Biodegradation Capacity and Activity Enzymatic of *Bacillus subtilis* Against Low-Density Polyethylene

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

ABSTRACT

Aims: The objective of this work was to determine the degradation capacity of low-density polyethylene by the bacterium *Bacillus subtilis* and analyze the production of extracellular laccase activity.
Methodology: The experiments was realized in 50 mL of culture medium, added with a fragment of known dry weight (1 cm^2 colorless polyethylene bag squares), and were incubated at 28°C, pH 6.5, for 6 months under static conditions, determining the growth of the bacterium by dry weight (68, 75, and 91 mg), the production of extracellular protein (271, 234, and 326.1 mg/mL), and the degradation of the substrate by dry biodegraded (8.57%, 5.88%, and 11.76%).
Results: The production of extracellular laccase enzyme was analyzed in presence of polyethylene, finding an enzymatic activity of laccase of 2.06, 1.49, and 2,03 U/mL, while in the control without substrate, no enzymatic activity was observed, which suggests that this enzyme may participate in the degradation of polyethylene. In addition, some characteristics of the extracellular enzymatic activities were analyzed, such as stability at 4^oC and 28^oC, optimal pH and temperature, the effect of protein and substrate concentration.
Conclusion: The extracellular protein production and dry weight of the bacterium are higher in the presence of low-density polyethylene. The laccase activity is very stable at 4^oC and 28^oC, the most effective pH and temperature, were 4.5 and 28^oC, and present an incubation time of 5 minutes, and this data suggest that this enzymatic activitiy may participate in the degradation of low density polyethylene.

Keywords: Bacillus subtilis, polyethylene, biodegradation, extracellular laccases

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1. INTRODUCTION

Plastics are organic materials that are obtained through chemical reactions using different synthetic and/or natural raw materials and are part of a group of compounds called polymers. Initially, they were manufactured using polymers and vegetable resins, such as cotton cellulose, furfural from the husk of *Avena sativa*, seed oil and casein from milk, and the first fully synthetic plastic was Bakelite (1907), to replace the use of natural products, as well as obtaining a simple, inexpensive, hard, and aesthetic product, to replace other natural products that are difficult to obtain [1]. In 2017, the world production of plastics was 348 million tons, the main producers being: Asia (50.1%, with China being the largest producer with 29.4%), Europe (18.5%), North America (Mexico, the United States and Canada, 17.7%), Africa and the Middle East (7.1%), Latin America (4%), and the Commonwealth of Independent States (former Soviet Republics, 2.6%) [2], thus currently, these products are one of the world's major concerns due to the large number of environmental problems that they cause, mainly due to their excessive consumption, which when eliminated, become very difficult to eliminate waste. For example, for bottled beverages, 500 billion tons of plastic bottles are produced per year [3], and it has been described that Mexico City, “is a large body with clogged plastic veins”, since the approximately 22 million inhabitants, each day produce almost 13,000 tons of solid waste, of which 123 tons are plastic waste [2] and, due to its mismanagement, as well as the custom of discarding them in streets, gardens, sewers, etc., cause an obstruction of the drainage, floods and other problems in the city, so that their use worldwide daily life is already unsustainable, and try to reuse it [2], in addition to the fact that the use of plastic containers is generally single-use [4].

Plastics are widely used due to their multiple applications, polyethylene being the most widely used plastic, of which two types have been reported: high-density and low-density, which are in great demand worldwide. to produce plastic bags that serve as packaging for food and articles of all kinds, which leads to the excessive accumulation of these plastics in the world [2]. In addition, they are used in the manufacture of containers (bottles and garbage cans) [5], packaging such as bags, membranes, sheets and films [6], as well as products as varied as overalls, pipes and joints for hip replacement, so it is very common to see plastic debris anywhere in the world [1, 7], since these can remain in nature between hundreds and thousands years [2, 8], so that today plastic waste is a serious threat on a global scale [9]. Different investigations have widely documented the great negative impact that the pollution that these products cause in the world [10], for example: more than thirteen million tons of plastic end up in our oceans [11] In Mexico, one out of every five fish for human consumption contains microplastics in its viscera, which affects people's health and sources of work related to fishing and tourism [12]. In addition, PET nanoparticles interact with the calcium ion affecting the tissue contraction/relaxation function, which could affect the functioning of the intestine of rodents [13]. Also, plastic contamination has been reported in Mexican protected natural areas, which shows that this type of contamination is present in the Mexican Republic beyond clandestine dumps, garbage thrown in the streets and landfills full of products that supposedly they must be recycled [7]. This indicates that our consumption decisions have an impact on the cleanest, most remote, and protected places on the planet, and as is evident, plastic pollution on our planet negatively affects biodiversity and hinders the main strategy of conservation of ecosystem services [7].

On the other hand, different methods of degradation of low-density plastics have been reported, which can be physical, chemical, and biological. Among the physical are photo-degradation and thermodegradation, and of the chemical ones, oxo-degradation [14]. Also, the separation of microplastics by density has been used through the application of physicochemical processes with zinc chloride in wastewater collected from the public discharges of the sewerage system of the city of Riobamba (Ecuador) [15]. But biodegradation is the method that is being used more exhaustively for its elimination, by means of microorganisms that degrade it by means of enzymes, although this degradation takes place very slowly [10]. Therefore, the use of a wide variety of microorganisms for the degradation of this type of pollutant is being widely investigated, such as: The biodegradation of plastic and polypropylene with larvae of the Coleópter *T. molitor* [5], *Aspergillus flavus* fungus isolated in the presence from humus and domestic composting [16], and from an orange in a state of decomposition [17], the bacteria *Bacillus cereus* and *Aeromonas hydrophila* and the fungi *Penicillium* sp., and *Aspergillus* sp., isolated of sanitary landfills [18], the biodegradation of low-density polyethylene by fungi and bacterial consortia isolated from municipal garbage dumps [6], the biodegradation of polystyrene, PET, and polyphenyl sulfide plastic beads by *Pseudomonas* sp., *P. aeruginosa* and *Tichoderma* spp., [19, 20, and 21], the biodegradation capacity of five filamentous fungi against polyethylene [10], the biodegradation of low-density polyethylene by a microbial consortium [14], the degradation of high-density polyethylene of marine debris by *Aspergillus tubingenis* and *A. flavus* [22], the biodegradation of low-density polyethylene by *Microbulbifer hydrolyticus* IRE-31 [23], the biodegradation of polyvinyl chloride plastic films by a marine consortium [24] as well as the degradation of plastic by environmental bacteria in Norway [25].

In addition, some enzymes that apparently participate in the degradation of polyethylene have been studied, which hydrolyze the ester bonds, causing the release of terminal groups of carboxylic and alcoholic acids [26], like the activity of laccases and esterases produced by *F. culmorum* grown in the presence of different concentrations of di (2-ethyl hexyl) phthalate and Tween 80 [27], a laccase of *Trichoderma viride* [28], a recombinant laccase from *Streptomyces cyaneus* CECT 3335 [29], a purified laccase from *Geobacillus* sp. ID17 [30], the esterase activity of *Pseudomonas* sp., which degrades polyurethane and low-density polyethylene [31], the activity of fungal esterases on the degradation of polyesters [32], an esterase from *Sphingobium* sp., C3 that degrades dimethyl terephthalate [33], two enzymatic activities of esterase and phthalate hydrolase from *Gordonia* sp., which degrade phthalate esters [34], cutinases from *F. solani* and *Pichia* *pastoris* [35], polyurethanases from *Pseudomonas* [36], hydrolases, lipases, and cutinases from different microorganisms that degrade plastic [37], carboxylesterases [38], cutinase from *Escherichia coli* [39], PETase and MHETase from *Ideonella* sakaiensis 201-F6 [40], lipase, carboxymethylcellulose, xylanase and protease from *Alcaligenes faecalis* [41]. Therefore, the objective of this work was to evaluate the degradation capacity of low-density polyethylene from commercial bags by the bacterium *Bacillus subtilis,* as well as to analyze some laccase enzymatic properties, since it has been reported that microorganisms exist in nature, which using specific enzymes, are capable of decomposing it into its most basic components, as a response developed by these microorganisms in the last 70 years to adapt to an environment invaded by plastic, which will allow these plastics to be manufactured and then reused in a controlled manner, thus reducing dependence on fossil resources such as oil and gas, as well as contributing to the elimination of this important pollutant.

2. MATERIAL AND METHODS

2.1 Strain Used

The strain of *B. subtilis* was obtained from the Microbiology Laboratory of the Faculty of Chemical Sciences of the UASLP, San Luís Potosí, S.L.P., México.

2.2 Culture medium for the Degradation of Low-Density Polyethylene

This medium contains (g/L): Glucose (10), yeast extract (5), KH_2PO_4 (0.6), $MgSO_4 \cdot 7H_2O$ (0.5), K_2HPO_4 (0.4), $CuSO_4 \cdot 5H_2O$ (0.25), $FeSO_4 \cdot 7H_2O$ (0.05), $MnSO_4$ (0.05), $ZnSO_4 \cdot 7H_2O$ (0.001), and 400 µL de Tween 80 [42]. Subsequently, 50 mL were added to 125 mL Erlenmeyer flasks, as well as a disinfected plastic fragment of known dry weight (1 cm^2 polyethylene bag squares) and sterilized by humid heat at 15 pounds (121^{o}C) for 20 minutes. Subsequently, they were cooled to room temperature, seeding 1 $x10^6$ cells/mL in triplicate, and incubating for 6 months at room temperature, pH 6.5, under static conditions, monitoring their growth visually every week, and adding new culture medium under sterile conditions every 3 weeks.

2.3 Bacterium Growth by Dry Weight

After 6 months of incubation under static conditions, the bacterial culture supernatant was harvested in a graduated tube, previously weighed, and centrifuged at 3000 rpm/10 min, discarding the supernatant. The cell pack was dried at 80°C, for 24 h, and the tube with the sample was weighed, determining the dry weight of the sample by difference, comparing the growth with a culture control grown under the same conditions, without the low-grade polyethylene fragment. All experiments were performed at least 3 times in duplicate.

2.4 Biodegraded Weight of Low-Density Polyethylene

After the incubation period, the low-density polyethylene samples were taken with surgical forceps, and placed in previously tared Petri dishes, washed with 2% (v/v) sodium dodecyl sulfate for 24 hours, subsequently with ethanol (70%), tridesionized water, and dried at 60^{o}C for 24 hours, weighed, and by weight difference the biodegraded weight, and the percentage of biodegradation of the sample were determined.

1) Biodegradability of the final weight of the low-density polyethylene sample was determined in milligrams, at 6 months of incubation at 28^{o}C, pH 6.5 under static conditions by the action of the bacterium *B. subtilis* using the following formula:

Biodegraded weight of the sample = initial weight-final weight

2) After obtaining the biodegraded weight of the difference from the initial weight minus the final weight, it was converted to a percentage, using the following formula:

$$\text{Weight loss (\%)} = \frac{\text{initial weight-final weight}}{\text{starting weight}} \text{ X } 100$$

2.5 Determination of Protein

This was determined by the method of Lowry *et al*. (1951) [43].

2.5.1 Reagents

A.- Standard albumin solution (Sigma Chemical Co.) 1mg/mL (p/v).
B.- 2% (p/v) sodium carbonate (Na_2CO_3, Monterrey Products) in distilled water.

C.- 0.5% (p/v) copper sulfate ($CuSO_4{\cdot}5H_2O$, Chemical Products Monterrey), dissolved in 1% (p/v) sodium citrate (Jalmak).
D.- Mix 50 mL of reagent B with 1 mL of reagent C.
E.- Folin-Ciocalteu reagent (Sigma Aldrich). Dilute the reagent 1:1 with deionized water (prepare 5 minutes before use).

2.5.1 Technique

Take aliquots of the different samples (0.5 mL) and place them in a test tube. Add 0.5 mL of 1N NaOH, mix and incubate for 24 hours at room temperature. Subsequently, complete the volume to 1.0 mL with deionized water, add 5 mL of reagent D, mixing in a Vortex shaker (Mixer Gegie-2), incubate for 10 minutes at room temperature, add 0.5 mL of the reagent E, shaking the samples on a Vortex shaker, and incubate for 45 minutes at room temperature. A blank for protein was included, and the absorbance of the samples was read at 750 nanometers in a spectrophotometer (Genesys 10S Uv-Vis-Thermo Scientific), interpolating the reading in a standard curve, in which bovine serum albumin is used.

2.6 Determination of Enzymatic Activity

The enzymatic activity was determined spectrophotometrically in the culture supernatant, obtained from the filtration of the samples.

2.7 Laccase

The reaction mixture contained 900 µL of 2 mM 2,6-dimethoxyphenol as substrate (Sigma Chemical Co.), in 0.1 M acetate buffer pH 4.5, and 100 µL of enzyme extract (supernatant), incubating at $40^{o}C$ for 1 minute [44], and determining the laccase activity as the change in absorbance at a wavelength of 568 nm in a UV-Visible light spectrophotometer, using as a reference a blank prepared with tridesionized water according to the previous procedure. One unit of laccase activity was defined as the amount of enzyme that produces an increase of one absorbance unit per minute in the reaction mixture [45]. Results are expressed as the average of 3 independent determinations.

3. RESULTS AND DISCUSSION

3.1 Bacterial Growth by Dry Weight

The growth of the bacterium was analyzed in the presence of low-density polyethylene as a substrate, determining the dry weight and the production of extracellular protein. In Figure 1, it is observed that the microorganism had a higher growth in dry weight of 68, 75, and 91 mg, like control (75 mg) (which has no substrate), at 6 months of incubation, pH 6.5 at $28^{o}C$, under static conditions, which indicates that polyethylene stimulates little the growth of the bacterium. The data found in this work coincide with some reports in the literature, in which the growth of different microorganisms is reported in the presence of different plastic substrates, such as the growth of five filamentous fungi in the presence of polyethylene [10], greater growth with respect to the control of *Pseudomonas* sp., [19], the fungi *Muco*r sp., and *Aspergillus* sp., which increase their growth by 8.75% and 21.73% in presence of low-density polyethylene at 3 months of incubation [46], for the white rot fungus *P. ostreatus*, a growth of 619 mg was observed with 15 mg/L of tire dust, which were obtained from an industrial waste landfill located in Cartagena, Colombia [47]. Also, *A. alternata,* isolated from urban waste containers in 5 cities of the V region of Chile, demonstrated the ability to grow in different types of plastic, especially in polyurethane, polyvinyl chloride, and ethylene polyereftherate [48].

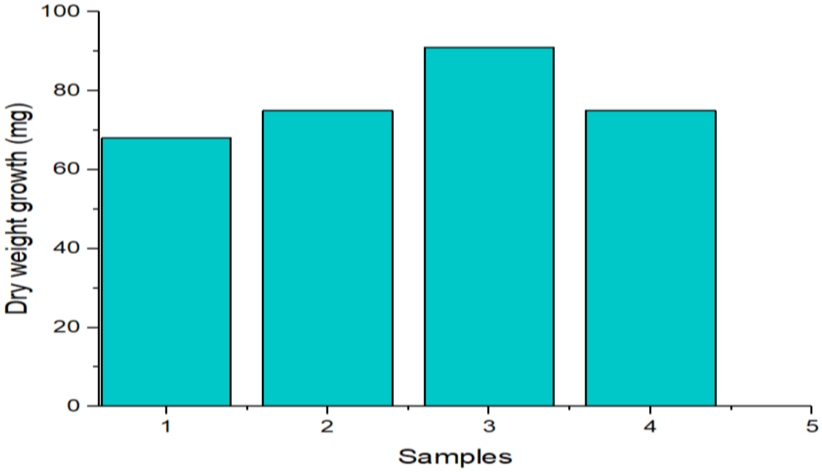

Fig. 1. Dry weight growth of *Bacillus subtilis* in presence of low-density polyethylene. 28°C. pH 6.5. 6 months of incubation. Static conditions (1 x 10^6 cells/mL). (1, 2, 3, problems, and 4.- control).

3.2 Extracellular Protein Production

Regarding the production of extracellular protein, a growth related to its production was found of 2.0, 1.72, and 2.4 times more than the control without substrate (Figure 2), which coincides with that reported for the fungus *F. culmorum* that produces a large amount of extracellular protein in the presence of 20 g/L of cutin [49].

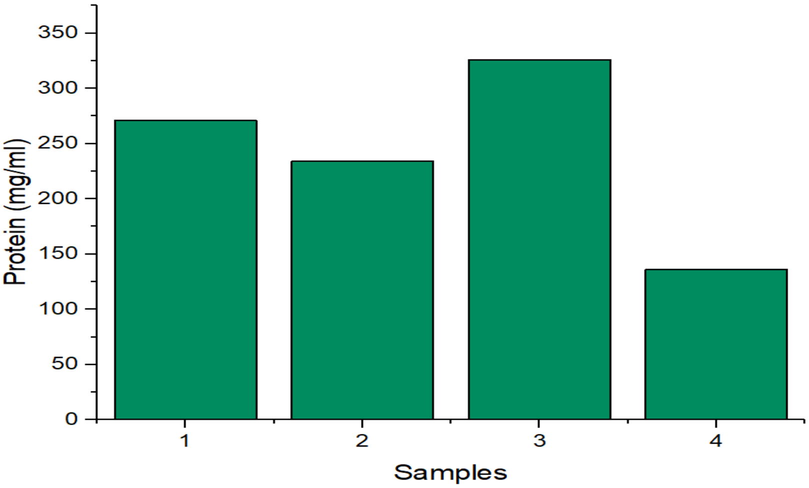

Fig. 2. Production of extracellular protein by *Bacillus subtilis* in presence of low-density polyethylene. 28°C. pH 6.5. 6 months of incubation. Static conditions (1 x 10^6 cells/mL). (1, 2, 3, problems, and 4.- control).

3.3 Biodegraded Weight of the Sample

In Figure 3, the biodegradation of low-density polyethylene is observed, with 8.57%, 5,88% and 11.76% of biodegradation based on the biodegraded weight of the substrate, under the conditions described above, results that coincide with that reported for three strains of the fungus *A. niger* isolated from plastic from the waste dump, from an orange in a state of decomposition, in presence of humus and domestic composting [16], which reduce 3.44%, 6.9% and 4.84% of the initial weight of polyethylene in a month, 10 days and a month, respectively [16, 17], for the fungi *Fusarium* sp., *Aspergillus* sp., *Trichoderma* sp., and *Mucor* sp., which reduce the dry weight of polyethylene from 1.0354 to 0.9533, from 1.0244 to 0.9715, from 1.096 to 0.9873, from 1.0047 to 0.9805 grams of dry weight, respectively [46]. But these results are slightly higher that the reported for the 2.88% biodegradation of low-density polyethylene by fungi and bacterial consortia isolated from municipal garbage dumps, at 70 days [6], for the 1.61% biodegradation of polystyrene at 15 days by *Pseudomona*s sp. [19] and the bacteria *P. microspora* E2712A and E3317B, which efficiently biodegrade polyurethane in liquid cultures at 16 days of incubation [50]. Also, the data found are lower than that reported for the biodegradation of the same substrate by the larvae of the Coleopter *T. molitor*, which biodegrade 64% in 45 days of incubation [5], for the bacterium *Bacillus cereus* and the fungus *Penicillium* sp., with a biodegradation of 17.91% of polyethylene terephthalate, at 4 months, although it was previously treated with UV light and thermodegradation [18], for *P. aeruginosa*, which biodegrades 21.7% and 27.3% of low-density polyethylene particles at 25°C and 35°C, respectively, after 30 days of incubation [51], and for the biodegradation of polyethylene terephthalate treated at 150°C for 8 hours, for *P. aeruginosa* (14.4%) and *Trichoderma* sp., (13.15%) during a period of 30-90 days [20].

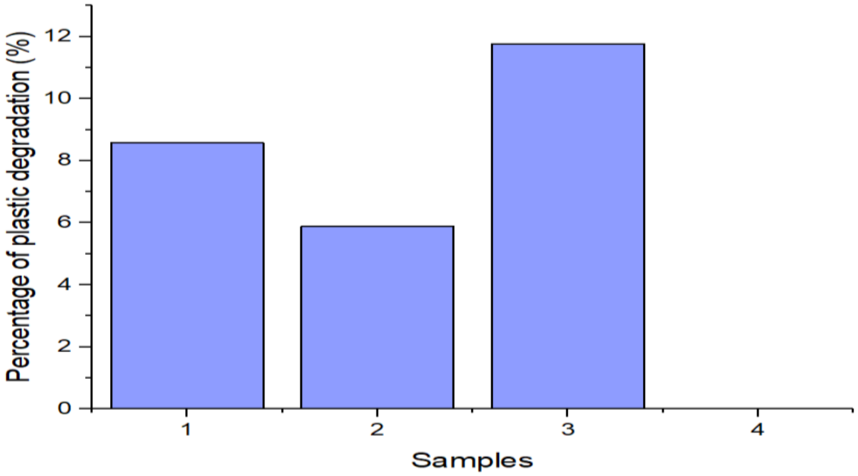

Fig. 3. Percentage of biodegradation of low-density polyethylene by *Bacillus subtilis*. 28^{o}C. pH 6.5. 6 months of incubation. Static conditions (1 x 10^6 cells/mL). (1, 2, 3, problems, and 4.- control).

3.4 Production of Extracellular Lacasse

In Figure 4, it shows the extracellular enzymatic activity of laccase produced in presence of low-density polyurethane, by the bacterium *B. subtilis*, under the conditions described above, finding an activity for laccase of 2.06, 1.49, and 2.03 U/mL. It should be mentioned that the controls without the substrate produced very little enzymatic activity. This is different for a laccase of *T. viride*, in which an activity of 7.31 U/mL with low-density polyurethane as substrate is reported [52], for 2 strains of *Alicycliphilus* sp., in which enzymatic activity of esterase is detected, but not of urease and protease [53], although they are lower than those reported for the production of esterase (12 U/mL) in presence of polyurethane by the bacteria *Bacillus* sp. AF8, *Pseudomonas* sp. AF9, *Micrococcus* sp. [10], *Arthrobacter* sp. AF11 and *Corynebacterium* sp. AF12 [54], a similar enzymatic activity of *F. culmorum*, where a value of 420.2 U/L is reported in the presence of 2 g/L of di (2-ethyl hexyl) phthalate at 200 hours incubation [55]. Also, for the esterase activity of different fungi isolated from sand contaminated with plastics, in which a higher esterase activity is reported with di (2-ethyl hexyl) phthalate and polyurethane foam as substrate [42].

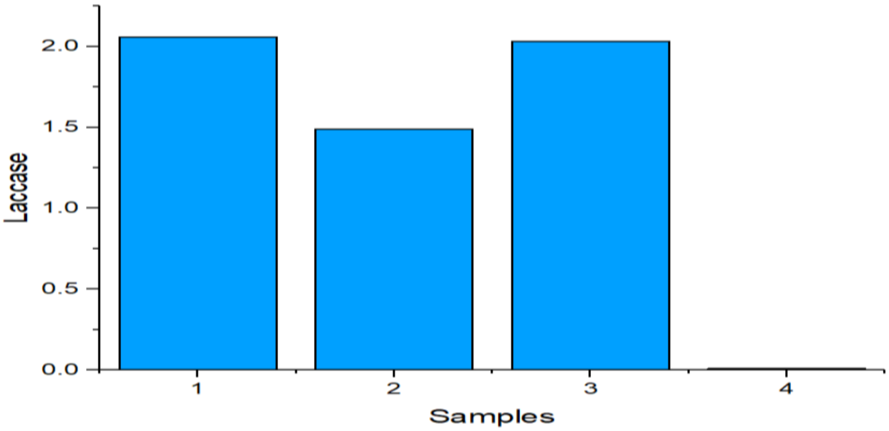

Fig. 4. Production of extracellular laccase (U/mL) by *Bacillus subtilis* with low-density polyethylene. 28^{o}C. pH 6.5. 6 months of incubation. Static conditions (1 x 10^6 cells/mL) (1, 2 3, Problems. 4. Control.

3.5 Analysis from Some Properties of Extracellular Laccase

Subsequently, some properties of the extracellular laccase activity were analyzed. For stability, it was found that laccase activity is very stable at 4^{o}C and 28^{o}C, conserving 90% and 82.5% of remaining activity (Figure 5), the most effective pH and temperature were 4.5 (Figure 6) and 28^{o}C (Figure 7), and an incubation time of 5 minutes (Figure 8). For the effect of protein concentration, was observed a linear reaction of laccase activity until 108.4 µg/assay of the concentrations analyzed (Figure 9), while the substrate concentration (2,6-dimethoxyphenol), the highest enzyme activity was observed at 0.542 µg/assay (Figure 10). In this regard, for a recombinant laccase from *S. cyaneus* CECT 3335, it has been reported that at temperatures of 60°C to 80ºC and pH of 3.0 the activity was greater than 75% of the maximum detected, and at concentrations greater than 0.1 mM of 2,6-dimethoxyphenol, this inhibit the enzymatic activity with 2,6-dimethoxyphenol as a substrate [29], and a purified laccase from *Geobacillus* sp. ID17, showed a similar stability at 55^{o}C, and an optimum pH of 7.5 [30], for a laccase from *T. viridae*, in which an optimal pH of 4.0-5.0 with low-density polyurethane as substrate, and optimum temperature of 30^{o}C and 40^{o}C is reported [50], a carboxylesterase from *E. coli* retains 100% of its activity after 23 days at 45^{o}C, and a pH of 9.0 [38], and for an extracellular depolymerase from *Penicillium oxalicum*, with an optimal temperature of 40^{o}C with aliphatic polyesters as substrates [56].

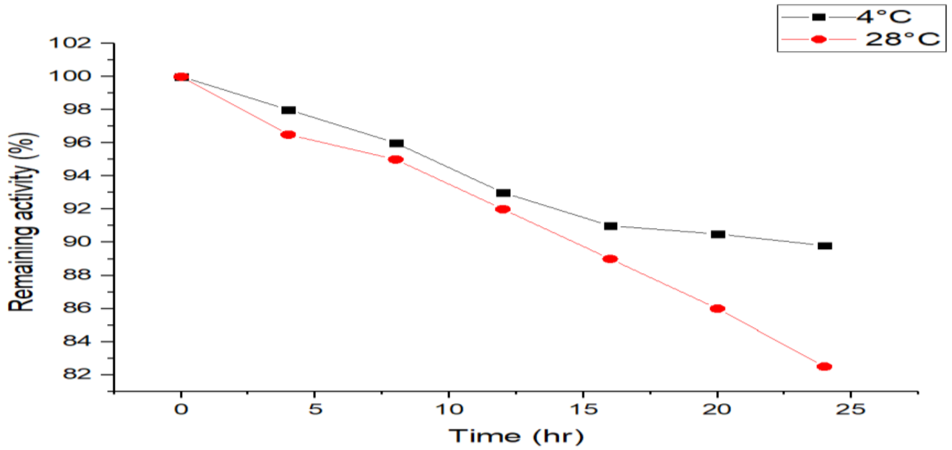

Fig. 5. Stability of the laccase extracellular activity of Bacillus subtilis at 4°C and 28°C.

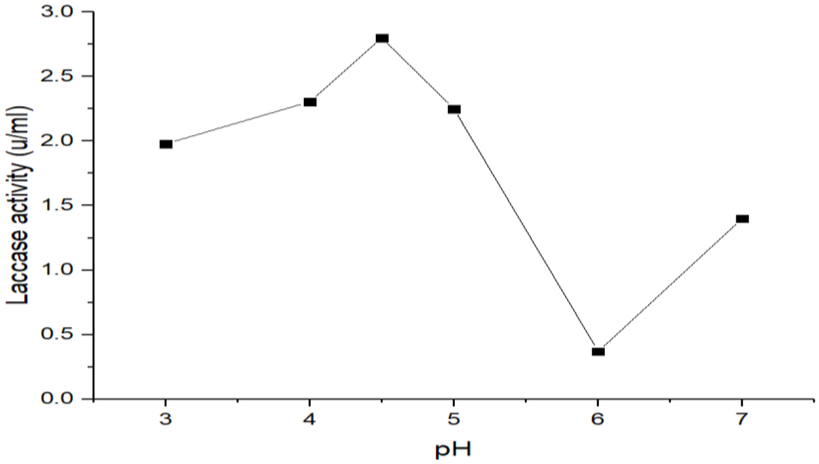

Fig. 6. Effect of the pH on the laccase extracellular activity of *Bacillus subtilis* at 28°C.

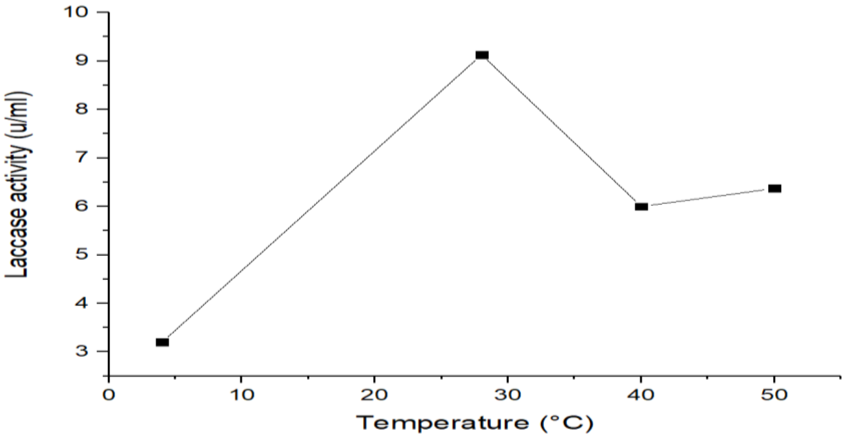

Fig. 7. Effect of the temperature on the laccase extracellular activity of *Bacillus subtilis*.

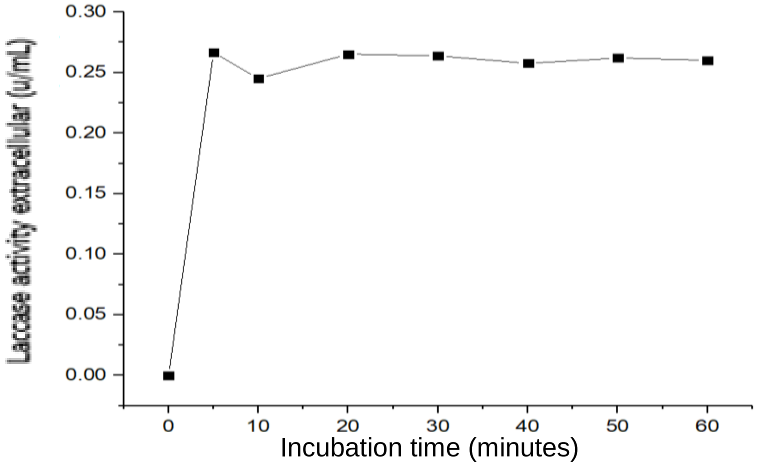

Fig. 8. Effect of the incubation time on the laccase extracellular activity of *Bacillus subtilis*.

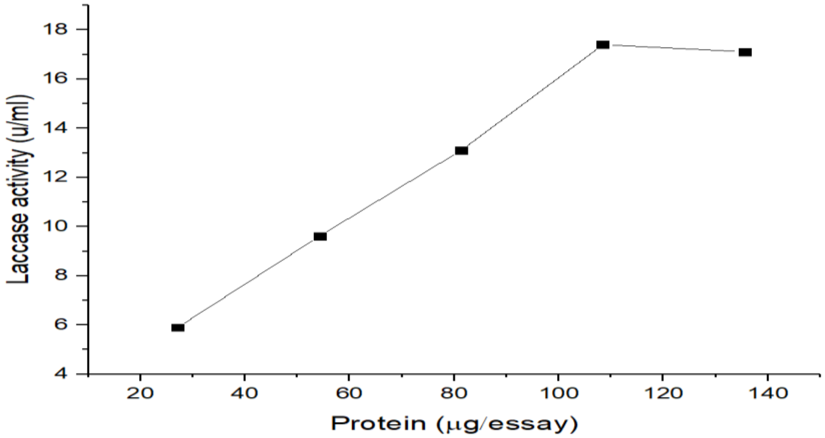

Fig. 9. Effect of the protein concentration on the laccase extracellular activity of *Bacillus subtilis*.

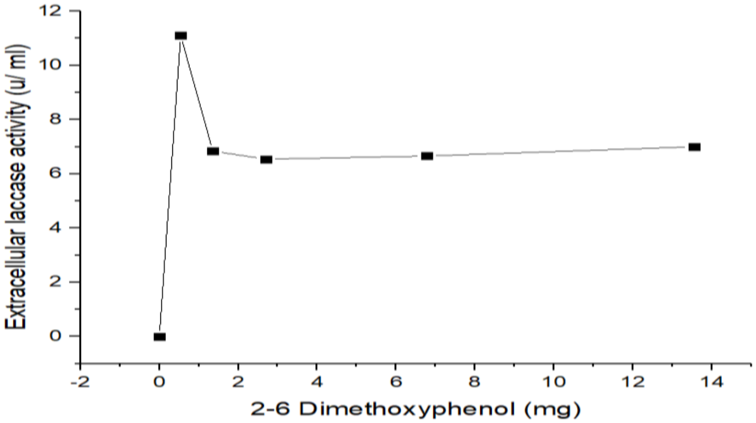

Fig. 10. Effect of the 2-6 Dimethoxyphenol on the laccase extracellular activity of *Bacillus subtilis*.

Finally, a summary of the results obtained for enzyme activity is shown in Table 1.

Table 1. Kinetics characteristics of the enzymatic extracellular activity of *Bacillus subtilis*

Parameter	Laccase
Stability to 4°C	90%
Stability to 28°C	82.5
pH	4.5
Temperature	28°C
Incubation time	5 minutes
Protein concentration	108.4 µg/ensayo
Substratum concentration	0.542 µg/ensayo*

*2,6-Dimethoxyphenol

Other enzymatic activities related to the degradation of polyurethane have also been reported, such as: polyurethanases from *Pseudomonas* [36], a phthalate hydrolase from *Gordonia* sp., which degrades phthalate esters [34], hydrolases, lipases, and cutinases of different microorganisms that degrade plastic [37], carboxylesterases [38], cutinase of *E. coli* [39], PETase and MHETase from *I. sakaiensis* [40], a lipase, carboxymethylcellulose, xylanase and protease from *A. faecalis* [41]. Finally, the data obtained suggest that the enzymatic activities of different microorganisms could be participate, in conjunction with other mechanisms in the degradation and/or the elimination of this type of contaminants.

4. CONCLUSION

1.- The extracellular protein production and dry weight of the bacterium are higher in the presence of low-density polyethylene.
2.- The biodegradation of the substrate based on the biodegraded dry weight was 8.57%, 5.88%, and 11.76%.
3.- The bacterium produced extracellular laccase activity in presence of polyethylene, with an activity of laccase of 2.06, 1.46, and 2.03 U/mL.
4.- The laccase activity is very stable at 4^{o}C and 28^{o}C, the most effective pH and temperature, were 4.5 and 28^{o}C, and present an incubation time of 5 minutes.
4.- The data obtained suggest that these enzymatic activity may participate in the degradation of low density polyethylene, but more studies are required to determine which microorganisms and enzymatic activities are the most efficient in the degradation of this substrate, as well as to optimize the production of the same for a faster and more efficient biodegradation

COMPETING INTERESTS

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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