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Biodegradation of polystyrene with laccase-producing enterobacteria isolated from a municipal waste dump

Biodegradación de poliestireno con enterobacterias productoras de lacasa aisladas de un basurero municipal

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Abstract
In this work, the ability to biodegrade two samples of polystyrene (PS) was evaluated: crystal and expanded with three bacterial strains isolated from PS waste collected in the municipal dump of Chimalhuacán, Estado de Mexico. The biodegradation potential of the isolated strains was determined by substrate weight loss assays. All bacterial strains were found to be able to biodegrade both types of PS. The study also aimed to determine if the isolated bacteria produce laccase since this enzyme has been reported with the ability to degrade polyethylene and other plastics. In addition, it was observed that when using copper sulfate as an inducer of laccase activity during biodegradation, there was an increase in both the enzymatic activity, as well as in the weight loss of the PS samples. The strain *Enterobacter* sp. UAMI-C3 proved to be the most efficient in the degradation of expanded PS with a weight decrease of 0.85% in 30 days with laccase activity at 9.803×10^{-3} U/mL. Therefore, so it is likely that laccase is involved in the biodegradation of this synthetic polymer.
Keywords: Polystyrene, biodegradation, *Enterobacter*ia, laccase.

Resumen
En este trabajo se evaluó la capacidad de biodegradar dos muestras de poliestireno (PS): cristal y expandido con tres cepas bacterianas aisladas de los residuos de PS recolectados en el basurero municipal de Chimalhuacán, Estado de México. El potencial de biodegradación de las cepas aisladas se determinó por ensayos de pérdida de peso del sustrato. Se encontró que las tres cepas bacterianas eran capaces de biodegradar ambos tipos de PS. El estudio también tuvo como objetivo determinar si las bacterias aisladas producen lacasa, ya que esta enzima ha sido reportada con la capacidad de degradar polietileno y otros plásticos. Se observó que al emplear sulfato de cobre como inductor de la actividad lacasa durante la biodegradación, hubo un aumento tanto en la actividad enzimática, así como en la pérdida de peso de las muestras de PS. La cepa *Enterobacter* sp. UAMI-C3 demostró ser la más eficiente en la degradación de PS expandido con una disminución de peso del 0.85% en 30 días con actividad lacasa de 9.803×10^{-3} U/mL. Por lo anterior, es probable que la lacasa participe en la biodegradación de este polímero sintético.
Palabras clave: Poliestireno, biodegradación, *Enterobacter*ias, lacasa.

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1 Introduction

Synthetic polymers are commonly used as packaging materials due to their low-cost production and properties such as light weight and resistance. Until 2015, a production of 381 million metric tons of all types of plastics was estimated and an increase of 75% was projected for 2020 (Palmay-Paredes *et al*., 2021). Expanded polystyrene (PS) is the most widely used plastic material in food packaging, better known under its commercial name as Styrofoam or unicel. It shows high thermal resistance which allows transportation of foods, including those recently prepared. However, the serious drawback of the expanded PS is that it is a single use disposable material (Shah *et al*., 2008; Lee and Liew, 2021; Machona *et al*., 2022).

Of all the plastic residues produced, only 9% is recycled while other 12% undergoes incineration. The remaining plastic wastes are discarded in the landfills or municipal dump wastes which contribute to the increase in the environmental pollution (Lee and Liew, 2021).

Recent years have been marked by the search of different methods to reduce plastic waste pollution. Such solutions should be inexpensive and environmentally benign. In this context, biodegradation which relies on the usage of bacteria capable of degrading plastic by the action of their enzymes and to use it further as substrates is a promising alternative for plastic waste treatment (Amobonye *et al*., 2021; Lee and Liew, 2021; Yeom *et al*., 2022).

In general, biodegradation process involves adhesion of bacteria to the plastic surface followed by the enzymatic digestion of polymer chains into low molecular weight structures. The latter can be used as carbon source for bacterial growth and biofilm formation (Mohan and Srivastava, 2010; Amobonye *et al*., 2021; Lee and Liew, 2021). Plastic materials, such as PS, polyethylene (PE), polyvinyl chloride (PVC), are composed of complex structures which contain rigid C-C bonds. Such linkages are difficult for bacteria to hydrolyze (Yeom *et al*., 2022).

In recent years, diverse studies have been carried out to investigate the biodegradation rate of different plastic materials using microorganisms, such as *actinobacteria*, *algae*, *bacteria*, and *fungi*. However, it was observed the process was very slow (Amobonye *et al*., 2021).

Very few studies involved investigating biodegradation of PS. For example, Mor and Sivan (2008) showed that *Rhodococcus ruber* C208 formed a biofilm on PS sheets and the polymer was used as a substrate by the bacteria. The results confirmed 0.8% loss of substrate weight in 8 weeks. In another study, Subramani and Sepperumal (2017) demonstrated that a bacterial strain of *Pseudomonas* sp., which was isolated from the waste sample of PS, had a capacity to biodegrade this synthetic polymer. The GC-MS analysis confirmed the presence of compounds with low molecular weight.

It has been also known from previous works that laccase is an enzyme that plays a role in PE degradation (Iiyoshi *et al*., 1998; Fujisawa *et al*., 2002). It was shown that addition of transition metal ions such as copper and manganese induced the production of laccase (Mongkolthanaruk *et al*., 2012). For example, Santo *et al*. (2013) evaluated copper sulphate as laccase inductor in *R. ruber* C208 for PE biodegradation. A 13-fold increase in the production of the enzyme was observed and biodegradation was higher by 40% when the medium was added with 20 μM $CuSO_4$.

In another work by Mukherjee and Das (2014), *Enterobacter asburiae* XJUHX-4TM isolated from the sewage water contaminated with colorants was shown to produce 66% more laccase upon the addition of malachite green to the cultivation medium.

Finally, *Enterobacter cloacae* KSB4 strain isolated from soil samples was also reported to produce laccase (Devasia and Nair, 2016).

In that context, the objective of the present study was to evaluate biodegrading capacity of three bacteria strains isolated from the PS residues disposed at municipal waste dump, as well as to investigate the relationship between laccase production and biodegradation rate of the crystal and expanded PS.

2 Material and methods

2.1 Isolation of bacterial strains

Approximately 10 g samples of expanded PS were collected from the municipal dump waste in Chimalhuacán, Estado de Mexico.

The PS samples were embedded in the nutritive broth (BD Bioxon, Mexico) and incubated at 40 °C for 4 days. Following that time, colonies of the individual bacteria strains were isolated using the streak plate method on nutritive agar and incubated at 40 °C for 24 h. The isolated cultures were stored in the nutritive broth mixed with glycerol (ratio 1:1) at -20 °C.

Fresh culture of each bacteria were stored in the nutritive broth at 4 °C, for use in biodegradation evaluations.

2.2 Identification of the isolated bacteria

Analysis of bacterial phenotype was performed using the Gram-staining technique. Following the isolation of genetic material (PowerSoil DNA kit, Mo Bio Laboratories, USA), the 16S rRNA gene sequence of the isolates was amplified by PCR using the specific primers: E9F (5'-GAGTTTGATCCTGGCTCAG-3') and E1492R (5'-ACCTTGTTACGACTT-3'), as previously reported by Forney *et al*. (2004). The reaction mixture for the PCR was carried out according to reported by Meléndez-Sánchez *et al*. (2022).

Once nucleotide sequences from the bacterial strains were known, a standard nucleotide - nucleotide homology comparison with the 16S rDNA sequences deposited at GenBank, NCBI, was performed using a basic search tool for local alignment (BLAST).

In this way, the identified bacteria according to their 16S rDNA sequences were registered at GenBank, NCBI.

2.3 Preparation of polystyrene samples for biodegradation assay

Two different PS samples were used. The first one was a 1 g commercially available crystal PS (PM 192,000; Sigma-Aldrich, EUA). The second sample comprised a commercial cup made of expanded PS that was cut into pieces of 2 x 3 cm, which weighed approximately 0.5 g in total. All the PS samples were disinfected by incubation in 70% ethanol (v/v) for 30 min and later rinsed with sterile distilled water. They were dried in an oven with mechanic convection (Felisa model FE-291AD) at 50 °C for 24 h till constant weight was reached.

2.4 Inoculum preparation

Prior the experiment, nutritive broth was sterilized at 121 °C for 15 min. 50 mL of the sterile nutritive broth was inoculated with 5 mL of bacterial culture stored in the nutritive broth (see Section 2.1) and incubated at 35 °C for 24 h. Nutritive broth was centrifuged at 3220 x g for 15 min at 4 °C. The cell pellet was collected and resuspended in 20 mL of a sterile fermentation medium (FM). The FM was prepared following the procedure reported by Mor and Sivan (2008) with slight modifications and contained (g/L): NH_4NO_3 (1.0), K_2HPO_4 (1.0), $MgSO_4{\cdot}7H_2O$ (0.2), KCl (0.15), $CaCl_2{\cdot}2H_2O$ (0.1), $FeSO_4{\cdot}6H_2O$ (0.001), $ZnSO_4{\cdot}7H_2O$ (0.001), $MnSO_4$ (0.001), yeast extract (0.1), gelatin peptone (0.1) and Tween 80 (0.02 mL).

2.5 Evaluation of biodegradation of PS

The fermentations were carried out by transferring a 0.5 g sample of expanded PS or 1 g sample of crystal PS previously disinfected (see Section 2.3) to 100 mL of sterile FM in a 125 mL Erlenmeyer flask and followed by inoculation with 5 mL of bacterial suspension with 0.2 Abs (see Section 2.4). Fermentation was carried out at 35 °C, 200 rpm for 30 days. To determine substrate weight loss, the PS samples were washed with sodium dodecyl sulfate as described by Mor and Sivan (2008), rinsed with distilled water, and dried at 50 °C for 24 h. The difference in dry weight of PS before and after experiment was measured gravimetrically. Fermentation control contained PS in media without inoculation. The fermentations were performed in triplicate.

For each fermentation, one mL aliquots were withdrawn every 5 days to quantify laccase production.

In addition, the effect of copper sulfate ($CuSO_4$) on the PS weight loss in biodegradation was evaluated. For that purpose, 100 μL of 50 mM $CuSO_4$ were added to FM.

2.6 Quantification of laccase activity

One mL from fermentations were used to measured laccase activity. First, samples were centrifugated at 4637 x g for 5 min. Then 0.8 mL of the supernatant were mixed with 0.2 mL of 1.76 mM guaiacol solution (2-methoxyphenol; Sigma-Aldrich, EUA). The mixture was incubated at 35 °C and 200 rpm during 24 h. The observed effect corresponded to the oxidation of guaiacol by laccase. The absorbance at 470 nm was measured spectrophotometrically (Shimadzu UV-1800 spectrophotometer, Tokio, Japan) every hour. One unit of laccase is expressed as an amount of enzyme necessary to oxidize 1 μM of guaiacol per minute at 35 °C and pH 7.0.

2.7 Construction of phylogenetic tree

To construct phylogenetic tree, bacteria sequences identified in the present study and those reported as laccase producers (Mor and Sivan, 2008; Mongkolthanaruk *et al*., 2012; Sheikhi *et al*., 2012; Mukherjee and Das, 2014) or biodegradable plastics were used (Auta *et al*., 2018; Chediu and Uchechukwu, 2022; Skariyachan *et al*., 2016; Urbanek *et al*., 2017)

For that purpose, MEGA 7 program (Kumar *et al*., 2016) was used. Kimura's model of two parameters (Kimura, 1980) together with NeighborJoin and BioNJ algorithms were employed to construct the phylogenetic tree using 16S rDNA sequences of the isolated bacteria and those deposited at GenBank (NCBI).

2.8 Statistical analysis

Comparative studies of the mean values obtained in gravimetric assays for the PS samples as well as laccase activity tests were carried out using the Tukey test of multiple comparisons, with the significance level of 95% using Minitab17 statistical program.

3 Results and discussion

3.1 Identification of bacterial strains

The isolates were identified as Gram-negative rods. Table 1 shows the results of the analysis carried out to identify strains isolated in this study. The 16S rDNA sequences obtained in this work were up to 1273 pair base (pb) long which was sufficient size to be compared with data reported at NCBI. On average, the length of full rDNA 16S gene is up to 1500 pb (Clarridge, 2004).

Table 1. Identification of the isolated bacteria strains and their GenBank access number.

Isolated strain	Sequence length (pb)	Similarity (%)	Identified genus	GenBank description	Access number
UAMI-C1	1273	96	*Enterobacter* sp.	*Enterobacter* sp. strain UAMI-C1	MK975880
UAMI-C2	1251	97	*Enterobacter* sp.	*Enterobacter* sp. strain UAMI-C2	MK975881
UAMI-C3	1205	97	*Enterobacter* sp.	*Enterobacter* sp. strain UAMI-C3	MK975882

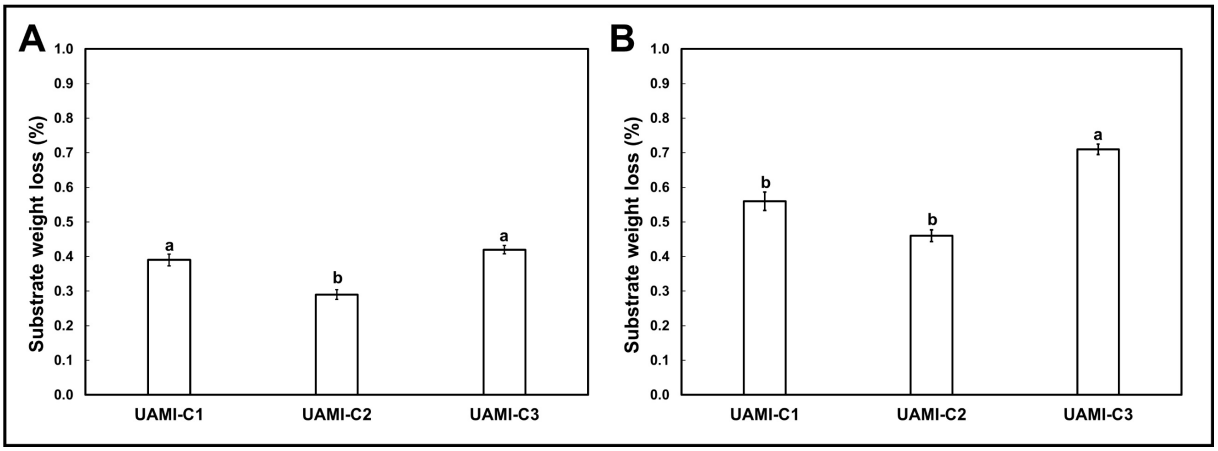

Figure 1. Substrate weight loss in biodegradation using crystal PS without (A) and with (B) copper ions for the three bacterial. The mean values that do not share the same letter are significantly different (Tukey test, 0.05).

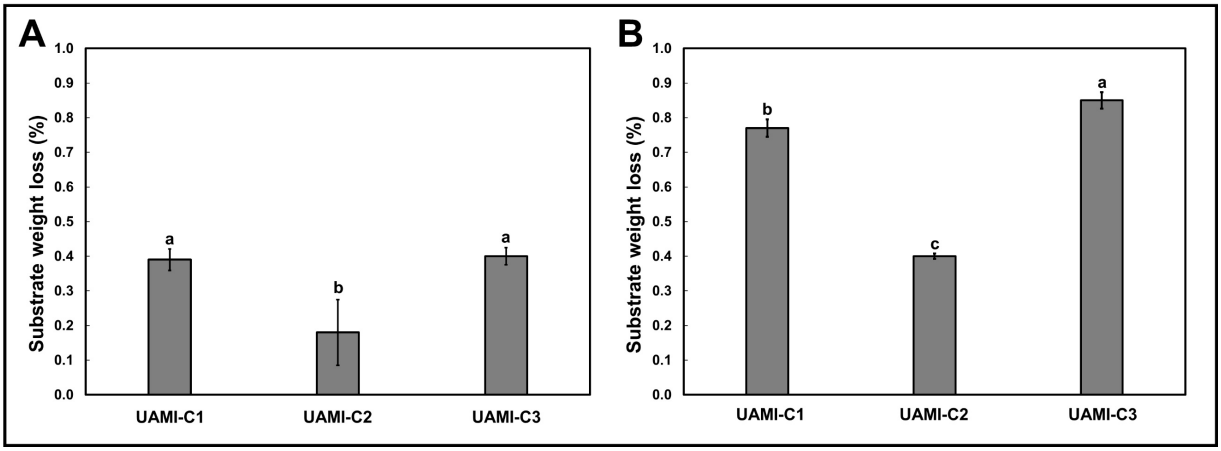

Figure 2. Substrate weight loss in biodegradation using expanded PS without (A) and with (B) copper ions for the three bacterial. The mean values that do not share the same letter are significantly different (Tukey test, 0.05).

Likewise, it was found that the three bacteria strains used in the present study shared up to 97% genetic similarity with sequences of *Enterobacter* sp. genus.

Moreover, it has been previously reported that *Enterobacter* genus is capable of producing laccases (Mukherjee and Das, 2014; Devasia and Nair, 2016). This study is based on the assumption that the isolated bacteria species could also be the laccase producers. It is noteworthy that laccase was linked to PE biodegradation in various studies (Iiyoshi *et al*., 1998; Fujisawa *et al*., 2002; Santo *et al*., 2013).

3.2 Substrate weight loss in biodegradation of crystal and expanded PS

Figures 1 and 2 shows the data obtained for 30-day biodegradation expressed as substrate weight loss using

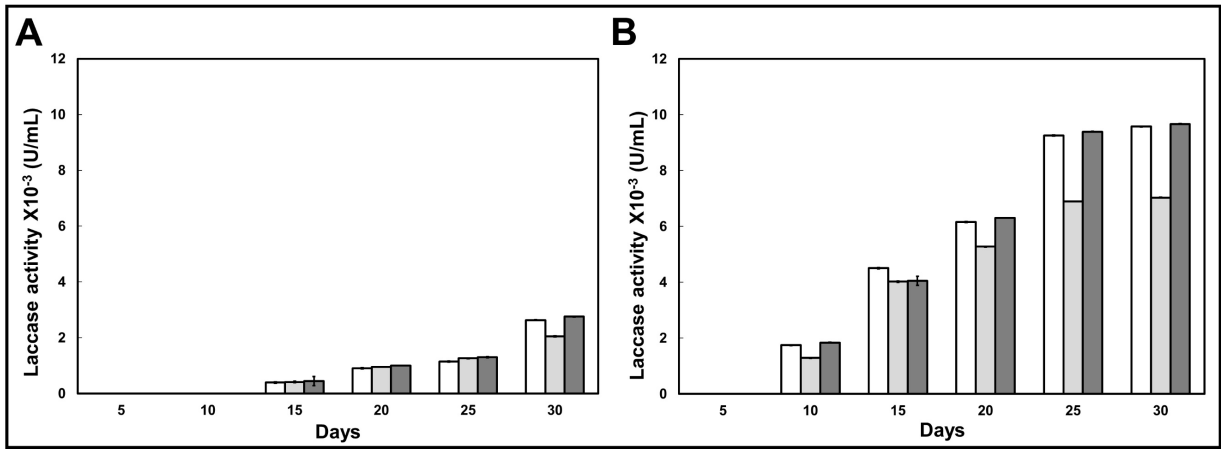

Figure 3. Laccase activity during biodegradation of crystal PS for 30 days without (A) and with (B) $CuSO_4$. (□) UAMI-C1; (■) UAMI-C2; (■) UAMI-C3.

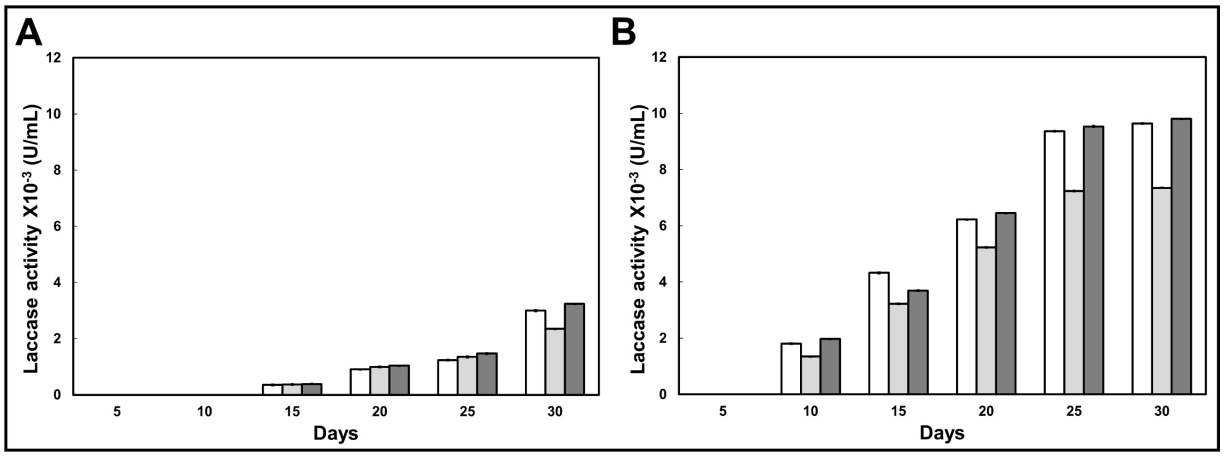

Figure 4. Laccase activity during biodegradation of expanded PS for 30 days without (A) and with (B) $CuSO_4$. (□) UAMI-C1; (■) UAMI-C2; (■) UAMI-C3.

three bacterial and two substrates: crystal and expanded PS. In the experiments when no metal ions were added to the FM, all bacterial were capable of decomposing PS regardless its type, i.e., crystal or expanded. The highest loss of substrate weight was observed for UAMI-C1 and UAMI-C3 strains. When biodegradation was carried out with the addition of copper to the FM, an increase in substrate weight loss was observed for all bacterial species and both PS types in comparison with control experiments (no $CuSO_4$ added). The most notable results were obtained for UAMI-C3 strain with 0.71 ± 0.038 % weight loss of crystal PS and 0.85 ± 0.024 % reduction in substrate weight of expanded PS. For comparison, Mor and Sivan (2008) reported the decrease in substrate weight by 0.8% in 8 weeks when *R. ruber* C208 and crystal PS were used in biodegradation tests. In the present study, the biodegradation time was reduced by half to achieve the same result. Furthermore, it is important to mention that when $CuSO_4$ was added to the FM, a higher substrate loss rate was observed for expanded PS (Figure 2B) compared to crystal PS (Figure 1B). These results can be explained by the fact that the structure of expanded PS has broader surface that facilitates bacterial interaction to form biofilm, as reported by Mor and Sivan (2008).

3.3 Laccase activity in the biodegradation of PS

As can be seen in Figures 3 and 4, during the biodegradation evaluation of the two PS samples (crystal and expanded), it was possible to measure laccase activity (U/mL). In addition, an increase in laccase activity was observed when $CuSO_4$ was added (Figure 4). Therefore, all bacteria demonstrated the capacity to produce laccase. The highest enzyme activity

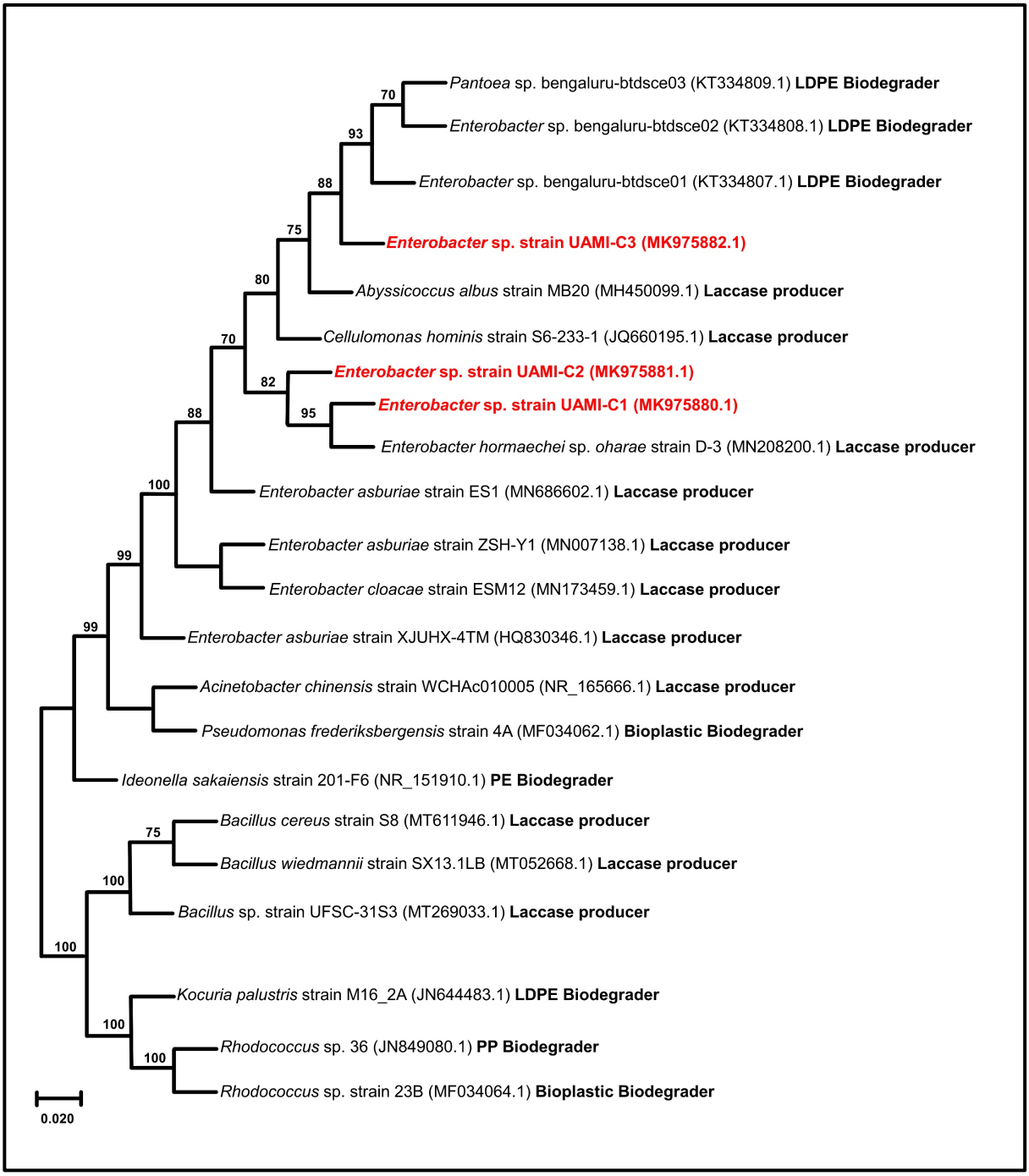

Figure 5. Phylogenetic tree to compare isolated bacteria (red) in this work with laccase-producing and plastic-biodegrading bacteria. The access number of NCBI is in parentheses. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. LDPE: Low Density Polyethylene; PE; Polyethylene; PP: Polypropylene.

in biodegradation was observed for UAMI-C3 strain at 9.673 $\times 10^{-3}$ and 9.803 $\times 10^{-3}$ U/mL for crystal and expanded PS, respectively.

Other research groups have reported laccase producing microorganisms and the effect of transition metal ions on the induction and activity of this enzyme. Mongkolthanaruk *et al*. (2012) isolated *Enterobacter* sp. from soil and residual waters from textile and paper industries, and they did not observe the effect of copper and manganese on the laccase production.

On the contrary, Santo *et al*. (2013) reported that addition of copper to *R. ruber* C208 in biodegradation assay of the PE substrate increased laccase production by 13-fold and polymer decomposition by 25%.

3.4 Phylogenetic comparison of the bacteria

The relationship between isolated bacteria strains and those previously reported to exhibit capacity to biodegrade PS or to produce laccase was determined using phylogenetic analysis. As seen in Figure 5, the first clade of the tree corresponds to separation into Gram-positive (bottom part) and Gram-negative (upper) bacteria. Additionally, the isolated bacteria and identified in the present study are closely related to strains of genus *Enterobacter* which have the ability to biodegrade different types of plastics, as well as laccase producers. In particular, UAMI-C3 has an 88% correlation with *Enterobacter* sp. Bengaluru-btdsce01 and *Enterobacter* sp. Bengaluru-btdsce02 reported as a low-density polyethylene biodegrader (Skariyachan *et al*., 2016). Whereas UAMI-C1 and UAMI-C2 were correlated with 95% and 83% respectively with *Enterobacter hormaechei* sp. *oharae* strain D-3 reported as a laccase producer (Chediu and Uchechukwu, 2022). It is therefore possible that the laccase-producing strains are also PS-biodegrader.

Conclusions

The isolation of three bacterial strains from the municipal dump waste capable of biodegrading PS by laccase activity was achieved. The bacteria were identified as *Enterobacter* sp. and all three species were demonstrated to hydrolyze crystal and expanded PS. The biodegradation rate was enhanced by addition of $CuSO_4$ to the fermentation medium. It was found that laccase activity was induced by $CuSO_4$ and the *Enterobacter* sp. UAMI-C3 strain exhibited the highest enzymatic activity. With the results obtained, we can see that it is very likely that the bacteria isolated in this work have the capacity to produce the enzyme laccase and that this may be associated with the biodegradation of the two PS samples due to the fact that a weight loss was obtained. Moreover, based on the phylogenetic analysis and the observed positive effect of $CuSO_4$ on laccase production in isolated bacteria, it is highly probable that laccase played a role in biodegradation process of PS. The obtained results encourage further search for the efficient methods to control plastic pollution by employing bacterial enzymatic systems.

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