Original Research Article   http://dx.doi.org/10.20546/ijcmas.2016.506.003

Comparative Study of Plastic and Polymer Degrading *Bacillus megaterium* and *Aspergillus niger* Isolated from Dumped Plastic Waste

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A B S T R A C T

Keywords

Biodegradation,
Plastics,
Bacillus sp.,
Aspergillus,
PCR.

Article Info

Accepted:
07 May 2016
Available Online:
10 June 2016

Plastics and their use has become a part in all sectors of economy. The word plastic comes from the Greek word 'plastikos', which means, able to be molded into different shapes. This study has covered the major concerns about the natural and synthetic polymers, their types, uses and degradability. The present study deals with the isolation, identification and degradative ability of plastic degrading microorganisms from soil. Different types of changes are produced by the microorganism during morphological and biochemical analysis. Synthetic plastic sample collected from the dumped soil of garden was used in this study. The morphology of the isolates was observed and genus was identified by biochemical tests as *Bacillus sp.* and *Aspergillus sp.* In the study, pieces of plastics and synthesized PES membrane were inoculated in the liquid culture medium and kept for 1 month to observe the percentage of weight loss by bacteria and fungi. The percentage of weight loss due to degradation was found more by *Bacillus*. This shows greater potential of degradation compared to *Aspergillus*. The preliminary screening of biodegradation capability was done by Fourier Transform Infra-Red (FTIR) Spectroscopy and SEM (Scanning electron microscopy) analysis. Target DNA from isolated strain was submitted for 16S rRNA sequencing after which BLAST and phylogenetic analysis have to be carried out.

Introduction

Plastics are defined as the polymers (solid materials) which on heating become mobile and can be cast into moulds. They are non-metallic moldable compounds and the materials that are made from into any desired shape and sizes. The word plastic comes from the Greek word 'plastikos', which means, 'able to be molded into different shapes'. The plastics we use are

Made from, inorganic and organic raw material such as carbon, silicon, etc., Basic material used for making plastic are extracted from coal, oil and natural gas. Plastics and their use has become a part in all sectors of economy.. Plastics are also used in packaging of products such as food, pharmaceuticals, cosmetics, detergents and chemicals. Approximately 30% of plastics are used worldwide for packaging

applications and the most widely used plastics used for packaging are polyethylene (LDPE, MDPE, HDPE, and LLDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyurethane (PUR), polybutylene terephthalate (PBT) and nylons tons (Gautam *et al.*, 2007).

Polyethylene is widely used for various one-trip applications like food packaging, retail industry uses and agricultural uses. These applications lead to a large quantity of plastic waste, causing serious environmental problems. PE is the most problematic plastic that is resistant to microbial attack. Polyethylene subjected to 26 days of artificial UV irradiation before being buried in soil evolved less than 0.5% carbon by weight after 10 years (Kyriako and Briassoulis, 2007). Polyethersulfone (PES) is a heat-resistant, transparent, amber, non-crystalline engineering plastic. PES is a tough and rigid resin similar to conventional engineering plastics, such as polycarbonate, at room temperature. Biodegradation (i.e. biotic degradation) is a chemical degradation of materials (i.e. polymers) provoked by the action of microorganisms such as bacteria, fungi and algae. The most common definition of a biodegradable polymer is "a degradable polymer wherein the primary degradation mechanism is through the action of metabolism by microorganisms" (Sivan, 2011). Biodegradation is considered a type of degradation involving biological activity. Biodegradable polymers used for producing biodegradable agricultural films include copolyesters, poly (vinyl) alcohol, poly (vinyl) chloride, acylated starch-plastic, modified starch and vegetable oil.

The purpose of this study was to isolate microorganisms from dumped soil area and screening of the potential polyethylene degrading microorganisms and identifying

the high potential microorganism that degrades the plastics.

Bacteria important in the biodegradation process include *Bacillus* (capable of producing thick-walled endospores that are resistant to heat, radiation and chemical disinfection), *Pseudomonas*, *Klebsiella*, *Actinomycetes*, *Nocardia*, *Streptomyces*, *Thermoactinomycetes*, *Micromonospora*, *Flavobacterium*, *Comamonas*, *Escherichia*, *Azotobacter* and *Alcaligenes*(some of them can accumulate polymer up to 90% of their dry mass). Temperature is one of the most important factors affecting microbial growth. Also important are sources of carbon and nitrogen, and pH. Fungi active in the biodegradation process are *Sporotrichum, Talaromyces, Phanerochaete, Ganoderma, Thermoascus, Paecilomyces, Thermomyces, Geotrichum, Cladosporium, Phlebia, Trametes, Chaetomium,* and *Aerobasidium*. The biodegradation process can be divided into (1) aerobic and (2) anaerobic degradation. The purpose of this study was to isolate microorganisms from dumped soil area and screening of the potential polyethylene degrading microorganisms and identifying the high potential microorganism that degrades the plastics.

Materials and Methods

Sample Collection

Plastic sample was collected from the dumped garden soil in Mannachanallur at Tiruchirappalli.

Isolation of Plastic Degrading Microorganisms

To isolate the plastic degrading bacteria and fungi, serial dilution was done in the range of 10^{-1} to 10^{-10}. 10^{-4} to 10^{-7} dilutions of the

sample were then spread on nutrient agar and incubated at 37°C for 1-2 days for bacterial growth. Simultaneously, 10^{-2} to 10^{-5} dilutions were spread on potato dextrose agar and incubated at 28°C for 5-7 days for facilitating fungal growth. After incubation, the numbers of colonies were counted The single colonies were picked and repeatedly streaked on nutrient agar medium and potato dextrose agar. Grown colonies were investigated for their morphological characteristics, cultural and biochemical characteristics following Bergey's Manual of Determinative Bacteriology (1994).

Identification of Isolates

Identification of the isolates were subjected to Gram staining and specific biochemical tests following Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986).

Morphological Identification

Bacterial smear was prepared for each of the isolates by Gram Staining and Motility test was done.various Biochemical tests for bacteria was performed by the methodology (Kloos and Jorgensen, 1985) tests such as the Indole Production Test, Methyl Red Test, Voges-Proskauer's Test, Citrate Utilization Test, Triple Sugar Iron Test, Catalase Test, Urease Test, Oxidase Test, Nitrate Reduction Test Gas production from glucose was assessed by inoculating the isolated strains in MRS broth containing glucose in a Durham tube in inverted condition and incubated at 37°C for 48-72 hrs. The upward movement of inverted Durham tube indicates positive reaction (gas production).

Fungal Identification Procedures

Lactophenol Mount (Leck 1999)

A small drop of lactophenol (LP) was placed on a clean glass microscope slide. small portion of the colony was placed into the drop of LP. A clean cover glass was placed over the suspension and observed microscopically.

Germ Tube Test (Sheppard 2008)

A germ tube represents the initiation of a hypha directly from the yeast cell. Germ tube formation is influenced by the medium, inoculum size and temperature of incubation. Commercially available germ tube solution (Remel Lenexa kansa) are to be used as the medium for the test.cells were suspended in the serum and the stick was discarded. The sample was incubated at 35° C for 2.5-3 hours and then examined with a microscope using to confirm the presence or absence of germ tubes.

Pre Treatment of Plastics

Plastic sample was treated for 6 days with nitric acid (99.0%) and inoculated in the pure bacterial culture obtained. The flasks were then incubated at 30°c and 150rpm for 1 month. After incubation, PE pieces were analyzed for any chemical or structural change by FTIR.

Polymer Membrane Preparation

Polyethersulfone (PES) (15 wt %) was dissolved in NMP (75 wt %) with stirring, and either Polyethylene glycol (PEG) (10 wt %) was added to the polymer solution. Slight heating (~45°C) was used during dissolution of PEG. The homogenous polymer solution was left. The polymer solution was cast with a thickness of 200 μm on a glass substrate (casting speed 25 mm/s) and subjected to humid air (RH = 50-60%) for 1 min. The proto-membrane was solidified in a coagulation bath containing water (20°C) for 1 hour. The resulting

membranes were washed and soaked in the water for 24 h before drying.

Microbial Degradation Studies

Determination of Weight Loss

Pre-weighed samples of 1-cm diameter were aseptically transferred to the conical flasks containing culture broth. Control was maintained with plastic discs in the microbe-free medium and all the flasks were kept in a shaker. After one month of shaking, the plastic discs were collected, and then weighed for estimation of final weight. From the data collected, the bacterial and fungal pure culture was calculated.

Sturm Test for the Analysis of Biodegradation by CO_2 evolution (Muller *et al.,* 1992)

CO_2 evolution as a result of LDPE, PU, PHB and PHBV biodegradation was determined by sturm test (Muller *et al.,* 1992). The samples were added to the culture bottle (Test bottle) with medium. Pure bacterial culture obtained was used for degradation of the samples. The test was performed at room temperature (30˚C) for 4 weeks. After 4 weeks of culturing, the change was observed with rising of amount of samples because of CO_2 production. Evolution of CO_2 as a result of degradation was trapped in the bottles.

Analytical Methods

Fourier Transform Infra-Red (FTIR) Spectroscopy

Fourier Transform Infra-Red (FTIR) analysis was done to detect the degradation of samples after culturing the liquid media, on the basis of changes in the functional groups. The pieces were mixed with KBr and made into a tablet which was fixed to the FTIR sample plate. Spectra were taken at 400 to 4000 waves –number cm^{-1} for each sample.

Scanning Electron Microscopy

The surface morphology of the degraded samples was analyzed through Scanning Electron Microscopy to check for any structural changes in the samples were mounted on the Al stubs by silver paint. Silver coating was carried out in vacuum by evaporation. The images of the samples were compared with those recorded on the original untreated samples.

Isolation of DNA from Isolated Bacteria

From the isolated bacterial cultures DNA was isolated by standard procedures (Marmurs method) and then it was subjected to Agarose Gel Electrophoresis. Agarose gel (0.8%) was prepared and then the isolated DNA sample and molecular weight DNA marker were loaded. The samples were run for 1 h at 100V and DNA was visualized by exposure to UV transilluminator.

Quantification of DNA

About 4μl of the DNA sample was mixed with 2996μl of TE buffer and its absorbance was read at 260nm by using UV – Visible spectrophotometer. 3mL of TE buffer served as a blank. An optical density was calibrated (Sambrook *et al*., 1989). Based on the OD value, DNA sample was quantified.

The Quantity of DNA (ng/μl) =

$$\frac{\text{Optical Density at 260nm X 50 X Dilution factor}}{1000}$$

PCR Amplification for 16S rRNA

The DNA was amplified by using universal 16S rRNA primers forward primer; 8F and reverse primer; 1492R (McCaig *et al.*,

1999). PCR reaction were performed in a 50μL containing 0.1ng of template DNA, PCR Master Mixer, 10pmol concentration of each primer and 0.025U of Taq DNA polymerase enzymes. The final volume was adjusted with sterilized Mili-Q water. A PCR thermocycler was used to amplify the reactions through an initial denaturation step consisting of 94°C for 2 min followed by 25 cycles at 94°C for 1 min, 52.3^{o}C for 1 min and with an extension of 72°C for 1 min followed by an final extension temperature at 72°C for 2 min (Chandler *et al*., 1997). Then amplified PCR products were stored at -20°C for further purification and downstream application.

Primers for 16S rRNA Amplification

SetA 8F	16S rRNA	AGAGTTTGATCCTGGCTCAG
SetA 1492R	16S rRNA	TACGGCTACCTTGTTACGACTT

Agarose Gel Electrophoresis OF PCR Product

About 5 μl aliquot of PCR amplified product was loaded on 1.4% agarose in 1X TAE buffer at 50 V for 45 min and the PCR products were visualized in a UV transilluminator. Lambda DNA double digested with EcoR I and Hind III was used as a marker.

Purification of the Amplified PCR Product

Purification of amplified PCR product was done using a DNA purification kit (HiYiled^{TM} Gel /PCR DNA extraction Kit- Real Genomics, Taiwan). To 45μl of the amplified PCR product, 500μl of DF buffer (supplied in the Kit) was mixed by vortexing. The mixture was mixed well by pipetting and allowed to stand for 1min. The mixture was added to a DE column.The column was transferred to a sterile centrifuge tube and 30μl of elution buffer was added in the center of the column and kept for 30sec until the elution buffer was absorbed by the matrix. The column was spun for 2 min at 12,000 rpm to elute the purified DNA and stored at -20^{0}C.

Phylogenetic Analysis of 16S rRNA

The 16S rRNA sequencing of isolated bacteria was carried out at. The sequencing reactions were run on AMI-PRISM automated sequencer- ABI-3730 DNA analyzer (Applied Bio systems, USA). The nucleotide sequence was initially analyzed at Blast-n site in NCBI server and corresponding sequences were down loaded. The alignment of the sequences was done using ClustalX software program. The phylogenetic tree was constructed using the aligned sequences by the neighbor joining method using MEGA 4 software (Tamura *et al*., 2007).

Result and Discussion

This study has covered the major concerns about the natural and synthetic polymers, their types, uses and degradability. Another area examined has been the biodegradation of plastics by the liquid culture method. The present study deals with the isolation, identification and degradative ability of plastic degrading microorganisms from soil. Different types of changes are produced by the microorganism during morphological and biochemical analysis. Synthetic plastic sample collected from the dumped soil of garden was used in this study and was subjected to serial dilution. After appropriate incubations a bacterial colony and fungal mycelium were observed. By frequent sub culturing, the single pure cultures were isolated and stored at 4°C (plate 1). The morphology of the isolates was observed and genus was identified by biochemical tests as *Bacillus sp* (Table 1) and *Aspergillus*

sp *(plate2)*. *Bacillus* is the most common bacteria associated with plastic biodegradation (Bhardwaj *et al.,* 2012).

Table.1 Characterization of the Bacterial Isolate

S.No	Parameters	Results
1	Gram's staining	**+(rod)**
2	Shape	Rod
3	Motility	Non Motile
4	Indole	-
5	Methyl red	-
6	VP	+
7	TSI	K/A
8	Citrate	-
9	Urease	+
10	Oxidase	-
11	Catalase	-
12	Nitrate	+
15	Strain name	***Bacillus sp.***

Table.2 Table.1 Characterization of the Bacterial Isolate

Sample	*Bacillus*			*Aspergillus*		
	Initial wt (mg)	**Final wt (mg)**	**% degradation**	**Initial wt (mg)**	**Final wt (mg)**	**% degradation**
Pretreated plastic	568	554.7	2.34	-	-	-
Untreated plastic	511	505.8	1.018	550	548	0.364
Polymer membrane	91.5	91	0.5	-	-	-

Plate.1 Pure culture *of Bacillus* species

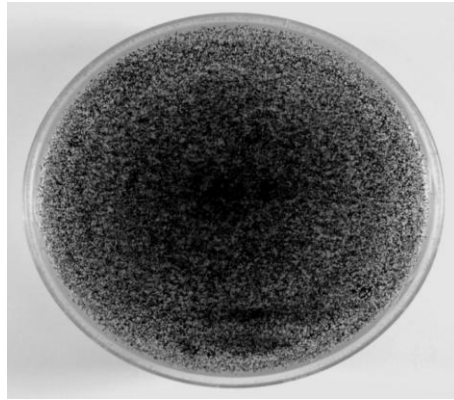**Plate 2** 10-^{5} dilution of dumped soil plated on fungal growth

Plate.3 Plastic inoculated into Bacillus liquid culture for Biodegradation studies

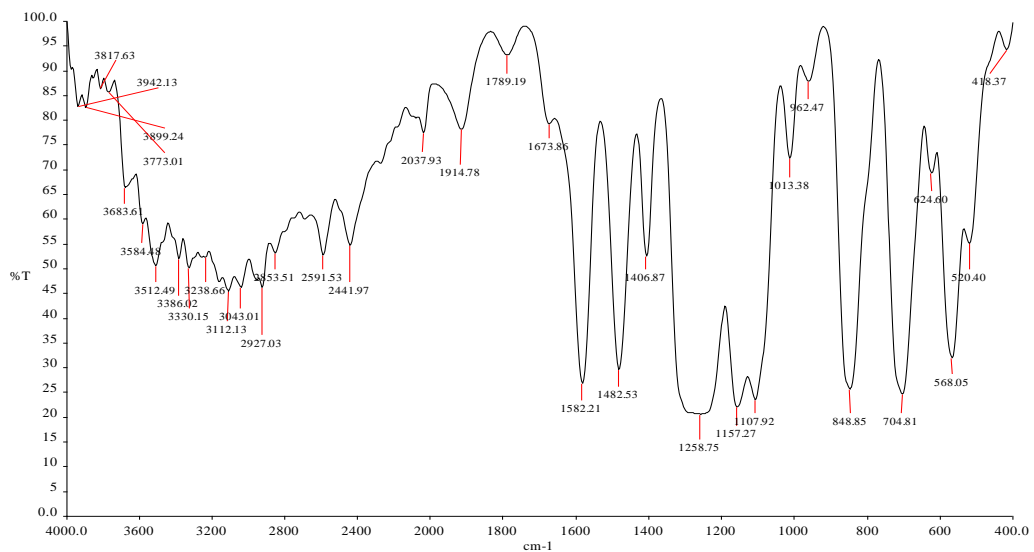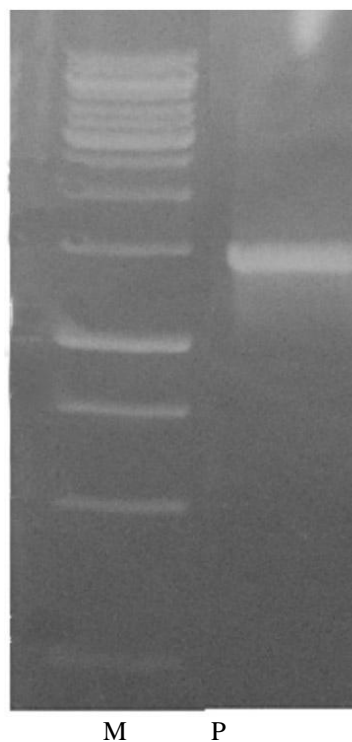**Fig.1** FTIR Spectra of Polymer Membrane Degraded by *Bacillus* sp

Fig.2 PCR Amplification of 16s rRNA

M   P

M-Marker(1kb ladder,P-PCRProducts(1500bp)

In the present study, pieces of plastics and synthesized PES membrane in the liquid culture medium containing bacterial and fungal isolates and kept for 1 month to observe the percentage of weight loss by bacteria and fungi The percentage of weight loss due to degradation was found more by *Bacillus*. (plate2*)*This shows it has the greater potential of degradation compared to *Aspergillus* (Table 2). Usha *et al*., 2011 who have reported a higher degradation of plastic by bacteria compared to fungi. The evolution of CO_2 showed the breakdown of plastics by microbial activity.

The preliminary screening of biodegradation capability was done by Fourier Transform Infra-Red (FTIR) Spectroscopy (figure 3) for surface changes (Deyal *et al*., 2012).

The changes in the spectra confirmed the degradation occurred.   In case of control,

peak appeared at 1315.93 cm^{-1} but it disappeared after microbial treatment. In peak at 1768.72 cm^{-1} in control was reduced to 1760.23 cm^{-1}, thus indicating the breakdown of C=O bond. In case of test, the formation of new peaks in the region of 1417-827 cm^{-1} indicates the formation of the C-C due to the breakdown of C-H bonds.

Further SEM (Scanning electron microscopy) analysis confirmed the degradation of plastic and polymer by revealing the presence of porosity of the bacterial degraded surface compared to control.Thus, surface analysis of plastics after microbial treatment show many differences in morphology.

A distinct PCR product of the 1500 bp (Figure 4) size was produced when target DNA from isolated strain was used. Further, the amplicon was submitted for 16S

rRNA sequencing after which BLAST and phylogenetic analysis have to be carried out.

In conclusion, the present study dealt with the isolation, identification and degradative abilities of plastic degrading microorganisms from soil. Synthetic plastic sample collected from the dumped soil of garden was used in this study. The morphologies of the isolates were observed and genus was identified by biochemical tests as *Bacillus sp* and *Aspergillus sp.* Polyether sulfone (PES) polymer membrane was prepared and it was subsequently used to study biodegradation by *Bacillus*. In the present study, pieces of plastics and synthesized PES membrane were inoculated in the liquid culture medium containing bacterial and fungal isolates and kept for 1 month to observe the percentage of weight loss by bacteria and fungi. The percentage of weight loss due to degradation was found more by *Bacillus.* This shows it has the greater potential of degradation compared to *Aspergillus.*

The preliminary screening of biodegradation capability was done by FTIR Spectroscopy. The changes in the spectra of the degraded samples confirmed the degradation occurred. Further, SEM analysis confirmed the degradation of plastic and polymer by revealing the presence of porosity and fragility of the bacterial degraded surface compared to control. A distinct PCR product of 1500 bp was produced when target DNA and the amplicon was submitted for 16S rRNA sequencing after which BLAST and phylogenetic analysis have to be carried out for species identification.

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