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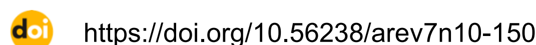
BIOTECHNOLOGICAL POTENTIAL OF PRIESTIA MEGATERIUM STRAIN E1

POTENCIAL BIOTECNOLÓGICO DO ISOLADO PRIESTIA MEGATERIUM CEPA E1

POTENCIAL BIOTECNOLÓGICO DEL AISLADO DE CEPA E1 DE PRIESTIA MEGATERIUM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Larissa Marques Negrett[1], Kauanny Wictoria Plenz[2], Rodrigo Matheus Pereira[3], Maricy Raquel Lindenbah Bonfá[4]

ABSTRACT
The increasing production of petroleum-derived plastics and their improper disposal have led to severe environmental impacts. In this context, biopolymers synthesized by bacteria, such as polyhydroxyalkanoates (PHAs), emerge as a promising solution for the development of bioproducts with a wide range of industrial applications. Therefore, the aim of the present study was to evaluate the PHA production and enzymatic potential of the bacterium Priestia megaterium strain E1, previously isolated from agricultural soil in the municipality of Dourados, MS, Brazil. For this purpose, qualitative tests were conducted for PHA detection using lipophilic dyes, specifically Sudan Black B and Nile Red. The evaluation of enzymatic potential was performed using the Cup Plate technique with solid minimal medium supplemented with skim milk for proteolytic activity and soluble starch for amylolytic activity. Plates were incubated in a BOD incubator at 30 °C and analyzed after 24, 48, and 72 hours. The enzymatic index (EI) was calculated based on the ratio between the degradation halo diameter and the colony diameter. Data were analyzed through mean, standard deviation, and analysis of variance followed by Tukey's test ($p > 0.05$) in triplicates. PHA production was confirmed by the intense blue coloration with Sudan Black B and by fluorescence under ultraviolet light after cultivation in a medium containing Nile Red. Regarding enzymatic activity, the strain exhibited significantly higher performance in protease production, reaching an average EI of 2.97 after 72 hours. For amylase, no degradation halos were observed within the first 24 hours; however, from 48 hours onwards, the average EI was 1.5 mm, indicating amylolytic potential.The results demonstrate that Priestia megaterium strain E1 possesses relevant characteristics for microorganisms of biotechnological interest. Its

[1] Undergraduate student in Bachelor of Biotecnology. Faculdade de Ciências Biológicas e Ambientais (FCBA). Universidade Federal da Grande Dourados (UFGD). E-mail: marquesnegrettlarissa@gmail.com
Orcid: https://orcid.org/0009-0000-9617-4889
[2] Undergraduate student in Bachelor of Biotecnology. Faculdade de Ciências Biológicas e Ambientais (FCBA). Universidade Federal da Grande Dourados (UFGD). E-mail: kakawictoria8@gmail.com
Orcid: https://orcid.org/0009-0005-8399-2803
[3] Dr. in Agriculture Microbiology. Programa de Pós-Graduação em Microbiologia Agropecuária (PPG-MICRO). Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP). E-mail: rodrigopereira@ufgd.edu.br
Orcid: https://orcid.org/0000-0001-6025-5118
[4] Dr. in Food Science. Programa de Pós-Graduação em Ciência de Alimentos (PPGCA). Universidade Estadual de Campinas (UNICAMP). E-mail: maricybonfa@ufgd.edu.br
Orcid: https://orcid.org/0000-0002-6978-4769

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sustainable potential makes it an essential candidate in the transition toward cleaner production processes, contributing to the reduction of environmental impact.

Keywords: Environmental Biotechnology. Biocatalysts. Bioconversion.

RESUMO

A crescente produção de plásticos derivados do petróleo e o descarte inadequado, vêm ocasionando impactos ambientais severos. Diante desse contexto, os biopolímeros sintetizados por bactérias, como os polihidroxialcanoatos (PHAs), surgem como uma solução promissora para a produção de bioprodutos, com uma variedade ampla de aplicações industriais. Neste contexto, o objetivo do presente trabalho foi avaliar a produção de PHA e potencial enzimático da bactéria Priestia megaterium cepa E1, isolada previamente em solo agrícola, no município de Dourados, MS. Para isso, foram conduzidos testes qualitativos para detecção de PHA por meio de utilização de corantes lipofílicos, como Sudan Black B e o Red Nile. A avaliação do potencial enzimático foi conduzida pela técnica de Cup Plate, utilizando meio mínimo sólido suplementado com Skim milk para análise da atividade proteolítica e amido solúvel para análise da atividade amilolítica. As placas foram incubadas em estufa tipo BOD a 30°C e avaliadas após 24, 48 e 72 horas. O índice enzimático (IE), foi calculado com base na razão entre o diâmetro do halo de degradação e o diâmetro da colônia, sendo os dados analisados por meio da média, desvio padrão e análise de variância seguida do teste de Tukey ($p>0,05$) das triplicatas. A produção de PHA foi confirmada tanto pela coloração azul intensa com Sudan Black B, quanto pela fluorescência observada sob luz ultravioleta após cultivo em meio contendo o corante Red Nile. Em relação à atividade enzimática, a cepa apresentou desempenho significativamente superior para a produção de protease, alcançando IE médio de 2,97 em 72 horas. Para à amilase nas primeiras 24 horas não houve presença de halos de degradação, entretanto a partir de 48 horas o IE médio foi de 1,5 mm, indicando potencial amilolítico. Os resultados obtidos demonstram que a Priestia megaterium cepa E1 possui características relevantes para microrganismos de interesse biotecnológico, seu potencial sustentável os torna essenciais na transição para processos produtivos mais limpos, contribuindo para redução do impacto ambiental.

Palavras-chave: Biotecnologia Ambiental. Biocatalisadores. Bioconversão.

RESUMEN

La creciente producción de plásticos derivados del petróleo y su eliminación inadecuada han causado graves impactos ambientales. En este contexto, los biopolímeros sintetizados bacterianamente, como los polihidroxialcanoatos (PHA), surgen como una solución prometedora para la producción de bioproductos con una amplia variedad de aplicaciones industriales. En este contexto, el objetivo de este estudio fue evaluar la producción de PHA y el potencial enzimático de la bacteria Priestia megaterium cepa E1, previamente aislada de suelo agrícola en el municipio de Dourados, Mato Grosso do Sul. Se realizaron pruebas cualitativas para detectar PHA utilizando colorantes lipofílicos como Sudan Black B y Red Nile. El potencial enzimático se evaluó mediante la técnica Cup Plate, utilizando un medio mínimo sólido suplementado con leche descremada para la actividad proteolítica y almidón soluble para la actividad amilolítica. Las placas se incubaron en una incubadora BOD a 30 °C y se evaluaron después de 24, 48 y 72 horas. El índice enzimático (IE) se calculó con base en la relación entre el diámetro del halo de degradación y el diámetro de la colonia. Los datos se analizaron mediante media, desviación estándar y análisis de varianza seguido

de la prueba de Tukey (p>0,05) por triplicado. La producción de PHA se confirmó tanto por tinción azul intensa con Sudan Black B como por fluorescencia observada bajo luz ultravioleta después del cultivo en medio que contenía colorante Nile Red. Con respecto a la actividad enzimática, la cepa mostró un rendimiento significativamente superior para la producción de proteasas, alcanzando un IE promedio de 2,97 en 72 horas. Para amilasa, no hubo halos de degradación en las primeras 24 horas; sin embargo, después de 48 horas, el IE promedio fue de 1,5 mm, lo que indica potencial amilolítico. Los resultados obtenidos demuestran que la cepa E1 de Priestia megaterium posee características relevantes para microorganismos de interés biotecnológico. Su potencial sostenible los hace esenciales en la transición a procesos de producción más limpios, contribuyendo a la reducción del impacto ambiental.

Palabras clave: Biotecnología Ambiental. Biocatalizadores. Bioconversión.

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1 INTRODUCTION

The replacement of polymers derived from fossil resources is of paramount importance, mainly due to their low biodegradability. According to a report by the International Resource Panel of the United Nations (UN, 2019), the global use of petroleum-derived materials could double by 2060, reaching about 190 billion tons. This significant increase tends to intensify waste generation, aggravating the pollution of soils and oceans (Isobe; Shinsuke, 2022).

In this context, the search for sustainable alternatives that can contribute to environmental preservation becomes essential. Among these alternatives, the use of microorganisms such as the bacterium *Priestia megaterium*, a species with a consolidated history and broad application, stands out. Studies report its use in the synthesis of polyhydroxyalkanoates (PHA), in the production of vitamin B12, and various enzymes, highlighting the potential of multifunctional microorganisms to optimize industrial processes (Biedendieck et al., 2021; Ashour; Abd-Elhalim, 2024).

In this sense, bacterial biopolymers, such as PHA, are stored as intracellular granules and show variations in the composition of hydroxyalkanoate monomers. They are produced from the fermentation of renewable raw materials, and their synthesis occurs when the cell is under stress. This allows for the production of various bioproducts, such as bioplastics, which are used in the production of various disposable utensils like bags, cups, and packaging, contributing to the reduction of greenhouse gas emissions and offering a significantly shorter degradation time compared to fossil-derived plastic (Khalil et al., 2023; Habitzreuter et al., 2023; Tennakoon et al., 2023; Martins; Gupta, 2024).

In addition to the potential for biopolymer production, the use of microorganisms in enzyme production has also stood out in the industry. These macromolecules, mainly obtained by fermentation on renewable substrates, act as highly efficient and specific biological catalysts, accelerating essential chemical reactions. Among the most relevant are protease, amylase, cellulase, and xylanase, widely recognized for their scalability, low cost, and their goals aligned with sustainability (Niyonzima; Venna; Sunil, 2020; Aarti, et al., 2020).

Several studies point to the genus *Bacillus* as one of the main sources of enzymes of commercial interest. Microbial protease, for example, catalyzes the breakdown of proteins into peptides and amino acids that are used in the detergent, textile, and food industries. Amylases, in turn, act on the degradation of starch into smaller sugars, and the products of this degradation depend on the type of amylase involved. These compounds are used in the

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production of pharmaceuticals, sweeteners, and in the manufacturing of biofuels (Far et al., 2020; Vojnovic, et al., 2024; Khan, 2025).

The sustainable potential of both technologies, coupled with the advancement of the circular economy culture and the growing pressure for low-impact processes and a reduced carbon footprint, has encouraged companies to invest in research and the development of innovative solutions for the area. However, they still face market acceptance problems due to their high production cost and the long time required for large-scale synthesis. In this context, the use of biotechnological tools has proven promising, allowing for the optimization of processes and the intensification of production (Alaerts et al., 2018; Koller; Mukherjee, 2022; Tobias-soria et al., 2023).

Thus, the present study aimed to evaluate the biotechnological potential of the isolate *Priestia megaterium* strain E1, with an emphasis on evaluating PHA production and enzymatic activity.

2 METHODOLOGY

The experiments were carried out at the Federal University of Grande Dourados (UFGD) Dourados - MS, in the Multidisciplinary Block II, at the Environmental Biotechnology Laboratory. The bacterium *Priestia megaterium* strain E1 was isolated from soil with a history of fipronil application at the UFGD Experimental Farm (FAECA) in 2015 with great potential for degrading this chemical insecticide (do Prado et al., 2022). In addition, another research conducted in conjunction with the UFGD Bioinformatics laboratory verified the presence of genomic sequences associated with the polyhydroxyalkanoate metabolism present in the bacterium's genome (Fernandes, 2024).

2.1 ACTIVATION OF *P. MEGATERIUM* STRAIN E1

To promote adequate growth and purity, the bacterium was reactivated in solid MMG medium (minimal glycerol medium), where glycerol was the main carbon source. This specific medium is used to evaluate the ability of bacteria to grow and produce polymers from simple substrates. Thus, the medium was prepared with the proportions 7.0 $g \cdot L^{-1}$ KH_2PO_4 (Monobasic phosphate); 2.0 $g \cdot L^{-1}$ Na_2HPO_4 (Dibasic sodium phosphate); 1.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$ (Magnesium sulfate heptahydrate); 0.1 $g \cdot L^{-1}$ $CaCl_2$ (Calcium chloride); 0.08 $g \cdot L^{-1}$ $FeCl_3 \cdot 6H_2O$ (Ferric chloride hexahydrate); 0.02 $g \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$ (Zinc sulfate heptahydrate); 0.8 $g \cdot L^{-1}$ Yeast Extract; 23.8 mL of glycerol and diluted in 1 L of distilled water.

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Then, the pH of the solution was adjusted to between 6.0 and 6.5. For the solidification of the medium, 20 g·L^{-1} of bacteriological agar was used, initially melted in a microwave oven with frequent stirring to ensure better dissolution of the salts and then sterilized in an autoclave at 121°C for 20 minutes. After cooling, the medium was poured into standardized sterile Petri dishes with 35 mL in each. The P. megaterium sample was reactivated and incubated in a BOD (Biochemical Oxygen Demand) at 30°C for 24 to 48 hours, for successive subcultures and to guarantee the obtaining of pure colonies.

2.2 MORPHOLOGICAL AND STAINING CLASSIFICATION

In order to confirm the morpho-tinctorial classification of the strain, the Gram staining technique was used, distinguishing between Gram-positive and Gram-negative. Initially, a smear was made using the biological material, followed by fixing the slide by heat emitted through a Bunsen burner. Subsequently, crystal violet dye was added for a period of 60 seconds, and at the end of this time interval, the excess dye was rinsed and removed by a fine stream of distilled water. Next, a Lugol's solution was added to the slide for a period of 60 seconds, with the repetition of the previously mentioned rinsing procedure.

Then, the slide was exposed to an ethanol decolorizing agent for approximately 15 seconds, followed by a new washing step with distilled water. After washing, 0.1% safranin was added to the slide, which acted for a period of 30 seconds, culminating in another round of rinsing the slide. After the complete drying of the slide, with a drop of immersion oil applied over the coverslip, the microscopic analysis was performed using an optical microscope. The cells that exhibited a reddish coloration were designated as Gram-negative, while those that showed a bluish tone were classified as Gram-positive.

For the catalase test, a platinum loop was used to collect the target bacterium, followed by the creation of a smear on a slide. Then, a drop of 3% hydrogen peroxide was applied over the bacterium on the slide, and the immediate appearance of effervescent bubbles was evaluated, demonstrating the conversion of H_2O_2 into water and gaseous oxygen, considered a positive result. On the other hand, the absence of effervescent activity was interpreted as a negative result. It is worth noting that this test was conducted exclusively on bacteria previously identified as Gram-positive.

After confirmation by comparing with old records of the bacterium, it was tested for biopolymer production.

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2.3 SCREENING FOR PHA PRODUCTION BY SUDAN BLACK STAINING METHOD

For the confirmation of biopolymer production, the cell staining methodology using Sudan Black dye, specific for the detection of lipids, was performed (Hartman et al., 1940). The colonies were stained with a 0.02% Sudan Black B dye solution dissolved in 96% ethanol for 15 minutes. Then, the dye was completely removed with distilled water by washing, subsequently, a 96% ethanol decolorizing solution was added for 5 minutes and washed again with the help of distilled water. At the end, the PHA-producing colonies remained blue as they are not decolorized by the ethanol solution. This occurs because the Sudan Black B dye dissolves and specifically binds to the lipids present in the cell, resulting in the characteristic coloration.

2.4 SCREENING FOR PHA PRODUCTION BY NILE RED STAINING METHOD

After preparing the previously described MMG medium and cooling, 1 µL of Nile Red was added to each mL of culture medium and subsequently poured into Petri dishes. The bacterium was inoculated by the Streak Plate method and incubated in a BOD at 30 ºC for 48 hours. The evaluation of PHA production was done through the detection of fluorescence under ultraviolet (UV) light in a photodocumenter (Loccus) to detect the intracellular accumulation of the biopolymer (Spiekermann et al., 1999).

2.5 SCREENING FOR ENZYME PRODUCTION

The determination of the bacterium's enzymatic potential for protease and amylase production was carried out by the qualitative Cup Plate method (Dingle et al., 1953). In this technique, with the aid of a sterile loop, the isolated colony is applied to the center of the Petri dish containing the MMG medium with a change in the carbon source (20 $g \cdot L^{-1}$ of Skim Milk and 20 $g \cdot L^{-1}$ soluble starch), causing the colonies to grow at only one specific point on the plate. Thus, the isolate was subcultured in the minimal medium and incubated in a BOD at 30 ºC for 24, 48, 72 hours. The determination of the enzymatic potential was found by calculating the Enzymatic Index (EI), which measures the degradation zone, calculated by the ratio of the halo diameter around the colony to the colony diameter as expressed in equation (I):

$$EI = \frac{HALO\ DIAMETER}{COLONY\ DIAMETER} \qquad (1)$$

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According to this method, the isolates that obtain a higher EI in the culture media are those that have greater enzymatic activity. For the colonies considered as good protease producers, those that presented an EI ≥ 2.0 cm were considered. As for the amylase-producing strains, in accordance with Alves et al. (2018), those that presented an EI ≥ 1.0 cm were considered good producers.

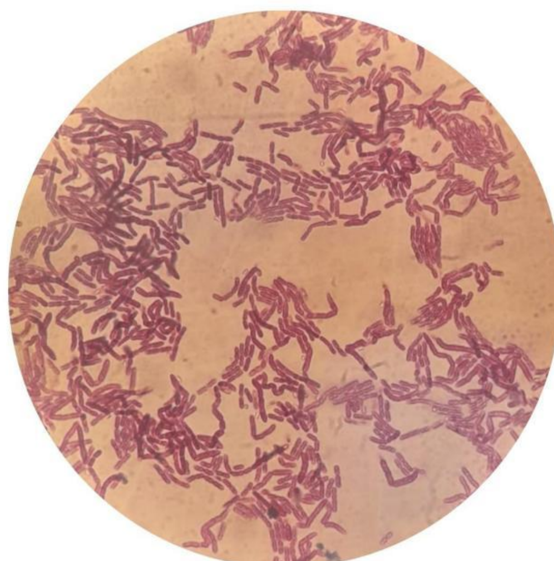
The results obtained for the enzymatic index of protease and amylase were expressed as the mean and standard deviation of the triplicates. To verify the existence of a significant difference between the incubation times and between the substrates, an analysis of variance (ANOVA) was performed, followed by Tukey's test at 5% significance. The statistical analyses were conducted using the Info Stat software version 2020.

3 RESULTS

After cultivating the bacterium in a minimal medium containing a simple substrate, the morpho-tinctorial characterization was performed. The observed morphology was bacillary, with the presence of spores, and Gram-positive, as shown in Figure 1.

Figure 1

Gram staining of P. megaterium strain E1 at 1000x magnification



Source: The authors, 2025.

The biochemical catalase test was also positive, confirmed by the immediate formation of bubbles after the addition of 3% hydrogen peroxide, in addition to the presence

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of spores, which reinforces the identification of the strain as belonging to the genus Priestia, as observed in Table 1.

Table 1

Morphological, staining, and biochemical screening results for P. megaterium strain E1

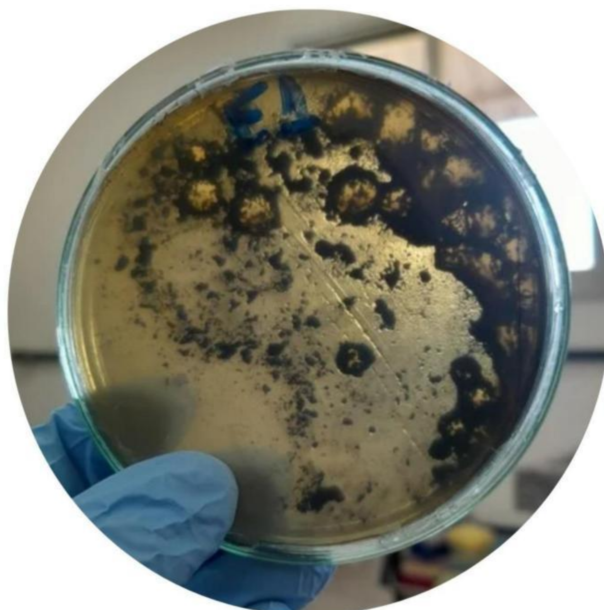
Isolate	Gram	Morphology	Catalase	Spores
P. megaterium strain E1	+	Rod	+	+

Legend: + = positive.
Source: The authors, 2025.

For the evaluation of polyhydroxyalkanoates (PHA) production, the Sudan Black B staining method was used, in which the colonies remained bluish even after washing with 96% ethanol, as recorded in Figure 2, indicating positive results for PHA accumulation.

Figure 2

Visualization of P. megaterium strain E1 colonies after Sudan Black B staining, with positive results for PHA accumulation



Source: The authors, 2025.

Subsequently, the bacterium was inoculated into a minimal medium supplemented with Nile Red dye. After the growth period, the plates were observed under UV light, revealing fluorescence in the colonies, positive for intracellular PHA accumulation.

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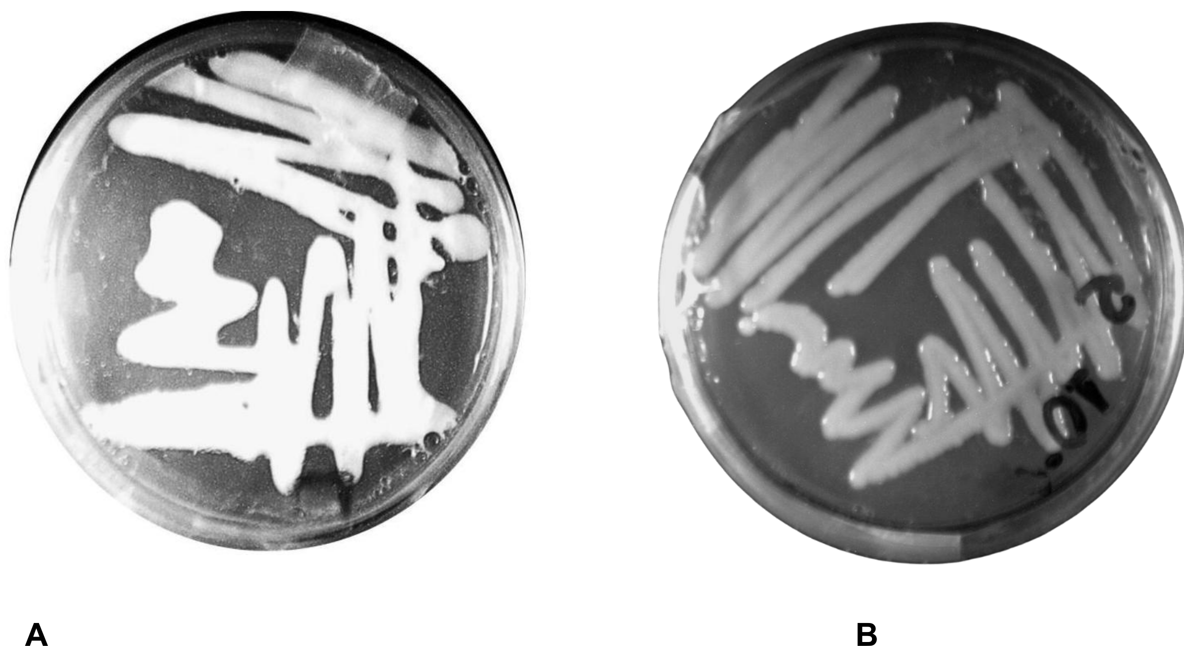
Figure 3

A. Fluorescence of P. megaterium strain E1 compared to B. the negative control strain, cultivated in Nile Red-supplemented medium under UV light



Source: The authors, 2025.

Table 2 presents the enzymatic index (EI) results for P. megaterium strain E1 at different incubation periods in protease and amylase media.

Table 2

Enzymatic index of P. megaterium strain E1

ENZYMATIC INDEX			
Time (h) / Treatments	**24**	**48**	**72**
Protease	$2,00 \pm 0,25_{Aa}$	$2,48 \pm 0,35_{ABa}$	$2,97 \pm 0,29_{Ba}$
Amylase	$0,00 \pm 0,80_{Ab}$	$1,50 \pm 0,19_{ABb}$	$1,84 \pm 0,16_{Bb}$

Legend: Results were expressed as mean ± standard deviation. Uppercase letters in the same row indicate significant differences between incubation times for the same enzyme type, while lowercase letters in the same column indicate significant differences between enzyme types at the same time, according to Tukey's test ($p>0.05$).
Source: The authors, 2025.

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As observed in Table 2, in the medium containing Skim milk, the bacterium showed a mean EI of 2.00 ± 0.25 already within the first 24 hours, with an increase in the subsequent times, reaching 2.97 ± 0.29 at 72 hours. In the medium supplemented with soluble starch, no enzymatic halo formation was observed after 24 hours, demonstrating an absence of initial amylolytic activity; from 48 hours, the mean EI was 1.50 ± 0.19, increasing to 1.84 ± 0.16 at 72 hours. In both substrates, the 72-hour period was significantly superior to the others at 5% probability by Tukey's test, indicating an increase in enzymatic activity over the incubation time.

Furthermore, at all analyzed times, proteolytic activity was significantly higher than amylolytic activity, as indicated by the distinct lowercase letters in the comparison between columns, reinforcing the greater potential of P. megaterium strain E1 for producing proteolytic enzymes under the tested experimental conditions.

4 DISCUSSION

The *P. megaterium* strain E1 exhibited morpho-tinctorial and biochemical characteristics consistent with data presented in the literature. Gram staining revealed Gram-positive cells with bacillary morphology and the presence of spores, which confers resistance to the strain, characteristics compatible with the genus *Priestia*, previously classified as *Bacillus* (Biedendieck et al., 2021). Regarding the biochemical test, the formation of bubbles when the sample was exposed to hydrogen peroxide confirms the presence of catalase, an enzyme related to protection against oxidative stress, a recurrent characteristic in aerobic and facultative microorganisms as observed by Iwase and collaborators in 2013.

In the context of polyhydroxyalkanoates (PHA) production, staining methods using lipophilic dyes, such as Sudan Black B and Nile Red, provided consistent visual evidence of the strain's ability to accumulate intracellular granules of this biopolymer. The dark blue coloration observed with Sudan Black B, even after washing with ethanol, indicates the presence of reserve lipids compatible with PHA (Aljuraifani et al., 2018; Sachan et al., 2025).

As described by Spiekermann et al. (1999), the fluorescence observed under ultraviolet light after cultivation in a Nile Red-supplemented medium is a consequence of the dye's absorption by the cell, accumulated in intracellular granules that, when exposed to UV light, emit fluorescence, evidencing the presence of the biopolymer. The positive result of the present study reinforces that this technique is highly effective for screening PHA-producing bacteria and corroborates previous studies that highlight *P. megaterium* as one of the

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promising species in biopolymer synthesis (Shahid et al., 2017; Israni and Shivakumar, 2020; Cal et al., 2025).

Regarding the enzymatic index, the study by Alves and Paiva (2018), which used cerrado soil samples to bioprospect for amylase-producing isolates, observed that degradation halos were not visible in the first 24 hours, and the enzymatic index for amylolytic activity showed an EI between 0.60 and 3.33, a value comparable to that reported in the present study, which shows an average EI between 1.5 and 1.84 after a 48-hour incubation period.

In another study, conducted by Sapkota and collaborators (2019), bacteria were isolated that showed an EI between 1.2 and 1.6 for amylase production, and after screening, the isolates were identified as belonging to the genus Bacillus. This demonstrates that the P. megaterium strain E1 showed increasing potential for amylolytic production.

Various studies highlight the genus Bacillus as one of the main producers of hydrolytic enzymes, especially protease, due to its ability to act at different temperatures, substrate concentrations, and pH (Oliveira et al., 2022). The study by Masi and collaborators (2021), which evaluated isolates of the same genus from leather industry effluent samples, showed an EI ranging from 1.4 to 1.8; these values are comparable to the bacterium evaluated in the present study, which demonstrated an average EI between 2.0 and 2.97.

Thus, the general data suggest that the P. megaterium strain E1 possesses efficient mechanisms for protease production, making it a viable candidate for applications in the food, leather, detergent, and waste treatment industries, in addition to PHA production, contributing to the development of innovative and sustainable processes, enabling greater productive efficiency and environmental preservation (Razzaq et al., 2019; Herrmann et al., 2024; Naseem et al., 2024).

Given these results, complementary tests are recommended to confirm the enzymatic performance of the strain, including the quantification of enzymatic activity, as well as the characterization of PHA. The combination of these approaches strengthens the study's foundation, enabling future strategies for optimizing enzyme and biopolymer production, as well as the possibility of genetic modification with a focus on increasing productivity, an essential aspect for large-scale industrial application.

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5 CONCLUSION

Based on the analyses performed, the P. megaterium strain E1 isolate, originating from agricultural soil in the municipality of Dourados - MS, exhibited relevant characteristics for microorganisms of biotechnological interest, proving promising for both biopolymer and hydrolytic enzyme production. In general, its characteristic morphotintorial profile of the genus was confirmed, being a Gram-positive bacterium, bacillary in shape, with the presence of spores and positive for the catalase test. Furthermore, the isolate showed positive results for tests using Sudan Black and Nile Red dyes, confirming its ability to produce PHA.

The enzymatic index obtained for protease production ranged between 2.0 ± 0.25 and 2.97 ± 0.29, being significantly higher than the values observed for amylase at all analyzed periods. For both substrates, the 72-hour incubation period statistically differed from the others ($p>0.05$), indicating increasing enzymatic potential over time. These results reinforce the strain's potential for the efficient conversion of simple and low-cost substrates into high value-added materials, favoring sustainability in industrial processes and contributing to the reduction of environmental impact.

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