

Original Research Article

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Effect of Fungi Isolated from Different Plastic Polluted Sites on Low Density Polyethylene Material Degradation with Reference to SEM Analysis

Jaya Philip^{1*}, Tanuja², Harshita Michael¹, Vanshika Verma¹ and Anam Rahman¹

¹Department of Microbiology, Patna Women's College, Patna University, Patna, Bihar, India

²Department of Botany, T. P. S. College, Patliputra University, Patna, Bihar, India

*Corresponding author

ABSTRACT

Keywords

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The use of plastics and their products have completely changed the human society. They are enormously used in medicines, food, cosmetics, transportation, etc. Plastics are complex polymers which take hundreds and thousands of years to degrade remaining in the environment and disturbing the ecosystem which are a part of it. LDPE is one of the thermoplastic polymers abundantly used for commercial purposes accounting for about 60% of the total plastic production. Under normal environmental conditions, such polymers might take millions of years to degrade completely. But with the use of effective microorganisms, the potential degradation of the polymer such as LDPE can be done efficiently under optimum lab conditions. The aim of this study was to isolate fungi from plastic polluted sites of Patna and to study their ability to degrade LDPE with reference to SEM analysis. The fungi were identified by studying their macroscopic and microscopic characteristics. *Penicillium* sp. was selected on the basis of the positive screening and was further used to study the degradation of LDPE sheets during a course of 15 and 30 days separately. Finally, after the end of the incubation period the percentage weight reduction of the LDPE sheets were calculated and weight reduction up to 20% and 50% after 15 and 30 days of incubation respectively was observed. Also, the shift in the pH of the MSM broth from neutral to acidic was seen. Finally, the degradation of LDPE sheets was confirmed by performing the SEM analysis which showed porosity and fragility on the surface of the sheets.

Introduction

The invention of plastics in the 19th century (Michel *et al.*, 2012) and the wide range of plastic based products have revolutionized the human society. Almost every domain of our life involves their applications like transportation, production of food, medicine etc. as they are inexpensive and highly durable (Andrady and Neal, 2009). The word plastic originated from the Greek word

“plastiko” meaning “Able to demold into various shapes” (Joel, 1995). Plastic constitute of carbon, hydrogen, silicon, oxygen, chlorine and nitrogen and is composed of the monomers linked together by chemical bond.

From the last three decades, the reckless and continuous usage of plastic product for packaging, industry, transportation, etc., in the urban and rural areas have raised serious

problems of waste disposal of plastic and pollution caused by its use. The use of plastic has globally increased due to its durability, strength, inertness and low cost. But, the disadvantage of plastic is that it is difficult to be degraded completely through natural processes and persist in the environment for millions of years.

Plastic has toxic pollutants that damage the environment and cause land, water, and air pollution has direct and deadly effects on wildlife particularly the marine animals. According to a report published in 2019, plastic contribution to greenhouse gases is equivalent to 850 million tons of carbon dioxide (CO₂) to the atmosphere which are expected to increase to 1.34 billion tons by 2030 (Kiehl *et al.*, 1997). The burning of plastic produces toxic gases like CO₂ and Dioxins (Ali *et al.*, 2009) and health hazards causing cancer and lung diseases (Pramila and Vijay, 2011).

The chemicals used in the manufacture of plastics are known to be toxic and can enter the food chain creating potential health risks (Talsness *et al.*, 2009). In the year 1999-2000, as reported by Tiwari *et al.*, (2000) that India has imported more than 12000 tons of plastics.

Depending on their chemical composition and varying properties related to absorption and adsorption, these factors contribute to the persistence of plastics (Barnes *et al.*, 2009). Each polymer chain of plastic has several two thousand repeating units of these monomers, this complex nature of the polymers makes them highly stable and difficult to degrade (Jensen and William, 2008). The biodegradation of polythene is a slow process, microorganisms such as bacteria, fungi and actinomycetes are involved in the degradation of natural and synthetic plastic. Some of the crucial characteristics

responsible for plastics resistance to biodegradation include their long chain polymer structure and high molecular weight along with hydrophobicity (Wilkes and Aristilde, 2017). Thus, the long chain polymers have to be first depolymerized or broken down into smaller monomers (Shah *et al.*, 2008). The efficiency of biodegradation by microorganisms depended on a number of factors such as physical, chemical and biological agents of treatment, along with particular enzymes involved in the degradation of the polymer, the metabolic pathways responsible for the up taking the plastic fragments (Wilkes and Aristilde, 2017).

It was reported by Furst *et al.*, 1997 that common fungi participate in the biodegradation of plastics and similar results were obtained by El Shafei *et al.*, (1998). In the recent year's microorganisms have been studied for their contribution in the degradation of plastics due to their ability to survive under various conditions and their abundance in different habitats where they are able to form stable consortia, exchange genetic material, mutate, accumulate of nutrients and protect against toxic substances (Rummel *et al.*, 2017), these properties can be exploited in the degradation of plastic. Filamentous fungi have been reported to degrade polymers into simple compounds by using them as a carbon source (Kyaw *et al.*, 2012).

Thermoplastic such as Low-Density Polyethylene (LDPE) are low density plastics that ranges from 0.910-0.940 gm/cm³, non-reactive at room temperature and can withstand 80°C continuously and 95°C for a short period of time (Pramila *et al.*, 2011). LDPE is the one of the most abundant commercially produced polymers that accounts for about 60 % of the total plastic production.

They are resistant to biodegradation due to its long carbon chains (Contat-Rodrigo and Ribes Greus, 2002). Under normal environmental conditions it takes almost 10 decades to mineralize the polymers (Otake *et al.*, 1998). The use of polythene has increased over the years and its degradation is becoming a great challenge. Only a fraction of this polythene waste is recycled, and the others take hundreds of years to degraded (Barnes *et al.*, 2009).

Biodegradation by utilizing effective microorganism is an attractive alternative for waste disposal and potentially much more efficient and does not produce secondary pollutants. In certain situations, it may even be possible to obtain useful end products with economic benefit from microbial metabolism of pollutants such as ethanol for use in biofuels (Iranzo, *et al.*, 2001).

There has not been any convention methods developed for biodegradation on a commercial scale. However, research is still being conducted on the effectiveness in the biodegradation of polymers, using the metabolic potential of microorganisms, it is expected that it is not long before feasible biodegradation processes are developed. Microorganisms are capable of degrading the organic and inorganic materials and hence there is a lot interest in the microbial degradation of plastics and wastes (Raaman *et al.*, 2012).

Materials and Methods

Collection of soil sample from plastic dumping sites

For collection of soil samples, the glass vials were sealed with aluminum foil and autoclaved at 121°C, 15 psi for 30 minutes. The soil samples were collected 10 cm deep from the ground (Raaman *et al.*, 2012) in the

sterilized vials mainly from three different sites of Patna. The samples were brought to the laboratory safely.

Isolation of fungi from collected soil sample and their identification

The dilution of each soil sample was prepared and was inoculated by spread plate method on the PDA media. The plates were kept at 26°C±0.2 for 24-48 hours in the incubator. The colonies were observed and counted. The fungal colonies were identified by studying their colony characteristics as well as microscopic characteristics by performing the Lacto phenol cotton blue staining. The macroscopic characteristics were studied by observing the colony characteristics like, colony shape, colony margin, colony color etc. The fungal colonies were picked up by the help of sterilized inoculating needle and placed on the slide, one drop of LPCB stain was added on the slide and the teasing of the fungal culture was done gently and cover slip was placed on the slide carefully allowing no air bubble to form under the cover slip. The slides were observed under the microscope at 10x and 40x magnifications.

Screening of the polythene degrading fungi and the maintenance of pure culture

The screening of LDPE degrading fungi was done by performing the clear zone formation test. Clear zone formation test is a semi quantitative approach to screen microorganisms able to depolymerize the polymers (Nishida and Tokiwa, 1993). For this MSM agar with 2g of PEG powder added to it was prepared. The fungal cultures were streaked on the MSM agar and incubated at 26°C±0.2 for 24-36 hours in duplicates. The fungal cultures able to form the clear zone around their colonies were selected as potential isolates and these cultures were maintained both on PDA and SDA media.

Clear zone formation test is a semi quantitative approach to screen microorganisms able to depolymerize the polymers (Nishida and Tokiwa, 1993).

Biodegradation of LDPE by selected isolates

For biodegradation of LDPE, MSMB was prepared in the Erlenmeyer flasks and all the flasks containing the MSMB were inoculated with pre weighed LDPE sheets cut in the dimensions 1.5cm x 1.5cm and sterilized with 70% ethanol for 5 minutes (Mukred *et al.*, 2008) and then thoroughly rinsed with distilled water and two loops full of fungal cultures separately.

A control flask was also maintained with only LDPE sheet inoculated in the MSMB. All the flasks were incubated in the shaker incubator at 30°C and 120 rpm for one month.

Weight reduction analysis of LDPE degraded by fungal isolate

After the end of the incubation period the flask were removed from the shaker incubator and the LDPE sheet was filtered out of the media, was washed with 70% ethanol for 5 minutes and then with distil water and dried in air. The final weight of the LDPE sheets was taken and the weight reduction percentage (refer to Table 3) was calculated by the formula,

Weight reduction (%) = (Initial weight – Final weight) × 100 / Initial weight (Munir *et al.*, 2018)

Measure of change in the pH before and after the incubation

The change in the pH of the media was measured before and after the incubation of the media inoculated with the fungal isolate

containing the LDPE sheets incubated in the shaker incubator at 30°C and 120 rpm for 15days and 30 days respectively.

SEM analysis of the fungal degraded LDPE sheet

The SEM (Raaman *et al.*, 2012)for the degraded LDPE sheet was performed at IIT Patna, Bihta. The samples were carried in to the SEM lab in the insulated bags, then the samples were taken out by the help of an aseptic forceps and were placed in the coating machine and finally the gold plating of the sample was done after which the specimen was placed on the stage of the scanning electron microscopy and was fixed properly and was connected to the computer system, after which the magnification, focus, fine resolution and the working voltage was set up with the help of the stage controller and finally the images were scanned.

Results and Discussion

Isolation of fungi from soil samples collected from plastic polluted sites in Patna

The fungal isolates were obtained from the soil samples collected aseptically from plastic polluted sites of Patna mainly from the industrial area of Patliputra, stadium area of Rajendranagar and plastic dumping sites near PMCH.

The samples were brought to the laboratory under sterilized condition and after plating and incubation following observation was made. After studying the macroscopic characteristic (Table 1) and microscopic characteristics of the fungal colonies the isolates may be identified as *Aspergillus* sp, *Rhizopus* sp., *Penicillium* sp (Fig. 1 and 2) and *Mucor* sp. (Aneja, 2003), (Vaughan Gwynne H.C.I & Barnes, 2004).

Screening of polythene degrading fungi from the isolates obtained

The fungal colonies isolated and identified were streaked on MSM agar media supplemented with PEG powder and incubated at 26°C for 7 days in the incubator. Clear zone formation was seen in *Aspergillus*, *Rhizopus* and *Penicillium*. No zone was observed in case of *Mucor* sp (Table 2). Further, based on the results obtained the screening of the fungal isolates was done. *Penicillium* sp. Which showed maximum clear zone formation (Figure 3) was selected to study the ability to degrade LDPE sheets

Weight reduction analysis of LDPE degraded by fungal isolate

The final dry weight of the LDPE sheets placed into the MSM broth inoculated with the fungal culture was taken after incubation and the percentage weight reduction was calculated. As compared to the control the LDPE sheets showed a significant reduction of 20% after 15 days of incubation and 50%

weight reduction after 30 days of incubation with *Penicillium* species (Table 3).

Measure of change in the pH

The change in the pH of the media was measured before and after the incubation of the media inoculated with the fungal isolate and containing the LDPE. As compared to the control it was observed that there was a reduction in pH of the media after incubation of 15 days from neutral to 6.8. However, no further reduction was observed from 15 to 30 days (Table 4).

From the data provided it can be inferred that there was a drop in the value of pH after the incubation of the media with the fungal cultures. Similar results were obtained by Das. M.P *et al.*, who studied the degradation of LDPE by *Aspergillus* sp. and *Fusarium* sp. and observed a shift of the pH from neutral to acidic after incubation of the MSM broth with fungal cultures.

Table.1 Characteristics of fungal colonies isolated and identified on PDA media

Isolate	Colony Morphology					Microscopic Characteristics
	Texture	Margin	Colour		Elevation	
			Front	Back		
<i>Aspergillus</i> sp.	Cottony	Irregular	Black	Off White	Raised	Presence of Vesicles, Conidiophores, Conidia present in chains.
<i>Rhizopus</i> sp.	Cottony	Regular	White	Off White	Raised	Rhizoids, sporangiospore, Sporangia and spores present.
<i>Penicillium</i> sp	Cottony	Regular	Green	Creamy	Raised	Presence of chain of conidia bearing conidiophores, branched, brush like appearance
<i>Mucor</i> sp	Cottony	Irregular	White	Off White	Raised	Sporangium and sporangiospore present, no rhizoids seen

Table.2 Diameter of clear zone formation by the fungal isolates on MSM agar supplemented with PEG

Isolates	Clear zone formation	Diameter (cm)
<i>Aspergillus sp.</i>	+	1.1
<i>Rhizopus sp.</i>	+	1.1
<i>Penicillium sp.</i>	+	1.4
<i>Mucor sp.</i>	-	0.0

Table.3 Percentage weight reduction of LDPE sheets placed in MSM broth inoculated with *Penicillium*

Sample Polymer used	Isolate	Initial Weight	Final Weight		Percent weight reduction	
			15 days	30 days	15 days	30 days
LDPE Sheets	<i>Penicillium sp.</i>	30mg	24mg	15mg	20.0%	50.0%
LDPE Sheets	Control	30mg	30mg	30mg	0	0

Table.4 Change in the pH of the media after incubation of the media inoculated with the fungal isolate and containing the LDPE

Isolate inoculated in the media	pH before inoculation	pH after inoculation	
		15days	30days
<i>Penicillium sp.</i>	7.0	6.8	6.8
Control	7.0	7.0	7.0



Figure.1 Cultural characteristics of *Penicillium sp.* on PDA media



Figure.2 Microscopic view of *Penicillium sp.* under 40x magnification



Figure.3 *Penicillium sp.* showing maximum clear zone around colony growth

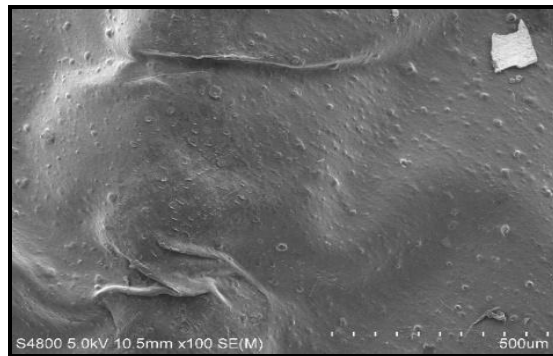


Figure.4 SEM image for control LDPE sheet showing no degradation

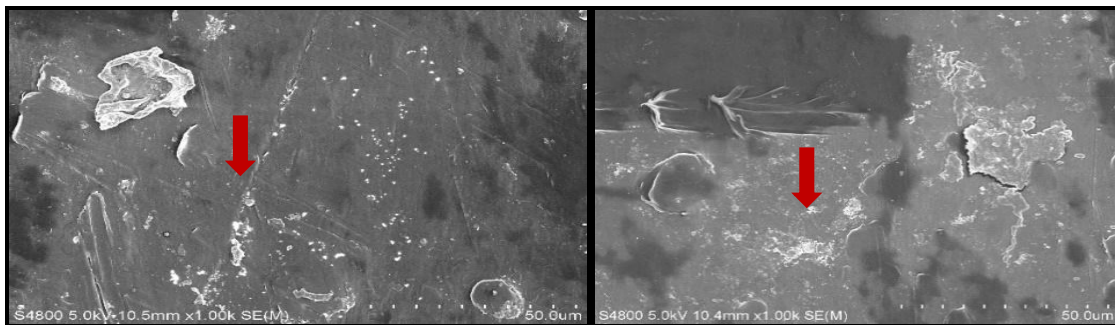


Figure.5 (a) SEM images showing degradation of LDPE sheet on two different sites by *Penicillium sp.* after 15 days

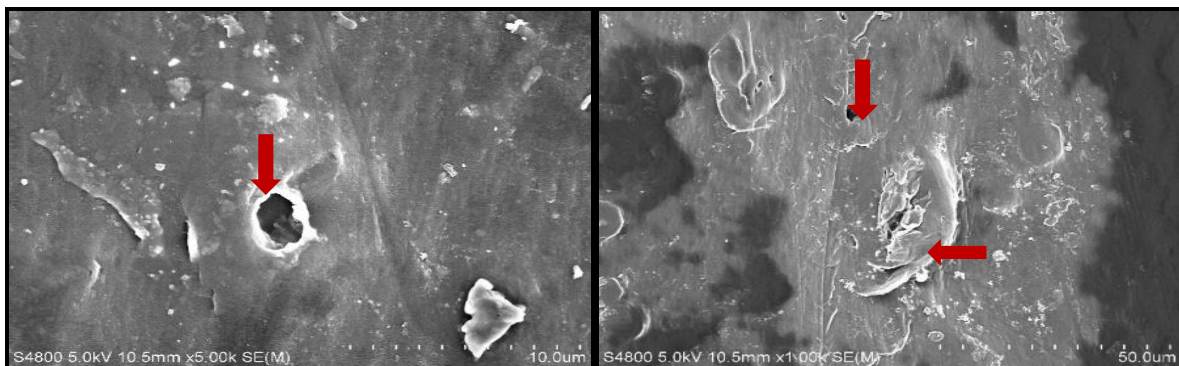


Figure.5 (b) SEM images showing degradation of LDPE sheet on two different sites by *Penicillium sp.* after 30 days

SEM analysis of the fungal degraded LDPE sheets

The LDPE sheet, which was degraded by *Penicillium sp.* showed significant degradation (Figure 5a and 5b) indicated by the presence of porosity and fragility on the surface of the LDPE sheets over the time period of 15 days and 30 days as compared to the control LDPE sheet (Figure 6). By comparing the images obtained from the SEM, it can be said that *Penicillium sp.* has the ability to perform degradation of LDPE sheet under suitable condition.

Thus the study focused on the effect of fungi isolated from different plastic polluted sites on low density polyethylene (LDPE) material degradation with reference to SEM analysis

The most efficient stain was identified as *Penicillium sp.* from plastic dumping sites. The isolate was selected based on its ability to form a clear zone around them in the media containing polymer (PEG) and was used further to check its ability to degrade LDPE sheets. The ability of this isolate to degrade the LDPE sheets was studied during the course of 15 and 30 days after inoculating the fungal isolate in MSMB with pre weighed LDPE sheets under the laboratory conditions.

The biodegradation of LDPE sheets was determined by the weight reduction. Finally, after the end of the incubation period the percent weight reduction of the LDPE sheets were calculated, and it was found that *Penicillium sp.* showed weight reduction up to 20% and 50% after 15 and 30 days respectively. Finally, the degradation of LDPE sheets was confirmed by performing the scanning electron microscopy which showed porosity and fragility on the surface of the sheets, thereby concluding that *Penicillium sp.* has the ability to perform degradation of LDPE sheet.

These observations can be helpful to find solution to degrade plastic in an eco-friendly manner by further optimizing the process.

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