IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 10, Issue 12 Ver. IV (Dec. 2016), PP 01-07
www.iosrjournals.org

Screening and Isolation of Polyethylene Degrading Bacteria from Various Soil Environments

Divyalakshmi S[1], Subhashini A[2]
[1](Department of Microbiology, Ethiraj College for Women, India)
[2](Department of Microbiology, Ethiraj College for Women, India)

Abstract: *Microbes from various environments, new degrading enzymes and genetic modifications are carried out continuously to overcome the environmental problems caused by plastics. The present study aims to screen and isolate polyethylene degrading bacteria from various soil environments like garbage soil, mangrove soil, and garden soil. The isolates obtained were screened for their ability to utilize polyethylene as sole carbon source in mineral salt medium (MSM). They were processed using shaker flask method containing MSM and pre-weighed polyethylene pieces. After 30 days of incubation at 37°C, the final weight of the polyethylene pieces were noted. Maximum weight loss of 13.6% was recorded. FTIR spectroscopy analysis was performed which showed changes in chemical properties of the polyethylene due to degradation. 16S rRNA sequencing was performed for the isolate that showed maximum changes in FTIR analysis. The isolate was found to be Staphylococcus arlettae (KX344032).*
Keywords: *Biodegradation, polyethylene, weight loss, FTIR, sequence homology.*

I. Introduction

A polymer is a macromolecule composed of repeating structural units connected by covalent bonds. The repeating structural units are called monomers which join to form the polymers. Examples of polymers include plastics, starch and proteins. Plastics are organic polymers of high molecular mass. The name plastic is derived from its property of plasticity. Plasticity is the property of any material by which the material is able to irreversibly deform without breaking. The term plastic is derived from the Greek word '*plastikos*' which means "capable of being shaped or molded" [1]. Organic and inorganic raw materials such as carbon, silicon, hydrogen, nitrogen oxygen and chloride are used for the manufacture of plastic and that are in use today. [2]

Plastics have displaced traditional materials like wood, stone, horn, leather, paper, metal, glass, ceramics etc. Apart from plasticity, they have other advantages like ease of manufacture, low cost, versatility, high hydrophobicity (imperviousness to water), durability etc. They have been increasingly used in many areas. About one-third of the plastics produced are used for packaging purposes. Other uses include piping, plumbing, automobiles, furniture, toys etc. They are also used in medical field for polymer implants. There is an increasing trend in plastic production worldwide.

From early 20th century, the environmental concern regarding to its slow degradation started as it takes thousands of years for its efficient degradation. To prevent accumulation of plastic it should be properly disposed. Improperly disposed plastic materials are significant source of environmental pollution. The plastic sheets or bags do not allow water and air to go into the earth which causes soil infertility. In sea, polyethylene sometimes cause blockage in intestine of fish, birds and marine mammals [3][4] [5]. Abundant plastic wastes have often been found during deep-sea investigation using research submersibles [6].

Incineration, recycling and land filling are some of the traditional methods for handling plastic wastes. However, these methods are costly and often create new environmental difficulties [7] [8]. When compared to other methods, biodegradation is pollution friendly. Usage of certain microorganism and enzymes to degrade polymers are classified as the biodegradation method of polymers [9]. Biodegradation is the break down or degradation on exposure to biological agents like bacteria, fungi, enzymes etc. thereby reducing the molecular weight. Fully biodegradable plastics are costly but under proper conditions microorganisms can be employed effectively to degrade plastics.

Aerobic metabolism results in carbon dioxide and water [5] whereas anaerobic metabolism results in carbon dioxide, water and methane as end products respectively [10]. Biodegradability is evaluated by weight loss, tensile strength loss, changes in percentage elongation and changes in polyethylene molecular weight distribution.

Polyethylene solid waste disposal problems pose various threats. So an attempt has been made to isolate the potent bacterium that degrades polyethylene from different soil environments.

II. Materials and methods

2.1 Plastic samples: Polyethylene bags were collected from local market. Plastic powder was collected from small scale plastic factory near Kodungaiyur. Used milk covers were collected, cut into small squares and used.

2.2 Soil sample collection: Soil sample was collected from five different sites- Pitchavaram mangrove forests, Kodungaiyur garbage dump yard, Pallikarnai garbage dump yard, local garbage dump yard near Porur, and from domestic garden after soil burial treatment. Small pieces of polyethylene were buried 10-20cm below in garden soil and allowed to be there for 20 days. After 20 days, the soil surrounding the polyethylene pieces were collected for sampling. Soil was collected from depth of 3-5cm in plastic bags and sealed immediately after sampling and processed quickly.

2.3 Isolation of microorganisms: 1g of all the five samples were suspended separately in 9ml sterile saline and serially diluted. 10^{-5} and 10^{-6} dilutions were plated on nutrient agar and incubated at 37°C for 24-48 hr to isolate different bacterial strains. The colonies with different colony morphology were subcultured onto nutrient agar plates.

2.4 Screening of plastic and polyethylene degrading organisms [11]: The isolated bacteria were screened for its ability to degrade plastic and polyethylene using mineral salt medium containing (per liter of distilled water) K_2HPO_4, 1g; KH_2PO_4, 0.2g; NaCl, 1g; $CaCl_2.2H_2O$, 0.002g; boric acid, 0.005g; $(NH_4)_2SO$, 1g; $MgSO_4.7H_2O$, 0.5g; $CuSO_4.5H_2O$, 0.001g; $ZnSO_4.7H_2O$, 0.001g; $MnSO_4.H_2O$, 0.001g and $FeSO_4.7H_2O$, 0.01g. [4]. Polyethylene and plastic powder were added to mineral salt medium at a final concentration of 0.1% (w/v) respectively. The medium was sonicated for 1 hour at 120 rpm in shaker, and autoclaved at 121°C and 15 lbs/inch^2 for 20 min.
The medium was poured into sterile plates and allowed to solidify. Wells were cut using well cutter and 20µl culture of isolated organisms was added to the well. The plates were then incubated at 30-37°C for 2-4 weeks and observed for growth around the wells.

2.5 Identification: From the isolated microorganisms, totally 12 organisms were screened based on their ability to utilize polyethylene as sole carbon source. They were identified on the basis of microscopic examination and biochemical analysis according to Bergy's manual [12].

2.6 Microbial degradation of polyethylene waste under laboratory conditions [13]: Polyethylene milk covers were cut, weighed (initial weight) and washed with sterile distilled water. They were then soaked in crude black phenol for 30 min and dried in laminar air flow for 15 min. This polyethylene film was inserted into 50ml of mineral salt medium aseptically. 2 loops of each bacterial isolates were inoculated into separate flasks containing mineral salt medium and polyethylene film. The flasks were incubated at 37°C in an incubator shaker for a month. After incubation the polyethylene films were washed with sterile distilled water and then sprayed with alcohol, air dried and weighed (final weight). Percentage degradation of polyethylene was determined by

$$\text{Percentage (\%) degradation} = \frac{\text{Initial weight-Final weight}}{\text{Initial weight}} \times 100$$

2.7 FTIR analysis: In FTIR, the Infra Red (IR) radiation is passed through the sample. Some IR is absorbed by the sample while some is transmitted (passed through). The resulting spectrum results in molecular absorption and transmission of the sample. The samples with high percentage (%) degradation were analyzed using FTIR and the results were noted.

2.8 16S rRNA sequencing: Species level identification of the isolate that showed maximum changes in FTIR was done by gene sequencing method.

Protocol for 16S rRNA sequencing :

1. Bacterial Genomic DNA isolation kit – InstaGene TM Matrix genomic DNA isolation kit catalog #732-6030.
2. PCR machine - MJ research PTC-225 Peltier Thermal Cycler.
3. Sequencing kit - ABI PRISM ® BigDye TM Terminator Cycle Sequencing kit with AmpliTaq® DNA polymerase.
4. Sequencer - ABI 3730×1.

Bacterial genomic DNA was isolated by using the Insta Gene TM Genomic DNA isolation kit catalog #732-6030. Using 16S rRNA universal primers gene fragment was amplified using MJ research PTC-225 peltier thermal cycler.

2.8.1 Polymerase chain reaction: 1 microlitre of template DNA was added in 20 microlitres of PCR reaction solution. 27F/1492R primers were used for bacteria and 35 amplification cycles were performed at 94°C for 45 seconds, 55°C for 60 seconds, and 72°C for 60 seconds. DNA fragments were amplified about, 1400 bp in the case of bacteria. Positive control (*Escherichia coli* genomic DNA) and negative control were included in the PCR. Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR clean up kit (Millipore).

2.8.2 Sequencing: The PCR product was sequenced using the 518F/800R primers. Details of PCR and Sequencing primers are given.

Sequencing Primer Details:

Primer name	Sequence details	Number of base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTCAATTCMTTTRAGTTT	20

Sequencing reactions were performed using a ABI PRISM ® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Single pass sequencing was performed on each template using 16S rRNA universal primers. Fluorescent labeled fragments were purified from the unincorporated terminators with an ethanol precipitation. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730×1 sequencer (Applied Biosystems). Sequenced data was aligned and analyzed with their closely related sequences using NCBI BLAST tool after the 16S rRNA sequences were screened for chimeric fragments with the DECIPHER program version 1.10.0. The sequenced data have been submitted to the Genbank. The phylogeny analysis of our sequence with the closely related sequence of BLAST results was performed. CLUSTALW was used for multiple alignments of sequences and resulting aligned sequences were subjected to phylogeny analysis. The software MEGA 7.0 was used for constructing Neighbor-Joining Phylogenetic Tree and Maximum Composite Likelihood as substitution model [14][15][16].

III. Results

To isolate the bacterial strains capable of degrading polythene, soil sample was collected from five different sites. Each soil was serially diluted and appropriate dilutions were plated on nutrient agar plates (Fig. 1). The colonies with different colony morphology were sub-cultured and were given a code (Table 1).

The isolated cultures were added to the wells cut on mineral salt medium (MSM) agar and the plates were then incubated at 30-37°C for 2-4 weeks. The isolates which showed growth on MSM agar plates were able to utilize plastic and polythene as the sole source of carbon. The results were noted and the screened organisms were further processed. The screened organisms were identified according to Bergy's manual of determinative Microbiology by performing gram staining technique and biochemical tests.

The screened isolates were inoculated into 50ml of MSM broth in a conical flask along with pre-weighed, sterilized polyethylene milk covers (Fig.2). Care was taken to cut non-dyed portion of the milk covers as the dye may interfere with FTIR results. The milk covers were cut into $2cm^2$ pieces, weighed, washed with sterile distilled water and soaked in crude black phenol for 30 min, dried in Laminar Air Flow (LAF) for 15 min. Initial weight for all the pieces were checked and was found to be 0.022g.

The flasks were incubated in a shaker water bath at 37°C for a month (Fig. 3). After incubation the polyethylene pieces were removed, washed with sterile distilled water, then sprayed with alcohol, air dried and weighed. The final weight of the polyethylene strips were noted (Table 2) and percentage degradation was calculated. The top three isolates with maximum weight loss- grd_5, grd_1, dy_4 were selected (Fig. 4). The micro destruction of the polyethylene pieces were studied under FTIR and the results were compared with the FTIR results of a non-degraded control polyethylene piece (Fig. 5).

The result clearly shows that the isolate GRD5 shows greater change in structure when compared with control. The gene sequence of the isolated strain grd_5 was analyzed with its closely related sequences using NCBI BLAST tool and identified. The strain grd_5 was identified as *Staphylococcus arlettae* based on the high degree of sequence similarity (99%). The sequence data was submitted to Genbank and can be accessible through the accession number KX344032 for the isolate grd_5. Phylogenetic tree exhibiting evolutionary relationship of the isolate is shown in Fig. 6.

IV. Figures and tables

Table1: Source of Soil and Number of Isolates

S. no.	Soil source	Code	No. of Isolates
1	Pitchavaram mangrove forest	Pmf	3
2	Kodungaiyur dump yard	Kod	7
3	Pallikarnai dump yard	Pll	11
4	Local dump yard	Dy	5
5	Garden soil	Grd	6

Table 2: Initial and Final Weight of Polythene Films

S. No.	Isolate	Initial Weight	Final Weight	Percentage Degradation
1	pmf_1	0.022	0.021	4.5%
2	pmf_2	0.022	0.021	4.5%
3	kod_4	0.022	0.021	4.5%
4	kod_5	0.022	0.020	9.09%
5	pll_1	0.022	0.021	4.5%
6	pll_2	0.022	0.020	9.09%
7	dy_4	0.022	0.022	9.09%
8	grd_1	0.022	0.019	13.6%
9	grd_2	0.022	0.021	4.5%
10	grd_3	0.022	0.022	0%
11	grd_5	0.022	0.020	9.09%
12	grd_6	0.022	0.022	0%

Figure 1: Isolated culture on Nutrient agar

Figure 2: Washed, cut, sterilized Polyethylene strips

Figure 3: polyethylene strip in MEM medium

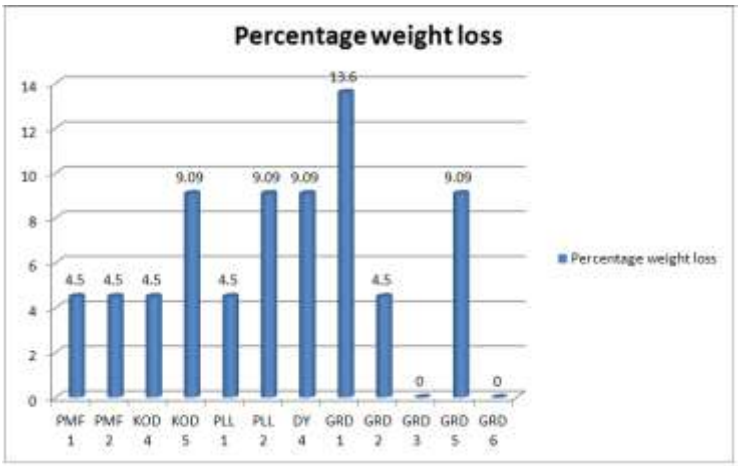

Figure4: percentage weight loss of the polyethylene strip after incubation

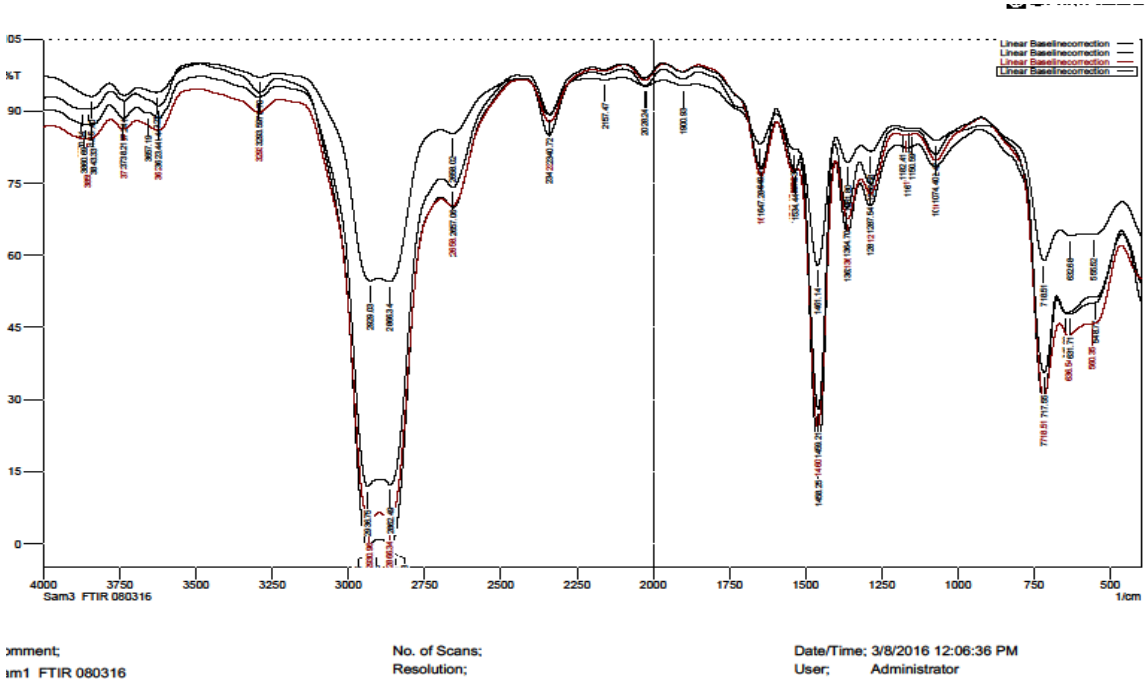

Figure 5: FTIR analysis of polyethylene strip – control, grd_1, dy_4 and grd_-5 (from bottom to top)

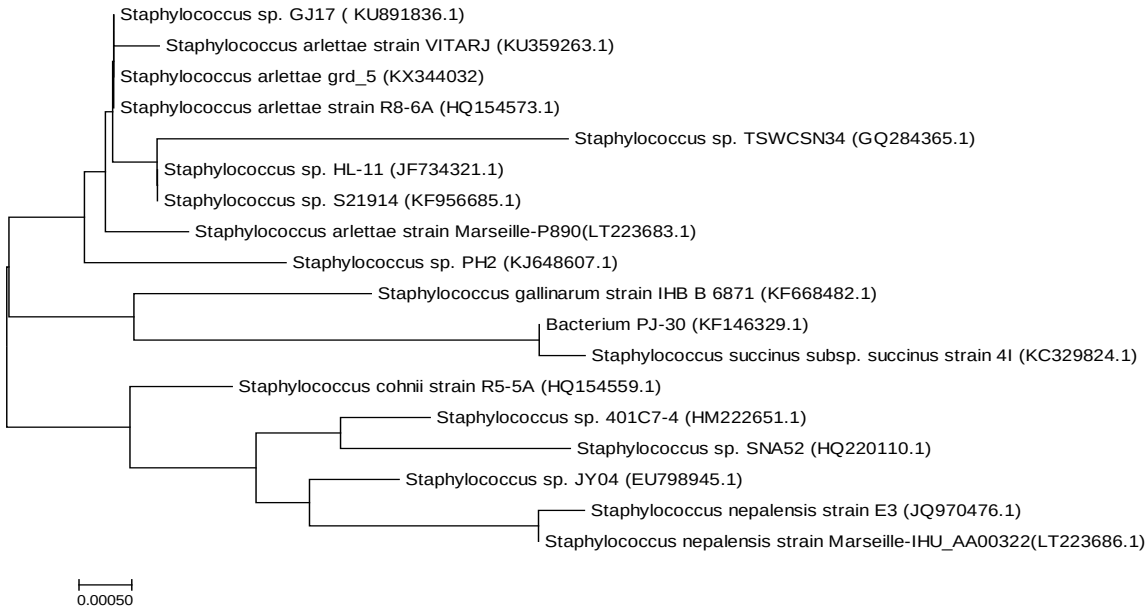

Figure 6: phylogram of *Staphylococcus arlettae* isolate grd_5 based on 16S rRNA gene analysis and constructed by MEGA version 7.0.

V. Discussion

Various soil environments were chosen to isolate polyethylene bacteria. Microbes capable of polyethylene degradation are native to many sites like Mangrove forests [11], polyethylene dumped garbage soil [17] and garden soil [18]. Soil burial treatment was performed in domestic garden soil as it increases the chances of plastic utilizing bacteria to adhere to the plastic film and the nearby soil. The soil samples were serially diluted and plated on nutrient agar. All the isolates may not have the ability to utilize plastic as sole carbon source and degrade it. Therefore they were screened to check their ability to utilize plastic and polyethylene as their sole carbon source.

The isolates, which were able to utilize plastic as their sole carbon source showed growth on MSM agar plates around the wells in which the culture was inoculated. This showed that these organisms have the ability to utilize plastics as carbon source. To check the efficiency of the organisms to degrade plastics, the organisms were inoculated into MSM broth with pre-weighed polyethylene piece as sole carbon source. MSM provides all the other minerals and nutrients required. As the organism utilizes the polyethylene carbon source, there is reduction in its weight. Thus reduction in weight of the polyethylene piece indicates that it has been utilized by the organism. Not all the screened organisms showed weight loss in shaker flask method. This may be due to shorter incubation period or low amount of inoculum. Also during screening, the plastic source was given in shredded form. But in shaker flask method the polyethylene piece was given in its original form. Thus the organism may take prolonged time to utilize it.

FTIR results showed that the isolate GRD5 has greatly changed the structure of the polyethylene piece. There was no formation of new groups in the polyethylene when observed by FTIR. But there was a clear change in intensity of many groups. The major changes in intensity were observed in the wavelength range of 2750-3000. This change corresponds to the change in alkane group (C-H). Other changes were found in the wavelength ranges of 1250-1500 and 3500-4000. These wavelengths correspond to compounds with aromatic rings and amines and/or alcohol respectively.

This isolate (grd_5) was obtained after soil burial treatment. This shows that normal garden soil is a good source of polyethylene degrading bacteria. Results of screening and percentage degradation of polyethylene pieces shows that the dump yard soil and mangrove forest soil are also good source of polyethylene degrading organism.

On sequencing, the isolate was found to be *Staphylococcus arlettae.* This organism showed vast changes in the intensity of absorption by polythene films representing the degradation of the polythene. In 2011, researchers screened polyethylene degrading microorganism from garbage soil which revealed greater potential of *Streptomyces* species when compared to other bacteria and fungi [19]. Similarly, gram positive non-sporing rods from landfills were found to degrade polyethylene efficiently in 2013 [20]. In 2014, the degradation ability of fungi isolated from polythene polluted sites against low density polythene (LDPE) was identified by Singh *et* Gupta [21]. The results were observed by the increase in fresh weight of fungal isolates along with the decrease in molecular weight of the polythene. This study showed that common soil fungi like *Aspergillus, Mucor, Penicillium, Fusaarium* etc has the ability to degrade LDPE upto 36% (Aspergillus).

The present study shows result is in accordance with Vatseldutt *et al* [17] where *Staphylococcus* sp. was found to degrade plastic effectively on the basis of weight loss of upto 52%. Other studies were also conducted to isolate fungi with ability to degrade polyethylene. Study by Singh *et* Gupta [18] in 2014 showed common soil fungi like *Aspergillus, Mucor, Penicillium, Fusaarium* etc has the ability to degrade LDPE upto 36% (Aspergillus).

Staphylococcus arlettae was also found to degrade azo dyes where the strain was isolated from textile effluent from an activated sludge process. The study showed that using this organism the sequential microaerophilic/aerobic stages were able to form aromatic amines by reductive break-down of the azo bond and to oxidize them into non-toxic metabolites [22].

Present study has explored that the isolated *Staphylococcus arlettae* strain is capable of degrading polythene films effectively and the change in intensities of alkane group was observed through FTIR analysis [23].

VI. Conclusion

From total 32 isolates, 12 organisms were screened based on their ability to utilize polyethylene as carbon source. In these 12 organisms, 10 organisms showed weight loss in plastic ranging from 4.54 % to 13.6% (average of 8.72%). Weight loss shows that polyethylene has been degraded. FTIR spectroscopy results show that the polyethylene pieces have been degraded with changes in chemical structure. Among the three isolates studied under FTIR, *Staphylococcus arletae* grd_5 showed greater changes in the structure of polyethylene piece in FTIR spectroscopy. Isolation of the genes for the enzymes required for degradation of plastic could pave way for recombinant DNA technologies where recombinant strains which could degrade plastic in a shorter period can be developed.

Acknowledgements

I would like to thank all those whose assistance proved to be a milestone in the accomplishment of my end goal. I am extremely grateful to my parents for their love, prayers, caring and sacrifices for educating and preparing me for my future.

References

[1]. F.R. Joel, Introduction to Polymer Science, *Polymer Science and Technology, Eds. 3,* 1995, 07458:4-9.

[2]. F.L. Adil, H. Fariha, S. Ziaullah, H. Abdul, F. Shah, A.S. Aamer, Optimization of culture conditions for the production of poly (3-hydroxybutyrate) depolymerase from newly isolated *Aspergillus fumigates* from soil, *Pakistan Journal of Botany., 43(2),* 2011,1361-1372.

[3]. E.R. Secchi, S. Zarzur, Plastic debris ingested by a Blainville's beaked whale, *Mesoplodon densirostris*, Washed ashore in Brazil. *Journal of Aquatic Mammals, 25(1),* 1999, 21-24.

[4]. L.B. Spear, D.G. Ainley, C.A. Ribic, Incidence of plastic in seabirds from the tropical Pacific 1984-91, Relation with distribution of species, sex, age, season, year and body weight, *Marine Environmental Research, 40,* 1996, 123-141.

[5]. A. Starnecker, M. Menner, Assessment of biodegradability of plastics under stimulated composting conditions in a laboratory test system, *International Biodeterioration & Biodegradation, 37(1-2),* 1996, 85-92.

[6]. K. Fugikura, S. Okutani, T. Maruyama, Deep-sea Life-Biological observations using research submersibles, *Tokai University Press,* 2008, 57-80.

[7]. C. Mario, Plastic materials and environmental externalities: structural causes and corrective policy, *Lethbridge Undergraduate Research Journal, 3(2),* 2008.

[8]. A.S. Mody, Biodegradable plastics, *Plastic packaging, 45,* 2000, 89-91.

[9]. R. Premraj, M. Doble, Biodegradation of polymers, *Indian Journal of Biotechnology, 4,* 2004, 186-193.

[10]. J.D. Gu, T.E. Ford, D.B. Mitton, R. Mitchell, Microbial corrosion of metals (*The Uhlig Corrosion Handbook,* 2nd Ed., Wiley, Newyork, USA, 2004).

[11]. K. Kathiresan, Polythene and plastic-degrading microbes from the mangrove soil, *Revista de Biologia Tropical, 51(3),* 2003, 629-634.

[12]. J.G. Holt, N.R. Krieg, P.H.A. Sneath, J.T. Staley, S.T. Williams, W.R. Hensyl, *Gram-Positive cocci. In: Bergy's Manual of Determinative Microbiology,* (Williams and Wilkins, 9th Edn., Baltimore, USA, 1994; 527-558.)

[13]. G. Gnanavel, M.J. Valli, M. Thirumarimurugan, T. Kannadasan, Degradation of plastics using microorganisms, *International Journal of Pharmaceutical and Chemical Sciences, 1(3),* 2012, 1040-1042.

[14]. N. Saitou, M. Nei, The neighbor-joining method: A new method for reconstructing phylogenetic trees, *Molecular Biology and Evolution, 4,* 1987, 406-425.

[15]. K. Tamura, M. Nei, S. Kumar, Prospects for inferring very large phylogenies by using the neighbor-joining method, *Proceedings of the National Academy of Sciences (USA) 101,* 2004, 11030-11035.

[16]. S. Kumar, G. Stecher, K. Tamura, MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets, *Molecular Biology and Evolution, 33,* 2016, 1870-1874.

[17]. Vatseldutt, S. Anbuselvi, Isolation and characterization of polythene degrading bacteria from polythene dumped garbage, *International Journal of Pharmaceutical Sciences Review and Research, 25(2),* 2014, 205-206.

[18]. P. Saminathan, A. Sripriya, K. Nalini, T. Sivakumar, V. Thangapandian, Biodegradation of plastics by *Pseudomonas putida* isolated from garden soil samples, *Journal Of Advanced Botany And Zoology, 1(3),* 2014,1-4.

[19]. R. Usha, T. Sangeetha, M. Palaniswamy, Screening of polyethylene degrading microorganisms from garbage soil, *Libyan Agriculture Research Centre Journal International; 2 (4),* 2011, 200-204.

[20]. D. Mahdiyah, B.H. Mukti, Isolation of polyethylene plastic degrading bacteria, *Biosciences International, 2(3),* 2013, 29-32.

[21]. J. Singh, K.C. Gupta, Screening and identification of low density polyethylene (LDPE) degrading soil fungi isolated from polythene polluted sites around Gwalior city (MP), *International Journal of Current Microbiology And Applied Sciences, 3 (6),* 2014, 443-448.

[22]. F. Elisangela, Z. Andrea, D.G. Fabio, R.M. Cristiano, D.L. Regina, C.P. Artur, Biodegradation of textile azo dyes by a facultative *Staphylococcus arlettae* strain VN-11 using a sequential microaerophilic/aerobic process., *International Biodeterioration and Biodegradation, 63(3),* 2009, 280-288.

[23]. R. Kavitha, K.M. Anju, V. Bhuvaneswari, Biodegradation of low density polyethylene by bacteria isolated from oil contaminated soil. *International Journal of Plant, Animal and Environmental Sciences, 4(3),* 2014, 601-610.