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Analysis of Bacterial Isolates Capable of Partially Degrading Polyethylene Terephthalate

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HIGHLIGHTS

- Eight bacterial isolates from five genera were positive to lipase test.
- Eight bacterial isolates were tested against pretreated PET for 60 days.
- Bacterial degradation in PET was detected by FT-IR and SEM.
- *Pseudomonas soli* was selected as the best PET degrader.

Abstract: In this work, we studied 46 bacterial isolates to determine their potential for polyethylene terephthalate (PET) degradation. Only eight lipase-positive isolates were pre-selected to continue analysis. The isolates belonged to the genera *Micrococcus*, *Acinetobacter*, *Pseudomonas*, *Bacillus*, and *Pseudochrobactrum*. In addition to lipase, other enzymatic activities such as protease, carboxymethyl cellulase, xylanase, and chitinase were detected. PET sheets were pretreated with temperature, UV light, HNO_3, and *Stenotrophomonas pavanii*. All isolates were tested against pretreated PET for 60 days. All isolates displayed lipase activity, and some hydrolase activities. The chemical changes of PET sheets were determined using Fourier-transform infrared spectroscopy. Peaks observed at 3000-3500 cm-1 corresponding to hydroxyl group suggested degradation. The physical changes of PET observed with the naked eye and with SEM consisted of erosion, bubbles, scales, bumps, pits, and a shiny surface. *Pseudomonas soli* was selected as the best PET degrader, according to the notable changes in the infrared spectrum and macroscopic changes compared to the other genera and the control. In addition, this isolate did not show xylanase activity and it lacks the chitinase gene too. It is possible that not only lipase activity could be associated with the PET degradation, but also another enzymatic activity not detected in this study. Although the genus *Pseudomonas* has been reported as a plastic degrader, the *P. soli* species is novel in

the degradation of PET, as well as the other genera mentioned here. Finally, these findings contribute to the knowledge of bacterial species for PET degradation.
Keywords: PET; hydrolases; degradation; lipases; *Pseudomonas*; *Bacillus*.

INTRODUCTION

Polyethylene terephthalate, commonly known as PET, is a plastic polymer highly used to produce plastic bags, containers, and disposable materials for packaging, among others [1]. Due to its outstanding mechanical properties, thermal stability, tightness for gases and liquids, transparency, and low price, PET is used chiefly for beverage bottles, textile fibers, and food containers [2]. The PET is a semi-crystalline thermoplastic structured by a macromolecule chain formed from monomeric units designed bis (2-hydroxyethyl) terephthalate (BHET). PET is synthesized by the polycondensation of aromatic terephthalic acid (TPA) and ethylene glycol or by transesterification of dimethyl terephthalate and ethylene glycol [2]. PET is a very stable polymer and resistant, so it has become the central component of plastic waste in the environment. The mass production of virgin polymers worldwide was calculated to be 8300 million metric tons (Mt) from 1950 to 2015 [3]. Approximately 6300 Mt was plastic waste; 9% was recycled, 12% was incinerated, and 79% was accumulated in landfills [4]. Plastic pollution adversely affects the biota, not only for their enormous accumulation in the ecosystems but for the hazardous additives they contain, which may contaminate drinking water, causing severe public health and safety concerns [5]. However, considering the PET structure, the C-O-backbone places it in the category of hydrolyzable plastics [6]. Fortunately, some microorganisms possess PET hydrolytic enzymes (PHEs) capable of hydrolyzing the ester bonds with which monomers are linked, becoming a product amenable to natural degradation [2]. Due to the nature of the PET composition, chemical, physical and biological reactions are often necessary to increase the polymer hydrophilicity. These processes provide a functional group, such as alcohol or carbonyl groups, enhancing bacterial attachment for the degradation process. The steps involved in the biodegradation of plastics begin with the initial attachment of microorganisms to form a biofilm onto the plastic surface. Microbial colonization leads to the next step, called biodeterioration, which occurs through the action of secreted enzymes having an essential impact on the plastic surface. The third step, called biofragmentation, consists of converting the polymer into a more straightforward form, broken down into oligomers, dimers, and monomers, through destabilization of the carbon skeleton. The microorganisms can assimilate the monomers released. When polymers are degraded to CO2, H2O, and CH4, the final step is mineralization [7]. In this work, we screened for some bacterial isolates able to degrade PET by cultivating environmentally deteriorated PET samples, expected to possess active PHEs. The hypothesis says that the more enzymatic activities (protease, xylanase, chitinase, lipase, and cellulase) a bacterium has, the greater its PET degrading capacity will be.

MATERIAL AND METHODS

Microorganisms

Bacterial isolates were obtained from deteriorated PET screw caps buried in the small squares of soil that form in the cobblestone floor from a parking lot of a shopping center located at 25.673932 N, -100.423704 W in the municipality of Santa Catarina, Nuevo Leon, Mexico. Also, deteriorated PET bottles were collected from the soil of a vacant lot located at 26.060432 N, -98.315513 W in Reynosa, Tamaulipas, Mexico. The samples were taken manually with gloves and placed in plastic bags until their use. Screwcaps and 1g-PET sheets were placed in Erlenmeyer flasks with 100 ml saline minimal medium (SMM) containing 2 g/L KH_2PO_4, 7 g/L K_2HPO_4, 1 g/L NH_4NO_3, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.001 g/L $ZnSO_4 \cdot 7H_2O$, 0.001 g/L $CuSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.002 g/L $MnSO_4 \cdot H_2O$, adjusted to pH 7.2, [8]. Media were incubated in a rotary shaker at 120 rpm for 7 days at room temperature. After this, 1 mL of media from each flask containing screwcaps or PET sheets was transferred to fresh SMM containing three 2 x 2 cm PET sheets cut from soft drink bottles, each one according to the method described by Gaytán and coauthors [9]. Subsequently, every 7 days, 2 mL of the medium were transferred to a new Erlenmeyer flask with SMM and PET sheets for 28 days. After this period, 1 mL and PET sheets from each culture medium were inoculated in nutrient agar at 30 °C for 24 h to obtain potentially PET-degrading bacteria. Also, 37 *Bacillus* sp. isolates and *Stenotrophomonas pavanii* strain as control were provided by the Environmental Biotechnology Laboratory (CBG-IPN) to test their PET degrading capacity. They were observed under a microscope, and a Gram stain was performed.

Extracellular enzymatic activity

Lipase activity

The isolates were screened for lipase activity since lipolytic activity has been associated with PET degradation. The screening was conducted as a preliminary test to select only lipase-positive isolates. Rhodamine B agar plates were prepared as follows, the emulsion medium was prepared with 250 µL Tween 80, 30 ml olive oil, and 50 mL distilled water and emulsified in a blender; the emulsion was adjusted to pH 7. The base medium contained 4.5 g/L nutrient broth, 1.25 g yeast extract, 5 g agar, and 450 ml distilled water. The base medium and the lipoidal emulsion were autoclaved separately. Then rhodamine B (Sigma-Aldrich) was added to the lipoidal emulsion at a concentration of 0.024% w/v, and this was added to the base medium to a final volume of 1 liter. Isolates were plated in triplicate on rhodamine B agar plates and incubated at 30 °C for 48 h. Thereafter the plates were observed under a UV light lamp at 365 nm. A fluorescent halo identified lipase-positive isolates. Positive isolates were streaked onto individual Petri dishes for isolation and purification [10].

Proteolytic activity

The proteolytic activity was determined by testing in skim milk (Difco). The lipase (+) isolates were inoculated in nutrient broth at 37 °C for 24 h. Later, 100 µL of the culture were added to 5 mL of sterile skim milk in a test tube and incubated at 37 °C for 24 h. The appearance of a clot in the tube after 24 h indicated a positive proteolytic activity [11].

Cellulase activity

Cellulase activity was determined by the method described by Meddeb-Mouelhi and coauthors [12] with some modifications. Plates with minimal agar (0.1 %, NaNO3, 0.1 % K2HPO4, 0.05 % MgSO4, 0.1 % KCl, 0.05 % yeast extract, and 1.5 % Bacto agar) and 0.5 % carboxymethylcellulose sodium salt as substrate, were inoculated with the lipase (+) isolates in triplicate and incubated at 37 °C for 72 h. The plates were flooded with a 1 % Congo red solution for 20 min to visualize the inhibition haloes. Afterward, the solution was discarded, plates were stained with 1 M NaCl for 15 min. Later, plates were observed with a white background against the light.

Xylanase activity

The method described by Meddeb-Mouelhi and coauthors [12] was used with some modifications to determine xylan hydrolysis. Plates were prepared with minimal agar, as mentioned above. A 1 % solution, 0.45 µm filter sterilized of 4-O-methyl-D-glucurono-D-xylan dyed with Remazol brilliant blue was added according to the manufacturer's instructions (Sigma-Aldrich). Lipase-positive isolates were streaked by triplicate and incubated at 37 °C for 48 h. The plates were observed on a blue background.

Chitinase activity

Chitin hydrolysis was conducted according to the method described by Rosas-García and coauthors [13] with some modifications. Plates prepared with 10 g minimal medium, 2 g $(NH_4)2HPO_4$, 0.5 g K_2HPO_4, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$ for 1 L, supplemented with a solution of 5 % shrimp shells colloidal chitin. Lipase (+) isolates were inoculated in triplicate and incubated at 28 °C for 72 h. Plates were flooded with 1 % Congo red for 15 min, then the dye was discarded, and plates were allowed to dry to observe halos of chitinase activity [14].

Molecular identification of bacterial isolates with lipase activity

The DNA of bacterial isolates was extracted by the phenol-chloroform protocol [15]. Each isolate was inoculated in 15 mL of LB broth and incubated at 30 °C for 24 h at 120 rpm. Each sample (2 mL) was taken under sterile conditions and centrifuged in a microcentrifuge for 2 min at 13,000 rpm. The precipitate was washed twice with ultrapure water. Each sample was gently macerated with a pestle after the addition of 25 µl of TA extraction buffer (200 mM Tris HCl pH 8.25, 250 Mm NaCl, 25 Mm EDTA, 5% SDS), then homogenized in a vortex for 20 s and incubated for 20 min at room temperature, later 250 µL of phenol-chloroform 25/25 were added at 4 °C and mixed in a vortex at maximum speed for 5 min. Samples were centrifuged at 13,000 rpm for 30 min, the supernatant was transferred to a new tube, and 1 µL of RNAase (20 mg/mL) was added and incubated for 30 min at 37 °C. After that, 250 µL isopropanol at 4 °C were added,

mixed by inversion, and incubated for 30 min at -20 °C. The samples were centrifuged at 13,000 rpm for 5 min, the supernatant was discarded, and 250 µL of 70% ethanol at 4 °C were added. Tubes were mixed by slight inversion and centrifuged for 5 min, the supernatant was discarded, and the pellet was allowed to dry on absorbent paper. Once the pellet was dry, it was resuspended in 20 µL of sterile ultrapure water and stored at -20 °C until use. The gDNA from each isolate was used to amplify the 16S ribosomal RNA subunit gene. The PCR was performed using a reaction mix consisting of: 12.5 µL of Go Taq Green (2X) buffer (Promega M7122), 1 µL of 27F (5′-AGAGTTTGATYMTGGCTCAG-′3) and 1495R (5′-CTACGGCTACCTTGTTACGA- ′3) universal bacterial primers, 9.5 µL of sterile ultrapure water, then 24 µL of the mixture were transferred to each 0.2 mL tube, plus 1 µL of gDNA (50 ng/µL) to obtain a final volume of 25 µL, taken to the thermocycler. The program proceeded with an initial denaturation step at 94 °C/1 min, 30 cycles of a denaturation step at 94 °C/1 min, an alignment step at 60 °C/1 min, and an extension step at 72 °C/1 min, a final extension step was performed at 72 °C/3 min, and then the tubes were kept at 10 °C/∞. The PCR products were visualized in a 1.5% agarose gel. For this purpose, 5 µL of the PCR product, 2 µL of SyBr Gold (10X), and 2 µL of 100 bp molecular marker (Promega) were taken. Electrophoresis was performed in a horizontal chamber at 80 V/1 h, and the gel was visualized in an ultraviolet transilluminator. The 16S rRNA gene fragments obtained from PCR were purified with the commercial ExoSAP-IT® kit following the manufacturer's instructions. The purified products were stored at -20 °C until use. The amplicons were sent to the CBG-IPN service laboratory for sequencing. The sequences obtained in ABI format were compared by BLAST in the National Center of Biotechnology Information (NCBI) database. A similarity percentage above 95% was considered to identify isolates.

PET material

Soft drink PET bottles were collected, and the remains were discarded. Bottles were washed with dish soap, rinsed with distilled water several times, and dried with absorbent paper towels. The flat part of the bottle was cut into 2 x 2 cm sheets. The marker lines from each sheet were cleaned with 70% ethanol, dried at room temperature, and stored until use.

Physical and chemical pretreatment

The PET pretreatment was followed as reported by Sepperumal and coauthors [16]. Glass Petri dishes containing several PET sheets were exposed to 250 nm UV-C light for 5 days in a closed box [17, 18]. Then PET sheets were placed in an oven at 100 °C for 15 min and finally placed in a beaker with HNO_3 for 15 min. Pretreated PET sheets were cleaned with 5% SDS for 1 h under constant agitation and then disinfected in 70% ethanol for 1 h. The sheets were dried with sterile absorbent paper and stored in 50 mL Falcon tubes until use.

Biological pretreatment with *Stenotrophomonas pavanii*

Stenotrophomonas pavanii was cultured in LB broth and monitored until it reached an OD = 1. Erlenmeyer flasks were prepared in triplicate with 50 ml of LB medium and inoculated with 1 mL of *S. pavanii* (OD =1) with a physical and chemical pretreated PET sheet. The flasks were incubated in a rotary shaker at 150 rpm and 37 °C. Each PET sheet was removed from the culture at 28 days for macroscopic analysis and Fourier transform infrared spectrometry (FT-IR). **Biodegradation tests**

Physically, chemically and biologically treated PET sheets were disinfected with 5 % SDS and 70 % ethanol for 1 h and autoclaved inside test tubes. Each pretreated PET sheet was placed in 75 mL of LB medium, then 1 mL of each isolate (OD = 1) was inoculated. Flasks were incubated at 37 °C at 150 rpm for 60 days. Each isolate was tested in triplicate. The changes in chemical structure in PET material were evaluated at 0, 14, 30, and 60 days by FT-IR analysis [19]. The analysis was conducted with an ALPHA II compact FT-IR spectrometer (Bruker, UK), scanning from 500 cm^{-1} to 4000 cm^{-1} at room temperature. The analysis was conducted with an untreated PET sample, and pretreated PET inoculated with the isolates. Data were analyzed by the software Opus v 8.5.

Simple visualization and SEM observation of degraded PET

PET surface damage was observed by photographs obtained by simple visualization with a cell phone camera. Scanning electron microscopy (SEM), observation was conducted in an FEI Nova NanoSEM, using an acceleration voltage of 15kV and low vacuum electrons (eSEC) imaging technique of as-obtained 1 cm^2 PET pieces at 800X magnification.

Design of oligonucleotides for detection of enzymes in selected isolate

Oligonucleotides were designed to detect cellulase, xylanase, protease, chitinase, and phospholipase genes for isolate with the highest degrading ability. For this purpose, sequences of conserved regions and promoters of the corresponding isolate from NCBI were analyzed with the Primer Design tool. The designed oligonucleotides were analyzed with the Sequence Manipulation Suite SMS v.2 to confirm amplification.

RESULTS

Selection of microorganisms with lipase activity and enzymatic activity detection

Eight different isolates were pre-selected due to their positivity in the lipase tests. Three isolates (M52, P1, P2) were obtained from the collected soil samples and PET bottles, and four isolates (C18, C19, C21, C29) were obtained from the laboratory collection.

The enzymatic activity detected in the eight isolates shows differences. Only C18 showed chitinase activity, and the cocci M51 and M52 only exhibited lipase activity. All bacilli had protease, cellulose, and xylanase activities (Table 1).

Table 1. Enzymatic activity associated to PET degradation from eight bacterial isolates.

Isolate key	Morphology	Gram	Enzymatic activity				
			Lipase	Protease	Cellulase	Xylanase	Chitinase
M51	Cocci	(+)	+	-	-	-	-
M52	Cocci	(-)	+	-	-	-	-
P1	Bacilli	(-)	+	+	-	-	-
P2	Bacilli	(-)	+	+	+	-	-
C18	Bacilli	(+)	+	+	+	+	+
C19	Bacilli	(+)	+	+	+	+	-
C21	Bacilli	(+)	+	+	+	+	-
C29	Bacilli	(+)	+	+	+	+	-

Isolates molecular identification

The genera and species identified by sequencing of the 16S rRNA gene are shown in Table 2.

Table 2. Isolates molecular identification based on 16S rRNA gene sequencing.

Isolate key	ID BLAST	Long	Similarity percentage	GenBank/GB
M51	*Micrococcus aloeverae*	1356	98.56%	NR_134088.1
M52	*Acinetobacter beijerinckii*	1349	97.94%	NR_042234.1
P1	*Pseudomonas soli*	1386	98.59%	NR_134794.1
P2	*Pseudochrobactrum saccharolyticum*	1424	97.82%	NR_042473.1
C18	*Bacillus siamensis*	1317	97.76%	NR_117274.1
C19	*Bacillus amyloliquefaciens*	1378	97.41%	NR_041455.1
C21	*Bacillus subtilis*	1395	98.68%	NR_112116.2
C29	*Bacillus tequilensis*	1395	98.24%	NR_104919.1

Biodegradation tests

The isolate that showed the most significant change in the infrared spectrum was P1, corresponding to *Pseudomonas soli*. Marked changes are observed in the peaks corresponding to –OH, C=O, aromatic ring, and CH_2 groups. *P. soli* showed a prominent peak in the range of 3000-3500 cm^{-1} (-OH) and a decrease in the transmittance of the peaks corresponding to ester bonds (1700 cm^{-1}), aromatic ring (1000-1100 cm^{-1}), and CH_2 (>700 cm^{-1}) compared to the other isolates and the positive control of *S. pavanii* at 60 days of

treatment as indicated in Figures 1 and 2. *P. soli* was selected as the bacterium with the highest PET degrading ability.

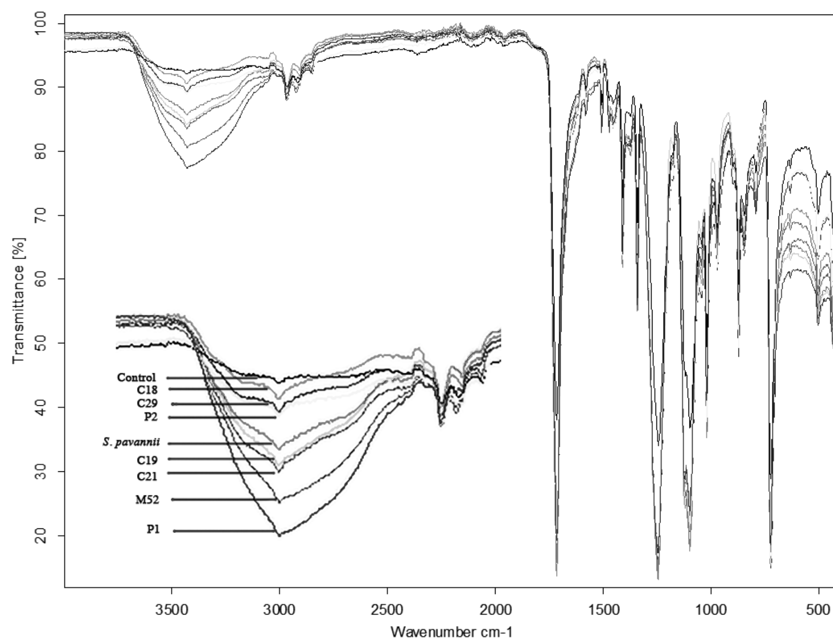

Figure 1. Infrared spectrum of PET degraded by each one of the eight isolates after 60 days of incubation.

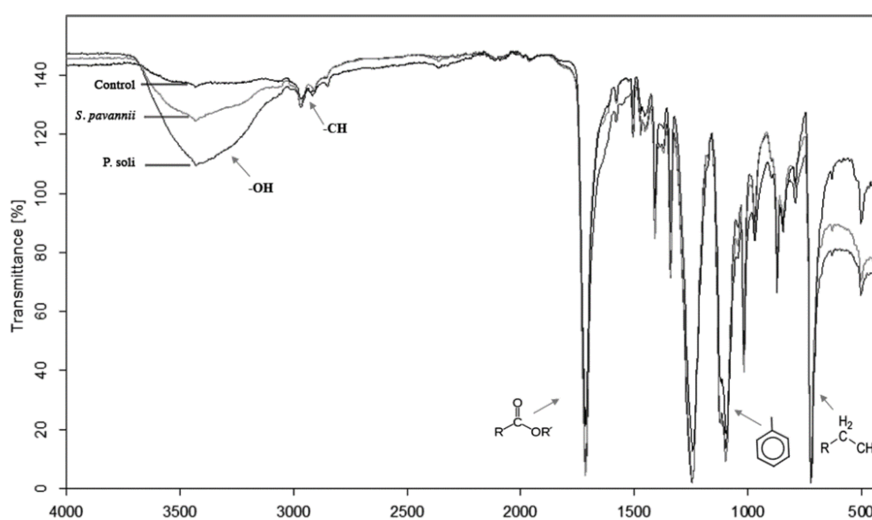

Figure 2. Infrared spectrum of the PET degraded by *S. pavanii* and *P. soli.*

Simple visualization and SEM observation of degraded PET

The PET sheets underwent various physical changes after being treated with UV + 100°C + HNO_3, as shown in Figure 3. Under simple visualization, (3B) PET sheets had a dusty white surface to the touch. The dust was loose since it was removed after biological treatment with *S. pavanii*, leaving erosion marks on the PET surface after 28 days of exposure (3C). When the PET is shaken in the culture medium (3D, 3E, and 3F), it acquires a pearly white appearance, and some dark spots are observed. The PET pretreated and exposed to *S. pavanii* (3G, 3H) shows a pearly white surface, bubble formation, and dark spots. After 60 days of exposure, the PET sheet still has bubbles, but the surface is gleaming (3I). The PET pretreated and exposed to *P. soli* looked similar to that of *S. pavanii*, but more dark spots were observed, and after 60 days PET sheet was shiny.

In the SEM images obtained by eSEC signal (Figure 4), the untreated PET sheets (Figure 4A) show scratches derived from the ordinary handling of the plastic since this PET came from soft drinks bottles. PET physically and chemically pretreated [UV, 100 °C, HNO_3] shows a dusty surface, with grooves and some clean areas (Figure 4B). In the PET pretreated + *S. pavanii* treatment for 28 days (Figure 4C), erosion and scales are observed. The PET sheets without previous treatment agitated in LB medium exhibit scaling and erosion

(Figures 4D, 4E, 4F). PET pretreated and exposed to *S. pavanii* shows an irregular surface with depressions (Figure 4G) and bulges (Figure 4H), and after 60 days, it looks scaled and with dust particles. The PET sheet pretreated + *S. pavanii* was exposed to *P. soli*, furrows, and bulges were observed (Figure 4J), scaling (Figure 4K), and pits and dust particles (Figure 4L).

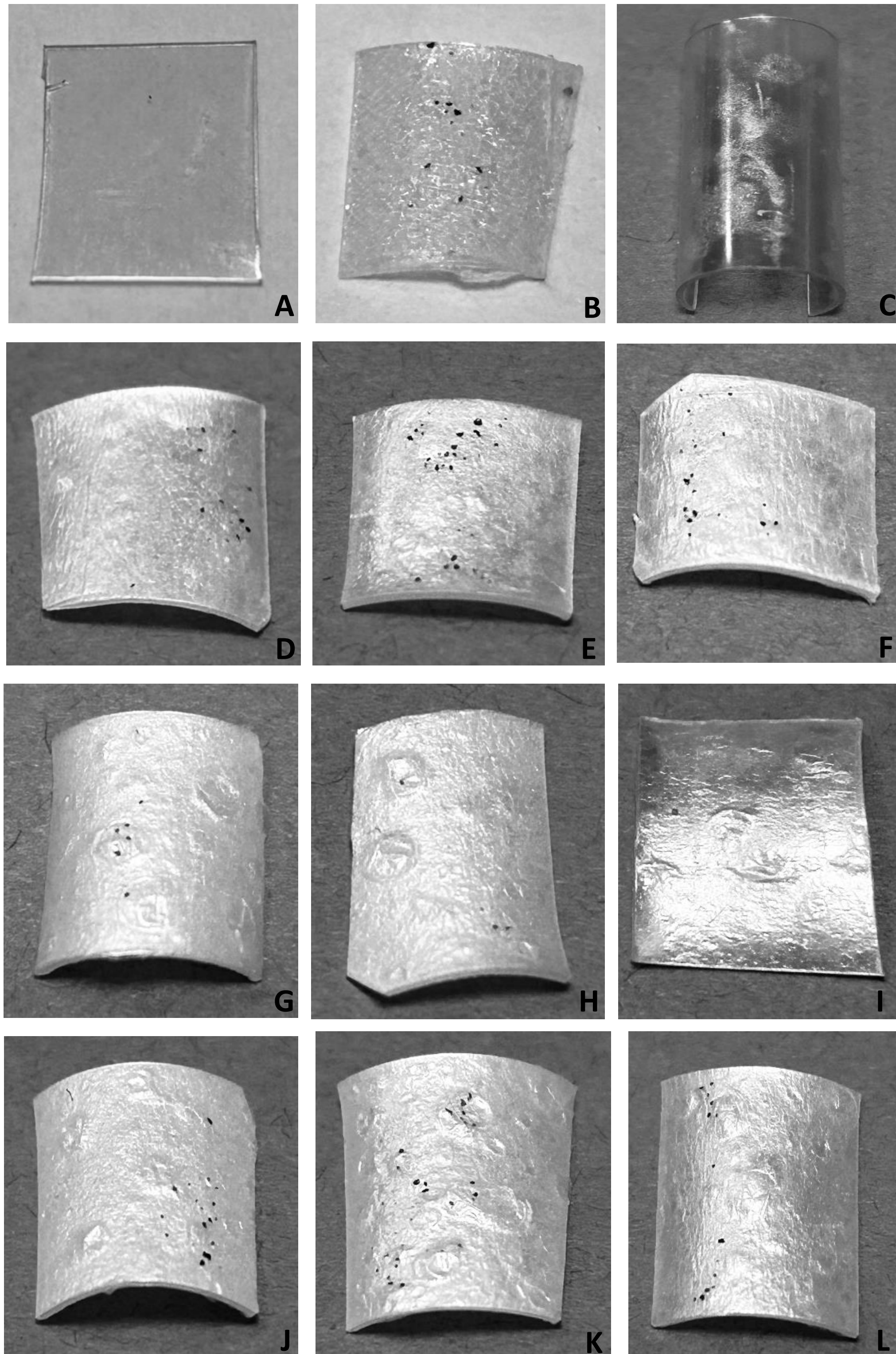

Figure 3. Visual observation of PET sheets after treatments. A) Untreated PET, B) PET physically and chemically pretreated [UV, 100°C, HNO_3]. C) PET pretreated + *S. pavanii* treatment for 28 days. D) PET agitated in LB medium for 14 days. E) PET agitated in LB medium for 30 days. F) PET agitated in LB medium for 60 days. G) PET pretreated + *S. pavanii* for 14 days. H) PET pretreated + *S. pavanii* for 30 days. I) PET pretreated + *S. pavanii* for 60 days. J) PET pretreated + *S. pavanii* and exposed to *P. soli* for 14 days. K) PET pretreated + *S. pavanii* and exposed to *P. soli* for 30 days. L) PET pretreated + *S. pavanii* and exposed to *P. soli* for 60 days.

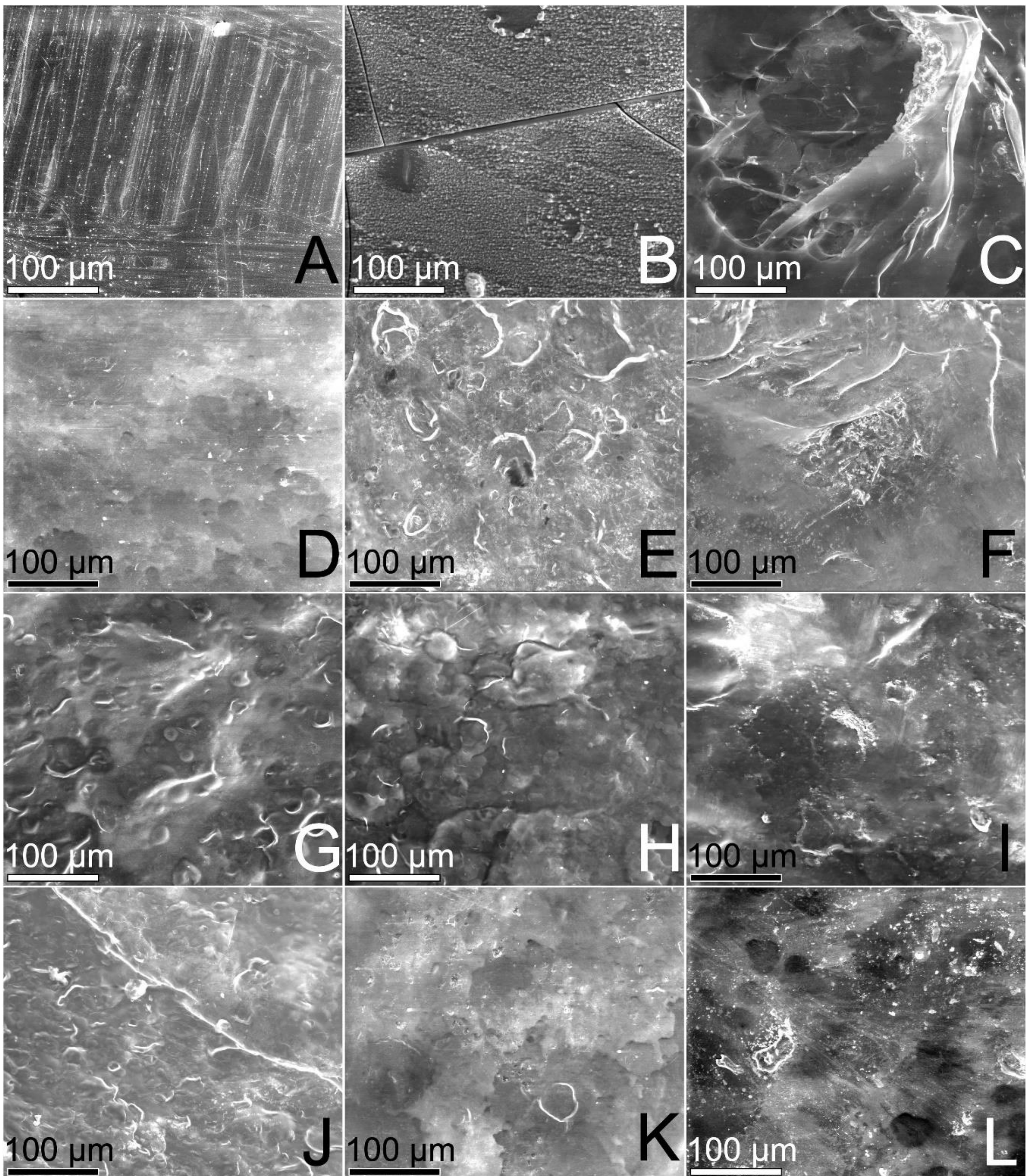

Figure 4. Scanning electron microscope observation of PET sheets after treatment. A) Untreated PET, B) PET physically and chemically pretreated [UV, 100°C, HNO_3]. C) PET pretreated + *S. pavanii* treatment for 28 days. D) PET agitated in LB medium for 14 days, E) PET agitated in LB medium for 30 days, F) PET agitated in LB medium for 60 days. G) PET pretreated + *S. pavanii* for 14 days, H) PET pretreated + *S. pavanii* for 30 days, PET pretreated + *S. pavanii* for 60 days. J) PET pretreated + *S. pavanii* and exposed to *P. soli* for 14 days, K) PET pretreated + *S. pavanii* and exposed to *P. soli* for 30 days, L) PET pretreated + *S. pavanii* and exposed to *P. soli* for 60 days.

Oligonucleotides design and amplification of hydrolases genes in *Pseudomonas soli*

The presence of the hydrolase genes was determined on *P. soli*. Table 3 indicates the searched enzymes and the oligonucleotide design. *Pseudomonas soli* harbors the cellulose, phospholipase, protease and xylanase genes. No amplification was observed for the chitinase gene (Figure 5).

Table 3. Oligonucleotide design for hydrolase enzymes in Pseudomonas soli

Enzyme/Oligonucleotide	Gen selected region	5'-3' sequence	Size producto (bp)
Cellulase/ Cellu1	24700-41684	F TCAACCAGTTGGGTATCGGC R CCGGTCTGTTCGAGGATCTG	514
Protease/ Prot2	1603811-1725811	F TTTCATTAAAGCGCGCCACC R TCCGGAGTGACATTGAGCAC	640
Chitinase/Chit3	24700-41684	F ATCATCGCCCTCACCGAAAG R ATGCAGGATCTTCATGCCGT	294
Xylanase/ Xyl4	24700-41684	F CGATAGCCCGTACCAGACAC R TCGATGACCCGCGAATAGTG	636
Phospholipase/ Phos6	10715-16799	F AGGACAAGGCCGACATCAAG R GTCGGTGACTGGACTTCTGG	531

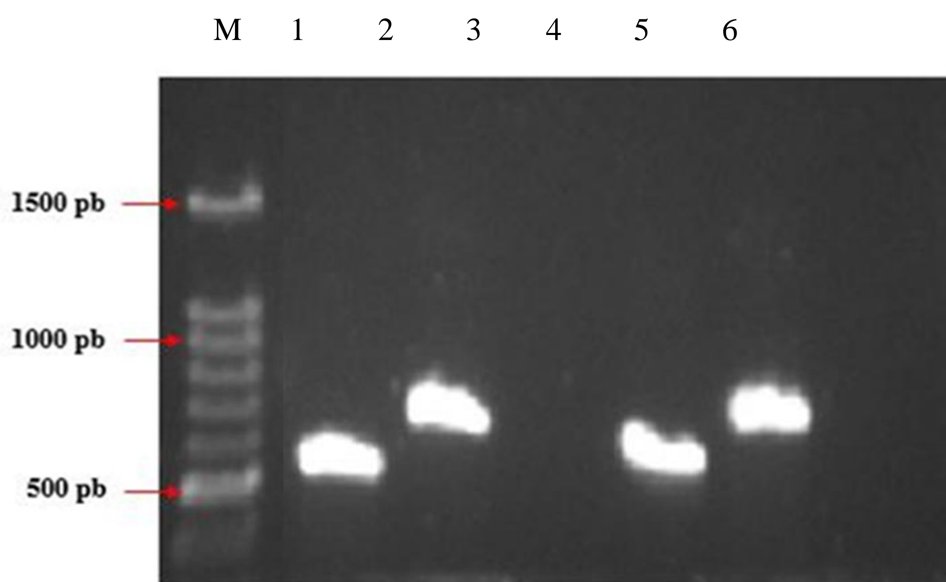

Figure 5. Amplification of hydrolases genes in *Pseudomonas soli*. 1%. Agarose gel. (M) molecular marker DNA ladder 100 bp (GoldBio), 1) Cellu1, 2) Xyl4, 3) Chit3, 4) Phos6, 5) Prot2, 6) Control

DISCUSSION

After analyzing 46 bacteria isolates, only eight showed the ability to degrade PET partially. Among them, *P. soli* was considered the most degrading bacterium compared to the other isolates tested. *Acinetobacter beijerinckii* showed a degradation peak close to that of *P. soli* at 3500 cm^{-1}. This strain has been isolated from human and animals, and from different environments [20]. There are no reports of *A. beijerinckii* as plastic degrader; however, it has been reported as a hydrocarbon degrader [21]. Although this strain was not selected as the best degrader in this work, it presents a degradation potential that can be explored. *Micrococcus aloeverae* is a halotolerant actinobacterium [22] that has been reported as a chemical insecticide degrader [23]. *Pseudochrobactrum saccharolyticum*, which is an alkaliphilic and halotolerant strain, has been reported as a Cr(VI) reducing bacterium [24], and according to the Technical Rules for Biological Agents it is classified in the risk group 1 because it is improbable that can cause and infectious disease in humans [25]. No information is reported so far as plastic degrader.

The *Bacillus* isolates analyzed in this work demonstrated the lowest degradation rate despite being reported as very efficient degraders in different plastic polymers [26]. *Bacillus subtilis* and *B. amyloliquefaciens* showed a low PET degradation, even though they possess almost all enzymatic activities (except chitinase activity). *Bacillus siamensis* was the only strain that exhibited all the enzymatic activities, including chitinase, and *B. tequilensis* did not produce chitinase activity. However, both strains showed less prominent peaks corresponding to the group OH. In several *Bacillus* species, the plastic degrading ability has been attributed to extracellular enzymes such as lipases, proteases, carboxymethylcellulases, xylanases, and chitinases [27]. These enzymes possess a typical α/β-hydrolase fold and several disulfide bonds,

promoting thermal stability and specific binding to PET. These enzymes are considered necessary in plastic degradation since they are assumed to degrade large polymers into smaller subunits that bacteria can incorporate and funnel to classical degradation pathways [28]. We looked for these enzymes in all the strains studied, expecting that the greater the number of genes, the greater the degradation rate.

Nevertheless, this is not the case since *Bacillus siamensis* exhibits all the enzymatic activities tested, but its activity to degrade PET was similar to that of the control (Figure 1). All the studied strains harbor lipase activity which, is associated with polymer degradation due to lipid and polymer chain structural similarities. Lipase enzymes are associated with lipid hydrolysis, breaking ester bonds (C=O), and due to the structural similarities of lipid and polymer chains, lipase activity is considered essential to select microorganisms with plastic degrading potential. Lipase activity could be actual since *Acinetobacter beijerinckii* only exhibited lipase activity and showed a degradation peak very similar to that of *P. soli*. However, not all lipases can degrade PET [10].

Some species of *Pseudomonas* have been reported as low-rate plastic degraders for polypropylene, polyvinyl chloride, and PET [29]. *Pseudomonas aeruginosa*, *P. putida*, and *P. syringae* can degrade a variety of synthetic plastics; however, PET is one of the polymers with fewer reports of biodegradation [29, 30]. *Pseudomonas soli* was reported for the first time by Pascual and collaborators [31] as a new strain able to produce xantholysins, which are lipodepsipeptides active against tumor cell lines. Interestingly, our *P. soli* strain harbors four hydrolase genes (lipase, protease, cellulase, and xylanase), lacks chitin gene and although xylanase gene is present, the enzyme is not produced. *Pseudomonas* spp. have not been very effective for PET degradation, nor lipases isolated from them [32]. Although it is not well known which enzymes participate in this process, their biofilm formation capacity indisputably helps in the degradation process. Bacterial cell adhesion to hydrocarbon was evaluated toward the hydrophobic surface of synthetic plastic, indicating that biofilm was formed and also *Pseudomonas* used it as a carbon source [7].

Proteases are also considered necessary in the structure and function of the biofilm to adhere to the material and allow colonization to the surface [33]. Other hydrolytic enzymes, such as cellulase, chitinase, and xylanase, break the glycosidic bonds and long-chain peptides, as well as degrade complex compounds such as chitin from shells of insects or mollusks [27]. In this way, bacteria can work more efficiently in the degradation process, which is facilitated by the pretreatment of PET. Mechanisms such as photo-oxidation, chemical hydrolysis, wear of the material, and biological treatment using *Stenotrophomonas pavanii*, which has been reported as a microorganism to pretreat PET [34], are essential. The combined use of these treatments serves to generate wear on the surface of the material, decrease crystallinity and hydrophobicity of the surface, creating porosities that help microorganisms to colonize and facilitate extracellular enzymatic actions to have access to the substrate [18, 35].

Plastic degradation processes using microorganisms have been demonstrated in different techniques. One of the most used is infrared spectroscopy. Multiple authors agree that PET degradation can be detected after the appearance of peaks between 3000 - 3500 cm^{-1}, as well as the decrease or appearance of corresponding peaks to ester bonds and aromatic rings by the formation of -OH and -COOH groups, in addition to stretching of C-O groups in estimated times of 30 to 60 days [17, 21, 27]. The infrared spectra from our results obtained 60 days after the bioassay agree with those observations. Our study observed a new peak around 3500 cm^{-1} of the PET in the presence of *P. soli*, confirming partial PET biodegradation after the pretreatment and *P. soli* action.

With the naked eye, it is possible to observe the bubbles formed in the PET surface as well as deterioration and a shiny texture, but SEM observation indicates that *P. soli* can produce not only scales and grooves but deep wells, as observed on the PET surface. The formation of bumps and dips on the PET surface is observed in biological degradation (Figure 3F, 3G, 3I, and 3J), and after 60 days, pits formation is more evident in Figures 3H and 3K corresponding to the activity of *S. pavanii* and *P. soli* respectively. We consider that the pretreatment facilitated the formation of pits by *P soli*.

This study shows that there are microorganisms that have the potential to degrade PET. Not all of the enzymes that have been associated with PET degradation are actually involved in the process. Other enzymes, such as dehydrogenases, oxidases, and cutinases, have yet to be analyzed. Moreover, they may have substantial participation in the degradation of PET. More studies are necessary concerning the type of enzymes that participate in this type of process and the study of the time it takes for a bacterium and the conditions of its environment to degrade PET. Having an enzymatic battery reported as useful for the degradation of PET does not ensure that said degradation is carried out. In addition, more studies are necessary for developing and producing a formulation or application form of these bacteria that can help in the degradation of the PET contaminating the ecosystem and offer an alternative solution that is friendly to the environment.

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