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Polyethylene degradation by microbes isolated from municipal solid waste

Nouzia Fathima.K [1]., Siluvai Kirubagari Aneeshia.C [2]

[1,2]Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi,
Affiliated to Manonmaniam Sundaranar University,
Tirunelveli, TN, India.

Abstract

There is an ever increasing problem due pollution caused in the environment by accumulation of plastic wastes. In this context, an attempt was made to study the biodegradation of polyethylene films, one of the major contributors to plastic waste in the environment. In the current study, biodegradation of low density polyethylene was analyzed using microbes present in the soil (municipal solid waste) for over a period of 2, 4 and 6 months. The methods include composting of polyethylene films with the municipal waste. The strains of microbes associated with degradation were isolated from sample, identified and characterized. The isolated microbes contained many bacterial species some of which are *Bacillus sp, Pseudomonas sp, Staphylococcus sp* and a few fungal species such as *Aspergillus, Penicillium.* The isolated strains were screened for their ability to degrade the polyethylene by plate assay method and CO_2 evolution test. Among the isolated strain, the one with the best potential to degrade polyethylene was found to be in *Aspergillus niger*. The percentage of degradation was measured using weight loss method and was found to be 20% in 6 months.

Keywords: *Polyethylene, Biodegradation, Municipal waste, Aspergillus niger, Weight loss*

1 .Introduction:

Plastics have recently been the highlight of all the environmental problems due to its lack safe disposal. Plastics are manmade long chain polymeric molecules (Scott, 1999).The word plastic comes from the Greek word "plastikos", which means 'able to be molded into different shapes' (Joel, 1995).

Plastics are considered to be the basic material for they are being used for various purposes in our day to day life (Gnanavel *et al.,* 2012). The annual global production of synthetic polymers has gone up more than 140 million tonnes with their utility increasing at a rate of 12% per annum (Shimao, 2001).

According to their chemical structure they are classified as polystyrene, polypropylene, low density polyethylene, high density polyethylene, polycarbonate (Kumari N.A *et al.,* 2013). Hence, plastics are persistent in the environment and are one of the sources of environmental pollution (Tokiwa and Ugwu, 2007).

Polyethylene is nothing but a polymer made up of long chains of repeating units of ethylene monomers. Polyethylene (PE) is a miracle material of the plastics industry with world wide application. PE usage, particularly of low-density polyethylene (LDPE) is growing daily. LDPE surpasses other PE types in many properties, making it extremely popular for a wide range of applications. LDPE film is strong, durable, thermally stable, odour free, heat sealable and resists chemical and biological attack (Fellows, 2000).

Their disposal both on the land and the aquatic environment has resulted in their accumulation due to little, if any, biodegradation, making the environment unaesthetic, with possible health implications to humans, animals, and other organisms (Siddiqui *et al.*, 2008).

The unique properties such as durability, lighter weight and versatility of these polymers cause them to remain in the landfills and natural water resources creating a severe threat to the environment (Hoffmann *et al.,* 2003; Jang *et al.,* 2002).

The drastic increase in production and lack of degradability of commercial polymers, particularly common plastics has created a huge public attention on a potentially high environmental accumulation and pollution problem that could persist for centuries (Albertsson *et al.,* 1987). They cause the environmental pollution by getting accumulated in

the environment this takes place because of their stable nature (Hemashenpagam *et al.*,2013).

Fortunately, society has recognized this problem, and efforts are underway of finding ways to reduce accumulation of plastics in the environment (Tokiwa and Ugwu, 2007).
The recent studies suggests a better approaches for eliminating plastic waste without causing other ill-effects to the environment. Among them degradation is one of the way. The degradation which employs biological factors such as microbes is called biodegradation.
Biodegradation is a process which include microorganisms like bacteria and fungi that can degrade the polythene and therefore the process of biodegradation is an upcoming trend in the field of degradation (Gu *et al.*, 2000).
Some of the well-known microbes have the capacity to degrade plastic polymers into their respective simple monomeric (Ghosh *et al.*, 2013). Microorganisms involved in the degradation of both natural and synthetic plastics include both bacteria and fungi (Gu *et al.*, 2000).
Microorganisms that can degrade plastic includes over 90 genera, from bacteria and fungi, among them; *Bacillus megaterium*, *Pseudomonas* sp., *Azotobacter*, *Ralstonia eutropha*, *Halomonas* sp., etc. (Chee *et al.*, 2010).
Degradation of plastics by microbes is done by their enzymatic activities which cleaves the polymer into oligomers and monomers after which they are further metabolized by the microbial cells. Aerobic metabolism leads to the production of carbon dioxide and water (Starnecker and Menner, 1996) and on the contrary anaerobic metabolism production of carbon dioxide, water and methane as the end products (Gu *et al.*, 2000).
In the present study, an attempt was made to examine the biodegradation of plastics in the natural environment i.e. by composting with municipal solid waste and to isolate plastic degrading microorganism. And finally to assess the biodegradability by comparing the potential of various isolated microorganism and to optimize the different parameters for enhanced biodegradation of polyethylene.

2. Materials And Methods:

2.1 Sample Collection:

Soil sample was collected from one of the waste dumping sites, Tuticorin Corporation located at Taruvaikulam. Polyethylene bags were collected from local market in Tuticorin. Care was taken to collect LDPE polyethylene bags.

2.2 Composting Of Sample:

2.2.1 Pretreatment of plastic sample:

The bags were cut into pieces of 5×20 cm^2 and pretreated before burying in the soil by washing with water then followed by ethanol and finally rinsed with water. The sample was shade dried and buried into soil sample. A duplicate was also kept for incubation. The weight of sample was taken before burying.

2.2.2 Incubation:

Small pieces of polyethylene were buried 10 cm below in soil and allowed to be there for 6 months. After 6 months, the soil surrounding the polyethylene pieces along with the pieces was collected for sampling.

2.3 Isolation Of Microorganisms:

1g of soil sample was suspended separately in 9 ml sterile saline and serially diluted. 10^{-5} and 10^{-6} dilutions were plated on nutrient agar and incubated at 37ºC for 24-48 hr. to isolate different bacterial strains. 10^{-3} and 10^{-4} dilutions were plated on Rose Bengal Chloramphenicol agar and incubated for 48 hr. to isolate different fungal strains. The colonies with different colony morphology were selected and sub cultured the respective media for further use.

2.4 Identification:

The isolated organisms were identified on the basis of microscopic examination and biochemical analysis according to Bergey's manual.

2.5 Molecular Identification Of The Isolates:

The isolated colonies, was checked for its purity before DNA extraction.

2.5.1 Preparation for colony PCR

The morphologically distinct colonies were picked from the pure culture plate and inoculated into 2ml of Nutrient broth and kept for incubation in shaker for 3 hrs at 250 rpm at 37ºC. After incubation, the tubes were centrifuged at 13,000 for 2 minutes. The supernatant was discarded and the pellet was

suspended in 50µl of Colony Lysis Solution (CLS). It was kept in water bath at 60ºC for 20 minutes. This lysed cell suspension was used as template for BOX and ERIC PCR amplification.

2.5.2Genomic DNA extraction

Overnight culture (10ml) was centrifuged at 10,000 rpm for 5 minutes in 1.5ml micro centrifuge tubes. The bacterial pellet was treated with 90µl of 10% SDS and 90µl of lysozyme (20mg/ml) in TE buffer (20mM Tris, 2mM EDTA). It was incubated for 90 minutes at room temperature. It was followed by the addition of 150µl of 5M NaCl; 100µl of CTAB prepared in 5M NaCl and kept in water bath for 30 minutes at 65˚C. Then the aqueous layer of DNA was extracted with the mixture of phenol, chloroform and isoamyl alcohol in the ratio of 25:24:1.It was centrifuged at 13,000 rpm for 15 minutes and the aqueous layer was collected. The aqueous layer was precipitate with 3M sodium acetate salt ($1/10^{th}$ volume) and equal volume of 70% ice cold ethanol, centrifuged at 7000 rpm for 5 minutes. The DNA pellet was again washed with 70% ice cold ethanol and the pellet was suspended in TE buffer (pH 8). The DNA products were resolved through 1% (w/v) agarose gel electrophoresis in 1 X TAE buffer using 1 Kb ladder as molecular weight marker and visualized by staining with ethidium bromide.

2.5.3 PCR amplification of 16S rRNA gene:

The PCR amplification of 16S rRNA gene was performed with universal eubacteria primer 27 F (AGAGTTTGATCTGGCTCG) and 1492R (TACGGYTACCTTGTTACGACTT)(Lane, D.J., 1991). The reaction mixture contained 50ng of template DNA 10µl, 10X PCR buffer 5µl, 1.5mM $MgCl_2$2µl, 2.5 mM each dNTPs 2µl, 10 pmol of forward 1µl and reverse primer 1µl,1.5 U of Taq DNA polymerase 1µl and milli Q 28µl. The total mixture volume was 50µl and the program starts with initial denaturation at 94˚C for five minutes, followed by denaturation at 94˚C for 30s, annealing at 55˚C for 1.30 minutes, extension at 72˚C for 2.30 minutes then the final extension at 72˚C for 5minutes. From denaturation to extension step, 35 cycles were repeated. The 16S product was resolved in 1.5% (w/v) agarose gel electrophoresis in 1 X TAE buffer using 1Kb ladder as molecular weight marker and visualized by staining with ethidium bromide.

2.5.4 DNA sequencing:

The 16S product was sequenced using 27F and 1492R primers in Chromous, Biotech, Bangalore, India. The partial sequences obtained were compare with the nucleotide sequence present in GenBank using BLAST search through the online option available at www.ncbi.nlm.nih.gov/BLAST.

2.6 Weight Loss Method:

One set of sample was taken, washed, shade dried and weighed for final weight determination. The weight loss percentage was calculated by the following formula (Kyaw *et al.*, 2012)

$$\text{Percentage Degradation} = \frac{\text{Initial weight-Final weight}}{\text{Initial weight}} \times 100$$

2.7 Screening Of Polyethylene Degrading Organisms:

2.7.1 Plate assay method (Seal KJ.*et al.*, (1988):

The basal media was plated. Small pieces of polyethylene bit of 3×3cm diameter were placed at the center of plate. The isolated strains were streaked on the bits. The plates were incubated for about 4 weeks. The degrading strain showed growth around the polyethylene bits

2.7.2 Preparation of LDPE powder (Shah, A. A., (2007)):

LDPE bag was cut into small pieces and boiled for 15 min in xylene till it became uniformly dissolved in xylene. The residue was crushed while it was warm by using pestle and mortar. The LDPE powder was washed with ethanol to remove remaining xylene. Excess of ethanol was allowed to evaporate. The powder is dried for some time in hot air oven to get fine dry powder.

2.7.3 Zone method (Kathiresan.K., 2003):

The isolated bacteria were screened for its ability to degrade polyethylene using mineral salt medium. Polyethylene powder were added to mineral salt medium at a final concentration of 0.1% (w/v) respectively. The medium was continuously shaken at 120 rpm in shaker for 1 hour, and then sterilized. After the medium was solidified in sterile petri plate, wells were cut and 20µl culture of isolated organisms was added to the well. The plates were then

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incubated at room temperature for 2-4 weeks and observed for growth around the wells.

2.7.4 CO_2 Evolution test (Muller,R.J *et al.*,(1992)):

The degradation of LDPE can be measured by the amount of CO_2 evolved on the basis of Strum test. LDPE powder were added to the test flask containing 100 ml of enrichment medium. The LDPE was incubated with the test organisms which served as the test and LDPE without the isolated strain served as the control. Both the flasks were incubated at room temperature for 48 hours. After incubation, the metabolic CO_2 from the test flask and atmospheric CO_2 from the control flask were calculated gravimetrically. The CO_2 evolved, as a result of degradation of polymeric chain was trapped in absorption flask containing KOH (1 M). To the absorption flask containing KOH, barium chloride solution (0.1 M) was added which resulted in formation of barium chloride precipitation (using CO_2 released from the breakdown of polymer). CO_2 produced was calculated gravimetrically by measuring the amount of CO_2 evolved by titrating against $BaCl_2$.

3. Result And Discussion:

3.1 Isolation:

After incubating for respective time, various colonies were formed. From the bacterial mixed culture white and transparent colonies were subcultured and streak on Nutrient Agar medium to identify the bacterial strains,where as fungal colonies on Potato Dextrose Agar medium to identify the fungal strains. Nine distinct isolates were obtained of which four were bacteria and five were fungi.

3.2 Morphological And Physiological Characteristics:

3.2.1 Bacteria:

The isolated bacteria were identified based on their morphological characteristics and colony morphology on nutrient agar plate.

3.2.2 Fungi:

The isolated fungi were characterized and identified by their morphological characteristics based on size, shape and colony morphology on potato dextrose agar plate.

3.3 Molecular Identification Of The Isolates:

The isolated strain were identified by 16s rRNA gene sequencing method, the results are tabulated in TABLE -1

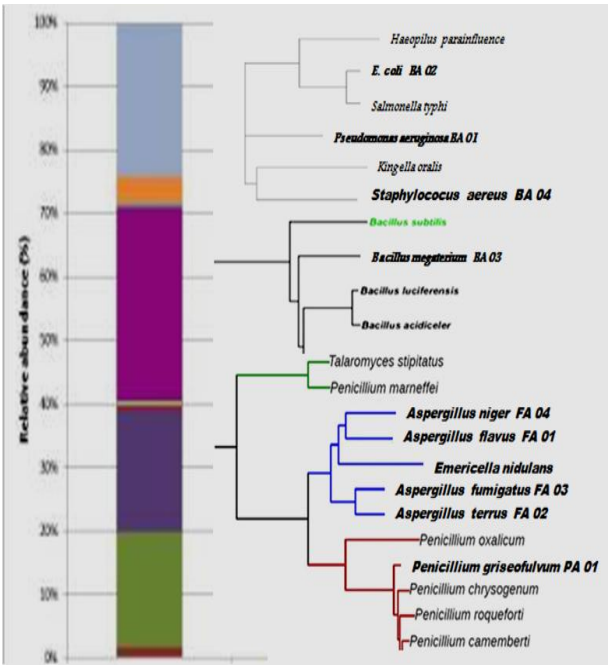

Fig -1 Phylogenetic tree of various isolated strain

Table 1- Isolated strains

S.No	Isolation code	Accession number	Closely related bacterial	Identity (%)
1	Strain 1	**BA 01**	*Pseudomonas aeruginosa*	80
2	Strain 2	**BA 03**	*Bacillus megaterium*	60
3	Strain 3	**BA 02**	*E. coli*	90
4	Strain 4	**BA 04**	*Staphylococcus aureus*	70
S.No	**Isolation code**	**Accession number**	**Closely related fungi**	**Identity (%)**
1	Strain 1	**FA 02**	*Aspergillus terrus*	40
2	Strain 2	**FA 01**	*Aspergillus flavus*	50
3	Strain 3	**FA 03**	*Aspergillus fumigatus*	40
4	Strain 4	**PA 01**	*Penicillium griseofulvum*	25
5	Strain 5	**FA 04**	*Aspergillus niger*	30

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3.4 Weight Loss:

The total degradation was determined by percentage weight loss (TABLE-2). There was no change in weight for first 4 months of incubation. Therefore there was a lag period of 4 months (Fig -2)

Table -2 Initial and Final weights of plastic strip

S.N0	DURATION OF INCUBATION	INITIAL WEIGHT	FINAL WEIGHT	% WEIGHT LOSS
1	DAY 30	0.15	0.15	0
2	DAY 60	0.15	0.15	0
3	DAY 120	0.15	0.14	6.66
4	DAY 180	0.15	0.12	20

In present study percentage of weight loss is about 20% in 6 months of incubation. Weight loss of plastic film were noticed after 4 months of composting and this loss was more apparent in the samples composted for 12 months (Ch.Vijaya *et al.*, 2007). Kathiresan *et al., 2003* reported that the biodegradation (weight loss) by the microbes was ranging from 2.19 to 20.54% for polyethylene.

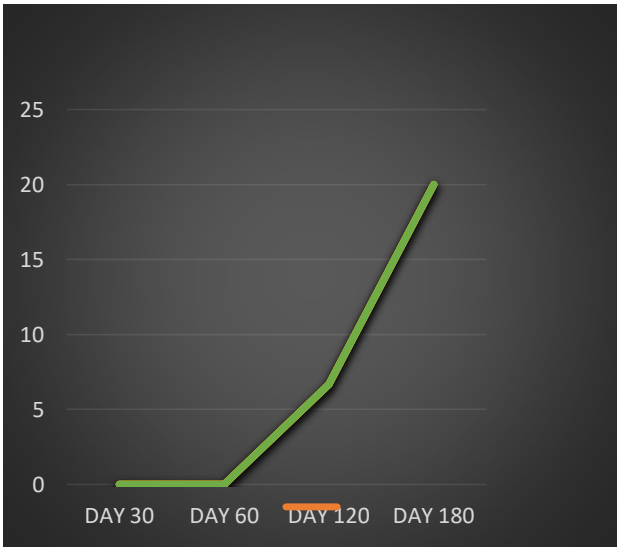

Fig-2 Percentage weight loss

3.5 Screening Of Polyethylene Degrading Organisms:

3.5.1Plate Assay Method:

The strains which have the capacity to degrade polyethylene have shown growth around the bit. The fungal strain 5 (FA 04) and strain 2 (FA 01) have shown more capacity to degrade while bacterial strain 4 (BA 04) has shown better results. The degradation has been visible around the edges of polyethylene.

3.5.2 Zone Formation Test:

The polyethylene containing mineral salt agar plates were inoculated with the isolated bacteria and fungi. Opaque zone was observed after 10 days of incubation at 25-30°C around the colony. On this screening one of *Bacillus* and *Staphylococcus* sp, two of *Aspergillus* sp showed high degradation activity. Similar type of organisms were reported earlier which associated with the polythene bag and plastic films in the soil (Kathiresan.K (2003).

3.5.3 CO_2 Evolution Test:

The total amount of CO_2 evolved as calculated gravimetrically and volumetrically for the isolates according to Strum test. The maximum amount of CO_2 was evolved by fungal strain (FA-04) which is *Aspergillus niger* (2.56g/l). Among bacteria strain 4 (BA- 04) (1.36g/l) showed highest result. The results are shown in (Fig- 3).

The findings were found to be similar with the work done by Shah *et al.*, (2009) who reported 1.85 g / l evolution of CO_2 after a 30 day period of incubation with fungal strain of *Fusarium* sp. on LDPE films.

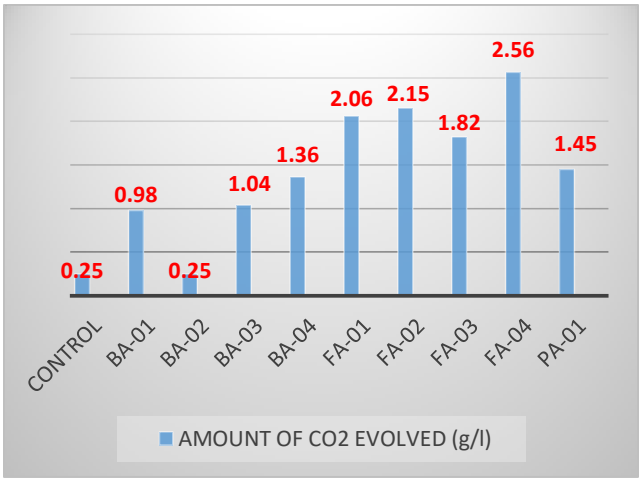

Fig-3 Amount of CO_2 evolved (g/l)

4. Conclusions:

Plastic degrading microorganisms were isolated from degraded plastic sample and degradation of plastic strips was determined by using weight loss method. The isolate which shows high opacity was selected and further used. The isolates obtained was subjected to standard biochemical test results showed the presence of *Bacillus megaterium*, *E.coli*,

Pseudomonas aeruginosa and Staphylococcus aureus for bacteria and *Aspergillus sp and Mucor sp.* were observed in fungi. The degrading ability of various strains was measured using various tests, based on which the fungal strain-5 (*Aspergillus niger*) and bacterial strain-4 (*Staphylococcus aureus*) showed highest capacity of degradation.

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