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Minimal Conditions to Degrade Low Density Polyethylene by *Aspergillus terreus* and *niger*

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ABSTRACT

Plastics pollution is a major worldwide concern because there is strong evidence about marine influence on the human trophic chain. Thus, we experimented with the biodegradation of low density polyethylene (LDPE) by *A. niger* and *A. terreus* in order to increase the degradation rate without any co-substrate or photothermal treatment. Our contribution is to show how to degrade LDPE under minimum nutritional conditions using both LDPE and sucrose as carbon sources. Up to 30% weight loss was obtained by *A. niger* and *A. terreus* which were isolated from an Ecuadorian mangrove. The evidence of cracks and biomass growth on the LDPE surface samples showed the potential of both fungi species to operate under low nutrient concentrations. The outlook of the present work was focused on understanding the fungi survival under minimal conditions.

Keywords: Laccase, plastics, polymer, SEM, Czapek

INTRODUCTION

Approximately 12.7 million of metric tons of plastics contaminate the ocean every year, and this amount is equivalent to around 4.6% of the total plastic waste generated from 192 coastal countries (Jambeck et al., 2015; Wilcox, Mallos, Leonard, Rodriguez, & Hardesty, 2016; Worm, Lotze, Jubinville, Wilcox, & Jambeck, 2017). It is estimated that up to 2.41 million tons of plastic currently flow from the global riverine system into the oceans every year (Lebreton et al., 2017). Mapping the plastic concentrations at oceans, it was revealed that the highest concentrations were found in sub-tropical latitudes, with the highest concentration on the North Atlantic gyre, amounting to 2,324 pieces/km^2 (Cole, Lindeque, Halsband, & Galloway, 2011). Meanwhile, the plastic production is still increasing up to almost 330 Mt per year worldwide (Amélineau et al., 2016; Worm et al., 2017). The major concern connected with plastics being dumped into oceans is that they fragment into particles that are ingested by small marine invertebrates (Jambeck et al., 2015). As a result, the annual dietary exposure for European shellfish accounts to 11,000 microplastics (<1 mm) per year (Van Cauwenberghe & Janssen, 2014). Thus, the impact of plastic pollution through ingestion and entanglement is known to affect at least 243 species of marine fauna, ranging from zooplankton to cetaceans, seabirds and marine reptiles (Worm et al., 2017). Besides, the absorbed toxic substances from plastics like plasticizers, coloring agents and flame retardants are transferred into the tissues and organs through ingestion, thus ending up in the food web (Bellas, Borerro, Martinez-Camara, Besada, & Martinez-Gomez, 2016; Eriksen et al., 2014). If global actions are not taken, the mismanaged plastic waste would seriously affect the biodiversity and the human health.

Synthetic polymers like low-and high-density polyethylene (LDPE, HDPE), polyvinyl chloride, polystyrene and polypropylene represent approx. 80% of the worldwide plastic waste (Pathak & Navneet, 2017). Specifically, the present work

focuses on LDPE, because it is a major cause of environmental pollution due to its high tensile strength, lightweight, resistance to water, and resistance to microbial attack (Esmaeili, Pourbabaee, Alikhani, Shabani, & Esmaeili, 2013; Ndahebwa Muhonja, Magoma, Imbuga, & Makonde, 2018). The degradation of plastics using microorganisms is being reported as a trend option because of the low energy consumption and further stabilization of the resulting biosolids. For biodegradation of plastic polymers, the most widely used bacterial strains are *Klebsiella pneumonia*, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas putida, Clostridium botulinum, Brevibacillus borstelensis, Ideonella sakaiensis* (Ahmed et al., 2018; Awasthi, Srivastava, Singh, Tiwary, & Mishra, 2017; Hadad, Geresh, & Sivan, 2005; Kyaw, Champakalakshmi, Sakharkar, Lim, & Sakharkar, 2012; Restrepo-Florez, Bassi, & Thompson, 2014; Tribedi & Sil, 2013a). The fungi which degrade synthetic polymers are *Rhizopus arrhizus, Penicillium simplicissimum, Aspergillus flavus, Penicillium funiculosum, Aspergillus niger, Penicillium funiculosum, Fusarium, Pestalotiopsis microspore, Curvularia senegalensis and Fusarium solani* (Bonhomme et al., 2003; Das & Kumar, 2014; Esmaeili et al., 2013; Hikmah, Setyaningsih, & Pangastuti, 2018; Ibrahim, Maraqa, Hameed, Saadoun, & Maswadeh, 2011; Jung, Yang, & Su, 2018; Leja & Lewandowicz, 2010). The degradation of synthetic polymers is performed by altering the chemical and physical properties of the polymer through enzymatic cleavage, mainly by laccases (Fujisawa, Hirai, & Nishida, 2001; Pathak & Navneet, 2017). The partial degradation of polyethylene is achieved after UV irradiation, thermal treatment or oxidation with acid; thus, these pre-treatments reduce the polymeric chain size and therefore the carboxyl, carbonyl, and hydroxyl groups are easily degraded by microorganisms (Sarmah & Rout, 2018).

The aim of this study was focused on polyethylene degradation, based on the fact that it represents nearly 64% of all the synthetic plastics produced (Tribedi & Sil, 2013). The following contributions are presented herein:

- Formulation of a culture media to growth *Aspergillus niger* and *terreus*, which were further used for the degradation of low density polyethylene (LDPE);
- Evaluation of the weight loss of LDPE as a result of the fungal degradation performed by *Aspergillus niger* and *terreus*, which were isolated from an Ecuadorian mangrove.

The significance of this research relies on a minimalistic perspective on the design of degradation strategies, in which neither LDPE thermal nor photo-oxidation pre-treatment were used.

METHODS

Sampling and isolation

The soil samples were collected at three points on the Santay Island mangrove (Guayaquil, Ecuador) as shown in *Figure 1*: coastal zone (P1), mid zone (P2) and landward zone (P3). Each sample of soil (1 Kg) consisted of 10 sub-samples, randomly collected from 10 different points over an

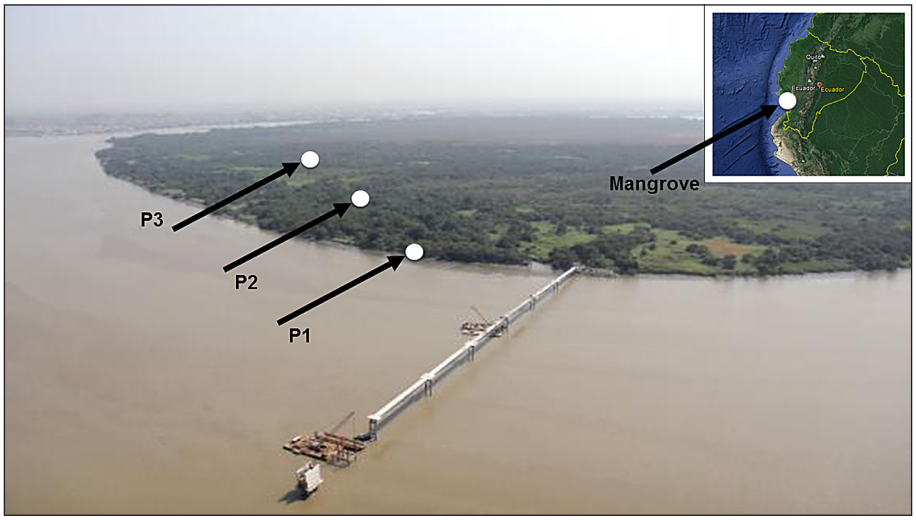


Fig. 1. Sampling points (P1, P2 and P3) where *Aspergillus niger* and *terreus* were isolated at the Santay mangrove in Guayaquil, Ecuador

area of approximately 10 m^2 at a depth of approx. 5 cm. After the sampling, a soil extract was prepared by pouring 20 g of the well-mixed soil sample into a 300 mL 1X PBS solution (8 g/L NaCl, 0.6 g/L KH_2PO_4, 1.2 g/L Na_2HPO_4 and 0.2 g/L KCl). A 1 mL of soil extract was poured into Petri dishes over Sabouraud agar (TM Media, India) as culture media, adding 0.5 mL of chloramphenicol to prevent the bacterial growth. After this inoculation, the Petri dishes were incubated at 37°C for 4 days. The organisms found were evaluated according to their morphological characteristics such as conidial heads, mycelial color, shape and roughness, using an optical microscope (Unico M280, USA) (McClenny, 2005). The organisms that were not of interest for the present work were discarded. The fungi colonies which presented morphological characteristics that were similar to the ones reported for *Aspergillus spp.* were isolated in Sabouraud agar medium for 7 days at 37°C.

Biodegradation of LDPE

The LDPE films with a thickness of 100 μm were cut into squares of 2 cm^2 and weighed using an analytical balance (Kerns, USA). They were placed over a Czapek agar (15 g/L of agar-agar, 0.5/L g of KCl, 1.0 g/L of K_2HPO_4, 2 g/L of $NaNO_3$, 30 g/L of sucrose, 0.01 g/L of $FeSO_4$ and 0.5 g/L of $MgSO_4$, and chloramphenicol). For the biodegradation test, *A. niger* and *A. terreus* (10 replicates for each sampling point P1, P2 and P3) were inoculated over the LDPE samples, as shown in Figure 1. The LDPE weight loss was determined after an incubation time of 77 days at 37°C. Small samples were washed with ethanol, cut into squares of 0.5 cm^2 and then fixed with osmium tetraoxide (OsO_4) for 24 hours. Then, a dehydration step was carried out by freezing the samples with 100% t-butanol solution for 6 hours. The samples were later coated with gold for 20 seconds. A scanning electron microscope (JEOL-JSM 5310, Japan) was used to observe the LDPE biodegradation (Amano & Diaz, 2015). The biodegradation process presented herein did not use any thermal or photo-oxidation treatment.

The following Equation (1) was used to compute the percent of loss weight of LDPE over a period of 77 days:

$$Removal,\% = \frac{m_O - m_f}{m_O} * 100 \qquad (1)$$

where: m_o – initial weight of LDPE;
m_f – final weight of LDPE after 77 days of biodegradation.

RESULTS AND DISCUSSION

The isolates of *A. niger* were sampled from three different points at the Santay Island mangrove (P1, P2 and P3). However, *Aspergillus terreus* was found only in P1. We tested if taking the fungi from different points would have any

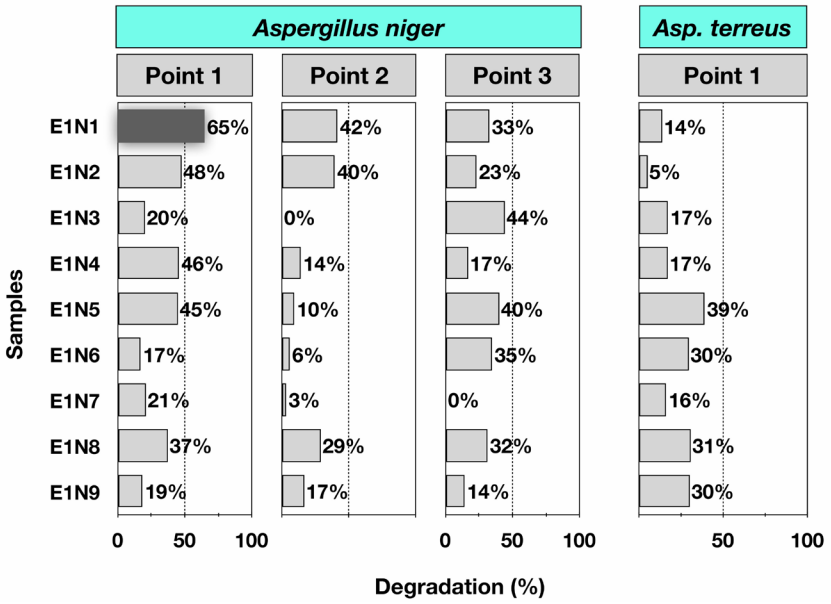


Fig. 2. Dry weight loss of LDPE in percent of *A. niger* and *A. terreus* which were isolated from three different geographical points (P1, P2 and P3)

influence on the degradation process. Figure 2 shows the LDPE weight loss percent over a period of 77 days. The results revealed that *A. niger* (from Point 1) could degrade up to approx. 35.3% of polymer, on average; meanwhile, *A. terreus* (from Point 1) reduced the weight of the polymer by an average of 22.14%. Both results show a high degradation potential if compared with the ones previously reported in literature (Table 1).

We compared the data only from Point 1 because *A. terreus* only grew in that particular point. According to Figure 3, the weight loss data from *A. niger* and *terreus* from Point 1 follows a normal distribution (Fig. 3B). A two-way T-student (Fig. 3A) determined that the loss of LDPE weight from both fungi were statistical different ($p < 0.01$).

Polyethylene is a synthetic polymer which is resistant to biodegradation as carbon source, because of its highly stable C-C and C-H covalent bonds. As consequence, microbial cells cannot easily penetrate the polymer surface due to the lack of readily oxidizable or/and hydrolyzable chemical groups (Gautam, Bassi, & Yanful, 2007; Hadad et al., 2005). However, the microbes still managed to do it. According to Figure 4, the microbial degradation of polyethylene starts at the polymer surface. Many authors agree that degradation occurs either by the secretion of extracellular or intracellular enzymes. It is argued that the penetration and distribution ability of *Aspergillus* hyphae is an example of fungal tip polarized growth, in which neurons, root hairs and pollen tubes are extended over the polymer (Esmaeili et al., 2013; Riquelme, 2013; Sarmah & Rout, 2018). In this way, the polyethylene integrity is fragmented, causing it to become more brittle and hydrophilic (Hakkarainen & Albertsson, 2004). As consequence, the diffusion of enzymes increases, and the transformation of polymers into monomers takes place as well. Once the size of the molecules is reduced, the oxidation process is performed, where the remaining molecules are transformed to carboxylic acid which is subsequently metabolized in the Krebs cycle, forming the citric acid as a metabolite (Restrepo-Florez et al., 2014). Figure 4 shows the colonization of *Aspergillus niger* and *Aspergillus terreus* over the LDPE films over a period of 77 days.

The aerobic degradation of plastic polymers is performed via hydrolysis by such enzymes as esterases, laccases, and lipases (Ahmed et al.,

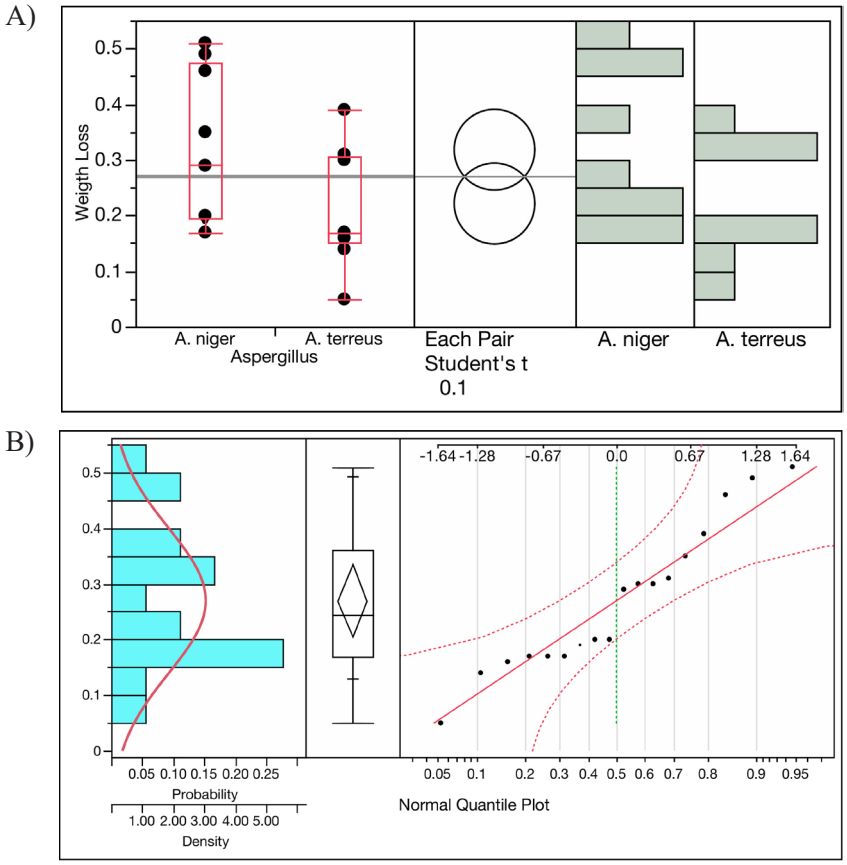
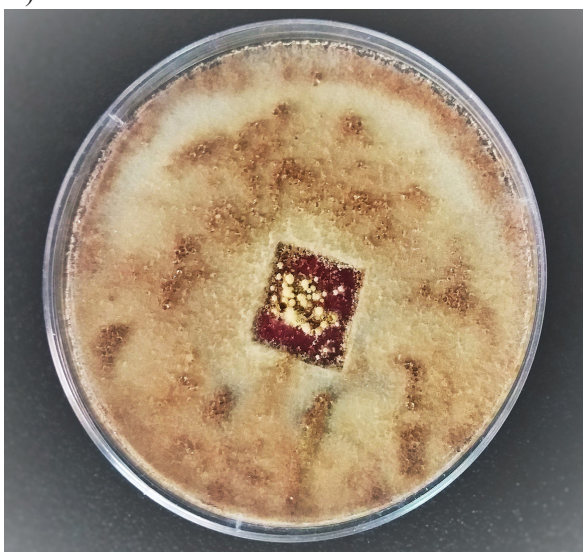


Fig. 3. Statistical analysis of weight loss data. (A) Two-way T-student and (B) normal quantile plot

A)



B)

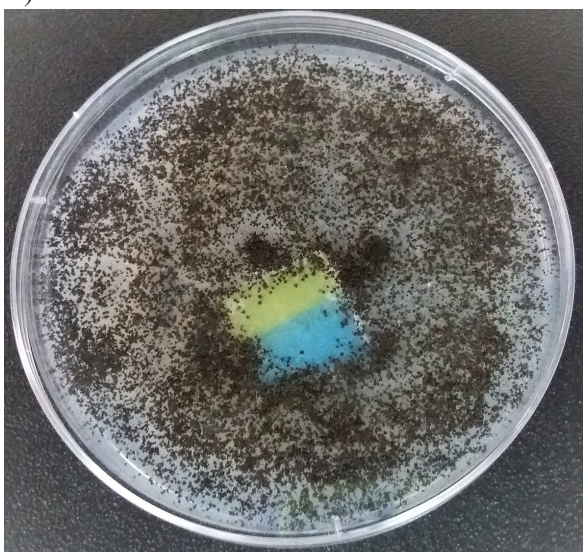


Fig. 4. Fungi growth over LDPE films (A) *Aspergillus niger,* (B) *Aspergillus terreus*

2018; Fujisawa et al., 2001; Ndahebwa Muhonja et al., 2018; Tokiwa, Calabia, Ugwu, & Aiba, 2009). In particular, fungal laccase is a polyphenol oxidoreductase that contains copper atoms in its active center and is involved in the degradation of lignin as well as plastic polymers (Brijwani, Rigdon, & Vadlani, 2010; de Vries & Visser, 2001; Tamayo Ramos, Barends, Verhaert, & de Graaff, 2011). One degradation mechanism of laccase occurs with the reduction of oxygen to water accompanied with one electron oxidation; this oxidation results in the generation of an oxygen-centered free radical compound that can be converted to quinone in a second enzyme catalyzed reaction (Brijwani et al., 2010). Because of this reaction, there is now an increasing application of laccase mediated surface activation, with a view to grafting materials of interest (Iqbal, Kyazze, Tron, & Keshavarz, 2018). Another degradation mechanism converts carbonyl groups from the polymer to alcohols by monooxygenases; the alcohol is then oxidized to an aldehyde by an alcohol dehydrogenase enzyme; later, an aldehyde dehydrogenase converts aldehydes to fatty acids, which thereafter are degraded inside cells by a β-oxidation pathway (Gautam et al., 2007). Moreover, *A. terreus* is a prolific producer of secondary metabolites and its biosynthetic repertoire includes the mycotoxins citrinin, citreovirdin, and patulin, which could be also involved in polymers degradation (Bennett, 2009). Figure 5 shows the growth of *A. niger* and *terreus* over LDPE films over a period of 77 days, where cracks were clearly formed in the plastic films.

The rate of polyethylene degradation would be boosted if a more oxidized surface were used as a substrate. For example, other authors applied starch as co-substrate. Co-substrates modify the properties such as crystallinity level and morphological changes of the original polymer; as a consequence, the biodegradation of the polymer would be facilitated (Awasthi et al., 2017). Another attempt at increasing the hydrophilicity and then facilitating the colonization of polyethylene on the polymeric surface involves by adding nonionic surfactants to the culture medium (Hadad et al., 2005). The biodegradation of polyethylene is positively affected by pretreatment with ultraviolet light (photo-oxidation), with additives, and with antioxidants, since the chemical structure of polyethylene is similar to that of linear alkanes monomers (Albertsson, Andersson, & Karlsson, 1987). Both the reduction of molecular weight and the polymers oxidation depend on the interaction effects between the biotic and abiotic factors (thermal treatments or photo-oxidation). Therefore, a photo-thermal or chemical pretreatment increase the surface hydrophilicity of the polymer by the formation of additional groups such as carbonyl groups that can be metabolized by microorganisms (Esmaeili et al., 2013). In the present work, we increased the concentration of sucrose to accelerate the fungi growth and to promote the colonization over the LDPE film. We found out that the Czapek media was an effective alternative to boost the degradation of plastic. In Table 1, we compare the LDPE loss weight of different studies and their pre-treatments, respectively. In

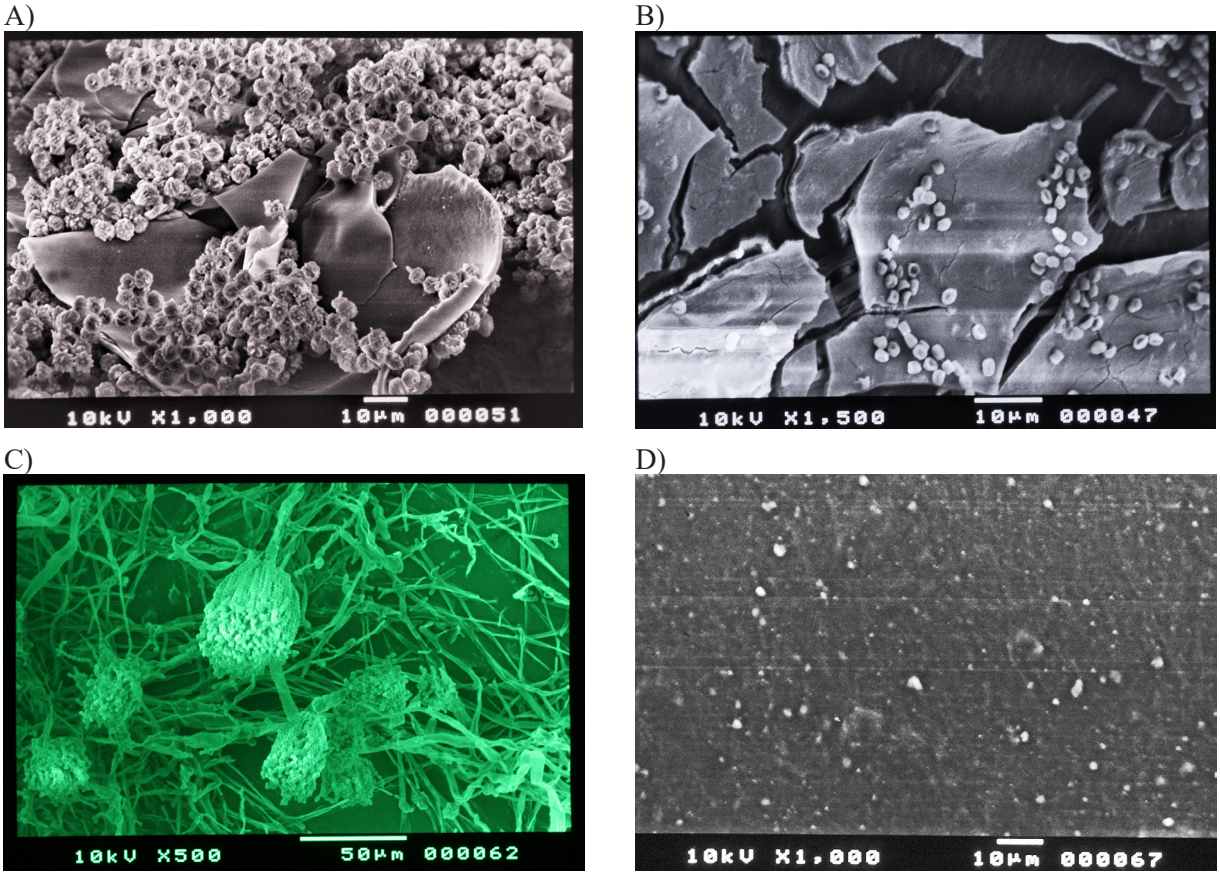


Fig. 5. SEM micrographs of fungi over LDPE films. (A) *A. niger* hypha over LDPE film. (B) *A. niger* over LDPE films after 77 days. (C) *A. terreus* conidia over LDPE. (D) LDPE sample with no fungi

Table 1. LDPE degradation from different microorganisms

Culture media to degrade LDPE	Pre-treatment	% loss weight	Microorganism	Reference
Rose Bengal broth medium	None	10% in 28 days	*Bacillus, Micrococcus*, *Listeria* and *Vibrio*	(Raaman, Rajitha, Jayshree, & Jegadeesh, 2012)
Mangrove soil	None	5% in 56 days	*Aspergillus niger, Aspergillus japonicus*	(Kumar, Hatha, & Christi, 2007)
Forest sandy soil	Thermal degradation at 55°C	60% in 540 days	-	(Chiellini, Corti, & Swift, 2003)
Mineral salt medium	None	9% in 60 days	*Fusarium sp.*	(Das & Kumar, 2014)
Farmland soil	25 days under UV light	29.5% in 126 days	*Aspergillus sp., Lysinibacillus sp.*	(Esmaeili et al., 2013)
Mineral medium	UV photo-oxidization	11% in 30 days	*Brevibaccillus borstelensis*	(Hadad et al., 2005)
Mineral salt medium	None	7.5% in 35 days	*Trichoderma* spp.	(Hikmah et al., 2018)
Mineral oil and nonionic surfactant Tween 80	None	5% in 45 days	*Pseudomonas sp.*	(Tribedi & Sil, 2013b)

summary, several synergistic biotic and abiotic factors results in different degradation times.

CONCLUSIONS

Aspergillus niger and *terreus* were isolated from a mangrove in Ecuador and they degraded up to 35.3% and 22.14% of LDPE films weight over a period of 77 days. In the present study, a rich in sucrose culture media was used, which is believed to enhance the fungal degradation process. It is argued that the microbial degradation of polyethylene starts at the polymer surface by the secretion of extracellular or intracellular enzymes, laccase being one of them. Furthermore, the penetration and distribution ability of *Aspergillus* hyphae is an

example of fungal tip polarized growth, which explains the rapid and vast colonization over the LDPE films.

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