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Identification and biotechnological potential of cellulases-producing rhizobacteria from the Amazon rhizosphere

Identificação e potencial biotecnológico de rizobactérias produtoras de celulases da rizosfera amazônica

Identificación y potencial biotecnológico de rizobacterias productoras de celulasas de la rizosfera amazónica

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Abstract
The Amazon rainforest hosts an extraordinary microbial diversity with significant yet underexplored biotechnological potential. This study aimed to identify and evaluate the production of endoglucanase, β-glucosidases and CMCase rhizobacteria isolated from the rhizosphere of *Pueraria phaseoloides* and *Inga edulis*, focusing on strains with potential applications in lignocellulosic biomass conversion. A total of 50 rhizobacterial isolates were reactivated and screened for cellulolytic activity using carboxymethylcellulose (CMC) agar. Thirty-one isolates (62%) formed hydrolysis halos, and 22 strains exhibited an enzymatic index (EI) ≥ 2.0, indicating high cellulolytic potential. Statistical analysis revealed significant differences between host plants, with isolates from *P. phaseoloides* showing higher average EI values than those from *I. edulis*. Molecular identification of the most active isolates (EI ≥ 2.0) revealed strains belonging to the genus *Bacillus,* including *Bacillus subtilis* and *Bacillus velezensis*. The strain B. subtilis INPA R-583, which showed the highest EI, was selected for enzymatic characterization. Kinetic analyses demonstrated stable and high endoglucanase (CMCase) activity, increasing β-glucosidase activity at later cultivation stages, and relatively low total cellulase (FPase) activity. These results indicate a functional but incomplete cellulolytic system, with strong potential for applications requiring endoglucanase activity. Overall, the findings highlight the Amazon rhizosphere as a valuable reservoir of cellulolytic bacteria and support the biotechnological relevance of native rhizobacteria for sustainable biomass conversion processes.
Keywords: Rhizobacteria; Forest amazon; Cellulose; Cellulases; Enzymes.

Resumo
A Amazônia abriga uma elevada diversidade microbiana com expressivo potencial biotecnológico ainda pouco explorado. Este estudo teve como objetivo identificar e avaliar rizobactérias produtoras de endoglucanases, exoglucanases e beta-glucosidases isoladas da rizosfera de *Pueraria phaseoloides* e *Inga edulis*, visando sua aplicação na conversão de biomassa lignocelulósica. Cinquenta isolados rizobacterianos foram reativados e submetidos à triagem da atividade celulolítica em meio sólido contendo carboximetilcelulose (CMC). Desses, 31 isolados (62%) apresentaram halos de degradação, sendo que 22 exibiram índice enzimático (IE) ≥ 2,0, indicando elevado potencial celulolítico. A análise estatística demonstrou diferenças significativas entre as plantas hospedeiras, com maior eficiência média dos isolados provenientes de *P. phaseoloides*. A identificação molecular dos isolados

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mais ativos revelou a predominância do gênero *Bacillus,* incluindo *Bacillus subtilis* e *Bacillus velezensis*. O isolado *B. subtilis* INPA R-583, que apresentou o maior IE, foi selecionado para caracterização enzimática. As análises cinéticas indicaram produção consistente de endoglucanase (CMCase), aumento tardio da atividade de β-glucosidase e baixa atividade celulase total (FPase). Os resultados evidenciam um sistema celulolítico funcional, porém incompleto, com elevado potencial para aplicações biotecnológicas que demandem endoglucanases. O estudo destaca a rizosfera amazônica como uma importante fonte de microrganismos nativos com potencial para processos sustentáveis de bioconversão.
Palavras-chave: Rizobactéria; Amazônia; Celulose; Celulases; Enzimas.

Resumen
La Amazonía alberga una extraordinaria diversidad microbiana con un alto potencial biotecnológico aún poco explorado. Este estudio tuvo como objetivo identificar y evaluar rizobacterias productoras de endoglucanasas, exoglucanasas y beta-glucosidasas aisladas de la rizosfera de *Pueraria phaseoloides* e *Inga edulis*, con énfasis en su aplicación en la conversión de biomasa lignocelulósica. Se reactivaron 50 aislados rizobacterianos y se evaluó su actividad celulolítica en medio sólido con carboximetilcelulosa (CMC). Treinta y un aislados (62%) presentaron halos de hidrólisis, y 22 mostraron un índice enzimático (IE) ≥ 2,0, lo que indica alto potencial celulolítico. El análisis estadístico evidenció diferencias significativas entre las plantas hospedadoras, siendo los aislados de *P. phaseoloides* más eficientes que los de *I. edulis*. La identificación molecular de los aislados más activos reveló la presencia de especies del género *Bacillus*, principalmente *Bacillus subtilis* y *Bacillus velezensis*. El aislado B. subtilis INPA R-583, con el mayor IE, fue seleccionado para la caracterización enzimática. Los ensayos cinéticos mostraron alta y estable actividad de endoglucanasa (CMCase), incremento tardío de β-glucosidasa y baja actividad celulasa total (FPase). Los resultados indican un sistema celulolítico funcional pero incompleto, con alto potencial para aplicaciones biotecnológicas basadas en endoglucanasas. El estudio refuerza la importancia de la rizosfera amazónica como fuente de microorganismos con valor biotecnológico.
Palabras clave: Rizobacteria; Amazonía; Celulosa; Celulasas; Enzimas.

1. Introduction

Cellulose, a natural polymer abundantly found in plants, is composed of long chains of glucose linked by β-1,4-glycosidic bonds. This structure imparts unique properties to cellulose, such as high mechanical strength, water insolubility, and chemical stability, making it a fundamental component of the plant cell wall and a valuable resource for industry. Plant biomass, which is rich in cellulose, hemicellulose, and lignin, represents a renewable carbon source for the production of biofuels and bioproducts. Cellulose, in particular, has been extensively studied and exploited due to its potential for bioethanol production (Etale et al., 2023).

The process of converting cellulose into fermentable sugars involves the action of cellulase enzymes, which degrade cellulose chains into smaller units. Endoglucanases initiate the process by cleaving internal bonds within the polymer chain, while exoglucanases act on the chain ends, releasing cellobiose. Finally, β-glucosidases convert cellobiose into glucose, which can then be fermented by microorganisms to produce ethanol (Davies & Henrissat, 1995). In addition to biofuel production, cellulose has multiple applications in other industrial sectors such as the paper and pulp industry, waste treatment, detergent production, textile manufacturing, animal nutrition, and food processing. Despite cellulose's great potential, the efficient conversion of this polymer into value-added products still faces challenges, including its structural resistance and the complexity of the lignocellulosic matrix. Nevertheless, continuous advances in biotechnology and enzyme engineering have made it possible to overcome these barriers, making cellulose an increasingly attractive raw material for industrial use (Abolore, Jaiswal & Jaiswa, 2024; Ejaz, Sohail & Ghanemi, 2021; Gaete et al., 2020).

Cellulase production is a common feature among various organisms, including bacteria, fungi, and some invertebrates (Belmont-Montefusco et al., 2020; Lo et al., 2003; Watanabe & Tokuda, 2010). The ease of cultivation and genetic manipulation of microorganisms makes them preferred sources for the industrial production of these enzymes. Rhizobacteria, which establish symbiotic relationships with plant roots, play a crucial role in promoting plant growth, nitrogen fixation, and protection against pathogens (Vieira-Jr et al., 2013). Moreover, some rhizobacterial species are capable of producing

cellulases, making them valuable tools for agricultural waste management. The production of cellulase by rhizobacteria opens new perspectives in biotechnology. The ability of these bacteria to degrade cellulose present in agricultural residues, such as straw and sugarcane bagasse, contributes to the reduction of waste volume while generating high-value products such as biofertilizers and biofuels (Ansiliero, Candiago & Gelinski, 2018; Dai et al., 2020; Ega et al., 2020; Pramanik et al., 2021; Soares et al., 2017).

The Amazon, one of the most biodiverse biomes on the planet, harbors an immense variety of microorganisms, including rhizobacteria. These bacteria, which establish symbiotic relationships with plant roots, play an important ecological role and possess great biotechnological potential. However, despite the Amazon's rich biodiversity, there remains a significant gap in detailed studies on cellulase-producing rhizobacteria in this region. Cellulases, capable of degrading cellulose, have numerous industrial applications, including the production of biofuels, bioplastics, and enzymes for the food industry. This study aimed to identify and evaluate the production of endoglucanases, exoglucanases and β-glucosidases rhizobacteria isolated from the rhizosphere of *Pueraria phaseoloides* and *Inga edulis*, focusing on strains with potential applications in lignocellulosic biomass conversion.

2. Methodology

This study was conducted as a laboratory-based quantitative investigation, developed under controlled conditions to evaluate the cellulolytic potential of rhizobacteria associated with leguminous plants. The methodological approach comprised the reactivation and screening of previously selected rhizobacterial strains, followed by qualitative and quantitative enzymatic assays, statistical analysis of the obtained data, and molecular identification of the most promising isolates. Descriptive statistics and inferential analyses were applied to ensure the reliability and interpretation of the results, providing a comprehensive assessment of cellulase production and taxonomic characterization of the selected microorganisms.

2.1 Selection and Reactivation of Microorganisms

A total of 76 rhizobacteria previously selected for their ability to produce hydrolytic enzymes, degrade petroleum, and surfactants were reactivated. Among these, 50 strains were viable and active in the Microorganism Collection of the Laboratory of Ecology and Biotechnology of Microorganisms (LEBMAM) at the National Institute for Amazonian Research (INPA). The selection criterion established was that the rhizobacteria had been isolated from root nodules of *Pueraria phaseoloides* (N = 24) and *Inga edulis* (N = 26) (Cáuper, 2018; Costa, 2017; Oliveira et al., 2022; Vaz, 2013).

The rhizobacteria were reactivated on Petri dishes containing solid YMA medium (yeast extract, mannitol, agar) and incubated at 28 °C for four days and subsequently transferred to fresh plates under the same conditions to prevent contamination (Vincent, 1970; Somasegaran & Hoben, 1985; Hungria et al., 1994). The isolates were stored in test tubes containing YMA medium (K_2HPO_4: 0.5 g L^{-1}; $MgSO_4$: 0.2 g L^{-1}; NaCl: 0.1 g L^{-1}; yeast extract: 0.5 g L^{-1}; mannitol: 10.0 g L^{-1}), following the recommendations of Vincent (1970) and Somasegaran & Hoben (1985).

2.2 Cellulolytic Activity Screening

The rhizobacteria were evaluated for cellulase production using carboxymethyl cellulose (CMC) agar medium (K_2HPO_4: 0.5 g L^{-1}; $MgSO_4$: 0.2 g L^{-1}; NaCl: 0.1 g L^{-1}; yeast extract: 0.5 g L^{-1}; CMC: 10.0 g L^{-1}; agar: 15.0 g L^{-1}) (Vincent, 1970; Hankin & Anagnostakis, 1975; Ruegger & Tauk-Tornisielo, 2004). The plates were incubated at 28 °C for five days, followed by a 2-h thermal shock at 50 °C. Degradation halos were visualized by incubating the plates with 10 mL of Congo red solution (2.5 g L^{-1}) in Tris-HCl buffer (0.1 M, pH 8.0) for 30 min, followed by washing with 5 mL of NaCl solution (0.5 M) in

Tris-HCl buffer (0.1 M, pH 8.0) (Nogueira & Cavalcante, 1996).

The assays were performed in quadruplicate on a single Petri dish containing CMC. The Enzymatic Index (EI) for all rhizobacteria forming degradation halos was calculated as the ratio between the average halo diameter and the average colony diameter across the four replicates (Hankin & Anagnostakis, 1975; Nogueira & Cavalcante, 1996; Florencio et al., 2012). Rhizobacteria showing an EI greater than 2.0 (Lealem & Gashe, 1994) were subsequently selected for molecular identification. EI values were initially subjected to descriptive statistical analysis and a Student's t-test to compare results between plant species, using BioEstat® software version 5.3.

2.3 Determination of Cellulolytic Enzymatic Activity

After rhizobacterial selection, enzymatic assays were conducted to evaluate cellulase production by the strain with the highest EI. The concentration of released sugars was determined from a standard curve obtained via linear regression. To induce cellulase production, the selected rhizobacterium was cultivated in liquid YMA medium containing 1% glucose, where mannitol (1%) was replaced by 1% microcrystalline cellulose (Avicel). Cultures were incubated at 28 °C under agitation (125 rpm) in 100 mL Erlenmeyer flasks until the cell density reached an optical density (OD_{600}) between 0.6 and 0.8, corresponding to approximately 10^7 cells mL^{-1} (Das & Mukherjee, 2007; Dias, 2012). Then, 1 mL of the bacterial culture grown in basal medium + 1% glucose was inoculated into 250 mL of Avicel medium (triplicates, pH ≈ 5.0, 28–30 °C). Samples were collected every 12 h, centrifuged, and stored for analysis.

The standard curve was constructed using glucose solutions ranging from 0.1 to 2.0 g L^{-1}, following the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The curve exhibited an $R^2 = 0.97$, indicating a strong linear correlation between absorbance and glucose concentration.

Total Cellulase Activity (FPase)

Total cellulase activity (FPase) was determined according to Ghose (1987) using Whatman No. 1 filter paper strips (1 × 6 cm) as substrate. Strips were incubated with 1 mL citrate buffer (pH 4.8) and 0.5 mL enzyme extract at 50 °C for 1 h. After incubation, 100 µL of the reaction mixture was mixed with 200 µL of DNS reagent, heated at 100 °C for 5 min, cooled, and diluted to 1.5 mL with distilled water. Absorbance was measured at 540 nm. Two types of controls were used: a reaction blank (without enzyme) and an enzyme blank (without substrate).

Endoglucanase Activity (CMCase)

Endoglucanase (CMCase) activity was quantified according to Ghose (1987), with adaptations for microvolumes (1-log reduction). The reaction contained 50 µL of enzyme extract in citrate buffer (pH 4.8) and 50 µL of 2% (w/v) CMC. The mixture was incubated at 50 °C for 30 min.

After incubation, 300 µL of DNS reagent was added and the mixture was reheated at 100 °C for 5 min. Upon cooling, the volume was adjusted with distilled water and absorbance was read at 540 nm. Reaction blanks (buffer + CMC) and enzyme blanks (enzyme + buffer, no substrate) were included. Enzymatic activity was calculated based on the amount of enzyme required to release 0.5 mg of glucose.

β-Glucosidase Activity

β-glucosidase activity was quantified according to Ghose (1987), using 50 µL of cellobiose and 50 µL of enzyme extract diluted in citrate buffer (pH 4.8). The reaction was incubated at 50 °C for 30 min. Released glucose was measured

using a glucose oxidase (GOD) enzymatic kit following the manufacturer's instructions, with absorbance read at 500 nm. Activity was calculated based on the critical dilution required to release 1 mg of glucose.

2.4 Molecular Analysis

Rhizobacteria showing the highest EI values were reactivated in 50 mL of liquid YMA medium at 28 °C with agitation (125 rpm) for 24 h. Genomic DNA was extracted using the Qiamp DNA Kit (250) (Qiagen, Germany) following the manufacturer's protocol and Chapaval et al. (2008). Samples were identified and authenticated at the molecular level by amplification of the 16S rRNA gene using primers 530F (5′-TGACTGACTGAGTGCCAGCMGCCGCGG-3′) and 1492R (5′-TGACTGACTGAGAGCTCTACCTTGTTACGMYTT-3′) as recommended by Borneman & Triplett (1997).

PCR products (4 µL + 1 µL loading buffer) were subjected to electrophoresis at 100 V for 60 min at room temperature in 2% agarose gel containing 2 µL GelRed™ (Biotium) in 1× TAE buffer. DNA fragments were visualized using an iBright™ Imaging System. Sequencing was performed on an ABI 3500 Genetic Analyzer with eight capillaries.

2.5 Taxonomy

Both DNA strands from each sample were analyzed using Geneious Trial Prime® 9.0.5 (Kearse et al., 2012). The identification of cellulolytic rhizobacteria was carried out by aligning consensus sequences against genome data deposited in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI).

3. Results

A total of 50 rhizobacterial isolates grew on YMA medium and were evaluated for their ability to produce cellulose-degrading enzymes using an enzymatic assay with CMC. The formation of degradation halos was observed in 31 strains (62%). On CMC medium, the isolates produced hydrolysis halos with enzymatic index (EI) values ranging from 1.0 to 5.58. Among these, 22 rhizobacteria were considered potential cellulase producers, as their enzymatic index (EI) values were ≥ 2.0 (Table 1).

Table 1 - Cellulolytic rhizobacteria isolated from *Pueraria phaseoloides* and *Inga edulis* in the central region of the Brazilian Amazon. *EI = enzymatic index (mean ± SD, n = 3).

Colleted	Host plants	Rhizobacterial isolate	Gram reaction	EI*
Ramal do Caldeirão km3 S 30 13' 41,9" W 600 13' 27,8"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R583	-	5.57 ± 0.62
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R779	-	4.69 ± 0.27
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R774	-	4.14 ± 0.62
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R762	-	3.94 ± 0.44
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R741	-	3.84 ± 0.58
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R746	-	3.56 ± 0.14
	Inga edulis Mart.	INPA R233	+	3.38 ± 0.08
Ramal do Caldeirão km3 S 30 13' 41,9" W 600 13' 27,8"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R571	-	3.34 ± 0.11

Manacapuru; Lago do Paru S 3°15'24,7" W 60°32'3,4"	*Inga edulis* Mart.	INPA R1001	-	3.29 ± 0.11
amal do Caldeirão km3 S 30 12' 26,9" W 600 12' 2,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R555	-	3.21 ± 0.24
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R735	-	3.16 ± 1.04
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R757	-	2.99 ± 0.36
Rio Preto da Eva	*Inga edulis* Mart.	INPA R296	+	2.94 ± 0.08
Brasileirinho	*Inga edulis* Mart.	INPA R178	-	2.94 ± 0.31
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R764	-	2.89 ± 0.13
Ramal do Caldeirão km3 S 30 13' 41,9" W 600 13' 27,8"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R565	-	2.82 ± 0.46
Brasileirinho	*Inga edulis* Mart.	INPA R236	-	2.71 ± 0.22
Manacapuru; Lago do Paru S 3°15'24,7" W 60°32'3,4"	*Inga edulis* Mart.	INPA R729	-	2.64 ± 0.26
Balbina; Km 42 Sítio Marisa S 2°3'5,2"W 59°40'11,1"	*Inga edulis* Mart.	INPA R792	-	2.56 ± 0.91
Ramal do Caldeirão km3 S 30 12' 26,9" W 600 12' 2,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R552	-	2.46 ± 0.56
Rio Preto da Eva	*Inga edulis* Mart.	INPA R266	-	2.07 ± 0.32
Rio Preto da Eva	*Inga edulis* Mart.	INPA R276	-	2.02 ± 0.15
Manacapuru; Lago do Paru S 3°15'24,7" W 60°32'3,4"	*Inga edulis* Mart.	INPA R722	-	1.90 ± 0.10
Rio Preto da Eva S 2°44'15,9" W 59°46'50,1"	*Inga edulis* Mart.	INPA R261	-	1.71 ± 0.01
Rio Preto da Eva S 2°44'15,9" W 59°46'50,1"	*Inga edulis* Mart.	INPA R274	-	1.53 ± 0.21
Brasileirinho	*Inga edulis* Mart.	INPA R046	-	1.36 ± 0.11
Brasileirinho	*Inga edulis* Mart.	INPA R034	-	1.31 ± 0.03
Manacapuru; Estrada do Acajatuba S 3°13'36,1" W 60°34'20,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R699	-	1.11 ± 0.04
Ramal do Caldeirão km3 S 30 12' 26,9" W 600 12' 2,5"	*Pueraria phaseoloides* (Roxb.) Benth.	INPA R547	-	1 ± 0
Manacapuru; Lago do Paru S 3°15'24,7" W 60°32'3,4"	*Inga edulis* Mart.	INPA R721	-	1 ± 0.57
Rio Preto da Eva S 2°44'15,9" W 59°46'50,1"	*Inga edulis* Mart.	INPA R293	-	1 ± 0

Source: Adapted from Cáuper (2018), Costa (2017), Oliveira et al. (2022), and Vaz (2013).

Based on the Lilliefors normality test, it was verified that the data distribution exhibited a normal pattern when comparing plant species and isolated bacteria, since the p-value was not significant (ns). Consistently, the Shapiro–Wilk test also indicated a normal distribution of data, as p-values were greater than 0.05 specifically above 0.3 for both enzymatic indices analyzed. A statistically significant difference was observed between enzymatic indices ($t = 3.05$; $df = 29$; $p = 0.0048$), confirming the alternative hypothesis that there is a significant difference in enzymatic production among bacterial isolates from different host plants. Therefore, bacteria isolated from *Pueraria phaseoloides* exhibited, on average, a higher ability to produce cellulolytic enzymes compared to those isolated from *Inga edulis*. These results suggest that the host plant may exert a direct influence on the functional profile of associated microbial communities. Studies on *Pueraria phaseoloides* indicate that this leguminous species hosts a diversity of nitrogen-fixing bacteria, highlighting its potential to enrich soil with biologically

available nitrogen (Wedage, Aberathne, Harischandra & Gunawardana, 2019). The p-value used in this inferential test was two-tailed, as the result could indicate three possible outcomes: (i) *Pueraria phaseoloides* isolates being more efficient, (ii) *Inga edulis* isolates being more efficient, or (iii) no significant difference between groups. At a 95% confidence level, the mean difference in EI between the two groups was –1.1025, with a confidence interval (CI) ranging from –1.8415 to –0.3634.

Molecular identification was performed exclusively on isolates that exhibited the highest enzymatic index (≥ 2.0) after growth in a synthetic medium containing carboxymethylcellulose, since the focus of the present study was the selection of strains with high potential for cellulolytic enzyme production rather than the assessment of bacterial diversity associated with the host plant. Consequently, only a limited number of isolates were subjected to molecular analysis, resulting in the identification of strains belonging to the genus *Bacillus* (Table 2). The isolates INPA R-583, INPA R-779, and INPA R-774 were identified as *Bacillus subtilis*, with similarity percentages ranging from 86.91% to 91.39%, while the isolate INPA R-762 was identified as *Bacillus velezensis*, showing 89.55% similarity when compared with sequences deposited in the NCBI and UNITE databases. All isolates were obtained from *Pueraria phaseoloides* and exhibited high enzymatic indices, with isolate INPA R-583 standing out by presenting the highest mean enzymatic index. The isolate INPA R-583, identified as *Bacillus subtilis*, was subsequently used for the determination of cellulolytic enzymatic activity.

Table 2 - Molecular identification of the isolates that had an enzymatic index ≥ 2.0 after growing on a synthetic carboxymethylcellulose medium. E.I. = enzymatic index (mean index ± SD, n = 3); (*) = percentage of similarity between our sequences and those available in the NCBI and UNITE databases.

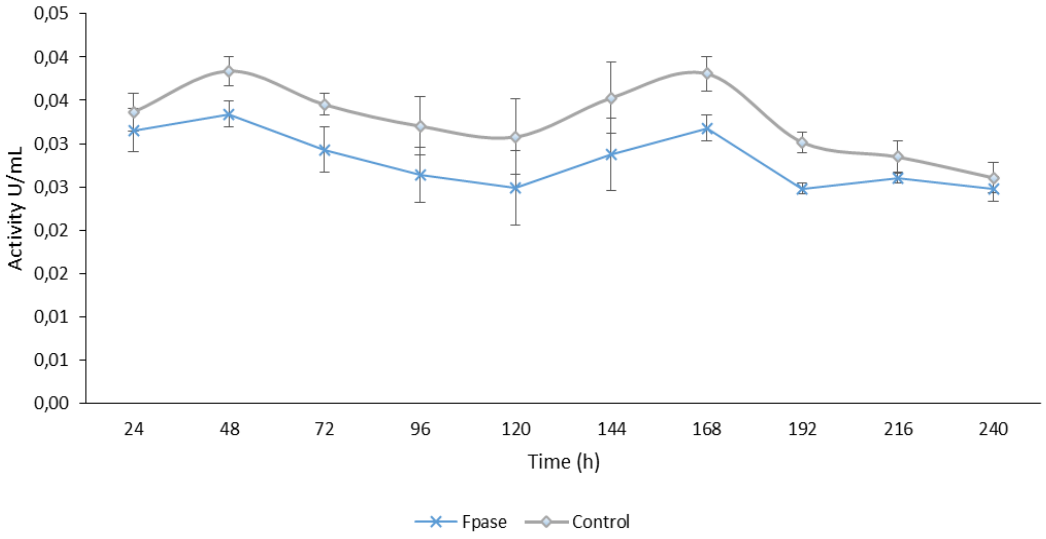
Isolates	Host plant	Genus/Species	Enzymatic Index (EI ± SD)	*Identity (**)	Accession number
INPA R-583	*Pueraria phaseoloides*	*Bacillus subtilis*	**5.57 ± 0.62**	91.39%	OP115602.1
INPA R-779	*Pueraria phaseoloides*	*Bacillus subtilis*	**4.69 ± 0.27**	86.91%	MN220534.1
INPA R-774	*Pueraria phaseoloides*	*Bacillus subtilis*	**4.14 ± 0.62**	90.39%	MG004175.1
INPA R-762:	*Pueraria phaseoloides*	*Bacillus velezensis*	**3.94 ± 0.44**	89.55%	CP021011.1

Source: Authors.

Determination of Cellulolytic Enzymatic Activity

The production of cellulolytic enzymes by the bacterium INPA R-583, identified as *Bacillus subtilis*, was monitored over 240 h of cultivation under submerged fermentation conditions. FPase (total cellulolytic activity), CMCase (endoglucanase), and β-glucosidase activities were evaluated and expressed as enzymatic activity units per milliliter (U/mL). FPase activity remained relatively low throughout the experimental period, with values ranging from approximately 0.02 to 0.035 U/mL. The highest activity was observed at 48 h, followed by slight fluctuations, remaining nearly constant up to 240 h (Figure 1). This low activity suggests limited synergistic action among the enzymatic complexes produced by the strain, which may result in reduced efficiency in the complete degradation of crystalline cellulose.

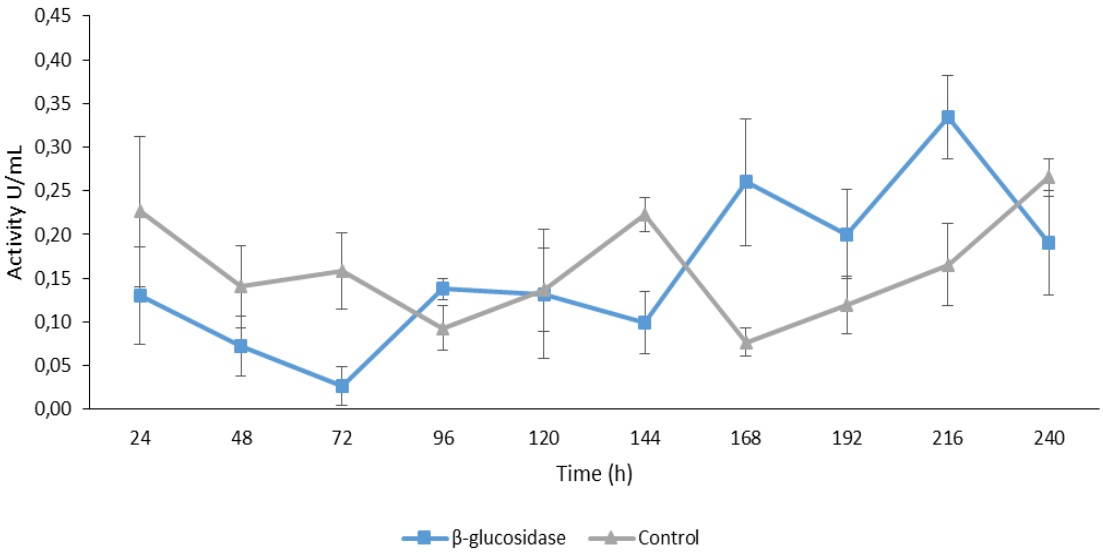
Figure 1 - Kinetic profile of FPase production in the culture supernatant of *Bacillus subtilis* (INPA R-583). Values represent the mean ± standard deviation (n = 3).



Source: Authors.

β-Glucosidase activity exhibited a distinct profile, with high levels observed during the early and late phases of cultivation. The first peak occurred at 24 h (≈0.13 U/mL), followed by a decrease at intermediate time points (48–144 h). From 168 h onward (~0.26 U/mL), a marked increase in activity was observed, reaching a maximum at 216 h (~0.33 U/mL), although with a high standard deviation, indicating variability among replicates or instability in enzyme production (Figure 2). This late-stage trend may indicate differential regulation of β-glucosidase expression, possibly associated with the availability of oligosaccharides derived from the action of other cellulases.

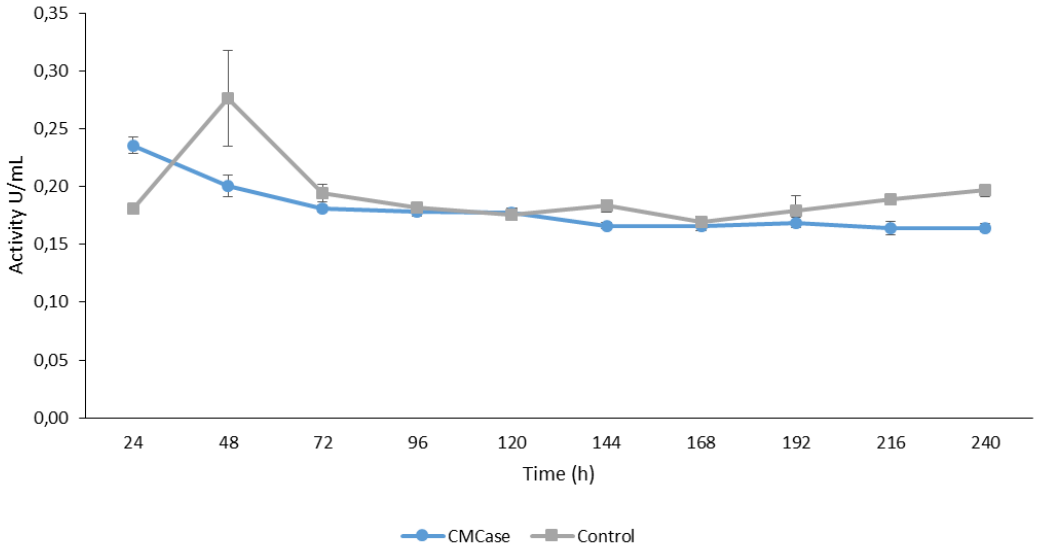
Figure 2 - Kinetic profile of β-glucosidase production in the culture supernatant of *Bacillus subtilis* (INPA R-583). Values represent the mean ± standard deviation (n = 3).



Source: Authors.

In contrast, CMCase activity showed the most consistent and highest profile throughout the experimental period. The maximum peak was recorded within the first 24 h (~0.24 U/mL), followed by a slight reduction at subsequent time points. Nevertheless, the activity remained relatively stable, ranging from 0.17 to 0.20 U/mL up to 240 h, with low standard deviations, demonstrating good enzymatic stability and process reproducibility (Figure 3).

Figure 3 - Kinetic profile of CMCase production in the culture supernatant of *Bacillus subtilis* (INPA R-583). Values represent the mean ± standard deviation (n = 3).



Source: Authors.

This result suggests that the *Bacillus subtilis* strain (INPA R-583) has a greater capacity for endoglucanase production, enzymes responsible for cleaving internal bonds in amorphous cellulose. Taken together, the data indicate that the evaluated bacterial strain exhibits higher potential for CMCase and β-glucosidase production, rather than for total cellulase activity (FPase), which may limit the complete conversion of cellulose into glucose. Nevertheless, the consistent production of endoglucanase and the increasing β-glucosidase activity over time indicate that, with adjustments in cultivation conditions or the use of microbial consortia, this strain may be promising for biotechnological applications aimed at the enzymatic hydrolysis of lignocellulosic biomass.

4. Discussion

The prospection of rhizobacteria associated with the roots of *Pueraria phaseoloides* and *Inga edulis* revealed the potential of these Amazonian plants as important reservoirs of cellulolytic microorganisms, reinforcing the role of the rhizosphere as a highly selective microenvironment for mechanisms involved in the degradation of structural components of the plant cell wall. Different plant species exert distinct ecological pressures on their microbial communities, modulated by factors such as exudate composition, carbon availability, root architecture, and symbiotic interactions (Badri & Vivanco, 2009; Bais et al., 2006; Mendes et al., 2013). Thus, it is not surprising that *Pueraria phaseoloides* supported a microbiota with a higher prevalence of cellulase-producing bacteria, possibly due to the chemical profile of its exudates and the typical physiology of legumes, which frequently stimulate microbial consortia specialized in the transformation of structural polymers (Dakora & Phillips, 2002; Philippot et al., 2013). The literature shows that plants with greater inputs of cellulosic and

hemicellulosic material tend to harbor microorganisms adapted to the utilization of these substrates, favoring bacteria that express glycoside hydrolases involved in the attack of amorphous and crystalline cellulose (Watanabe & Tokuda, 2010; Dai et al., 2020).

Soils of the Amazon region harbor highly complex and functionally diverse microbial communities, partly due to pronounced environmental heterogeneity and intense interactions among plants, microorganisms, and decomposers (Jesus et al., 2009; Navarrete et al., 2015). Several studies point to the Amazon as one of the largest sources of plant growth–promoting microorganisms and strains with unique enzymatic repertoires, resulting from selection in environments rich in organic matter and subject to variations in moisture, acidity, and nutrient availability (Rodrigues et al., 2024; Lopes et al., 2025). In this context, the identification of bacteria producing endoglucanases, β-glucosidases, and other enzymes capable of modulating carbon cycling reinforces the biotechnological potential of these communities, including applications in waste bioconversion, agricultural formulations, and enzymatic pretreatment systems (Kuhad et al., 2011; Lynd et al., 2002).

The differences observed among the enzymatic activities evaluated are also consistent with a coherent biological framework. The more consistent production of endoglucanases suggests that the analyzed strain possesses an efficient apparatus to initiate cellulose cleavage, particularly of the amorphous fraction, an essential step in the natural depolymerization process of this polysaccharide (Zhang et al., 2024; Horn et al., 2012). In many bacteria of the genus *Bacillus*, endoglucanase acts as the primary metabolic response to the presence of cellulosic polymers and is regulated by induction mechanisms dependent on fragments released during the initial degradation of the plant cell wall (Koeck et al., 2014; Schallmey et al., 2004). In contrast, the later and more variable expression of β-glucosidase is often interpreted as a consequence of the dynamics of oligosaccharide accumulation and catabolite repression exerted by glucose, which are well-known modulators of cellulose degradation pathways. This behavior suggests a functional enzymatic system subject to fine regulation, a feature widely described for natural strains of *Bacillus subtilis* and related organisms (Ega et al., 2020; Liu et al., 2023).

The lower overall efficiency of the strain's cellulolytic system, particularly in steps dependent on the degradation of crystalline cellulose, may reflect an incomplete genetic repertoire, a common characteristic of many bacteria isolated from natural environments (Koeck et al., 2014; Wilson, 2011). While filamentous fungi typically exhibit robust multienzyme systems, many rhizospheric bacteria display a more specialized profile, acting mainly in the early stages of depolymerization or in the final conversion of oligomers. This specialization contributes to the cooperative functioning typical of rhizosphere microbial communities (Berendsen et al., 2012; Cragg et al., 2015). Therefore, the ecological role of these bacteria may be more closely related to the initial fragmentation of cellulose and the release of simple sugars for other members of the microbial consortium, highlighting the importance of studies that consider microbial interactions rather than the isolated action of individual strains.

From a biotechnological perspective, these patterns indicate that strain INPA R-583 has relevant potential for processes that rely on stable endoglucanases, allowing its application in the initial pre-hydrolysis stage or as a component of enzyme mixtures formulated for lignocellulosic biomass treatment. The differential regulation of β-glucosidase and the low overall cellulase activity suggest that the strain's performance could be significantly enhanced in synergistic contexts, such as microbial consortia or systems supplemented with complementary enzymes. Furthermore, genomic analyses and physiological optimization studies may elucidate the regulatory architecture of the cellulolytic system and reveal strategies to maximize the expression of enzymes of interest (Lynd et al., 2008; Schallmey, Singh, & Ward, 2004).

The rhizosphere of *Pueraria phaseoloides* and *Inga edulis* represents a promising environment for the prospection of bacteria with cellulolytic attributes, and the results obtained reinforce the role of the Amazon as one of the main natural reservoirs of functional microbial diversity. Understanding the ecological and physiological bases that modulate the production

of these enzymes is essential both for advancing knowledge of plant–microorganism interactions and for the development of sustainable applications based on microbial resources from the region.

5. Conclusion

The rhizobacteria isolated from *Pueraria phaseoloides* and *Inga edulis* exhibited a strong capacity for cellulolytic enzyme production, with particular emphasis on the rhizobacteria associated with *Pueraria phaseoloides*, which showed significantly higher enzymatic indices. These microorganisms can be considered promising sources for biotechnological applications, with potential uses in cellulose degradation, biofuel production, and the generation of bioproducts.

This study contributes significantly to the understanding of Amazonian microbial biodiversity by highlighting the potential of rhizobacteria as biotechnological agents for the degradation of lignocellulosic residues. Moreover, it opens new perspectives for the exploration of native and poorly studied microorganisms, such as those associated with *Pueraria phaseoloides* and *Inga edulis*, which may play a crucial role in improving sustainability and driving innovation in industrial processes. Future studies should focus on optimizing cultivation conditions and characterizing the enzymes produced, as well as investigating the performance of these strains in large-scale industrial processes.

The results further indicate that the *Bacillus subtilis* strain INPA R-583 exhibits a favorable enzymatic production profile for endoglucanase and β-glucosidase, but limited total cellulase (FPase) activity, suggesting an incomplete or suboptimal system for the complete degradation of crystalline cellulose. The consistency of CMCase activity and the late activation of β-glucosidase represent positive attributes that support its biotechnological potential, particularly when combined with optimization strategies, genetic engineering, or co-cultivation approaches. Future studies aimed at completing or complementing the enzymatic system, as well as evaluating saccharification of real biomass substrates, will be essential to validate this strain as a candidate for lignocellulosic biomass hydrolysis processes.

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