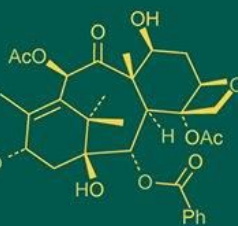
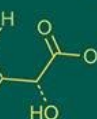


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Effect of potential fungi on low density polyethylene isolated from municipal solid waste with reference to scanning electron microscopy analysis

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Abstract

Polyethylene makes up a significant 64% of all synthetic plastics and is extensively utilized in the production of various items which leads to accumulation in environment and natural resources. Among numerous approaches to mitigate the accumulation of plastic waste bioremediation stands out as the most ecofriendly method. The aim of present study is the detection of a potential polyethylene degrading fungi for management of municipal waste. A total of thirty one fungi were isolated on screening media using low density polyethylene powder as sole carbon source. These isolates were then subjected to *in vitro* biodegradation studies using low polyethylene films for sixty days. Three fungal isolates (MF-14 MF-16 and MF-20) that exhibited more than 10% weight loss were further screened at varied pH conditions (3.5, 7, and 9.5) to assess the percent weight loss in the treated polyethylene strips with continuous shaking for sixty days. Maximum percent weight loss ($37.66 \pm 06.24\%$) was recorded with MF-16 at pH 3.5. A decrease in the tensile strength of the polyethylene strips was also observed with MF-16 showing the maximum percent loss in tensile strength ($92.30 \pm 06.98\%$). On the basis of 18S rRNA gene sequencing the taxonomic confirmation of fungal species revealed fungal isolate were of *Aspergillus* species. Biodegradation of polyethylene was further confirmed through Scanning Electron Microscopy (SEM) analysis.

Keywords: Low density polyethylene, biodegradation, environmental pollution, *Aspergillus*, polymerization

Introduction

Plastic is defined as a polymer that becomes malleable when heated and can therefore be molded into molds. Its composition comprises carbon hydrogen silicon oxygen chloride and nitrogen (Seymour, 1989) [27]. To date billions amount of plastic products produced that were finally discarded into the natural environment (Nielsen *et al.*, 2020) [16]. India produces approximately 12 million tonnes of plastics every year while 9205 tonnes were recycled per day (Kartikey *et al.*, 2016) [12]. Polythene makes up roughly 64% of the waste generated by synthetic polymers (Lee *et al.*, 1991) [14]. One of the most widely used materials in everyday life is polyethylene which is used to make shopping bags plastic bottles milk jugs films and bottles for toiletries (Romani *et al.*, 2019) [21]. Excessive production leads to accumulation of large amount of waste due to lack of proper disposal (Jambeck *et al.*, 2015) [11]. The accumulation of plastics in the environment due to their non-biodegradation nature results in widespread contamination (Yang *et al.*, 2021) [33]. The presence of polyethylene microplastics reduces the soil adsorption capacity and the particle size of aggregates (Hou *et al.*, 2021) [10]. Small plastic particles even accumulate around seed pores delaying the germination and growth of terrestrial vascular plants (Bosker *et al.*, 2019) [3]. Plastic in landfills takes thousands of years to decompose rendering the land unusable and endangering the health of its occupants (Sarker *et al.*, 2011) [25]. The literature describes three different forms of polymer degradation processes as photo-degradation thermo-oxidative degradation and biodegradation (Gweret *et al.*, 2015) [7]. The natural process of materials being broken down by microorganisms including fungi bacteria and algae is called biodegradation (Sangle *et al.*, 2012) [23]. The degradation of polyethylene involves several biochemical pathways that can be categorized into four main steps i.e bio-fragmentation biodegradation assimilation and mineralization.

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These processes rely on the actions of various active enzymes (Pathak, 2017) ^[17]. Hyphae produced by fungus cells have a high adhesive property on polyethylene surfaces and they can cling to nearly any kind of material (Sánchez, 2020) ^[22]. Microbial enzymes that lead to biodegradation of polyethylene are identified to be proteases, lipases, cutinases, laccases, manganese peroxidases, lignin peroxidases and alkane hydroxylases etc. (Ahmed *et al.*, 2018) ^[1]. To date, Fungi have been identified including *Aspergillus niger*, *Tricoderma viride*, *Paecilomyces varioti*, *Aspergillus terreus*, and *Penicillium onchrocloron* which have abilities to degrade low density polyethylene (Zeghal *et al.*, 2021) ^[34]. This study's main objective is to isolate and identify potential fungal population that have the capability to break down polyethylene. The research work focuses on evaluating the potential of fungi to degrade Low-Density Polyethylene which has been isolated from the soil of municipal waste disposal site. The results of this study are compared with those of other previously published research projects on the deterioration of plastic.

Materials and Methods

Survey and collection of polyethylene samples

Two municipal waste disposal sites Kot Balwal (Lat. 32.7266°N and Lon. 74.8570°E) and Bagwati Nagar (Lat. 32.7287°N and Lon. 74.8368°E) located in Jammu district of UT of Jammu & Kashmir India were surveyed for the collection of partially degraded polyethylene samples. At a depth of ten to fifteen cm the partially degraded plastic samples with adherent soil were collected in sterile zip-lock bags from the Kot Balwal and Bagwati Nagar municipal waste disposal sites. These samples were properly labelled sealed in zip lock bags and then transported to the laboratory and stored at a temperature of 4°C for further processing within 24 hours of collection.

Preparation of low density polyethylene powder

Low density polyethylene sheets were purchased from local market and were cut into small pieces (2×2 cm) and were immersed in xylene. These pieces were brought to a boil and allowed to dissolve entirely which took 5 to 15 minutes. A blender set to 3000 rpm was used to crush the residue that resulted. Two or three ethanol washes were performed on this crushed residue in order to remove any leftover xylene. The obtained polyethylene powder was kept for evaporation by using ethanol and dried overnight in a hot air oven at 60°C. Finally, the low density polyethylene powder was stored at room temperature (Das and Kumar, 2015) ^[5].

Isolation of polyethylene degrading fungi

One gram of soil sample from municipal waste site was suspended in nine millilitres of sterile distilled water. This suspension was serially diluted upto 10⁻⁴ and 10⁻⁵ after being incubated for ten minutes. In order to isolate the fungi capable of degrading low density polyethylene powder the spread plate technique was applied on synthetic medium agar plates containing 0.1% of low density polyethylene powder. The synthetic medium had the following composition with a pH of 7.0: Dipotassium phosphate (K₂HPO₄) 1 g; Potassium dihydrogen phosphate (KH₂PO₄) 0.2g; Sodium Chloride (NaCl) 1 g; Calcium chloride dihydrate (CaCl₂·2H₂O) 0.002g; Ammonium sulfate ((NH₄)₂SO₄) 1 g; Magnesium sulfate heptahydrate (MgSO₄·7H₂O) 0.5 g; Copper(II) sulfate pentahydrate

(CuSO₄·5H₂O) 0.001 g; Zinc sulfate heptahydrate (ZnSO₄·7H₂O) 0.001 g; manganese(II) sulfate monohydrate (MnSO₄·H₂O) 0.001 g and ferrous sulphate heptahydrate (FeSO₄·7H₂O) 0.01 g and Agar 15 g). The plates were then incubated at 25 °C for seven days. The pure fungal isolates were subcultured on potato dextrose agar and then preserved in slants at 4 °C (Das and Kumar, 2013) ^[4].

Biodegradation studies of polyethylene degrading fungi

After being preweighed and sliced into 2 × 2 cm pieces low density polyethylene films were cleaned with distilled water and acetone dried and then weighed with a weighing scale. After being exposed to UV light for 15-20 minutes in a laminar airflow the low density polyethylene films were sterilized. To allow for fungal biodegradation 50 millilitres of potato dextrose broth were added to each flask. In the microbe-free media low density polyethylene films were used to maintain control. To each flask one millilitre inoculum of two-day-old fungal isolates was added. Each treatment was kept in a different flask. Following inoculation all of the fungal isolates were shaken for sixty days at room temperature on an orbital rotary shaker at a speed of 140±20 rpm at room temperature. After the isolates had been incubated for sixty days the low density polyethylene films were carefully removed using tweezers and cleaned with 70% ethanol and sterile distilled water. The film was air dried at ambient temperature for 24 hours prior to analysis. The screening of polyethylene degrading fungal isolates was carried out assessing them based on the percentage of weight loss and the percentage decrease in tensile strength of the polyethylene (Sangle *et al.*, 2019) ^[24].

Determination of percent weight loss

After 60 days of incubation, the degraded low density polyethylene films were removed aseptically from each flask and put in separate petridishes and then cleaned with 70% ethanol and distilled water. Following washing 40 °C overnight oven drying was performed on all low density polyethylene films. The dried low density polyethylene films were then weighed and the formula below was used to get the percent weight loss:

$$\% \text{ Weight loss (W)} = \frac{\text{Initial weight (X)} - \text{Final weight (Y)}}{\text{Initial weight (X)}} \times 100$$

where X is the average initial weight (mg) and Y is the average final weight after sixty days of incubation.

Three fungal isolates that have the potential to degrade low density polyethylene films based on percentage weight loss (greater than 10% weight loss) were chosen for a repeat of the polyethylene biodegradation experiment in order to verify the results' repeatability based on different pH. The three potential polyethylene degrading fungal isolates (using low density polyethylene films + potato dextrose broth) were incubated in triplicate on a rotary shaker at room temperature at varied pH levels (3.5 7.0 and 9.5) along with the control (using polyethylene films + potato dextrose broth). Following sixty days of incubation the percentage of weight reduction in low density polyethylene films was determined. (Shahnawaz *et al.*, 2016; Sangle *et al.*, 2019) ^[28, 24]

Determination of percent tensile strength loss

Following a sixty days incubation period the reduction in the tensile strength (N/mm²) of the low density polyethylene films degraded by the potential fungal isolates at varied pH levels (3.5, 7.0, and 9.5) was measured using the American Society for Testing and Materials (ASTM 2012) method and the Universal Tensile Strength Testing Machine. The following formula was used to get the percentage reduction in tensile strength (% loss in tensile strength)

$$\text{Percent tensile strength loss} = \frac{\text{Initial tensile strength (X)} - \text{Final tensile strength (Y)}}{\text{Initial tensile strength (X)}} \times 100$$

where X is the average tensile strength of the untreated low density polyethylene films before the experiment (N/mm²) and Y is the average tensile strength of treated low density polyethylene films after sixty days. Following a sixty days incubation period the percentage of tensile strength loss of low density polyethylene films was calculated. (Shahnawaz *et al.*, 2016) [28]

Confirmation of polyethylene degradation by most efficient fungal isolates using Scanning electron microscopy (SEM) analysis

Scanning electron microscopy was performed on low density polyethylene films that had undergone sixty days of incubation with fungal isolates and exhibited both the maximum percent weight loss (after sixty days of incubation) the maximum percent loss in tensile strength (after sixty days of incubation) at varied pH levels (3.5, 7.0, and 9.5) and untreated polyethylene films (after sixty days of shaking in potato dextrose broth medium as a control). The samples were cleaned using a 2% (v/v) aqueous solution of sodium dodecyl sulphate (SDS) and rinsed with distilled water for scanning electron microscopy analysis. The prepared samples were covered in a layer of gold for forty seconds after being placed on the scanning electron microscopy sample stub using carbon tape. Finally high-resolution scanning electron microscopy (SEM) was employed for the analysis of degraded polyethylene films (Das and kumar, 2015) [51].

Morphological and Molecular Identification of fungal isolates

Standard identification methods including colony shape microscopic examination and lactophenol cotton blue (LPCB) staining were used to identify different fungal strains. Microscopically the structure of fungal hyphae and conidophore was observed. Molecular identification of most efficient fungal isolates was done by 18S rRNA sequencing method (Gontia *et al.*, 2014) [8]. Using a GeneAmp PCR system 9600 (Applied Biosystems) a total reaction volume of 40 µl was achieved by utilizing 1 µl Taq Polymerase (Applied Biosystems) 1 µl of forward and reverse primers at 10 pM each 27 µl deionized water 8 µl PCR buffer comprising dNTPs and MgCl₂ and 2 µl DNA template. The primer pairs F-566:5' and R-1200 respectively amplified the segments of the V4 and V5 sections which was approximately 650 bp long (Hadziavdic *et al.*, 2014) [9]. The polymerase chain reaction results were seen by electrophoresis on a 1% agarose gel with an instant addition of ethidium bromide. SeqMan Pro was used to combine the forward and reverse sequence files (Nayariseri *et al.*, 2013) [15]. The sequences obtained were compared to the sequences

contained in the NCBI database using the basic local alignment tool (BLAST). Following an alignment of the strains' 18S rRNA gene sequences with those of their closest neighbours the isolates were identified using the blast score and the percentage homology of the 18S sequences.

Results and Discussion

Isolation of polyethylene degrading fungi

In the current study, polyethylene-degrading microbial populations were isolated from partially disintegrated plastic samples with adhering soil in synthetic media using low density polyethylene films as the sole carbon source for sixty days. There were eighteen fungal isolates obtained from the Kot Balwal dumping site and thirteen fungal isolates obtained from the Bagwati Nagar dumping site for a total of thirty one fungal isolates from two municipal waste disposal sites. In prior study conducted by Sangle *et al.*, (2019) [24] 109 fungal isolates were recorded from rhizosphere soil from 12 eco-geographical regions of west coast of India.

Assessment of Percent weight loss

On the basis of percent weight loss of low density polyethylene films three fungal isolates MF-8 MF-14 MF-16) from Kot Balwal dumping site and two fungal isolates from Bagwati Nagar site (MF-20 MF-29) has shown more than 10% weight loss biodegradation activity (Table 1 & 2) and were further chosen for additional investigation to study the biodegradation activity at different pHs. However the percentage weight reduction recorded in potential fungal isolates was 25.66% (MF-14) 27.33% (MF-16) and 24.66% (MF-20) (Table 1). After sixty days of incubation percent weight loss of polyethylene films was calculated at different pH (3.5 7.0 and 9.5). Fungal isolates MF-14 and MF-16 showed their maximum biodegradation activity (29.66 + 03.81% and 37.66±06.24%) at pH 3.5 and MF-20 (24.66±02.01%) showed their maximum biodegradation at pH 7.0 (Table 3). All the potential fungal isolates showed their minimum biodegradation activity at pH 9.5. Numerous promising studies yielding similar results have been conducted to identify potential Fungi in order to enhance the rate of plastic degradation have been reported. Following a two-month testing period, the polyethylene yielded results of 7.5% (weight loss) with *Rodococcus ruber* (C208) (Sivan *et al.*, 2006) [29], and 6.657% weight loss (unheated) with a mixture of *Penicillium frequentans* and *Bacillus mycoides* (Seneviratne *et al.*, 2006) [26] and 21.87±6.37% weight loss VASB1/WL (Shanawaz *et al.*, 2016) [27]. The fungal consortium comprising of *Aspergillus oryzae*, *Aspergillus niger*, and *Aspergillus flavus*, exhibited maximum weight reduction of 26.15% (D'Souza *et al.*, 2021) [6].

Assessment of percent tensile strength loss

In the current study, using a universal tensile strength testing machine the percent tensile strength loss (N/mm²) of the low density polyethylene films that were deteriorated by potential fungal isolates at varied pH (3.5, 7.0 and 9.5) for sixty days was determined. The fungal isolate MF-16 showed maximum% loss in tensile strength (92.30%±06.98) of the low density polyethylene films followed by MF-20 (84.61%±05.17) and MF-14 (82.05%±03.14)(Table 4). Similar results were observed by Kyaw *et al.* (2012) [13] demonstrated 75% loss in tensile strength loss of low density polyethylene films after two

months of incubation with *P. aeruginosa* PAO1. Vijaya and Reddy (2008) [32] adhered to the ASTM standard while examining the degradation of high-density polyethylene high density polyethylene film through composite testing with municipal solid waste. Their study revealed a substantial reduction of 20% in high density polyethylene film tensile strength after one year of testing.

Scanning electron microscopic analysis of low density polyethylene film

The surface of untreated low density polyethylene film as control was smooth and free of fissures holes or pits (Figure 1a). In contrast SEM images showed that the surfaces of polyethylene films that had been degraded by the most effective fungal isolates (MF-14 MF-16 and MF-20) had holes cracks abrasions and pores (Figure 1). This was conclusive proof that the polyethylene had been biodegraded. Similar results were also recorded by Shahnawaz *et al.* (2016) [28] where Scions/crakes on the surface of the degraded polythene were observed by scanning electron microscopy analysis. Ren *et al.* (2019) [20] used scanning electron microscopy was used to examine the polyethylene film in detail and found that it had rough, cracked, and depressed surfaces that confirmed the biodegradation of polyethylene.

Morphological and molecular identification of fungal isolates

The three most efficient fungal isolates (MF-14 MF-16 and MF-20) were morphological and microscopically examined using lacto phenol cotton blue staining. All the three fungal isolates were identified as *Aspergillus* species (Figure 2 & Figure 3). similar study conducted by Raaman *et al.*, (2012) [17] where on the basis of staining LDPE degraded fungi were identified as *Aspergillus niger*, *Aspergillus japonicus*, *Aspergillus terreus*, *Aspergillus flavus*, and *Mucor sp.* On the basis of 18s rRNA gene sequencing the taxonomic confirmation of fungal species revealed fungal isolate MF-14 as *Aspergillus fumigatus* MF-16 as *Aspergillus flavus* and MF-20 as *Aspergillus fumigates*. Among the fungal species, members of *Fusarium* (Sivan, 2011) [30], *Chaetomium* and *Pullularia* (Sudhakar *et al.*, 2008) [31], *Penicillium*, *Zalerion* (Raddadi, 2019) [19] were known for biodegradation of low density polyethylene and HDPEs. On the basis of molecular identification process several fungal species were identified as *Aspergillus flavus*, *Eurotium repens*, *Mucor mucedo*, and *Rhizopus stolonifer* (Aiyeni *et al.*, 2022) [2].

Conclusion

The ability of fungal species to degrade polyethylene was thoroughly explored utilizing low density polyethylene as their carbon source in the current research effort. Subsequent analysis also proved the biodegradation potential of the fungal isolates. It draws attention to the possibility for naturally occurring fungi to assist in the biodegradation of municipal garbage especially polymers like various varieties of polyethylene which are renowned for their resistance to decomposition. Such research efforts can help to lessen the environmental impact of polyethylene trash and offer insightful information about more sustainable waste management techniques.

Table 1: Percent weight loss of low-density polyethylene films by fungal isolates isolated from Kot Balwal dumping ground after sixty days of incubation

S. No	Culture code	Initial weight (mg)	Final weight (mg)	Weight loss (%)
1.	MF-1	0.0300	0.0300	00.000
2.	MF-2	0.0300	0.0300	00.000
3.	MF-3	0.0300	0.0300	00.000
4.	MF-4	0.0300	0.0299	00.330
5.	MF-5	0.0300	0.0299	00.330
6.	MF-6	0.0300	0.0298	00.660
7.	MF-7	0.0300	0.0299	00.330
8.	MF-8	0.0300	0.0271	09.660
9.	MF-9	0.0300	0.0299	00.330
10.	MF-10	0.0300	0.0300	00.000
11.	MF-11	0.0300	0.0300	00.000
12.	MF-12	0.0300	0.0297	01.000
13.	MF-13	0.0300	0.0297	01.000
14.	MF-14	0.0300	0.0223	25.660
15.	MF-15	0.0300	0.0298	00.660
16.	MF-16	0.0300	0.0218	27.330
17.	MF-17	0.0300	0.0300	01.000
18.	MF-18	0.0300	0.0294	02.000

Table 2: Percent weight loss of low density polyethylene films by fungal isolates isolated from Bagwati Nagar dumping ground after sixty days of incubation

S. No	Isolates	Initial weight (mg)	Final weight (mg)	Weight loss (%)
1.	MF-19	0.0300	0.0300	00.000
2.	MF-20	0.0300	0.0226	24.660
3.	MF-21	0.0300	0.0298	00.660
4.	MF-22	0.0300	0.0300	00.000
5.	MF-23	0.0300	0.0300	00.000
6.	MF-24	0.0300	0.0299	00.330
7.	MF-25	0.0300	0.0299	00.330
8.	MF-26	0.0300	0.0299	00.330
9.	MF-27	0.0300	0.0300	00.000
10.	MF-28	0.0300	0.0300	00.000
11.	MF-29	0.0300	0.0274	08.660
12.	MF-30	0.0300	0.0299	00.330
13.	MF-31	0.0300	0.0300	00.000

Table 3: Percent weight loss of low-density polyethylene films (mg) by bacterial and fungal cultures at different pH after sixty days of incubation

Fungal isolates	Percent weight loss of low-density polyethylene films by bacterial and fungal cultures at different pH (3.5, 7.0, 9.5)		
	3.5	7	9.5
MF-14	29.66±03.81	22.66±02.64	09.33±01.23
MF-16	37.66±06.24	27.33±01.42	09.33±01.17
MF-20	21.66±01.48	24.66±02.01	08.66±01.18

Table 4: Percent tensile strength loss of low-density polyethylene films with three potential polyethylene degrading bacterial and fungal cultures in N/mm² after sixty days of incubation

S. No	Fungal isolates	Initial strength (N/mm ²)	Final strength (N/mm ²)	Percent tensile strength loss (%)
1.	MF-14	01.95±00.00	00.35±00.23	82.05±03.14
2.	MF-16	01.95±00.00	00.15±00.14	92.30±06.98
3.	MF-20	01.95±00.00	00.3±00.43	84.61±05.17

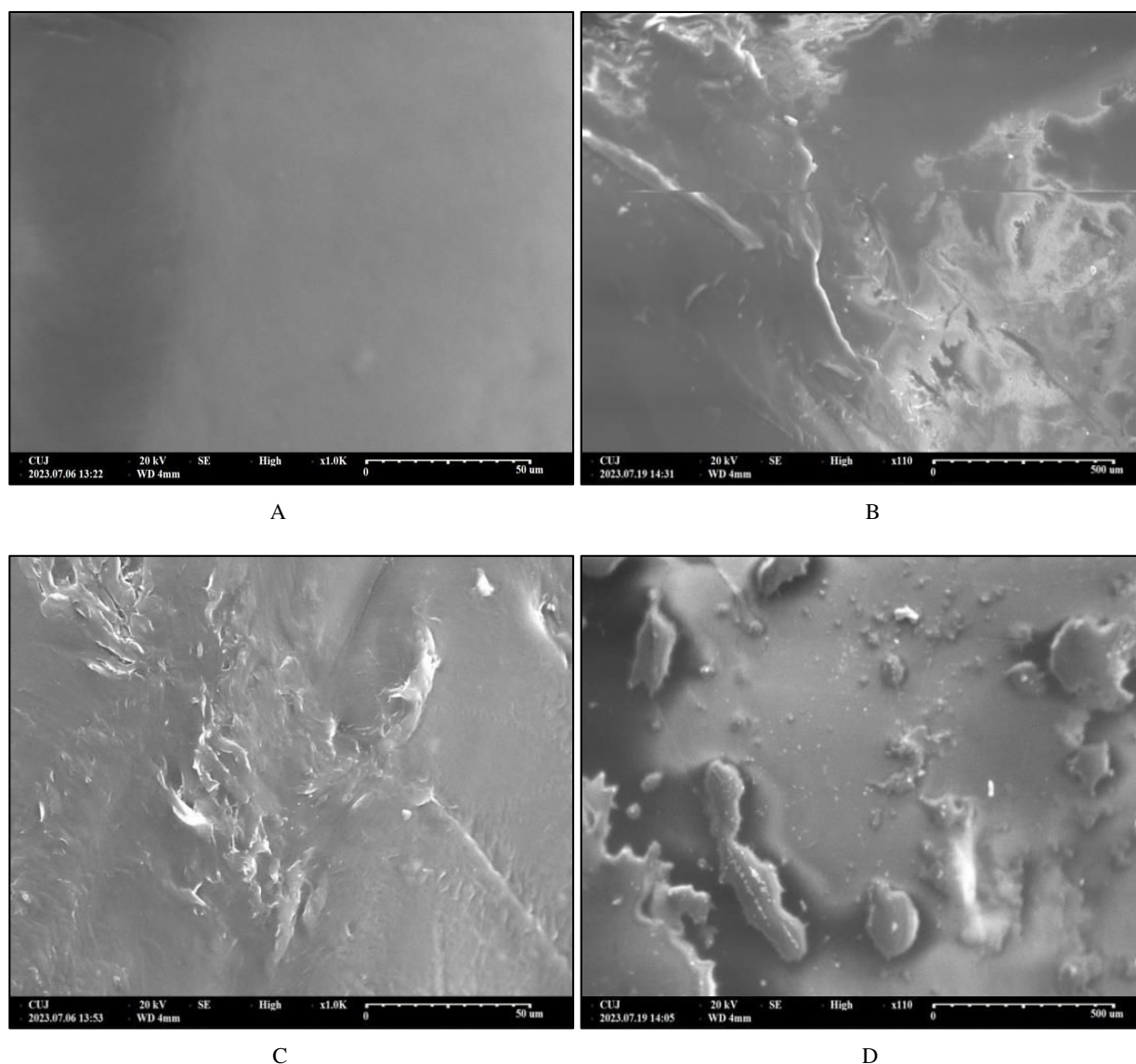
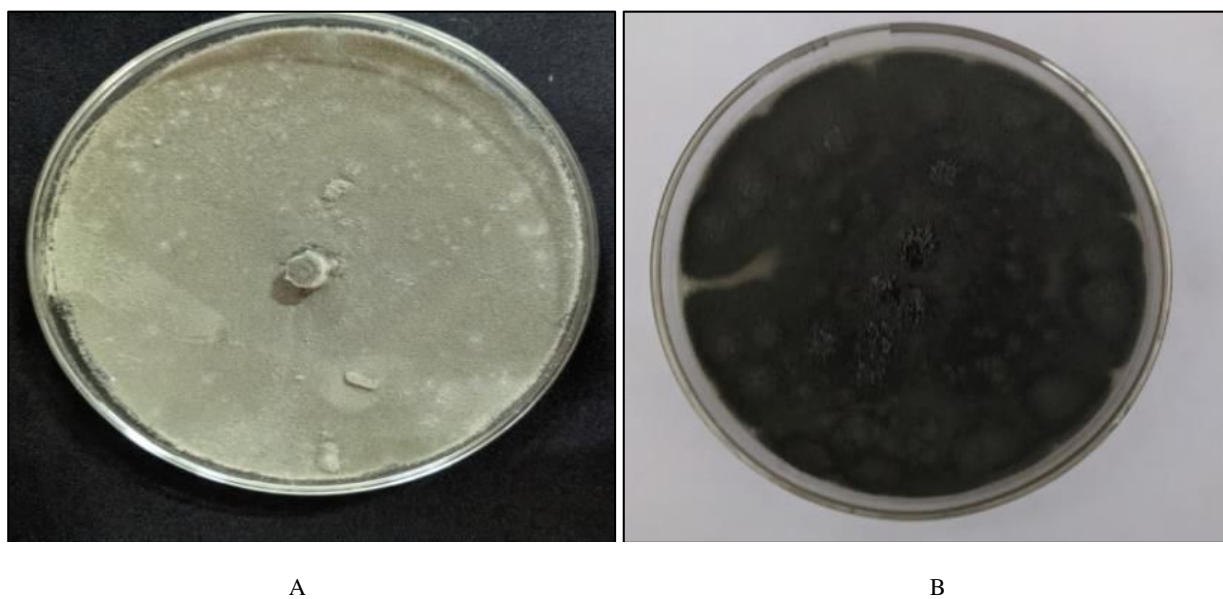
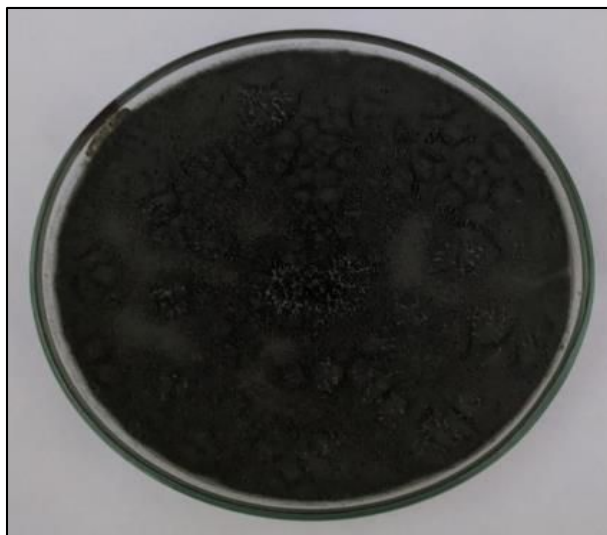


Fig 1: Scanning electron microscopy (SEM) images of the polyethylene biodegradation by most potential fungal isolates after sixty days of incubation

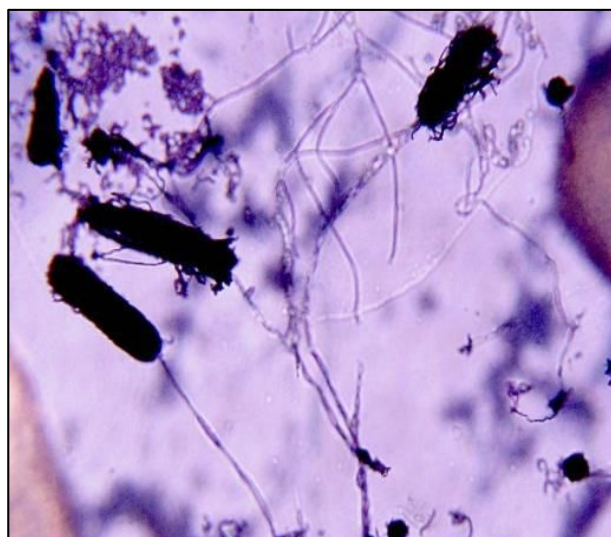
- A. Control-untreated polyethylene film
- B. Low density polyethylene film biodegradation by fungal isolate MF-14
- C. Low density polyethylene film biodegradation by fungal isolate MF-16
- D. Low density polyethylene film biodegradation by fungal isolate MF-20





C

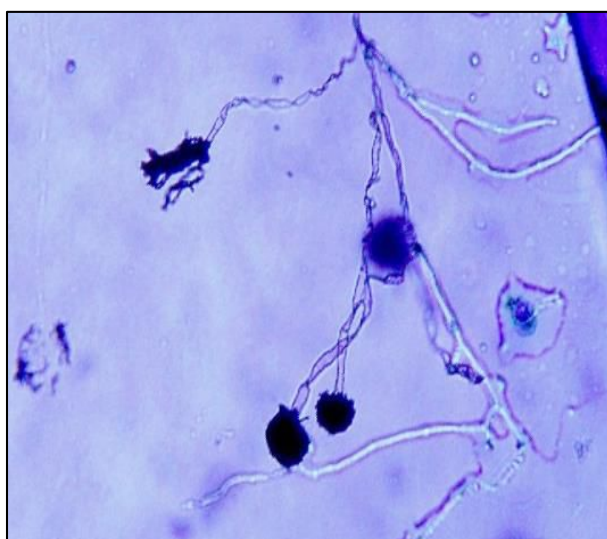
Fig 2: Colony morphology of three most efficient fungal isolates (A) MF-14 B) MF-16 C) MF-20)



A



B



C

Fig 3: Microscopic examination three most efficient fungal isolates (A) MF-14 B) MF-16 C) MF-20)

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