Bioprospecting Bacteria with Cellulolytic Potential in Cocoa Husks

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This study aimed to identify bacteria with cellulolytic degradation potential and evaluate their ability to degrade cellulose. Samples of cocoa fruit were collected, treated, and inoculated into a carboxymethylcellulose (CMC) medium, with cellulose as the sole carbon source. After microbial growth, the bacteria were isolated, morphologically characterized, and assessed for their cellulolytic activity. A total of 42 colonies were observed on the initial four plates. The results were promising, with the largest degradation halo measuring approximately 46 mm and an enzymatic index of 3.4. These findings highlight the biotechnological relevance of this research and its contribution to ongoing studies in the field.

Keywords: Cellulolytic Microorganisms. Cellulose. Bioprospecting.

Cellulose is widely used across several industries, including the paper, chemical, textile, food, and pharmaceutical sectors. In Brazil, the pulp and paper industry employs over 175,000 individuals across approximately 4,000 companies, with 32.5% of its production destined for export [1]. As the primary structural component of plant cell walls, cellulose provides rigidity and strength, making it a challenging compound to hydrolyze. However, certain bacteria and fungi produce enzymes capable of degrading cellulose, facilitating its conversion into fermentable sugars [2].

These microorganisms can be found in plant-based environments, such as cocoa residues. Brazil is the seventh largest cocoa producer globally, with an annual output of approximately 265,000 tons. This production generates a substantial amount of plant waste, primarily from cocoa husks, which account for about 80% of the total biomass. When improperly discarded, these residues can pose significant environmental risks [3].

Utilizing cocoa waste offers both economic and environmental benefits. The application of cellulolytic microorganisms has gained attention over chemical catalysts due to advantages such as operational safety, shorter processing time, lower energy consumption, economic feasibility, non-toxicity, and environmental sustainability [4]. Therefore, the main objective of this study was to prospect bacteria with cellulolytic potential in cocoa husks, assess the production of bioactive compounds capable of degrading cellulose, and determine the microbial load of bacteria found in the plant material. Additionally, the study aimed to evaluate the cellulose degradation potential of selected microorganisms with a view toward their industrial applicability and contribution to the development of biotechnological products.

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Materials and Methods

Treatment of the Plant Source

Cocoa (*Theobroma cacao*) was selected as the plant source for this study. Aseptic treatment was performed to reduce the microbiota in the cocoa husks. The samples were divided and subjected to two types of surface sterilization: the first was treated with 70% ethanol, and the second was treated sequentially with sodium hypochlorite and 70% ethanol, each for 60 seconds.

Preparation of the Culture Medium

The culture medium was prepared with the

following composition: 1 g of $(NH_4)_2SO_4$, 1 g of K_2HPO_4, 5 g of $MgSO_4$, 1 mg of NaCl, 15 g of agar, and 10 g of carboxymethylcellulose (used as the sole carbon source) per liter of distilled water [5].

Cultivation and Isolation of Colonies

For microbial cultivation, 1 g of the treated plant material was added to 9 mL of sterile saline solution (0.85% NaCl), shaken for 1 minute, and left to rest at room temperature for 1 hour. Afterward, 0.1 mL aliquots of the suspension were spread on CMC agar plates [6]. Microbial growth was assessed after 24–48 hours. Colonies showing clear zones (indicative of cellulolytic activity) were transferred to new CMC agar plates for enzymatic activity confirmation and colony purification [7].

For isolation, colonies were picked with a platinum loop and serially diluted in microtubes containing 0.9 mL of sterile saline until no turbidity was observed. The diluted samples were inoculated on CMC agar plates and incubated for 24–48 hours. The streaking method was used to reduce microbial density and facilitate the isolation of pure colonies through two sequential streaks on solid media [8]. Pure cultures were subsequently analyzed by Gram staining.

Wet Mount Slide

Wet mount slide observation was performed to differentiate the isolates as bacteria, fungi, or yeasts. Morphological features such as cell size and shape were used as parameters to aid in microbial identification [9].

Gram Staining

The Gram staining technique was employed to analyze bacterial morphology. Microorganisms staining pink or red were classified as Gram-negative, while purple or blue were classified as Gram-positive [10].

Analysis of Cellulolytic Activity

The cellulolytic activity was quantified using the Enzymatic Index (EI), which is calculated as the ratio between the diameter of the degradation halo and the diameter of the bacterial colony. This index was used to select strains with the highest potential for extracellular cellulase production [11].

The presence of cellulolytic activity was confirmed by the formation of orange zones around the colonies following the application of an iodine solution (prepared with 2 g of potassium iodide, 1 g of iodine in 300 mL of distilled water) poured onto the plate surface, and allowed to react for 2 hours, according to Florêncio and colleagues (2012) [12]. The diameter of the halos was measured using a caliper.

Results and Discussion

Isolation of Bacteria

This section discusses the entire methodological process of the present study, which employed a qualitative-quantitative, experimental approach. Through laboratory analyses, the study aimed to prospect for bacteria capable of producing cellulase and to assess their cellulolytic potential based on cellulose degradation in carboxymethylcellulose (CMC) medium, thereby enabling the qualification of the studied microorganisms.

The initial isolation of bacteria from cocoa husks was carried out on four culture plates, categorized according to the aseptic treatment applied to the fruit. The plates treated solely with 70% ethanol yielded 21 colonies, while the plates subjected to both ethanol and sodium hypochlorite treatments produced 22 viable colonies. These colonies were identified and documented in Table 1, with further characterization presented in Tables 2 and 3 to facilitate the tracking and analysis of each microorganism throughout the study.

Table 1. Total number of colonies isolated from cocoa husk samples according to aseptic treatment method.

Aseptic Tratment	Plate Number	Colony Number
Alcohol 70%	A1 or A2	A1-7
Sodium Hypochlorite	H1 or H2	H2-7

Table 2. Characterization of colonies in cocoa husks treated with 70% alcohol.

Colony	Form	Aspect	Colony	Form	Aspect
A1-1	Regular	Opaque	A2-1	Rhizoid	Opaque
A1-2	Irregular	Translucent	A2-2	Irregular	Opaque
A1-3	Regular	Opaque	A2-3	Regular	Opaque
A1-4	Circular	Opaque	A2-4	Regular	Translucent
A1-5	Circular	Opaque	A2-5	Regular	Opaque
A1-6	Irregular	Opaque	A2-6	Regular	Opaque
A1-7	Regular	Opaque	A2-7	Regular	Opaque
A1-8	Regular	Translucent	A2-8	Irregular	Opaque
A1-9	Regular	Opaque	A2-9	Irregular	Opaque
A1-10	Circular	Translucent	A2-10	Regular	Opaque
A1-11	Irregular	Opaque			

Table 3. Characterization of colonies present in cocoa husks treated with hypochlorite and 70% alcohol.

Colony	Form	Aspect	Colony	Form	Aspect
H1-1	Regular	Translulcent	H2-2	Regular	Translulcent
H1-2	Regular	Opaque	H2-3	Irregular	Translucent
H1-3	Regular	Translucent	H2-4	Filamentous	Translucent
H1-4	lrregular	Translucent	H2-5	Irregular	Translucent
H1-5	Irregular	Translucent	H2-6	Filamentous	Translucent
H1-6	Irregular	Opaque	H2-7	lrregular	Opaque
H1-7	Irregular	Opaque	H2-8	Regular	Translucent
H1-8	Irregular	Translucent	H2-9	Regular	Translucent
H1-9	Filamentous	Translucent	H2-10	Regular	Translucent
H1-10	Filamentous	Translucent	H2-11	Regular	Translucent
H2-1	Irregular	Opaque	H2-12	Irregular	Translucent

Plates treated only with 70% ethanol exhibited a predominance of opaque colonies (80.95%), whereas plates treated with sodium hypochlorite and ethanol showed a higher frequency of translucent colonies (77.27%). This discrepancy may be associated with the effectiveness of the aseptic treatments: the ethanol-only procedure appears less effective, resulting in a greater microbial load, which may lead to more visibly opaque colonies due to denser bacterial growth.

Gram Staining

Gram staining was performed to analyze the morphology of the isolated microorganisms. This method allows differentiation based on the structural and chemical properties of the cell wall, classifying the bacteria as either Gram-positive or Gram-negative. The results of this analysis are summarized in Table 4 and visually represented in Figure 1.

Wet Mount Slide

Wet mount slide microscopy was conducted to differentiate between bacteria, fungi, and yeasts (Figure 2). The analysis confirmed the predominance of bacterial structures, characterized by their prokaryotic morphology (absence of a defined nucleus). This distinguishes them from yeasts—which typically exhibit budding—and from filamentous fungi, which present hyphal structures.

Analysis of Cellulolytic Activity

For the degradation test in a solid medium, the colony diameter and the diameter of the CMC degradation halo were measured. The Enzymatic Index (EI) estimated the extracellular enzyme production, calculated as the ratio between the degradation halo diameter and the colony diameter (Table 5).

The isolated bacteria's EI values ranged from 1.07 to 3.4. In a similar study by Behera and colleagues (2014) [13], bacteria incubated aerobically at 37ºC showed EI values ranging from 1.18 to 2.5 under comparable conditions.

The presence of a degradation halo indicates the ability of the microorganism to produce cellulase, as it reflects the breakdown of carboxymethylcellulose. Among the isolates, A1-2 exhibited the largest

Figure 1. Plate A1-3 slide analyzed under the microscope for determination of gram stain, predominantly Gram-negative monococcus.

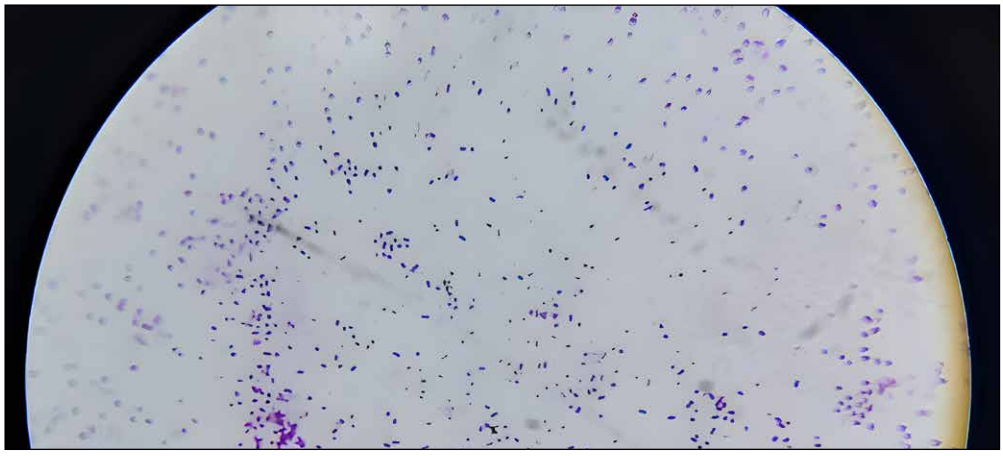


Table 4. Gram tests and morphological characterization of isolated colonies.

Colony	Morphology	Gram	Colony	Morphology	Gram
A1-1	Bacillus	+	A2-2	Monococcus	+
A1-2	Bacillus	+	A2-3	Monococcus	+
A1-3	Monococcus	+	A2-4	Bacillus	+
A1-4	Monococcus	-	H1-1	Monococcus	+
A1-7	Streptococcus	+	H1-3	Monococcus	-
A1-8	Diplococcus	-	H2-2	Streptococcus	+
A1-9	Bacillus	-	H2-5	Diplococcus	+
A1-10	Bacillus	+	H2-6	Bacillus	-
A2-1	Streptococcus	+	H2-7	Monococcus	-

Figure 2. Wet slide: microorganisms prospected on a coverslip being analyzed through microscopy.

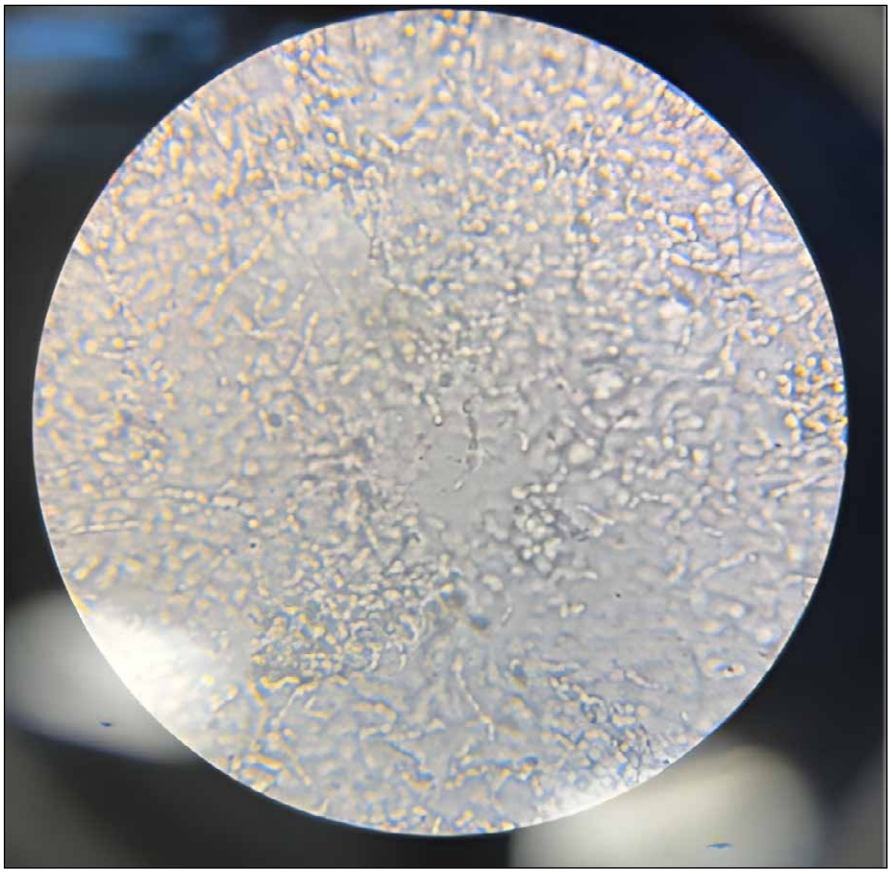
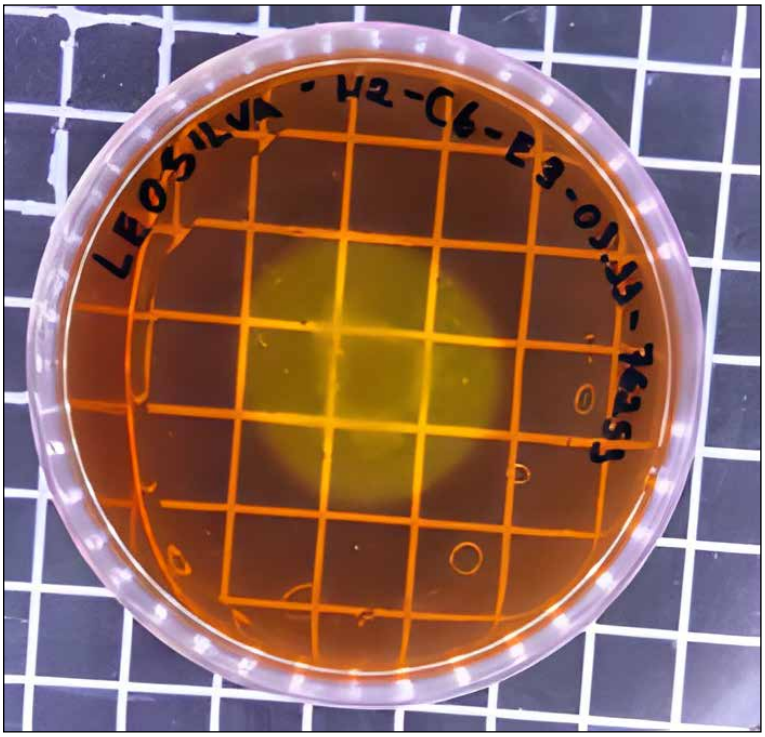


Table 5. Cellulolytic activity of microorganisms isolated from cocoa husks.

Colony	Colony Diameter (mm)	Halo Diameter (mm)	Enzyme Index
A1-1	21	46	2.19
A1-2	27	29	1.07
A1-3	20	44	2.2
A1-5	10	34	3.4
A1-7	22	43	1.95
A1-9	16	32	2
A1-10	26	34	1.3
A2-1	15	32	2.13
A2-4	23	40	1.73
H1-3	17	31	1.82
H2-3	22	36	1.63
H2-5	16	42	2.62
H2-6	22	39	1.77
H2-7	20	34	1.7

Figure 3. Plate H2-6 halo degradation test with the aid of iodin.



colony diameter at 27 mm, followed by A1-10 with 26 mm. In terms of halo size, isolate A1-1 displayed the largest halo at 46 mm, followed by A1-3 at 44 mm (Figure 3).

The highest enzymatic index was observed in colony A1-5 (EI = 3.4), followed by H2-5 (EI = 2.62). Isolates A1-1 (EI = 2.19), A1-3 (EI = 2.2), A2-1 (EI = 2.13), and A1-9 (EI = 2.0) also met the recommended threshold of EI ≥ 2.0, as proposed by Lealem & Gashe (1994) [14], to indicate strong cellulolytic activity. These results suggest that the studied microorganisms possess a high capacity for cellulose degradation, with most colonies producing degradation halos greater than 30 mm.

Conclusion

Based on the results presented in this study, bioprospecting is a promising approach for advancing biotechnology and industrial applications. The successful isolation of cellulolytic bacteria from a natural source—cocoa husks—demonstrated significant degradation potential. Isolate A1-1 achieved the largest halo diameter (46 mm), A1-2 exhibited the largest colony diameter (27 mm), and isolate A1-5 presented the highest enzymatic index (3.4). These findings reinforce the potential use of these microorganisms in developing biotechnological processes focused on cellulose degradation.

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