Bioprospection of Enzyme-Producing Bacteria Applicable to the Hydrolysis of Cacao Biomass (*Theobroma cacao*)

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This article examines the potential of cocoa agro-industrial waste for biofuel production, aiming to provide a sustainable alternative to fossil fuels and promote waste reuse. It explores the bioprospecting of cellulase-producing microorganisms in cocoa-growing areas, focusing on the microbial diversity of Brazilian soil and its role in lignocellulose degradation. The method involved the induction of cellulolytic activity in a medium with CMC, isolation and characterization of the bacteria, production of an enzymatic extract, and quantification of reducing sugars via the DNSA method. The objective is to identify efficient cellulase-producing microorganisms to optimize the conversion of cocoa residues into biofuel, promoting a greener economy in the energy sector. A total of four bacterial strains (3A, 5, 6, and 8) were isolated, Gram staining was performed for each, and sugar quantification yielded the following enzymatic activity results: strain 3A – 0.42; 5 – 0.2; 6 – 0.24; and 8 – 0.27 U/mL.
Keywords: *Theobroma cacao*. Enzymes. Cellulase. Biofuel.

Brazil is a country whose economy is primarily based on agriculture, which consequently generates large amounts of waste annually. Among these, the residues from the cocoa agro-industry stand out, with 584 valid records of cocoa seedlings and seed producers integrated into the National Register of Seeds and Seedlings, with Bahia representing 13.4% of this total [1]. In 2018, this industry produced approximately 255.1 thousand tons of cocoa beans, which represent only 10% of the fruit's total mass, resulting in the generation of more than 2.5 million tons of waste [2].

Cocoa residues are lignocellulosic in nature, with lignocellulose being the main component of plant biomass. It is mainly composed of cellulose, hemicellulose, and lignin. The use of lignocellulosic materials as raw material for bioethanol production is a strategic tool for the sustainable production of fuels from renewable sources and an effective alternative for reusing organic solid waste [3].

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The project on bioprospecting enzyme-producing microorganisms aims to explore the microbial diversity present in soil, especially in cocoa cultivation areas. These bacteria have significant potential for expanding industrial applications and reducing costs, exhibiting higher growth rates than fungi, although their production potential remains underexplored [4]. Moreover, soil is a crucial biodiversity reservoir, possessing diverse functional characteristics of microbial groups [5]. The biodiversity of Brazilian soils is invaluable, benefiting the environment and enabling sustainability in various sectors. However, the degradation of this biome and desertification significantly reduce microbial diversity [6].

The present study aims to prospect for cellulase-producing microorganisms for application in sustainable biofuel production. This work aims to provide innovative solutions for the utilization of agro-industrial waste, contributing to the transition toward a greener and more sustainable energy sector.

Materials and Methods

The method employed was designed to ensure maximum reliability and precision in microbiological assays. A rigorous asepsis protocol was adopted, with the sterilization of all materials being a crucial step. Sterilization was performed

using autoclaving, a method recognized for its effectiveness in eliminating microorganisms, including bacteria, fungi, viruses, and bacterial spores. The experiments were conducted at the Microbiology and Bioprocess Laboratory "Moacyr Dunham de Moura Costa."

Cellulolytic activity was induced and monitored using carboxymethylcellulose (CMC) as a carbon source [7]. The culture medium was prepared following the protocol of Wood & Bhat (1988), with adaptations [8]. Plating was performed under laminar flow using Petri dishes that had been previously sterilized in an autoclave. Fifteen milliliters of CMC culture medium were distributed in each dish. The inoculation of cocoa samples into the CMC medium followed the method described by Alves and colleagues (2021), with adaptations [9].

Bacterial isolation involved selecting distinct colonies from primary plates and then transferring them into a saline solution to obtain a uniform suspension. Serial dilutions may be performed to reduce bacterial concentration. The suspension is then spread onto new plates, resulting in isolated colonies that can be characterized. The enzymatic extract was produced by fermenting the isolated bacterium in a liquid medium using an orbital shaker incubator for 24 hours at 27°C and 120 rpm.

The amount of reducing sugars present in the enzymatic extract was determined using 3,5-dinitrosalicylic acid (DNSA) reagent, following the DNS test protocol adapted from Miller (1959) [10]. Reducing sugars react with DNSA, reducing it to a colored compound. The intensity of the color is proportional to the amount of sugar present in the sample. The results were expressed in enzymatic activity units (U), defined as the amount of enzyme required to produce 1 µmol of glucose per mL per minute under assay conditions.

Results and Discussion

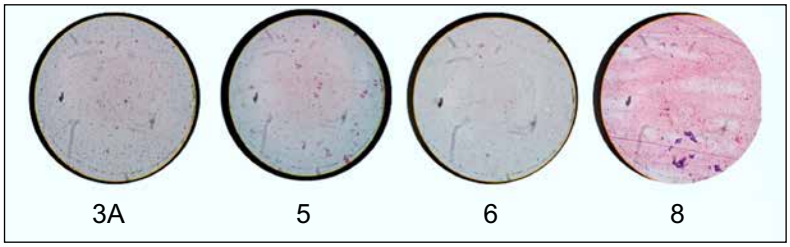
This study reports the isolation of four distinct bacterial strains, designated 3A, 5, 6, and 8, obtained from cocoa samples. Initial analysis, based on microscopic observation and Gram staining, revealed that all strains exhibit a coccoid morphology and are Gram-negative, as shown in Figure 1.

The determination of reducing sugars in the enzymatic extract yielded the results shown in Table 1. Strain 3A exhibited the highest level of free glucose in the reaction, comparable to results found in the literature, such as those reported by Malik [12], who found values between 0.2 and 0.5 U/mL under the same temperature and incubation time conditions prior to enzyme optimization. Enzymatic activity can be improved by increasing incubation time during the DNS test and avoiding freezing the enzymatic extract between fermentation and enzymatic assays. Furthermore, the cellulolytic potential can be enhanced by using multiple enzymes simultaneously.

Table 1. Results of cellulase enzymatic activity assay.

Sample	Result (U/mL)
3A	0.42
5	0.20
6	0.24
8	0.27

Figure 1. Gram-stained slides of the cellulolytic strains identified.



The analyses provide crucial information regarding the enzymatic characteristics of the isolated strains.

Thus, the findings underscore the biotechnological potential of the isolated bacterial strains, with particular emphasis on strain 3A, which exhibited the highest glucose release, suggesting noteworthy enzymatic activity. The consistent observation of Gram-negative cocci among all isolates raises the possibility of a correlation between morphological features and enzymatic performance, although further studies are required to validate this association. Comparative analysis with previous reports, such as those by Malik[12], supports the applicability of these strains in cellulose degradation processes, particularly when employing optimization strategies such as extended incubation times or the synergistic use of multiple enzymes. These preliminary results justify the continuation of this investigation, with a focus on enhancing enzymatic efficiency and assessing strain performance across various environmental and industrial conditions.

Conclusion

This project stands out due to its relevance in various aspects. It primarily addresses the urgent need to find sustainable alternatives to fossil fuels, exploring the untapped potential of agro-industrial waste as a renewable energy source. By focusing on the bioprospecting of cellulase-producing microorganisms, the study advances national biotechnology by seeking innovative and low-cost solutions for biofuel production. Additionally, it contributes to reducing the environmental impact of the cocoa agro-industry by transforming waste problems into value-generating opportunities and promoting sustainability. In summary, the project represents a significant step toward a greener economy, driving the development of clean technologies and the appreciation of Brazilian microbial biodiversity.

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References

1. MAPA - Ministério da Agricultura e Pecuária. Diagnóstico da produção de sementes e mudas de cacau no Brasil, 2023.
2. IBGE, 2019.
3. Pitarelo AP, Silva TA, Zamora PGP, Ramos LP. Efeito do teor de umidade sobre o pré-tratamento a vapor e a hidrólise enzimática do bagaço de cana-de-açúcar. Química Nova. 2012;35(8):1502–1509.
4. Sethi S, et al. Optimization of Cellulase Production from Bacteria Isolated from Soil. ISRN Biotechnol. 2013;2013:1–7.
5. Freitas JR, Mantovani W. An overview of the applicability of functional diversity in Biological Conservation. Braz J Biol. 2017;78(3):517–524.
6. Junior SLF. Isolamento e caracterização de bactérias celulolíticas de ambientes extremos [dissertação]. Universidade de Mogi das Cruzes; 2011.
7. Thiele S, Vendruscolo DCE, Fiorini A, Santos FS, Fernandes VB, Scheufele BF. Estabelecimento de protocolo para identificação de bactérias com potencial celulolítico. Rev Bras Energias Renováveis. 2019;8(2):494–506.
8. Wood TM, Bhat KM. Methods for measuring cellulase activities. Biomass Part A: Cellulose and Hemicellulose. 1988;87–112.
9. Alves BA, et al. Bioprospecção e caracterização de bactérias produtoras de celulase a partir do solo do Cerrado brasileiro. Res Soc Dev. 2021;10(8):e34010817426.
10. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31:426–428.
11. Yang W, et al. Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production. 2014.
12. Malik WA, Javed S. Enhancement of cellulase production by cellulolytic bacteria SB125 in submerged fermentation medium and biochemical characterization of the enzyme. Int J Biol Macromol. 2024;263(Pt 2):130415.