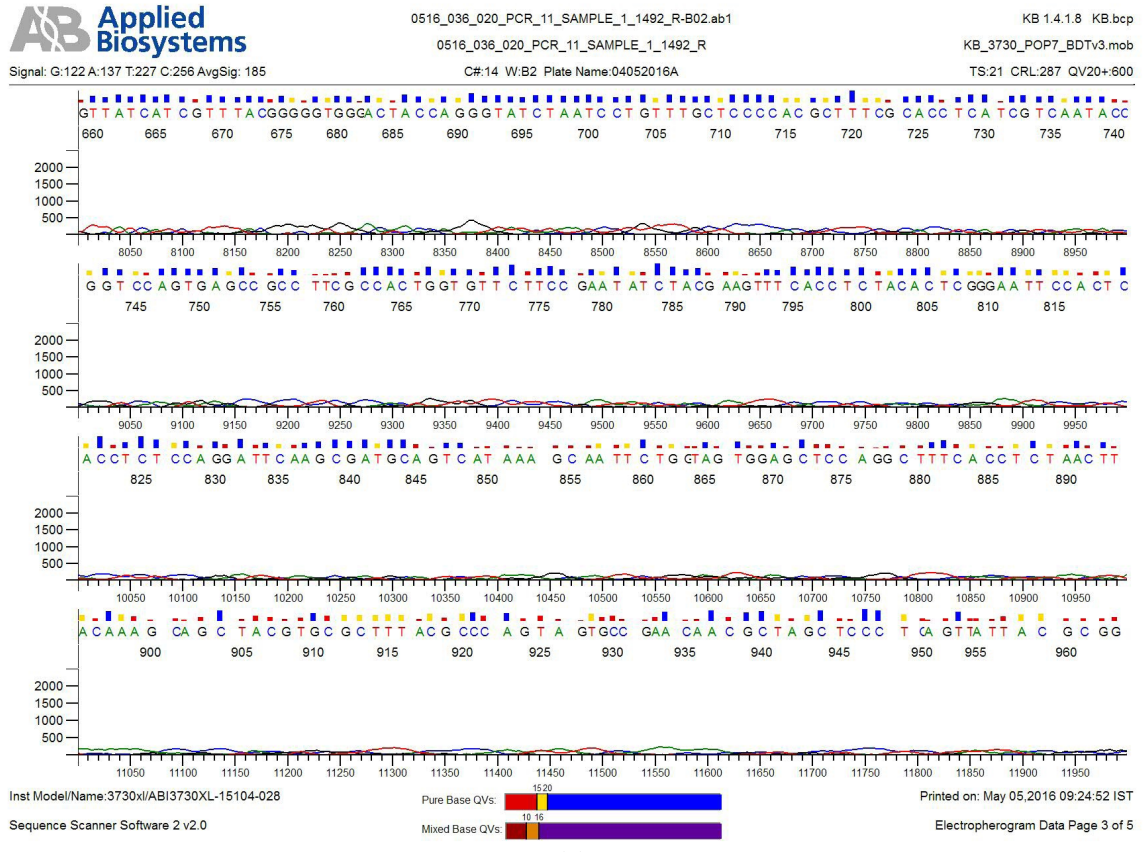


(b)

(Fig. 9) Contd....

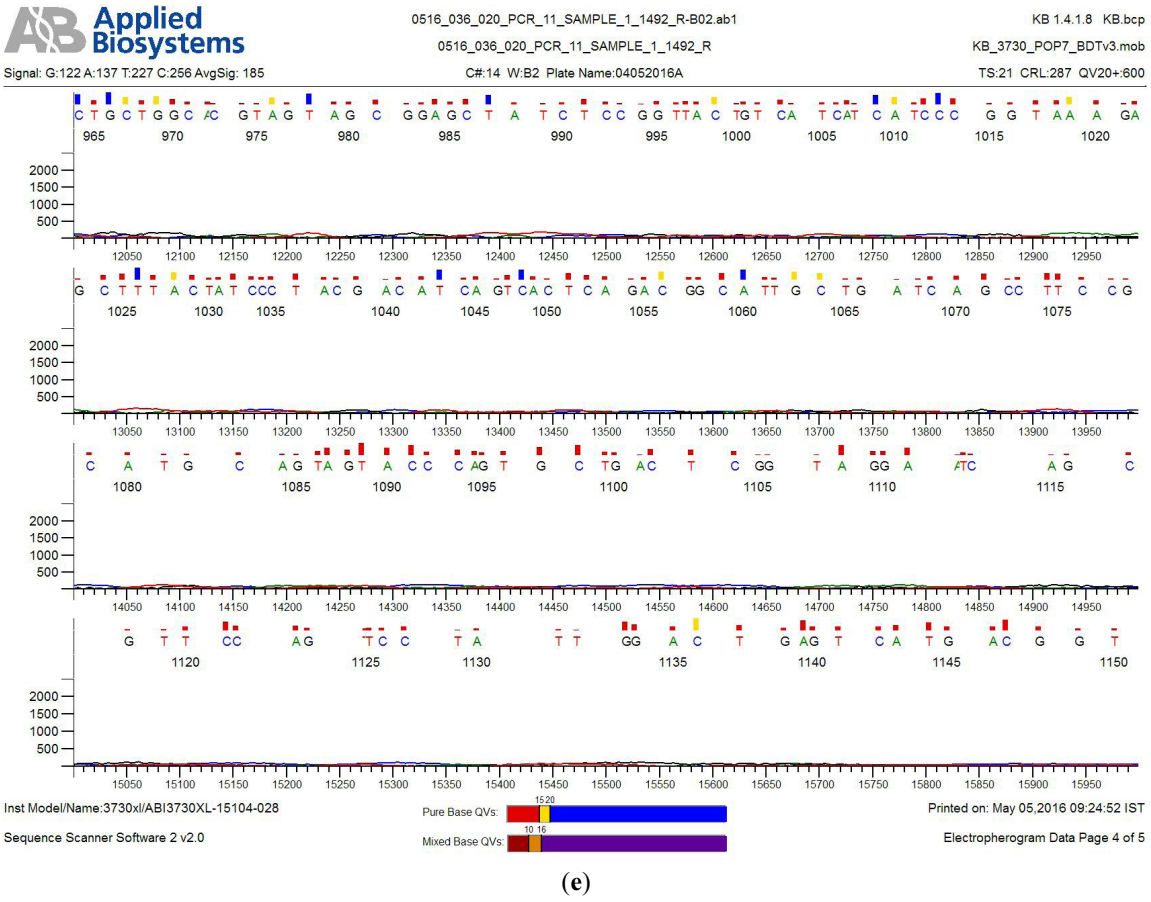


(c)

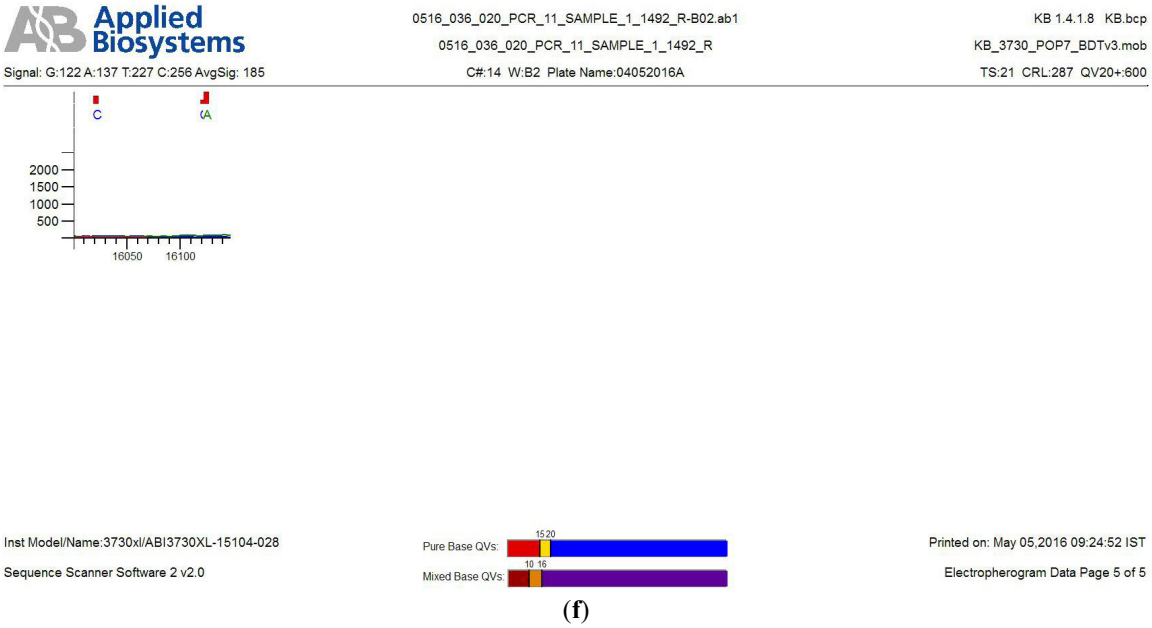


(d)

(Fig. 9) Contd....



(**e**)



(**f**)

Fig. (9). Comparison of our bacterial sequence with known other bacterial sequence (**a**, **b**, **c**, **d**, **e**, **f**).

The figure showing molecular identification *Caulobacter* species has shown more similarity to the sample bacteria *Sphingomonas* species. *Caulobacter* also has the ability in degradation of plastic. Hence, the experimental results led to a model for plastic degradation and suggest that many aspects of the model for plastic degradation developed based on studies.

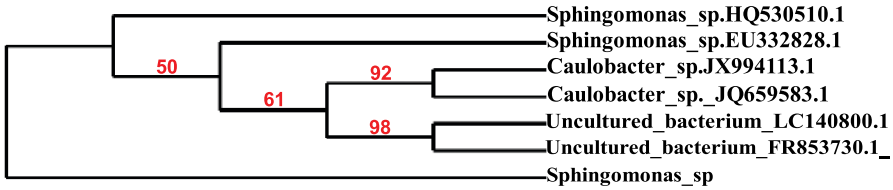


Fig. (10). Phylogenetic tree of bacterial sequence.

3.4. Phylogenetic Tree

Bacteria which gave a high degradation rate were sequenced with other similar bacteria and the phylogenetic tree derived from the comparison is represented in Fig. (**10**).

Plastic strips were incubated for 35 days in a shaker incubator with three fungal species and two bacterial species indicated a 65% decrease in the weight of the plastic strips. The SEM analysis study indicated surface degradation of plastics. The study indicated that the degradation is more in the presence of Sphingomonas bacterial species. But the unidentified fungal species showed more degradation rate than bacterial species. Hence, the study clearly indicated that these microbes can be effective for Eco friendly degradation of plastic which gave 55% of degradation of plastics.

The study was based on looking for an option for disposal of plastics and assessing polymer degradation. The synthetic plastic sample was collected from the dumped soil was used for study, and its degradability was checked by using plastic degrading microbes from soil.

Microbial degradation of a solid polymer like polyethylene requires the formation of a layer on the polymer surface to enable the microbes to efficiently utilize the non-soluble substrates by enzymatic degradation activities and found to be powerful degrading agents in nature. The initiation of degradation was based on the formation of microbial colonies on the organic substrate. Thus, the total degradation period depends on the duration of the microbial colonization.

In our present study, microbial counts in the degrading materials were recorded up to 0.073×109 per gram for total heterotrophic bacteria compared to that of the literature, which indicated that the microbial count was less than this count. Similarly with respect to the final count, which recorded up to 0.05660×10^{2} per gram for total heterotrophic fungi. The probabilistic identification of unknown bacterial isolates against identification matrices of known strains was done using PIBWIN (Probabilistic Identification of Bacteria) program.

Micro organisms play an important role in the degradation of materials. In the process of degradation, extra cellular and intracellular enzymes are involved. These enzymes break complex polymers into smaller molecules of short chains. Microbes were recovered from the soil and after screening; they were identified as *Sphingomonas species*, *Pseudomonas aeruginosa*, *Aspergilus niger*, *Aspergillus flavus*. These organisms were used for the degradation of pre treated plastic.

The degradation were analysed by the decrease in the weight of polythene strip after 35 days of incubation , *Sphingomonas species* indicated plastic degradation of 56% (black) and 31% (white), *Pseudomonas aeruginosa* showed plastic degradation of 42% (black) and 24% (white), *Aspergilus niger* showed plastic degradation of 38% (black) and 26% (white), *Aspergillus flavus* showed plastic degradation of 27% (black) and 16% (white) and unknown fungal species indicated the plastic degradation of 64% (black) and 45% (white).

Bacillus cereus isolated from the dumpsite soil will grow on minimal medium containing polyethylene as carbon source and degrade the polyethylene. Degradation was measured by weight loss of polyethene [14]. In the present work, *Sphingomonas* species indicated maximum degradation of plastic by 56% and *Aspergillus flavus* showed the degradation of 27%. Also, unidentified fungi contributed the degradation of 64%. Further studies are based on the identification of this unknown fungus. Hence, microbes contributes to degradation of polyethene, therefore biodegradation can play a vitol role in the reduction of polyethene waste from the environment.

CONCLUSION

Eco friendly plastic degradation is being acknowledged as an important practical strategy for the management of elimination of plastic wastes from our environment which is considered as a major environmental problem. The microbes which are isolated from the various different sources, such as cooking oil, grease and petroleum has given a remedy for this environmental problem. The isolated microbes contain two bacterial species and three fungal species. The chemically treated plastic strips were inoculated with the specific media containing bacterial and fungal species. After the incubation period of 35 days, weight loss % of degradation was determined. It was observed degradation of plastics was up to 56% by microbes. The fungal culture indicated more degradation than the bacterial culture. Also during the degradation, change in the pH was analyzed; pH was changed from 7.0 to 8.0. SEM analysis also indicated a positive impact of surface degradation of these plastic strips when compared to the control. SEM analysis indicated degradation in polyethylene plastic samples at different magnifications. Sequence analysis of S*phinogomonas species* was compared with the similar known bacterial species and the phylogenetic tree was generated based on the sequence analysis. Further studies will be made to identify the unknown fungal cultures which indicated 64% degradation of plastic compared to *Sphingomonas species*.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Scott G. Photo-biodegradable plastics: Their role in the protection of the environment. Polym Degrad Stab 1990; 29(1): 135-54.

[2] Mueller RJ. Biological degradation of synthetic polyesters-enzymes as potential catalysts for polyester recycling. Proc Biochem 2006; 41(10): 2124-8.

[3] Albertsson AC, Karlsson S. The influence of biotic and abiotic environments on the degradation of polyethylene. Prog Polym Sci 1990; 15(2): 177-92.

[4] Urbanek AK, Rymowicz W, Mironczuk AM. Degradation of plastics and plastic-degrading bacteria in cold marine habitats. Appl Microbiol Biotechnol 2018; 102(18): 7669-78.

[5] Gu JD, Ford TE, Mitton DB, Mitchell R. Microbial Degradation and Deterioration of Polymeric Materials. In: Review, The Uhlig Corrosion Handbook, 2nd ed. New York: Wiley 2000.

[6] Huang SJ, Roby MS, Macri CA, Cameron JA. The effects of structure and morphology on the degradation of polymers with multiple groups. In: Vert M, Ed. Biodegradable Polymers and Plastic. London: Royal Soc Chem 1992.

[7] Glass JE, Swift G. Agricultural and synthetic polymers, biodegradation and utilization, ACS Symposium Series, American Chemical Society. Nucleic Acids Res 1989; 433(1):105-110.

[8] Cruz-Pinto JJC, Carvalho MES, Ferreira JFA. The kinetics and mechanism of polyethylene photooxidation. Angew Makromol Chem 1994; 216(1): 113-33.

[9] Albertsson AC, Barenstedt C, Karlsson S. Abiotic degradation products from enhanced environmentally degradable polyethylene. Acta Polym 1994; 45(2): 97-103.

[10] Priya T, Adria H, Salman A, Siddiqui MH, Sayeed U, Khan MKA. Role of microbes in degradation of synthetic plastics and manufacture of bioplastics. J Chem Pharm Res 2016; 8(3): 211-216.

[11] Kandler O, Weiss N. Regular, Non-Sporing Gram-Positive Rods. Bergey's Manual of Systematic Bacteriology. Baltimore. Williams and Wilkins 1986; 1208-1034.

[12] Robert C Edgar. Muscle: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004; 32(5): 1792-1797.

[13] Dereeper A, Guignon V, Blanc G, *et al.* Phylogeny fr: Robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 2008; 36(suppl_2): 465-469.

[14] Sowmya HV. Biodegradation of polyethylene by *Bacillus cereu*. Adv Poly Sci Technol Int J 2014; 4(2): 28-32.