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HIGH DENSITY POLYETHYLENE MICROPLASTICS BIODEGRADATION BY BACTERIA ISOLATED FROM A LANDFILL IN CALI, COLOMBIA

Biodegradación de microplásticos de polietileno de alta densidad mediante bacterias aisladas de un relleno sanitario en Cali, Colombia

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Key words: Mixed cultures, weight loss method, scanning electron microscopy, *Pseudomonas aeruginosa*, *Streptomyces*, *Tsukamurella*.

ABSTRACT

Microplastics cause pollution problems because they are difficult to degrade and tend to be easily transported and accumulate in the environment. Biodegradation is a promising solution by exploiting the ability of fungi or bacteria to accelerate the degradation process of microplastics, but this is a topic with several knowledge gaps. To evaluate the ability of microorganisms to degrade high-density polyethylene microplastics, four bacterial strains isolated from a sanitary landfill were selected, in a minimal salt medium with microplastics as the sole carbon source. The selected strains were identified as *Tsukamurella* sp. strain JP3, *Tsukamurella* sp. strain JP5+, *Pseudomonas aeruginosa* (JP5–) and *Streptomyces* sp. strain JP8. The bioassays were performed with single strains and mixed cultures. Biodegradation was determined by calculating the percentage weight loss of the microplastics and observing changes in their surface structure using scanning electron microscopy (SEM). Biodegradation rates between 6.81 and 18.14% were obtained, with JP5+JP8 being the treatment with the highest percentage, but with no significant differences between treatments. However, SEM showed microbial damage and growth on the surface of microplastics. Therefore, the bacterial strains used would have potential for biodegradation of high-density polyethylene, but it is advisable to continue investigations to determine the conditions and appropriate times to improve biodegradation with these strains.

Palabras clave: cultivos mixtos, método de pérdida de peso, microscopía electrónica de barrido, *Pseudomonas aeruginosa, Streptomyces, Tsukamurella*.

RESUMEN

Los microplásticos generan problemas de contaminación ya que son difíciles de degradar y tienden a transportarse y acumularse fácilmente en el ambiente. La biodegradación es una solución prometedora que aprovecha la capacidad de hongos o bacterias para acelerar el proceso de degradación de los microplásticos, pero se trata de un tema con varios vacíos de conocimiento. Con el fin de evaluar la capacidad de los microorganismos para degradar microplásticos de polietileno de alta densidad, se seleccionaron

cuatro cepas bacterianas aisladas de un vertedero sanitario, en un medio mínimo de sales con microplásticos como única fuente de carbono. Las cepas seleccionadas se identificaron como *Tsukamurella* sp. cepa JP3, *Tsukamurella* sp. cepa JP5+, *Pseudomonas aeruginosa* (JP5–) y *Streptomyces* sp. cepa JP8. Los bioensayos se realizaron con cepas individuales y cultivos mixtos. La biodegradación se determinó calculando el porcentaje de pérdida de peso de los microplásticos y observando los cambios en su estructura superficial mediante microscopía electrónica de barrido (MEB). Se obtuvieron tasas de biodegradación entre el 6.81 y el 18.14%, siendo JP5+JP8 el tratamiento más eficiente, pero no se presentaron diferencias significativas entre tratamientos. Sin embargo, la MEB mostró daños y crecimiento microbiano en la superficie de los microplásticos, excepto en el testigo. Por lo tanto, las cepas bacterianas utilizadas tendrían potencial para la biodegradación del polietileno de alta densidad, pero es recomendable continuar las investigaciones para determinar las condiciones y tiempos adecuados para mejorar la biodegradación con estas cepas.

INTRODUCTION

Plastics are long-chain synthetic polymers composed of various organic compounds derived mainly from coal, natural gas and oil (Barnes et al. 2009, Ahmed et al. 2018, Rodríguez et al. 2020, Amobonye et al. 2021). Plastics are widely used in daily life and in different industries, which has led to their massive production since the 1950s, and each year their production increase, so that in 2015 were produced more than 400 million tons, and it is expected that this number will double by 2035. Low-density polyethylene (LDPE) and high-density polyethylene (HDPE) are the most commonly produced and utilized plastics, primarily for the manufacturing of plastic bottles, containers, and bags (RSTA 2019, Rodríguez et al. 2020, Ali et al. 2021, Amobonye et al. 2021).

However, plastics are difficult to degrade because their chemical composition gives them a very stable structure, preventing them from quickly entering the biosphere's degradation cycles (Ahmed et al. 2018, Wierckx et al. 2018, Jaiswal et al. 2020). Furthermore, plastic waste is often mishandled, resulting in its presence in various environments such as soil, sediments, and the water column of rivers, lakes, and oceans, leading to the accumulation of plastics in these locations, and environmental pollution problems (Barnes et al. 2009, Ahmed et al. 2018, Ganesh-Kumar et al. 2020). In addition, microplastics (pieces of plastic smaller than 5 mm in size) pose a greater risk than larger plastics because they are more easily transported by water or wind, accumulate more in different environments, and affect ecosystems and living organisms in different ways (Ogunola et al. 2018, Kutralam-Muniasamy et al. 2020, Du et al. 2021, Zhang et al. 2021). For example, microplastics can reduce soil fertility; release chemicals that are added to plastics as dyes, additives, or catalysts, which can be toxic; and enter the food chain through accidental ingestion by wildlife, which can cause serious disease in both the animals that consume them and their predators, including humans (Chae and An 2018, Qi et al. 2018, Boots et al. 2019, RSTA 2019, Ganesh-Kumar et al. 2020, Ali et al. 2021, Du et al. 2021, Zhang et al. 2021).

Various methods are commonly used to eliminate plastic waste, such as incineration, landfilling, and recycling. However, these methods have disadvantages such as: high cost, generation of toxic substances or gases, high energy consumption, or the need for very specific structures and machinery (RSTA 2019, Rodríguez et al. 2020, Amobonye et al. 2021). Furthermore, these methods are generally not suitable for microplastics (Du et al. 2021), so there is a need to explore the development of new alternatives. Among these, biodegradation has emerged as a promising solution due to its energy efficiency, low cost, and environmental friendliness. This technology utilizes the ability of microorganisms, especially those indigenous to contaminated sites, to degrade organic compounds using various enzymatic systems, ultimately resulting in the mineralization of contaminants, producing mainly CO_2, H_2O and microbial biomass (Shah et al. 2008, Rajendran et al. 2015, Rodríguez et al. 2020, Amobonye et al. 2021).

Studies on the biodegradation of plastics have identified more than 250 strains of bacteria and fungi that are capable of degrading different types of plastics, of which the most evaluated are polyethylene (mainly LDPE), polyurethane and polystyrene. These strains have been found in environments such as soils, activated sludge from treatment plants, and marine and freshwater sediments (Bhuvaneswari 2018, Park and Kim 2019, Rodríguez et al. 2020,

Matjašič et al. 2021). However, it is important to note that the degradation rates of reported microorganisms vary significantly depending on the experimental or environmental conditions, such as temperature, pH, exposure to light, and the type of plastic being degraded. Most studies report degradation rates of around 10-20% after one to six months of exposure to microorganisms (Bhuvaneswari 2018, Park and Kim 2019, Rodríguez et al. 2020, Ali et al. 2021, Amobonye et al. 2021, Matjašič et al. 2021).

Despite the research that has been done, there are still knowledge gaps that require further research. For example, there are no studies in specific regions that could aid in the identification of new microorganisms that have the potential to degrade. Furthermore, few studies utilize microplastics instead of large plastics, which is needed to know whether microorganisms that degrade large plastics interact with and degrade microplastics similarly. Similarly, few studies have evaluated whether better biodegradation rates can be obtained with consortia or mixed cultures compared to those obtained with pure cultures (Skariyachan et al. 2017, Rodríguez et al. 2020, Matjašič et al. 2021). Colombia is one of the countries where the biodegradation of plastics has been poorly studied, although some research conducted in the country has shown that plastics, especially microplastics, are contaminants that could have negative effects on wildlife, the ecosystem, and even human health (MASP and GC 2019, Kutralam-Muniasamy et al. 2020).

Therefore, researching the ability of microorganisms to biodegrade microplastics is crucial in order to identify promising organisms for biodegradation and to generate knowledge that can promote the development of a methodology for addressing microplastic contamination in the future (Rodríguez et al. 2020, Matjašič et al. 2021). In this project, we evaluated the ability of native microorganisms from soil contaminated with plastic waste to degrade HDPE microplastics.

MATERIALS AND METHODS

Sampling

Sampling was conducted at the old Navarro landfill in Cali, Colombia (3º22'52.9" N 76º29"20.7" W), which started operating in 1968, and closed in 2008 (Otagrí 2015). Approximately 200 g of soil samples were collected using sterile augers at a depth of 20 cm at three locations within the landfill. The samples were placed in airtight plastic bags, and transported at 4 ºC to the Microbiological Research Laboratory (LIM) of the Biology Department of the Universidad del Valle.

Seeding and isolation of HDPE degrading microorganisms

80 g of the samples from each collection point were weighed to isolate the microorganisms, placed in a single hermetic plastic bag, and mixed homogeneously. The sample was then sieved to separate rocks and solid waste from the sample. 10 g (with dry weight correction) of the sample were taken and added in triplicate to Erlenmeyer flasks containing 90 mL of a liquid minimal salt medium (MSM). The medium contained 2.34 g K_2HPO_4, 1.33 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, and 1 mL trace element solution (21.8 mg/L $CoCl_2 \cdot 6H_2O$, 21.6 mg/L $NiCl_2 \cdot 6H_2O$, 24.6 mg/L $CuSO_4 \cdot 5H_2O$, 1.62 g /L $FeCl_3 \cdot 6H_2O$, 0.78 g/L $CaCl_2$ and 14.7 mg/L $MnCl_2 \cdot 4H_2O$), per L of distilled water (Park and Kim 2019). In addition, 1% (w/v) pieces of HDPE microplastics (approximately 3 x 3 mm) were added as the sole carbon source, sterilized in 70% ethanol (Mouafo-Tamnou et al. 2021); these microplastics were obtained from HDPE detergent containers that were cut into small pieces. A negative control consisting of 90 mL of MSM containing 1% (w/v) microplastics was also prepared. The flasks were incubated at 30 ºC and 120 rpm for four weeks.

10, 5 and 1 mL of the incubated media and microplastics were transferred using a laboratory loop to three Erlenmeyer flasks containing 90 mL of fresh MSM. Additionally, a negative control was performed as described above. The flasks were then incubated at 30 ºC and 120 rpm for 10 days. Microorganisms were then isolated using the streak plate method in MSM with microplastics added as the sole carbon source on their surface. The Petri dishes were incubated at 30 ºC for one week.

Characterization and selection of microbial strains

The colonies with distinct morphologies were replicated in MSM until pure cultures were obtained. Each one was assigned a code with the letters JP and a number. To select the three strains for the bioassays, we monitored the growth time and colonization of the microplastics, selecting those with the shortest time in these parameters. After this follow-up, the strains JP3, JP5 and JP8 were selected for the bioassays. These strains were cultured on nutrient agar (BD Difco) and potato dextrose agar (PDA; Condalab),

and their macroscopic characteristics (shape, color, border, texture, and elevation of the colonies) as well as their microscopic characteristics (cell morphology and Gram stain) were described.

Additionally, it was necessary to separate a JP5 strain consisting of a possible consortium of Gram-positive bacilli (referred to as JP5+) and Gram-negative bacilli (referred to as JP5–), which could not be separated by the streak plate method. To achieve separation, Mannitol-Egg Yolk and Polymyxin agar (MYP agar; Merck) was used to isolate Gram-positive bacteria, and MacConkey agar (Condalab) was used for Gram-negative bacteria. Each separated bacterium was then cultured on nutrient agar for characterization.

Identification of selected strains

For identification of JP3, JP5+, and JP5– strains, fresh cultures were used in LB medium (Condalab), incubated at 30 ºC and 130 rpm, from which 1.5 mL was aliquoted into Eppendorf tubes, centrifuged at 14 800 rpm for 6 min, and the pellet was resuspended in 100 µL sterile distilled water. DNA extraction was performed using the Monarch Genomic DNA Purification Kit (New England BioLabs) according to the manufacturer's instructions.

PCR was performed using the 41F and 1387R primers (Marchesi et al. 1998, Hongoh et al. 2003), followed by nested PCR using the 63F and 1061R primers (Marchesi et al. 1998, Ku and Lee 2014). The following mix was used for both rounds of PCR: 2.5 µL 10X buffer, 1 µL 10 mM deoxynucleotide triphosphates (dNTPs), 1 µL of each primer (R and F) at 10 mM and 0.1 µL Taq DNA polymerase. In addition, for the first round of PCR, 0.5 µL of DNA and 19 µL of Milli-Q H_2O were added for a final volume of 25 µL, while for the nested PCR, 1 µL of PCR product and 18.4 µL of Milli-Q H_2O were added for a final volume of 25 µL. Amplification was performed under the following conditions: 1 cycle for 2 min at 95 ºC, 35 triphasic cycles at 95 ºC for 30 s, 52 ºC for 30 s, and 68 ºC for 1:30 min, and 1 cycle for 10 min at 68 ºC. Samples were sent to Genewiz for 16S rRNA gene sequencing, and finally a similarity search was performed using the National Center for Biotechnology Information (NCBI) BLAST database to identify isolates.

On the other hand, for the identification of the JP8 strain, the macroscopic and microscopic morphological characteristics that it presented when grown in PDA were used and compared with the characteristics described in the guides by Jayashantha (2015) and Li et al. (2016).

Formation of mixed cultures

The selected strains were tested for antagonism to determine if they inhibited each other. For this purpose, a horizontal line was drawn in the center of a Petri dish with MSM from pure cultures of each of the strains, and equidistant vertical lines were drawn on the horizontal line with the strains to be contrasted. In addition, three negative controls were performed with the strains individually seeded in a horizontal line (Moreno-Benavides et al. 2019). Petri dishes were incubated at 30 ºC for five days.

Mixed cultures were prepared by incubating pure cultures in LB broth for 48 hours at 30 ºC and 140 rpm. These cultures were centrifuged at 4000 rpm for 10 min, washed twice with 10 mL of MSM, and the resulting pellets were resuspended in 5 mL of MSM (Moreno-Benavides et al. 2019). The pellets were weighed, and this wet weight of biomass was used to calculate the concentration of the inoculum (g/mL). From this, different volumes of MSM were added to ensure that all strains had the same concentration. Finally, the strains were added in equal proportions to prepare the mixed cultures.

HDPE degradation bioassays

To Erlenmeyer flasks containing 9 mL of MSM, 1 mL of pure or mixed culture was added. The control was prepared with 10 mL of MSM and 0.1 mL of 1% (w/v) sodium azide (NaN_3) to prevent any possible growth in the medium (Park and Kim 2019). 10 mg of microplastics were added to all flasks, which were previously weighed on an analytical balance (Ohaus, Pioneer Pa214; precision 0.0001 g). Each assay was performed in quintuplicate, and flasks were incubated at 30 ºC and shaken at 130 rpm for 82 days.

Determination of biodegradation rate

The microplastics were removed from the flasks using the membrane filtration method with nitrocellulose filters (Advantec MFS, Inc.) with 0.45 µm pores. Subsequently, they were dried at 33 ºC for 14 h and reweighed to determine the HDPE degradation percentage using the weight loss method with the formula (Divyalakshmi and Subhashini 2016):

$$\text{Degradation percentage } (\%) = \left(\frac{P_0 - P}{P_0}\right) \times 100$$

where P_0 is the initial weight of microplastics and P is the final weight of microplastics.

Statistical analysis

Using R software, a Shapiro-Wilks test and a Levene test were performed to assess normality

and homogeneity of variances of the percentages obtained. The results of these tests indicated that the data did not follow a normal distribution and that there was homogeneity of variances. Therefore, the Kruskal-Wallis test was performed to estimate the statistical significance with a p-value < 0.05 of the degradation percentages.

Analysis of superficial morphological changes in microplastics

Superficial morphological changes were observed using scanning electron microscopy (SEM). One replicate of each treatment was randomly selected, and a 3% glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.0 was added to the microplastics, and left at 4 ºC for 48 h. This solution was then discarded and replaced with ultrapure water, the microplastics were placed at 4 ºC for 10 min, and the procedure was repeated with fresh ultrapure water. The ultrapure water was discarded, 30% ethanol was added and the samples were left at 4 ºC for 20 min. The procedure was repeated with ethanol concentrations of 40, 50, 60, 70, 80, 90, 95 and 100%, the latter being repeated twice. Finally, 98% hexamethyldisilazane (HMDS) was added twice for 30 s (Vejarano et al. 2019).

The samples were dried in an extraction chamber and placed on supports in a desiccator with silica gel. Subsequently, the samples were coated with a 1 nm thick gold layer by sputtering (SPI-Module™ Sputter Coater), and observed in a scanning electron microscope (Phenom Pro X, backscattered electron detector and accelerating voltage of 15 Kv) at magnifications of 500 and 5000 x.

RESULTS

Characterization and identification of selected strains

After characterization and identification of the selected strains, it was found that JP5–, which was observed as a Gram-negative bacillus, corresponds to *Pseudomonas aeruginosa* (NCBI: KX094932.1). These bacteria presented an oval, translucent, shiny colony with a whole edge and convex elevation (**Fig. 1a**). The remaining strains belonged to two genera of actinomycetes, *Tsukamurella* and *Streptomyces*. Strains JP3 and JP5+, Gram-positive bacilli belonging to the genus *Tsukamurella* and were named *Tsukamurella* sp. strain JP3 and *Tsukamurella* sp. strain JP5+, respectively. *Tsukamurella* sp. strain JP3 presented a cream-colored colony, irregular shape and edges, dry appearance, and wrinkles or ridges in the center of the colony (**Fig. 1b**), and *Tsukamurella* sp. strain

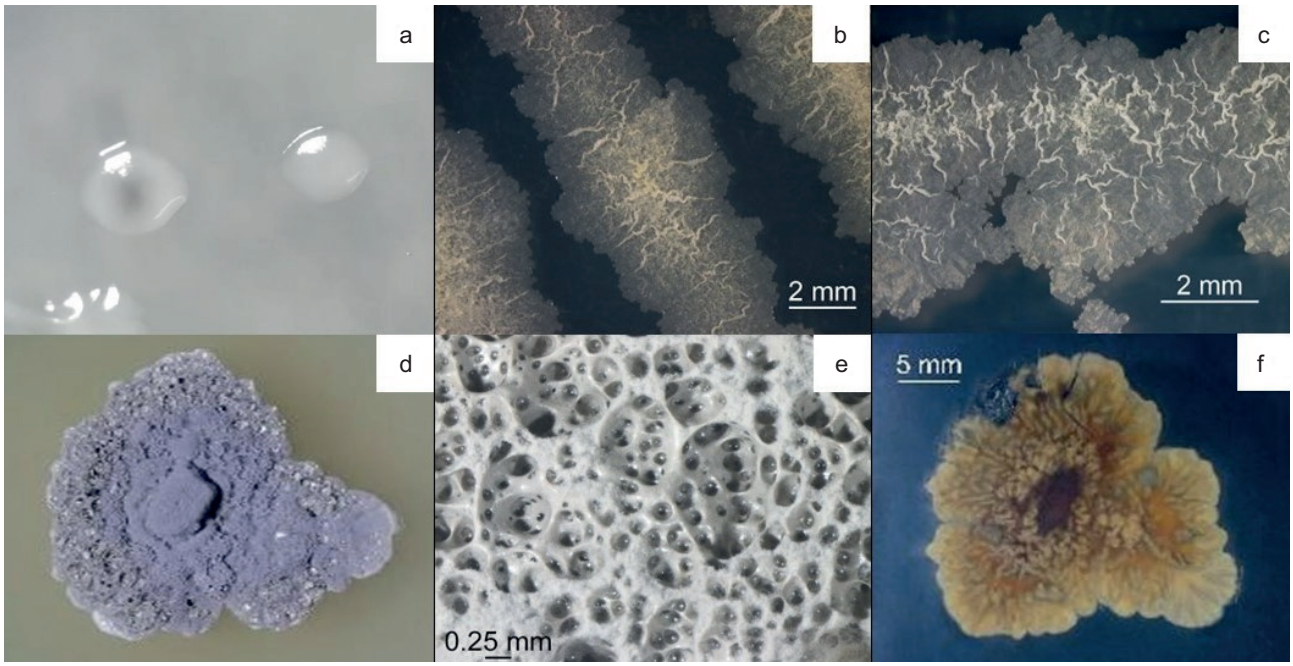

Fig. 1. Macroscopic morphological characteristics of the selected strains to: a) JP5– (*P. aeruginosa*), b) JP3 (*Tsukamurella* sp. strain JP3), c) JP5+ (*Tsukamurella* sp. strain JP5+), and d) JP8 (*Streptomyces* sp. strain JP8). e) Enlargement of the texture of the colony's verse of JP8, and f) Obverse of the colony.

JP5+ showed a pale cream-colored colony, irregular shape and edges, dry appearance, and visible wrinkles or ridges within the colonies (**Fig. 1c**). Finally, JP8 was identified as *Streptomyces* sp. strain JP8, a Gram-positive bacterium with filamentous growth and numerous small oval spores. The colony had a gray color on the verso with white edges (**Fig 1d**), plush texture forming small holes on the colony surface (**Fig. 1e**) and light orange color and rough texture on the obverse (**Fig 1f**).

Formation of mixed cultures

To ensure that the selected isolates did not inhibit each other when performing the mixed cultures for the HDPE degradation bioassays, an antagonism test was performed. The test did not reveal any inhibition between the evaluated strains, as shown in **figure 2**, where the absence of inhibition halos in the union zone of the contrasted strains can be observed. Therefore, strains JP3, JP5 (combination of JP5+ and JP5–) and JP8 were used to form mixed cultures, resulting in the following combinations: JP3+JP5, JP3+JP8, JP5+JP8, and JP3+JP5+JP8.

HDPE degradation bioassays

The results of the HDPE degradation bioassays are presented in **figure 3**, which shows the average degradation percentages of microplastics after 82 days of incubation. The control showed a low weight loss of 3.52%, while the treatment with JP3 (*Tsukamurella* sp. strain JP3) obtained a percentage of 14.79%; JP5 (*Tsukamurella* sp. strain JP5+ and *P. aeruginosa*) had 11.72% degradation; and the treatment with the lowest percentage was JP8 (*Streptomyces* sp. strain JP8) with 6.81%. On the other hand, the combinations of JP3+JP5 (*Tsukamurella* sp. strain JP3 + *Tsukamurella* sp. strain JP5+ and *P. aeruginosa*) showed a weight loss of 11.23%; JP3+JP8 (*Tsukamurella* sp. strain JP3 + *Streptomyces* sp. strain JP8) showed a weight loss of 12.44%; the treatment that had the highest weight loss was JP5+JP8 (*Tsukamurella* sp. strain JP5+ and *P. aeruginosa* + *Streptomyces* sp. strain JP8) with 18.14%; and JP3+JP5+JP8 (*Tsukamurella* sp. strain JP3 + *Tsukamurella* sp. strain JP5+ and *P. aeruginosa* + *Streptomyces* sp. strain JP8) had a weight loss percentage of 11.77%. However, statistical analysis revealed no significant differences between the treatments or the control group (p-value = 0.4883).

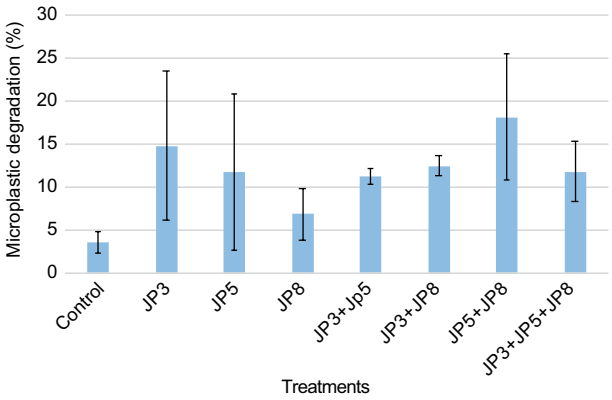

Fig. 3. Average percentage of microplastic degradation after 82 days of incubation with five replicates of the treatments: JP3: (*Tsukamurella* sp. strain JP3), JP5: (Combination of JP5+ (*Tsukamurella* sp. strain JP5+) and JP5– (*P. aeruginosa*)), and JP8: (*Streptomyces* sp. strain JP8). Error bars are obtained from the standard error.

Analysis of superficial morphological changes in microplastics

After performing the degradation bioassays, the morphological changes produced by the different treatments on the surface of the microplastics were visualized by SEM, as shown in **figure 4**. Note that the control microplastics presented a flat, smooth,

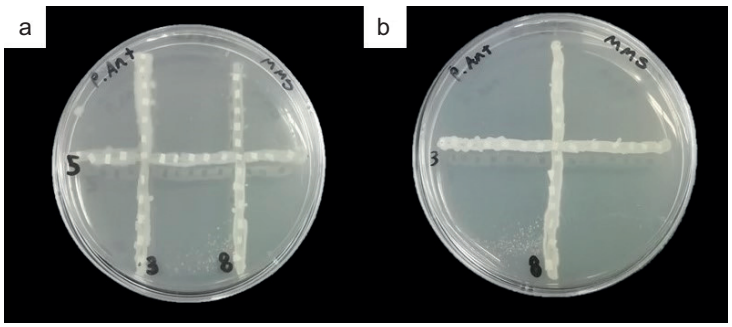

Fig. 2. Antagonism test of the selected strains. a) JP5 (horizontal) vs. JP3 and JP8. b) JP3 (horizontal) vs. JP8.

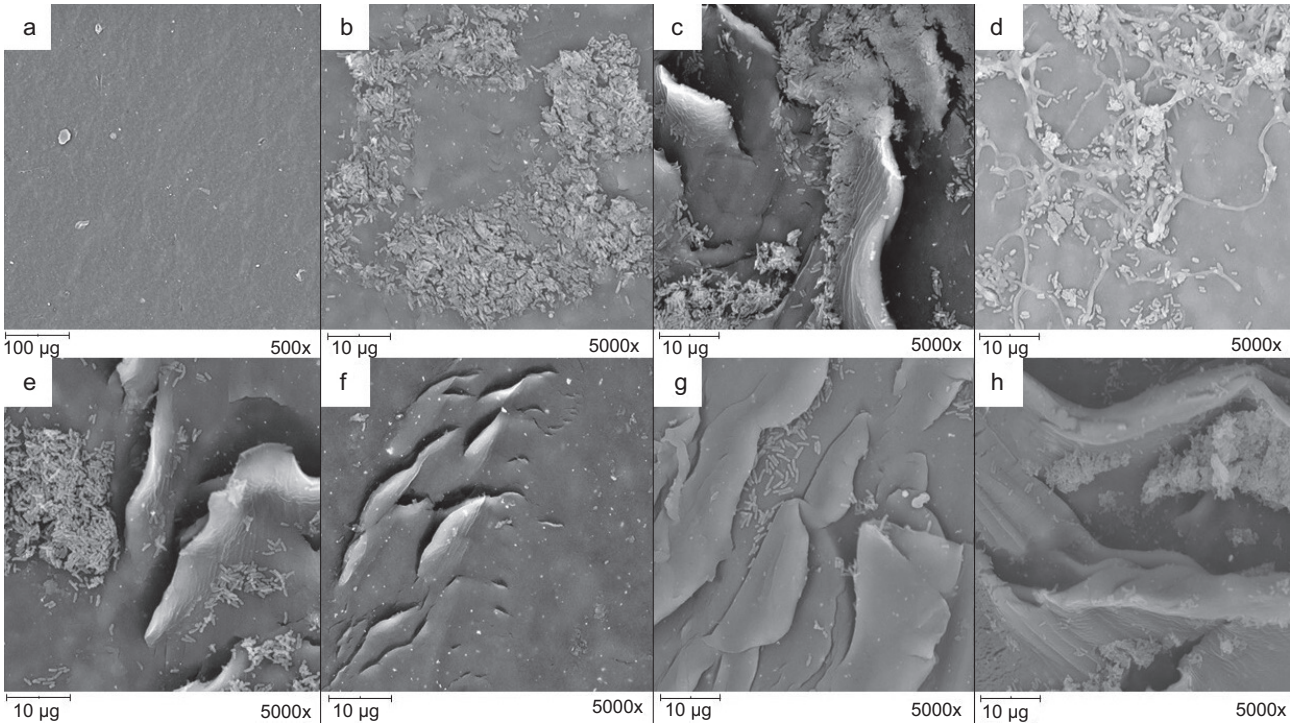

Fig. 4. Scanning electron microscopy. a) Control surface (without inoculation) at 500x. b) Surface of microplastics exposed to *Tsukamurella* sp. strain JP3 (JP3) c) to *Tsukamurella* sp. strain JP5+ and *P. aeruginosa* (JP5) d) to *Streptomyces* sp. strain JP8 (JP8) e) to *Tsukamurella* sp. strain JP3, *Tsukamurella* sp. strain JP5+ and *P. aeruginosa* (JP3+JP5) f) to *Tsukamurella* sp. strain JP3 and *Streptomyces* sp. strain JP8 (JP3+JP8) g) to *Tsukamurella* sp. strain JP5+, *P. aeruginosa* and *Streptomyces* sp. strain JP8 (JP5+JP8) h) to *Tsukamurella* sp. strain JP3*, Tsukamurella* sp. strain JP5+*, P. aeruginosa* and *Streptomyces* sp. strain JP8 (JP3+JP5+JP8), observed at 5000 x.

and homogeneous surface without any damage or microbial growth (**Fig. 4a**). In contrast, the microplastics treated with *Tsukamurella* sp. strain JP3 (JP3) showed abundant colonization of the surface of the microplastics, resulting in the formation of large cell agglomerations (biofilms) and small cracks on the plastic surface (**Fig. 4b**). *Streptomyces* sp. strain JP8 (JP8) exhibited similar behavior, with the presence of small cracks and crevices were present, and the growth of the microorganism on the surface of the microplastic, with filaments and numerous spores along the surface (**Fig. 4d**).

On the other hand, in **figure 4c**, it is observed that *Tsukamurella* sp. strain JP5+ and *P. aeruginosa* (JP5) caused significant damage to the microplastics, as evidenced by the numerous wrinkles, cracks and deep pits that resulted in large plastic parts detaching from the surface. Additionally, there was abundant bacterial growth, with the development of biofilms, both on the surface of the plastic and inside the pits. Similar observations were made in **figures 4e** and **4h**, where *Tsukamurella* sp. strain JP3, *Tsukamurella* sp. strain JP5+ and *P. aeruginosa* (JP3+JP5), and *Tsukamurella* sp. strain JP3, *Tsukamurella* sp. strain JP5+, *P. aeruginosa* and *Streptomyces* sp. strain JP8 (JP3+JP5+JP8), respectively, produced large cracks, pits and irregularities along the surface of the microplastics, as well as abundant colonization and proliferation of the microorganisms, leading to the formation of biofilms.

Finally, **figures 4f** and **4g**, display the effects of the treatments with *Tsukamurella* sp. strain JP3 and *Streptomyces* sp. strain JP8 (JP3+JP8), and *Tsukamurella* sp. strain JP5+, *P. aeruginosa* and *Streptomyces* sp. strain JP8 (JP5+JP8), respectively. The plastics in both figures exhibit pits and several cracks on their surfaces, with more significant damage observed in **figure 4g**. However, unlike the previous figures, there was little growth of microorganisms, with distant biofilms and/or single cells distributed on the surface. Based on the aforementioned characteristics, it can be concluded that the treatments causing the most damage were JP5, JP3+JP5, JP5+JP8 and JP3+JP5+JP8.

DISCUSSION

Contaminated sites exert pressure on native microbiota, leading to adaptation to these environments by developing metabolic pathways that allow microorganisms to utilize contaminants as a source of carbon and energy (Ali et al. 2021, Amobonye et al. 2021). For this reason, groups of potential microorganisms for biodegradation of plastics are typically found in sites contaminated with these wastes, such as landfills (Matjašič et al. 2021). This was observed in the study conducted by isolating several microorganisms from soil samples from a landfill, using HDPE microplastics as the only carbon source.

Several types of microorganisms, such as fungi, bacteria and algae, have been reported to be capable of degrading plastic (Rajendran et al. 2015, Matjašič et al. 2021). Among these, a group that has become increasingly important in biotechnology is the actinomycetes, microorganisms that mainly inhabit the soil and many of them are known to have the ability to degrade plastic materials. Additionally, they synthesize secondary metabolites such as herbicides, pesticides and antibiotics (Usha et al. 2011, Rodríguez-Fonseca et al. 2021). This study obtained two genera of actinomycetes, *Tsukamurella* and *Streptomyces*.

Tsukamurella is an environmental saprophytic bacterium in the form of a bacillus that can be isolated from soil, water, sludge, arthropods and sponges (Safaei et al. 2018). There are no known reports on the assessment of plastic degradation capacity of this genus; however, Hou et al. (2022) isolated a species of *Tsukamurella* from activated sludge and wastewater contaminated with plastics, but it was not used in the tests. Therefore, this study may be the first report on the subject. However, this genus of bacteria has been used in studies of rubber waste degradation (Basik et al. 2021) and pharmaceutical wastewater bioremediation (Rozitis and Strade 2015), making it a genus with potential for use in bioremediation of various contaminants.

Furthermore, it has been reported that some *Tsukamurella* species have the ability to synthesize biosurfactants (Choi et al. 1999, Nanda and Berruti 2021). These molecules have an amphipathic structure that can improve the biodegradation of plastics due to the increase in surface area they provide to hydrophobic substances such as HDPE, which in turn increases their solubility in water and the availability of organic compounds (Becerra and Horna 2016, Nanda and Berruti 2021). Taking this into account, it is possible that *Tsukamurella* sp. strain JP3 (JP3) and *Tsukamurella* sp. strain JP5+ (JP5+), not only have the ability to degrade HDPE but also produce biosurfactants that aid in this biodegradation, which is consistent with the SEM observations, which show that the treatments causing the most damage to the microplastic surface contained these bacteria (**Fig. 4c, 4e, 4g** and **4h**).

On the other hand, the genus *Streptomyces* belongs to bacteria that are mainly found in the soil, grow in filamentous form, so their morphology is similar to that of filamentous fungi (Sharma 1999, Jayashantha 2015). This genus is very important for biotechnology, and has been studied for the biodegradation of plastics, where promising results have been shown in terms of degradation and weight loss of the sample (Rodríguez-Fonseca et al. 2021). However, it is important to note that most studies have focused on the degradation of biodegradable plastics, such as polyhydroxyalkanoate or polyethylene succinate (Pathak and Navneet 2017, Bahl et al. 2020). However, *Streptomyces* has been reported to degrade some nondegradable plastics such as polyethylene, polyester, polyethylene terephthalate and polyurethane (Rajendran et al. 2015, Rodríguez-Fonseca et al. 2021). For the degradation of HDPE, Farzi et al. (2017) reported that *Streptomyces* species degraded 18.26% of 212 μm HDPE powder in 18 days, a high performance obtained mainly by the use of particulate plastic. Therefore, the present study would be one of the first reports on the degradation of HDPE in the form of small pieces by *Streptomyces*.

The presence of *P. aeruginosa* (JP5–) in this study is not unexpected, as this genus is frequently reported as being highly efficient in the biodegradation of plastics. Matjašič et al. (2021) in their review found that 21% of the articles reported on the degradation capacity of *Pseudomonas*, which consistently achieved high percentages of degradation. *P. aeruginosa* is a bacterium that has been isolated from landfills (Deepika and Madhuri 2015, Gupta and Devi 2020), activated sludge, sewage (Hou et al. 2022), and worm gut (Lee et al. 2020), and it has been reported to degrade polyethylene, polypropylene, polyurethane, polystyrene, polyvinyl chloride and polyphenylene sulfide (Kyaw et al. 2012, Shah et al. 2013, Rajendran et al. 2015, Lee et al. 2020).

Pseudomonas are known for their metabolic versatility and their ability to regularly convert nondegradable substrates into easily assimilated metabolites or those susceptible to enzymatic catalysis (Echeverri et al. 2010). In addition, bacteria of this genus have been reported to be able to produce several enzymes involved in plastic biodegradation, including oxidoreductases, hydroxylases, hydrolases

and lipases (Danso et al. 2019, Gupta and Devi 2020, Amobonye et al. 2021). One of these, serine hydrolase secreted by *P. aeruginosa*, is an enzyme that can mediate the depolymerization of polyethylene by hydrolysis (Lee et al. 2020). Thanks to this, *P. aueruginosa* has been reported as an effective degrader of LDPE and HDPE (Kyaw et al. 2012, Lee et al. 2020); which is consistent with the results obtained in this research, as all treatments that showed the highest damage to microplastics contained this bacterium, suggesting good degradation by this microorganism. Additionally, other researchers have also achieved positive biodegradation results using different strains of *P. aeruginosa*. For example, Sangeetha-Devi et al. (2019) discovered that out of 140 isolates from plastic waste found in coastal environments, two strains of *P. aeruginosa* (VRKPC5 and VRKPCH4) degraded 13.73 and 7.33% of HDPE, respectively, being among the ten isolates that showed the most efficient biodegradation.

Plastic degradation is evidenced by changes on the polymer surface, such as cracking and breaking, as reported by Shah et al. (2008). It is also usually evidenced by weight loss, which is one of the most commonly used quantitative methods to measure plastic degradation (Skariyachan et al. 2017, Shabbir et al. 2020), as it indicates the loss of mass, usually due to the action of enzymes secreted by microorganisms that degrade or modify the structure of microplastics (Gajendiran et al. 2016, Auta et al. 2018). Although weight loss was observed in this research, there were no significant differences between the treatments evaluated, possibly due to a short incubation period. Degradation of plastics is a slow process due to their low bioavailability because of the stable structure of polymers such as HDPE, which also has high crystallinity and molecular weight and lacks easily hydrolyzable functional groups, factors that hinder biodegradation (Rajendran et al. 2015, Jaiswal et al. 2020, Mouafo-Tamnou et al. 2021, Zhang et al. 2021). Therefore, a longer incubation period would be required to observe significant differences.

Additionally, the experimental conditions may not be the optimal for the strains used; it should be taken into account that factors such as temperature and pH can have a favorable or unfavorable influence on microbial growth and degradation rate (Rajendran et al. 2015, Matjašič et al. 2021, Miri et al. 2022). However, it should be noted that it is necessary to evaluate different experimental conditions with the strains used to know the optimal ones for them. For example, Skariyachan et al. (2017) evaluated the degradation rates of *Bacillus vallismortis*, *Pseudomonas protegens*, *Stenotrophomonas* sp., and *Paenibacillus* sp. at various temperatures and pH values. They found that higher efficiencies were obtained at 55 ºC and pH 8, which was contrary to their initial expectations. Therefore, it is suggested that future studies evaluate different growth factors and conduct trials with longer incubation times to determine if higher degradation percentages can be obtained with the strains used, and if there are significant differences between treatments.

Additionally, the small percentage of degradation of the control (3.52%) may be attributed to the leaching of additives, such as plasticizers, added during manufacturing (Kale et al. 2015), which causes a slight weight loss in the material.

Although there were no significant differences in weight loss, the morphological changes in the microplastics observed with SEM validated the biodegradation. The surface damage on the microplastics exposed to the strains and the formation of biofilms indicates that the selected microorganisms were capable to adhere to the surface of the HDPE and secrete enzymes that break the C-C bonds, generating a superficial erosion that is one of the main causes of mass loss (Gajendiran et al. 2016). Subsequently, the smaller molecules are integrated as a carbon source for bacterial growth (Shah et al. 2008, Auta et al. 2018, Shabbir et al. 2020). In addition, According to Kyaw et al. (2012), the formation of biofilms on the surface of microplastics can reduce the hydrophobicity of the polymer, thereby enhancing and facilitating biodegradation. Consequently, the presence of biofilms on microplastics could be a contributing factor in biodegradation by the strains used. Previous studies have reported similar changes on the surface of polyethylene, polypropylene and polyethylene terephthalate microplastics when exposed to different bacterial and fungal strains, and similarly, no changes were observed on the surface of the control plastics (Paço et al. 2017, Auta et al. 2018, Shabbir et al. 2020, Torena et al. 2021).

CONCLUSIONS

In conclusion, this study represents one of the first reports of HDPE microplastics degradation by *Tsukamurella* and *Streptomyces*. The obtained results indicate that *Tsukamurella* sp. strain JP3, *Tsukamurella* sp. strain JP5+, *P. aeruginosa* and *Streptomyces* sp. strain JP8 are potentially useful microorganisms for the biodegradation of HDPE microplastics by showing significant surface

damage, biofilm formation and weight loss. However, further research is necessary to determine the optimal incubation conditions and time required to achieve significant degradation percentages using pure strains or mixed cultures. This shows the importance of continuing to study of possible plastic degraders, their optimal conditions for greater biodegradation and the enzymes involved in this process. This will help identify strains with significant potential for developing new technologies to address the issue of plastic waste pollution in the future.

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