Colonization and Biodegradation of Photo-Oxidized Low-Density Polyethylene (LDPE) by New Strains of Aspergillus sp. and Lysinibacillus sp.

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Colonization and Biodegradation of Photo-Oxidized Low-Density Polyethylene (LDPE) by New Strains of *Aspergillus* sp. and *Lysinibacillus* sp.

**ABSTRACT** The primary objective of this study was the isolation of low-density polyethylene (LDPE)-degrading microorganisms. Soil samples were obtained from an aged municipal landfill in Tehran, Iran, and enrichment culture procedures were performed using LDPE films and powder. Screening steps were conducted using linear paraffin, liquid ethylene oligomer, and LDPE powder as the sole source of carbon. Two landfill-source isolates, identified as *Lysinibacillus xylanilyticus* XDB9 (T) strain S7-10F and *Aspergillus niger* strain F1-16S, were selected as super strains. Photo-oxidation (25 days under ultraviolet [UV] irradiation) was used as a pretreatment of the LDPE samples without pro-oxidant additives. The PE biodegradation process was performed for 56 days in a liquid mineral medium using UV-irradiated pure LDPE films without pro-oxidant additives in the presence of the bacterial isolate, the fungal isolate, and the mixture of the two isolates. The process was monitored by measuring the fungal biomass, the bacterial growth, and the pH of the medium. During the process, the fungal biomass and the bacterial growth increased, and the pH of the medium decreased, which suggests the utilization of the preoxidized PE by the selected isolates as the sole source of carbon. Carbonyl and double bond indices exhibited the highest amount of decrement and increment, respectively, in the presence of the fungal isolate, and the lowest indices were obtained from the treatment of a mixture of both fungal and bacterial isolates. Fourier transform infrared (FT-IR), x-ray diffraction (XRD), and scanning electron microscopy (SEM) analyses showed that the selected isolates modified and colonized preoxidized pure LDPE films without pro-oxidant additives.

**KEYWORDS** *Aspergillus*, *Bacillus*, biodegradation, isolation, polyethylene

**INTRODUCTION** Polyethylene is an inert plastic material that is broadly used in packaging and other industrial and agricultural applications. Plastic wastes are discarded every day after brief use, and they accumulate in the environment because of their high durability. The dramatic increase of low-density polyethylene (LDPE)
waste, decreasing landfill capacity, and a lack of development of procedures for safe waste disposal have led to considerable endeavors by researchers to identify ways to decrease the amount of LDPE waste. In the case of PE, obstacles that interfere with microbial attacks include its three-dimensional structure, high molecular weight, hydrophobic nature, and lack of functional groups such as carbonyl groups that are utilized by microorganisms and increase polymer biodegradation. In other words, a synergistic effect occurs between PE pretreatment operations (such as photo-oxidation, which increases polymer hydrophilicity followed by the production of functional groups) and its biodegradation (Albertsson, Andersson, and Karlsson 1987). The ability of several fungi isolates to utilize LDPE as the sole source of carbon has been previously reported (Orhan and Buyukgungor 2000; Volke-Sepulveda et al. 2002; Sahebnazar et al. 2010). Biological treatment of LDPE/starch blend films with Phanerochaete chrysosporium after a 6-month incubation period in soil caused a reduction in the mechanical properties and a change in the Fourier transform infrared (FT-IR) spectrum between 1600 and 1800 cm⁻¹ (Orhan and Buyukgungor 2000). Significant morphological and structural changes of thermally oxidized and biologically treated LDPE with Aspergillus niger and Penicillium pinophilum were observed after 31 months (Volke-Sepulveda et al. 2002). Landfill source fungi (A. terreus and A. fumigatus) in the biodegradation process of ultraviolet (UV)-irradiated LDPE for 100 days in a compost environment changed the structural and morphological properties of the films (Sahebnazar et al. 2010). Several researchers have isolated biofilm-producing strains of bacteria (Rhodococcus ruber and Brevibacillus borstelensis) that utilized preoxidized LDPE as the sole source of carbon (Gilan, Hadad, and Sivan 2004; Hadad, Geresh, and Sivan 2005). The hydrophobic nature of their cell surface enables them to form a dense biofilm on the PE surface and to improve their biodegradation capacity. The ability of several Bacillus species to utilize PE with and without pro-oxidant additives has also been evaluated (Abrusci et al. 2011).

In this study, microorganisms were isolated from a typical aged landfill through enrichment and isolation procedures. The ability of these two isolates to degrade pure UV-irradiated LDPE without any pro-oxidant additives was investigated separately and also as a treatment of the mixed isolates culture in liquid medium containing LDPE as the sole source of carbon for 56 days.

MATERIALS AND METHODS

Materials

An Iranian petrochemical company provided the LDPE granules (LF0200; at a density of 0.920 g·cm⁻³), and the ethylene oligomer (C₂₀–C₄₀). The LDPE films (20 μm thick) were made from the LDPE granules using a blowing film extruder.

Enrichment Cultures and the Isolation of Microorganisms

The enrichment procedure was performed to isolate microorganisms that utilize PE as the sole source of carbon. Different soil samples (11 in total) were randomly collected from landfills in which PE wastes had been buried for different periods. In the remainder of this paper, no specific permission was required for soil sampling or the described field studies conducted by the first author of this study. Additionally, the study area was not privately owned and the field studies did not involve endangered or protected species.

The following three methods for the enrichment culture were performed using LDPE films and powder:

1. Soil samples, 10 g each, were placed into test tubes containing 4 ml of synthetic mineral (SM) medium containing (g/L): NH₄NO₃, 1.0; MgSO₄·7H₂O, 0.2; K₂HPO₄, 1.0; CaCl₂·2H₂O, 0.1; KCl, 0.15; and approximately 300 mg of polyethylene film. The test tubes were incubated for 20 weeks at 30°C (Gilan, Hadad, and Sivan 2004).

2. Each soil sample (10 g) was placed in 250-ml Erlenmeyer flasks containing 50 ml of SM medium, and 1 g of PE powder (LF0200) was added to each flask as the sole source of carbon. The cultures were incubated on a rotary shaker (120 rpm) at 30°C for 12 weeks.

3. This method is the same as method 2 except the flasks were incubated without shaking at 30°C for 12 weeks.

Initial isolation of microorganisms was conducted using linear paraffin (as an available model substrate) as the sole source of carbon. After termination of the enrichment procedure, LDPE films were removed from the tubes and added to 100-ml Erlenmeyer flasks containing 15 ml sterilized physiological saline solution. Flasks were shaken for 10 min to wash off the soils and
microbial biofilm from PE surfaces. Then, 0.1 ml of these suspensions was used to inoculate plates containing SM-agar supplemented with 1% linear paraffin and 0.1% Tween 20 (Merck, Gernsheim site, Gross-Gerau district, Germany) (medium A). Plates were incubated at 28 ± 2°C for 48 h.

To isolate microorganisms from enrichment flasks (methods 2 and 3 of enrichment procedure), 0.1 ml of soil suspension from each flask was spread cultured to the surface of plates containing medium A. Plates were incubated at 28 ± 2°C for 48 h. In all tests, control plates were also inoculated (medium A without paraffin as the carbon source).

Microorganisms were selected through comparison of growth ability in medium A. The isolates that were initially selected based on paraffin utilization ability as the sole source of carbon were further screened for their ability to grow on medium A containing 2% ethylene oligomer as the sole carbon source. Selected microorganisms from this step were then transferred into flasks containing SM medium and different concentrations of liquid ethylene oligomer (3%, 4%, and 5%) as the sole source of carbon step by step. In each step, bacterial isolates were selected according to growth ability by the measurement of the turbidity of the culture (optical density [OD] at 600 nm), and fungal isolates were chosen based on measurement of the dry biomass to test for higher concentrations of ethylene oligomer. Finally, the ability of the chosen microorganisms from the last step (medium with 5% ethylene oligomer) to grow in mineral medium containing PE as the sole source of carbon was evaluated. Selected microorganisms were cultured in Erlenmeyer flasks containing liquid mineral medium (SM) and 0.1% PE powder, and they were incubated for 15 days. Flasks were inoculated with 1 plug (0.5 × 0.5 cm) of each fungal isolate that was grown on malt extract agar (MEA; Merck) medium and 1 ml of a mid-exponential phase of each bacterial isolate grown in NB (nutrient broth; Merck) medium for each fungal and bacterial isolate separately.

**Identification of the Selected Isolates**

Microscopic observations and biochemical tests (growth, physiology, the utilization of carbohydrates, existence and nonexistence of enzyme activity, growth in presence of salt) according to proposed methods of Parry, Tarnball, and Gibson (1988) were performed for the purpose of identifying the genera of the selected bacterial isolates. In addition, the taxonomic identification of the bacterial isolates, including advanced biochemical characterization and polymerase chain reaction (PCR) amplification of the 16S rDNA, was performed at the Iranian Biological Resource Center (IBRC). The partial nucleated sequence of the 16S rDNA from isolate S7-10F was determined by the Macrogen in South Korea (using the ABI system 3730 XL) and was deposited in the NCBI database under GenBank accession no. JF838304.

The identification of the fungal isolate (F1-16S) was performed by recognizing the diagnostic morphological features of genera using macroscopic and microscopic examinations (Watanabe 2002). The molecular identification methods using the PCR to amplify a complete sequence of the rRNA operon encompassing the 5.8S rRNA gene and the flanking internal transcribed spacers (ITS1-5.8S-ITS2) was done at the Iranian Biological Resource Center (IBRC) and was recorded in the NCBI database under GenBank accession no. KF582941.

**Ultraviolet Irradiation of Polyethylene**

The LDPE films were irradiated for 25 days under UV light (two 55-W lamps; Osram, Munich, Germany) in a laminar-flow cabinet and were cut into pieces measuring approximately 2 × 2 cm, weighed, disinfected in 70% ethanol, and air dried for 15 min in a laminar-flow cabinet.

**Evaluation of LDPE Biodegradation in Liquid Medium**

This test was conducted using three different treatments: treatment 1, culture of selected bacterial isolate (strain S7-10F); treatment 2, culture of selected fungal isolate (strain F1); treatment 3, mixed culture of both fungal and bacterial isolates. Synthetic mineral medium (SM) containing 1% malt extract for treatment 2 and 1% glucose for treatments 1 and 3 was prepared and distributed in 500-ml Erlenmeyer flasks (each containing 100 ml of SM medium), and 0.3% Tween 80 (Merck) was also added to each flask.

The fungal isolate was cultured on MEA slant tubes and incubated at 30°C until complete growth was
obtained. Next, its spores were washed off in another sterilized tube using sterilized physiological saline solution. Five milliliters of spore suspension of the fungus (1 × 10⁶ spore·ml⁻¹) was used to inoculate 100 ml SM medium containing 0.1 g UV-irradiated LDPE (2 × 2 cm) that had been dried overnight at 60°C, weighed, and disinfected (30 min in 70% ethanol), for treatments 2 and 3. Flasks of treatments 1 and 3 (100 ml SM medium containing 0.1 g of UV-irradiated LDPE) were inoculated with 5 ml of a mid-exponential phase culture of the bacterium grown in NB medium. Cell density of the inoculums was adjusted to 1.5 × 10⁸ colony-forming units (CFU)·ml⁻¹. All flasks were incubated on a rotary shaker at 30°C for 56 days. Each treatment consisted of three flasks.

Flasks were harvested periodically (every 2 weeks), and the cultures were filtered by using weighed Whatman Grade No.1 filter paper to determine the fungal biomass and the remaining LDPE pieces. Changes in the fungal biomass, pH of the medium, and the growth of the bacterial isolate as turbidity of the bacterial culture were measured. To measure the dry fungal biomass, the LDPE pieces were separated from the fungal biomass using sterile forceps. The filter papers containing fungal biomass were washed with distilled water three times and then were dried in an oven at 50–60°C for approximately 24 h to obtain the constant weights. The dry fungal biomass was calculated through the weight differences between initial filter paper weights and their final weights containing fungal biomass (Lestan and Lamar 1996; Boonchan, Britz, and Stanley 2000).

After 56 days of incubation, the process was terminated, and the LDPE pieces were washed in distilled water and then dried. LDPE samples were analyzed for biodegradation.

**Analysis of LDPE Films**

**Fourier Transform Infrared (FT-IR) Analysis**

The structural change in the LDPE surface was investigated using the EQUINOX 55 FT-IR spectrometer (BRUKER, UK). For each LDPE film, a spectrum was taken from 400 to 4000 cm⁻¹. The carbonyl and double bond indices were calculated based on the relative intensities of the carbonyl band at 1715 cm⁻¹ and the double bond band at 1650 cm⁻¹ to that of the methylene scissoring band at 1460 cm⁻¹ (Albertsson, Andersson, and Karlsson 1987).

**X-ray Diffraction (XRD) Analysis**

The x-ray diffraction patterns of the films were measured with a x-ray diffractometer (model D5000; Siemens, Munich, Germany) that is operated fully automatically using Cu Kα radiation (λ = 1.5418 A°). The scattered radiation was registered in the angular interval (2θ) from 2° to 40°. A current of 30 mA and a voltage of 40 kV were used. All diffraction patterns were examined at room temperature and under constant operating conditions.

**Scanning Electron Microscopy (SEM)**

The polyethylene samples were removed from the soil and were dried in a desiccator for 24 h under the vacuum. The samples were vapor-fixed at room temperature for 3 days in a sealable glass container containing two beakers, one containing 10 ml of 25% glutaraldehyde in H₂O and the other containing 5 ml of 5% OsO₄ in 0.1 M phosphate buffer at pH 7.0. After fixation, the container was aerated for 20 h (Sivan, Szanto, and Pavlov 2006). The samples were gold-coated using a sputter coater (model SCDOOS; BAL-TEC, Switzerland) and were examined using a scanning electron microscope (model X LP30; Philips, Netherlands).

**RESULTS AND DISCUSSION**

**Isolation and Identification of the Isolates**

In this study, three different enrichment procedure methods were performed using LDPE films and powder. In the initial step of the isolation, 144 isolates that consisted of 128 bacterial and 16 fungal isolates were selected by comparing their ability to growth in SM-agar containing linear paraffin (as an available model substrate) as the sole source of carbon. In addition, the screening of these isolates was performed by comparing their growth ability in SM-agar containing linear paraffin supplemented with 2% ethylene oligomer, resulting in the selection of 53 isolates. In total, five gram-positive and sporforming Bacilli and five fungal isolates were screened based on their growth ability and the measurement of pH changes in a liquid mineral medium (SM) (which decreased by the oligomer oxidation of the isolates) containing 5% ethylene oligomer. These isolates were cultured in Erlenmeyer flasks containing liquid mineral medium in the presence of 1% PE powder as...
the sole source of carbon. After 2 weeks, the dry fungal biomass and bacterial growth (as the turbidity of the culture [OD at 600 nm]) were measured. Of these isolates, one bacterial and one fungal isolate showed the highest amount of growth (growth intensity) and pH changes in the medium in comparison with the other strains and were selected as the final super strains used in the biodegradation assay (Figures 1, 2, 3, and 4). Moreover, these two isolates were the most dominant isolates during the isolation and screening procedures. The selected bacterial isolate (S7-10F) was identified as a gram-positive aerobic endospore-forming Bacilli after microscopic observation, and biochemical tests (growth, physiology, the utilization of carbohydrate, existence and nonexistence of activity of enzyme) were conducted according to the proposed methods of Parry, Tarnball, and Gibson (1988). The results are shown in Table 1. The taxonomic identification of the bacterial isolate (S7-10F), in accordance with Bergey’s Manual of Systematic Bacteriology (Claus and Berkely 1986) and 16S rDNA sequencing, indicated a 99.4% resemblance to Lysinibacillus xylanilyticus XDB9 (T), and the S7-10F strain was identified as Lysinibacillus xylanilyticus (GenBank accession number JF838304). Lysinibacillus xylanilyticus XDB9 (T) is a xylan-degrading bacterium that was first isolated from forest humus in Korea. This strain cannot hydrolyze starch and grows in a medium with 0–5% (v/w) NaCl (Lee et al. 2010).
However, our strain (strain S7-10F) was isolated from an aged landfill soil in Tehran that can hydrolyze starch and also tolerate 7% NaCl in the culture medium. These differences define this strain as a new *Lysinibacillus xylanilyticus* strain.

The morphological characterization of the selected fungal isolate (F1-16S), including the color of the colonies cultured on agar and the dimensions of the conidiophores and conidia, indicated that this isolate resembled *Aspergillus niger*. The molecular identification of the fungal isolate (F1-16S) was done at the Iranian Biological Resource Center (IBRC), and the comparison of the sequence from the new isolate with the GenBank sequences was performed using a nongapped, advanced BLAST search in NCBI. BLAST search comparisons between the new isolate of *Aspergillus* sp. and all opportunistic fungi available in the GenBank database indicated a similarity of 99–100% to species of *Aspergillus niger*. This strain was designated the F1 strain in this study. Macroscopic and microscopic observations of the F1 strain are shown in Figure 5. Gilan, Hadad, and Sivan (2004) isolated a PE-degrading bacterium (*Rhodococcus ruber* strain C208) from landfill soils using an enrichment procedure. In our study, several soil samples were taken from an area approximately 30 years old. In these soils, several compatible microorganisms live that can utilize durable materials such as PE. Domestic and other types of degradable wastes are utilized by microorganisms easily. Therefore, microorganisms that live in a typical aged landfill are compatible for the utilization of durable or nonbiodegradable materials such as polyethylene as the sole source of carbon. In the enrichment method 1, we used test tubes and PE films and prepared the situation similar to the conditions that exist in landfills. In methods 2 and 3, we used polyethylene powder instead of PE films, and these tests were performed using Erlenmeyer flasks. PE powder is more available than the film, and its availability helps the microorganisms to attach and start utilizing the source. Method 2 also used implemented shaking, which made the powder more available in the culture and helped us to isolate aerobic PE-degrading microorganisms.

In method 3, we prepared a suitable situation for the isolation of facultative anaerobic microorganisms similar to the organisms found in soils located in subsurface layers in landfills. Surprisingly, both of our super strains were isolated from an aged soil sample in which PE wastes had been buried for over 25 years. In the initial isolation step, most of the isolates were isolated from the oldest landfill soil sample, which had been used in enrichment methods 1 and 3. Our fungal and bacterial isolates were isolated from this soil using enrichment methods 1 and 3, respectively.

These results clearly show that these two methods created the closest situation to the conditions found in landfills, and the methods helped us choose the correct microorganisms with the ability to degrade PE as the sole source of carbon.

The initial isolation of microorganisms was performed using linear paraffin as an available model

### TABLE 1 Results of Biochemical Tests (Growth, Physiology, Utilization of Carbohydrate, Existence and Nonexistence of Activities of Enzymes)

<table>
<thead>
<tr>
<th>Test</th>
<th>Isolate: <em>Lysinibacillus xylanilyticus</em> strain S7-10F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>+</td>
</tr>
<tr>
<td>Endospore forming</td>
<td>+</td>
</tr>
<tr>
<td>Form of endospore</td>
<td>Sphere</td>
</tr>
<tr>
<td>Position of endospore</td>
<td>Terminal</td>
</tr>
<tr>
<td>Aerobic growth</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>+</td>
</tr>
<tr>
<td>Catalase test</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>+</td>
</tr>
<tr>
<td>Glucose (product of acid)</td>
<td>+</td>
</tr>
<tr>
<td>Maltose (product of acid)</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose (product of acid)</td>
<td>+</td>
</tr>
<tr>
<td>Xylose (product of acid)</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>+</td>
</tr>
<tr>
<td>Lecithinase</td>
<td>–</td>
</tr>
<tr>
<td>Citratase</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>–</td>
</tr>
<tr>
<td>Growth in presence of 2% salt</td>
<td>+</td>
</tr>
<tr>
<td>Growth in presence of 5% salt</td>
<td>+</td>
</tr>
<tr>
<td>Growth in presence of 7% salt</td>
<td>+</td>
</tr>
<tr>
<td>Growth in presence of 10% salt</td>
<td>–</td>
</tr>
</tbody>
</table>

**Note.** –, negative; +, positive.

![FIGURE 5 Macroscopic (a: colony surface of the F1 strain grown on malt extract agar plates) and microscopic (b: sporangium and released spores of the F1 strain) images of the F1 strain (*Aspergillus niger*).](image)
substrate because paraffin is classified as a very-short-chain PE. Microorganisms preferentially use linear n-paraffin, whereas the corresponding branched isomers are almost completely inert to biodegradation. The mechanism for paraffin biodegradation is comparable to PE-based degradation (Albertsson, Andersson, and Karlsson 1987). Paraffin is an available, very short PE that has the same structure as PE, but its availability allows microorganisms to utilize it as a carbon source.

Using paraffin in the initial steps of isolation, we identified the microorganisms that had the ability to break down the C—C bond and oxidize the chain. If a microorganism cannot utilize paraffin as the sole source of carbon, it will not be able to utilize PE as the carbon source. Next, we supplemented the cultures with an ethylene oligomer whose structure is more similar to PE; therefore, utilization by the microorganisms is harder than linear paraffin. Among the microorganisms that had the ability to utilize paraffin as the sole source of carbon, just a few of them grew on the medium supplemented with ethylene oligomer as the carbon source. We increased the concentration of the oligomer in the culture medium step by step and screened the capable microorganisms. Selected isolates that were screened based on their growth ability in mineral medium containing 5% oligomer (an approximately 5% concentration caused growth inhibition of the microorganisms) were compared for their growth ability to utilize PE as the sole source of carbon. One bacterial and one fungal strain showed the highest growth ability in the medium and were selected as the final super strains.

Using three similar substrates (paraffin, ethylene oligomer, and polyethylene, which were different from each other in their molecular weights and their availability in the culture) in the screening procedures, we adopted the microorganisms with the culture containing these substrates, and we chose capable microorganisms more accurately.

### Polyethylene Biodegradation Assay

This test was run in three treatments and the biodegradation of UV-irradiated LDPE was investigated using the fungal isolate, the bacterial isolate, and a mixture of both fungal and bacterial isolates. On days 0, 14, 28, and 56, the flasks were harvested and the cultures were filtered using filter paper to obtain fungal biomass and LDPE pieces. Dry fungal biomass profiles of the F1 strain in the F1 strain culture (treatment 2) and a mixed culture of the F1 and S7-10F strains (treatment 3) are shown in Figure 6. As shown in Figure 6, fungal biomass in treatments 2 and 3 increased during the initial days of incubation because of the utilization of malt extract as the carbon source. Then, the biomass leveled off for 14 days and increased again until the end of the process, which indicates the utilization of the carbonyl groups as the sole source of carbon by the fungus. Changes in the growth of the S7-10F strain represented by turbidity of the bacterial culture in treatments 1 and 3 are presented in Figure 7. According to this figure, bacterial growth in treatments 1 and 3 showed a similar pattern to the fungal biomass profile. Initial growth of the bacterium in these two treatments may be due to the presence of glucose as the carbon source in the medium. The growth reached the stationary phase in the first 28 days and then increased gradually. Thien, Nhi, and Thuan (2005) reported that the first log phase of the bacterial growth may be due to
glucose consumption in the culture medium. In their study, the number of bacteria did not change dramatically and move to the stationary phase. The secondary log phase of the growth began by utilizing the starch of the starch-blend PE films they used in their experiments. These results indicate that a simple carbon source such as glucose and malt extract can be easily utilized by the microorganisms. However, utilization of PE is a slow process and requires the biofilm to form on its surface. The growth profile of both bacterial and fungal isolates in treatment 3 showed slight changes in comparison with treatments 1 and 2 (cultures of the fungus and bacterium separately). This finding may be due to the competition effects of fungal and bacterial isolates in this treatment (Figures 6 and 7, treatment 3: mixed culture of strains S7-10F and F1). The antagonistic effect of these two isolates was tested before the biodegradation assays, and no antagonistic effect was observed between them. Furthermore, none of the isolates produced any metabolites with a growth inhibitory effect against another isolate. We expected to observe the highest rate of PE degradation in the mixed culture of these isolates in comparison with treatments containing each of these two isolates separately. However, no synergistic effect for PE degradation was found, which may be due to the competitive interaction among these two isolates that occurs due to limited resource. This study was the first time that PE biodegradation was investigated in the presence of a mixed culture of bacterial and fungal isolates. In this treatment, both isolates were alive until the end of the test, but their growth potential was decreased, and they did not utilize PE to the same level that could potentially be achieved in the absence of another isolate.

Surprisingly, in our other study (Esmaeili et al. 2013), LDPE biodegradation of the mixed culture of these isolates in soil was higher, which suggests the ecological nature of the soil in comparison with the liquid medium. Soil is a biogeochemical complex that contains numerous microsites where different microbes live without having any negative effects on neighboring microbes. Therefore, both of the isolates utilize PE as a sole source of carbon, and the overall rate of biodegradation was high.

Figure 8 shows pH changes in mineral medium containing UV-irradiated LDPE as the source of carbon in different treatments during 56 days of incubation. The pH value is a key factor for the survival and activity of microorganisms, but this parameter has not been monitored during the biodegradation process in most related studies. The pH profile exhibited an initial decrease in treatment 2 due to the presence of malt extract in the medium and its utilization by the fungus. Among the microorganisms, fungi live better in conditions with low or acidic pH, and they usually decrease the pH in the medium during the oxidation process of the hydrocarbons, which may be due to the formation of carbon dioxide and protons. In contrast, bacteria have a higher affinity for PE degradation in the presence of a mixed culture of bacterial and fungal isolates.
of secondary metabolites such as acidic products. The decreasing trend of pH in treatment 3 during the first 2 weeks indicates the dominant activity of the fungus in this treatment. The initial increase of pH in treatment 1 was due to the utilization of glucose by the bacterium. The pH changes in the medium were mediated after 28 days of incubation, and this parameter remained almost constant during the next 2 weeks, which is in agreement with Figures 2 and 3 in which the isolates showed the increasing growth rates in this period of time, and they started utilizing the PE films. Strain S7-10-F showed the second log phase during this period (Figure 3), which caused a decrement in pH, and this trend continued until the end of the incubation period.

The slight decreasing trends of pH values at the end of the process are attributed to the utilization of the carbonyl groups and the transformation of them to carboxylic acid by the isolates (Manzur, Limon-Gonzalez, and Favela-Torres 2004).

**FT-IR Analysis**

In the biodegradation of polyethylene, the initial abiotic step involves the oxidation of the polymer chain leading to the formation of carbonyl groups. These groups eventually form carboxylic groups, which subsequently undergo β-oxidation (Albertsson, Andersson, and Karlsson 1987) and are completely degraded via the citric acid cycle resulting in the formation of CO₂ and H₂O. β-Oxidation and the citric acid cycle are catalyzed by microorganisms. Monitoring the formation and disappearance of carbonyl and double bond bands using FT-IR is necessary to elucidate the mechanism of the biodegradation process. The FT-IR spectra of pure LDPE films without pro-oxidant additives before and after 25 days of UV irradiation are presented in Figure 9. As shown in Figure 9, the FT-IR spectrum of the LDPE film after 25 days of UV irradiation showed the appearance of a peak in the range of 1710–1750 cm⁻¹ due to the formation of carbonyl groups (abiotic oxidation). The intensity of the bands in the 1178 cm⁻¹ region increased, which is also related to the carbonyl groups (Manzur, Limon-Gonzalez, and Favela-Torres 2004). Figure 10 shows the FT-IR spectra of the UV-irradiated pure LDPE films without pro-oxidant additives before and after 56 days of incubation in liquid mineral medium in the presence of the bacterial isolate (strain S7-10F, treatment 1), the fungal isolate (strain F1, treatment 2), and the mixture of the two isolates (strains S7-10F and F1, treatment 3). The changes in the bands between 500 and 2000 cm⁻¹ are magnified in Figure 11. Based on Figure 11b showing the FT-IR spectrum of the UV-irradiated LDPE film exposed to strain S7-10F (treatment 1), new absorption bands between 1000 and 1700 cm⁻¹ (1227 and 1372 cm⁻¹) of the spectrum were observed, possibly because of the oxidized fractions such as moieties containing –OH groups resulting from the selected bacterial biodegradation (Corti et al. 2010). The intensity of the bands in the range of 1000–1700 cm⁻¹ (1221 and 1371 cm⁻¹) in the FT-IR
spectrum of treatment 3 (Figure 11d, mixed culture of strains S7-10F and F1) is also attributed to the oxidized fractions because of the action of the selected isolates (Figure 11d). The appearance of these bands only in the presence of the bacterial isolate (treatments 1 and 3, Figure 11b and d) may be attributed to the action of the S7-10F strain. Compared with the corresponding control (Figure 11a), the intensity of the carbonyl band at 1710–1750 cm$^{-1}$ was significantly decreased during the process with the selected isolates, and the highest amount of decrement was obtained in the presence of the fungal isolate (strain F1, treatment 2); and (d) UV-irradiated LDPE after incubation in liquid mineral medium in the presence of the mixed culture of both isolates (strains S7-10F and F1, treatment 3).

Initially, the carbonyl index increases due to abiotic factors; however, prolonged exposure to microorganisms leads to a decrease in the carbonyl index due to biodegradation (Sudhakar et al. 2008). The decrement trend of the carbonyl groups (which can be obtained by calculating the carbonyl index) indicates the utilization of PE by the microorganisms.

Table 2 shows the changes in the carbonyl index of films before and after biological treatment. As can be seen in this table, the carbonyl indices significantly decreased after 56 days of incubation in all treatments, and these indices between three treatments also significantly differed from one another. Strain S7-10F (Figure 11b) reduced the carbonyl index by 26.10%, whereas strain F1 (Figure 11c) and the mixture of both isolates (Figure 11d) reduced the index by 41.09% and 18.47%, respectively (Table 2).

Sudhakar et al. (2008) reported an initial increase in the carbonyl index due to the oxidation by the dissolved oxygen (abiotic factor) and a decrease in this index after prolonged exposure to organisms (biotic factor). An increase in the carbonyl index due to different abiotic oxidation treatments (such as UV irradiation) and its decrement in the presence of biotic environment have been reported in many related studies (Albertsson, Andersson, and Karlsson 1987; Chiellini, Corti, and Swift 2003; Gilan, Hadad, and Sivan 2004; Hadad, Geresh, and Sivan 2005).

Table 3 shows the changes in double bond index of films before and after biological treatment. There was no significant difference ($p = .05$) in double bond indices between time 0 and after 56 days of incubation of films in treatment 3 that indicated the competitive interaction among these two isolates in this treatment (Table 3, treatment 3). This index significantly increased in treatments 1 and 2 after incubation.

Manzur, Limon-Gonzalez, and Favela-Torres (2004) reported that the segments formed during the chain ruptured by the effect of biological treatment caused the formation of the vinyl group and double bond index (DBI) increment. Additionally, the DBI increment may be attributed to biotic dehydrogenation (Chiellini, Corti, and Swift 2003). Volke-Sepulveda et al. (2002) observed a significant DBI increase in

![FIGURE 11 The changes in the bands between 500 and 2000 cm$^{-1}$ of the FT-IR spectra of UV-irradiated pure LDPE films without pro-oxidant additives before and after 56 days of incubation in liquid mineral medium with different treatments: (a) blank (after 25 days of UV irradiation, no incubation); (b) UV-irradiated LDPE after incubation in liquid mineral medium in the presence of the bacterial isolate (strain S7-10F, treatment 1); (c) UV-irradiated LDPE after incubation in liquid mineral medium in the presence of the fungal isolate (strain F1, treatment 2); and (d) UV-irradiated LDPE after incubation in liquid mineral medium in the presence of the mixed culture of both isolates (strains S7-10F and F1, treatment 3).](image-url)
thermally oxidized LDPE samples during the incubation of *P. chrysosporium* for 3 months.

As shown in Tables 2 and 3, the carbonyl index (CI) decrement and DBI increment in all treatments were explained according to the proposed mechanism for PE biodegradation. According to this mechanism, formed carbonyl groups along the polymeric chain resulting from the action of abiotic factors were attacked microbially (CI decrease), which led to the release of unsaturated chains (DBI increase) (Volke-Sepulveda et al. 2002). The oxidized group was transformed to a carboxylic acid and then metabolized through β-oxidation (Manzur, Limon-Gonzalez, and Favela-Torres 2004).

Moreover, the ability of these isolates to oxidize and utilize ethylene oligomer as the sole source of carbon was evaluated in liquid mineral medium for each isolate separately. FT-IR analysis confirmed the ability of these isolates to oxidize and modify ethylene oligomer in culture conditions.

### XRD Analysis

The degree of crystallinity of the LDPE films was determined by x-ray diffraction. The XRD spectra of the non–UV- and UV-irradiated pure LDPE films without pro-oxidant additives are shown in Figure 12. As shown in this figure, the XRD spectra show distinguished peaks at 21.4 and 23.5 of the angular position 2θ. The intensity of the peaks of UV-irradiated film was higher than the peaks of the non-UV-irradiated film. This difference clearly demonstrated that oxidation pretreatment increased the degree of polyethylene crystallinity.

Figure 13 shows the XRD spectra of the UV-irradiated pure LDPE films without pro-oxidant additives before and after 56 days of incubation in liquid mineral medium in the presence of the selected bacterial and fungal isolates and after biological treatment with strain S7-10F (treatment 1), strain F1 (treatment 2), and the mixture of the two isolates (strains S7-10F and F1, treatment 3). The intensity of the peaks was significantly decreased after 56 days of incubation in liquid mineral medium in the presence of the selected bacterial and fungal isolates.

### Table 3

<table>
<thead>
<tr>
<th>Time</th>
<th>DBI ± SD</th>
<th>DBI ± SD</th>
<th>Δ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.168 ± 0.006</td>
<td>0.237 ± 0.013</td>
<td>41.07 (+)</td>
</tr>
<tr>
<td>56</td>
<td>0.168 ± 0.006</td>
<td>0.270 ± 0.013</td>
<td>60.71 (+)</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.168 ± 0.006</td>
<td>0.194 ± 0.014</td>
<td>15.48 (+)</td>
</tr>
</tbody>
</table>

Note. DBI = double bond index; Δ (%) = difference between double bond index of films before and after 56 days of biodegradation (shown as a percentage). Values accompanied by a similar letter are not significantly different according to Duncan's multiple-range test (p = .05). Each value represents the average of three replicates ± SD.

![XRD spectra of pure LDPE films](http://example.com/xrd.png)

**FIGURE 12** XRD spectra of pure LDPE films without pro-oxidant additives before and after 25 days of UV irradiation.

![XRD spectra of UV-irradiated pure LDPE films](http://example.com/xrd2.png)

**FIGURE 13** XRD spectra of UV-irradiated pure LDPE films without pro-oxidant additives before and after 56 days of incubation in liquid mineral medium with different treatments: (a) blank (after 25 days of UV irradiation, no incubation); (b) UV-irradiated LDPE after incubation in liquid mineral medium in the presence of the bacterial isolate (strain S7-10F, treatment 1); (c) UV-irradiated LDPE after incubation in liquid mineral medium in the presence of the fungal isolate (strain F1, treatment 2); and (d) UV-irradiated LDPE after incubation in liquid mineral medium in the presence of the mixture of the two isolates (strains S7-10F and F1, treatment 3).
(Figure 13). The decrease in these peaks especially in treatments 1 and 2 (Figure 13b and c) indicated that crystallinity and the crystal size for UV-irradiated films decreased during the process with the selected isolates (Abd El-Rehim et al. 2004). A decrease in the crystallinity of thermally pretreated LDPE that reacted with *B. sphericus* and *Arthrobacter parafineus* has also been reported (Sudhakar et al. 2008; Albertsson et al. 1995).

Manzur, Limon-Gonzalez, and Favela-Torres (2004) observed a significant decrease in the crystallinity of thermally oxidized LDPE incubated with *Aspergillus niger*. In contrast, Weiland, Daro, and David (1995) reported a crystallinity increase in thermally oxidized LDPE samples incubated with a fungal consortium (*A. niger, G. virens, P. variotii*, and *P. pinophilum*) for 21 months. These differences on crystallinity may be dependent on the types of biological and physiochemical treatment and on the length of the incubation period (Manzur, Limon-Gonzalez, and Favela-Torres 2004).

**FIGURE 14** SEM micrograph of LDPE films before and after 56 days of incubation in liquid mineral medium with different treatments: (a) blank (UV-irradiated LDPE film without incubation); (b) UV-irradiated LDPE film treated with the F1 strain; (c) UV-irradiated LDPE film treated with the strain S7-10F; and (d) UV-irradiated LDPE film treated with a mixture of F1 and S7-10F strains.
Scanning Electron Microscopy Analysis

SEM analysis was performed to monitor the changes on the surface of the films. The adhesion of the microorganisms to the polymeric surface is fundamental for biodegradation to occur (Volke-Sepulveda et al. 2002). Figure 14 shows SEM micrographs of the UV-irradiated LDPE films before (Figure 14a) and after (Figure 14b, c, and d) biological treatments. Figure 14b and d show fungal hyphae adhering to or penetrating the PE surface in treatments 2 and 3 resulting from the action of the F1 strain. Biofilm formation of bacterial cells was also observed on the surface of the PE incubated with the S7-10F strain in treatments 1 and 3 (Figure 14c and d). As shown in these images, penetration of fungal hyphae and the formation of a bacterial biofilm were the cause of cracks and cavities on the surface of the films in the biodegradation process of the PE. The presence of these features may be due to the absence of short branches or photodegradation products in the polymer matrix. Microorganisms that colonize the polymer surface most likely adhere to this surface due to extracellular polymer production, which is primarily constituted by polysaccharides. Portions of these polysaccharides synthesized by several fungi groups formed a type of capsule or sheath that was covalently bonded to the polymer wall and played an important role supporting and transporting depolymerization enzymes during polymer surface attachment (Volke-Sepulveda et al. 2002). The LDPE degradation by Aspergillus niger is consistent with previous results (Manzur, Limon-Gonzalez, and Favela-Torres 2004; Volke-Sepulveda et al. 2002). Aspergillus terreus participated in the degradation of modified and unmodified PE (El-Shefei et al. 1998). The degradation of UV-irradiated LDPE by A. fumigatus has also been reported (Sahebnazar et al. 2010). Several examples also exist in the literature confirming the ability of the genus Bacillus to degrade PE (Roy et al. 2008; Sudhakar et al. 2008; Abruci et al. 2011). Bacillus pumilus and B. halodenitrificans were able to degrade an abiotically aged LDPE containing pro-oxidants within 120 days (Roy et al. 2008). B. sphericus and B. cereus have also been shown to degrade unmodified and modified LDPE and HDPE with starch (Sudhakar et al. 2008).

The biodegradation of photo-degraded LDPE containing pro-oxidant additives by a mixture of Bacillus strains (B. megaterium, B. subtilis, and B. cereus) was evaluated within 90 days, and biofilm formation developed only in the photo-degraded material after 1 week of the bacterial treatment (Abruci et al. 2011).

CONCLUSION

In this study, the isolation of LDPE-degrading microorganisms was performed using landfill soils where PE wastes were buried for different periods of time. This procedure was conducted through an enrichment culture using LDPE films and powder. One bacterial isolate (identified as Lysinibacillus xylanilyticus XDB9 (T) strain S7-10F) and one fungal isolate (Aspergillus niger strain F1-16S designated as the F1 strain) were selected as super strains. The biodegradation of the UV-irradiated LDPE film was performed in liquid mineral medium in the presence of strain S7-10F (treatment 1), strain F1 (treatment 2), and a mixture of these two isolates (treatment 3) for 56 days. During the process, the fungal biomass and the bacterial growth increased, and the pH of the medium decreased, which indicates the utilization of the preoxidized PE by the isolates as the sole source of carbon. Carbonyl and double bond indices exhibited the highest amount of decrement and increment in the presence of the F1 strain (treatment 2), and the lowest values were obtained from the mixed culture of both fungal and bacterial isolates (treatment 3). These results may be due to the high ability of the F1 strain to utilize preoxidized PE and competition effects of the fungal and the bacterial isolates in treatments 2 and 3, respectively. FT-IR, XRD, and SEM analyses demonstrated the ability of selected isolates to modify and colonize preoxidized pure LDPE films without any pro-oxidant additives.

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REFERENCES


