ASSESSMENT OF CELLULASE PRODUCTION CAPACITY IN *Bacillus* sp. AND *Pseudomonas fluorecenses* STRAINS FOR THE BIOFUEL INDUSTRY

Dinalva Schein[1], Isabela L. Valente[1] & Marcio A. Mazutti[2]

[1] *Department of Chemical Engineering/Postgraduate in Chemical Engineering, Federal University of Santa Maria, Santa Maria, Brazil*
[2]*Professor Doctor, Department of Chemical Engineering, Federal University of Santa Maria, Santa Maria-RS, Brazil.*
** Corresponding author's email address: scheindinalva@gmail.com*

ABSTRACT

This research investigates the cellulase production capacity of four strains of *Bacillus* sp. and one strain of *Pseudomonas fluorecenses*, aiming to indicate their production. The cellulolytic indices (CI) of *Bacillus velezensis* AP03, *Bacillus subtilis*, *Pseudomonas fluorecenses* ATCC 13525, *Bacillus amyloliquefaciens* BGB037, and *Bacillus velezensis* BCRC 17467 were evaluated, followed by the assessment of cellulase activity using filter paper (FPase). The highest CI values were 2.14 for *B. velezensis* AP03 and 2.01 *for B. amyloliquefaciens*. FPase activity was evaluated, resulting in 0.066 UI mL^{-1} for *B. amyloliquefaciens* and *B. velezensis* AP03, and 0.068 UI mL^{-1} for *B. velezensis* BCRC 17467, demonstrating that the strains exhibit cellulase production indications even after 96 hours of fermentation.

Keywords: Biofuel. Celulase. FPase. Cellulolytic index.

1 INTRODUCTION

Cellulases have become highly sought-after enzymes in the industry due to their various applications in the textile, paper, and cellulose industries, food and beverage industry, but have gained significant prominence in the bioethanol industry from lignocellulosic biomass through enzymatic conversion [1].

Biomasses are composed of cellulose, hemicellulose, and lignin, requiring pre-treatment and the breakdown of cellulose into fermentable sugars, which can be carried out through enzyme action. Cellulases are composed of endocellulases, exocellulases, and β-glucosidases and act synergistically to completely hydrolyze cellulose into glucose monomers. Although some challenges are still encountered in the industry, such as costs and the need for a large quantity of enzymes for biomass degradation, biorefineries have positively viewed the production of biofuels as a sustainable solution for the environment [2].

Improvement in enzyme production is crucial for cost-effectiveness and in the selection of producing strains [3]. Although filamentous fungi are most commonly used in cellulase production, especially Trichoderma sp.[4], Bacillus species, such as *Bacillus pseudomycoides* [5], *Bacillus velezensis* [6], *Bacillus aestuarii* UE25 [7], have been investigated in the production of extracellular enzymes. An indicative parameter of enzyme production with cellulose degradation capacity is the analysis of total cellulase activity on filter paper (FPase) [8]. Therefore, this study aims to investigate and evaluate the capacity of four strains of *Bacillus* and *Pseudomonas fluorecenses* in FPase production, for subsequent optimization of cellulase production and application in biofuel production.

2 MATERIAL & METHODS

Different microorganisms were tested for cellulase production indicators, with Bacillus *velezensis* AP03, *Bacillus subtilis*, *Pseudomonas fluorecenses* ATCC 13525, *Bacillus amyloliquefaciens* and *Bacillus velezensis* BCRC 17467.

A 9 mm diameter disc of each microorganism was inoculated onto a Petri dish containing medium (g L^{-1}) composed of $MgSO_4.7H_2O$ (0.2); KH_2PO_4 (0.4); K_2HPO_4 (0.2); NaCl (0.1); yeast extract (0.4); carboxymethylcellulose (10.0); and agar (15.0), and then incubated at 30°C for 96 hours. After the incubation period, 0.1% Congo red was added for 30 minutes, followed by washing with 1 mol L^{-1} NaCl until discoloration. The diameter of the halo formed was measured using a caliper [9].

$$CI = \frac{D}{d}$$

Where: CI – Cellulolytic index; D- Diameter of hydrolysis circle(cm); d- Diameter of colony (cm)

Inocula for fermentation were prepared in nutrient broth and incubated for 24 hours. The fermentation medium was prepared in 250 mL Erlenmeyer flasks containing 100 mL of medium. The fermentation medium comprised $MgSO_4.7H_2O$ (0.5); KH_2PO_4 (1.0); NaCl (0.5); $FeSO_4.7H_2O$ (0.01); $MnSO_4.H_2O$ (0.01); NH_4NO_3 (0.03), with the subsequent addition of 1% (w/v) CMC. Following sterilization, 1% inoculum was added, with an optical density of 1.0 at 600 nm.

Fpase activity was determined using 1.0 x 6.0 cm of Whatman no. 1 filter and was performed by quantifying sugars using the 3,5-dinitro salicylic acid reaction [10]. For this, filter paper was placed,1 mL of the enzymatic broth and 2 mL of sodium acetate buffer

were added to the test tube and reacted for 60 minutes at 50°C. After the reaction, a 1 mL aliquot was withdrawn and mixed with 1 mL of water and 3 mL of DNS, then heated at 100°C for 5 minutes. After reaching room temperature, readings were taken using a spectrophotometer UV-Vis (Shimadzu UV-2600) at 540 nm. To zero the analysis, the enzymatic broth was replaced with water [11].

The quantification of FPases was determined by the following equation:

$$\mathrm{UI} = \frac{D\ x\ C\ x\ Vt}{t\ x\ Ve}$$

Where: D - dilution; C - concentration (μmol mL^{-1}); Vt - the total volume of the reaction (mL); t - reaction time (min); Ve - the volume of the enzymatic solution (mL).

3 RESULTS & DISCUSSION

Observing the enzymatic indices observed for each of the evaluated bacteria, it was found that the analyzed strains are cellulase producers, with indices ranging from 2.14 in Bacillus *velezensis* AP03, 1.67 in *Bacillus subtilis*, 1.74 in *Pseudomonas fluorecenses* ATCC 13525, 2.01 in *Bacillus amyloliquefaciens* and 1.93 in *Bacillus velezensis* BCRC 17467. Figure 1 depicts the halos formed by the production of extracellular cellulases.

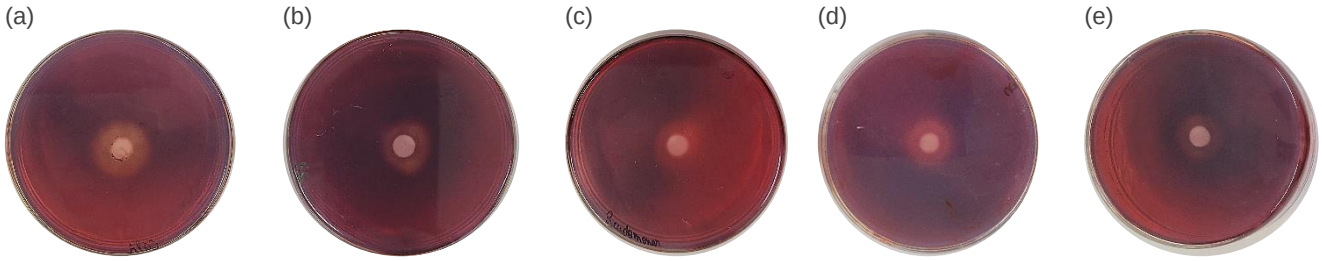

Figure 1 - Cellulolytic index from (a) Bacillus *velezensis* AP03; (b) Bacillus *subtilis* CCGB/LFB-1249; (c) Pseudomonas *fluorecenses* ATCC 13525; (d) Bacillus *amyloliquefaciens* BGB037 and (e) Bacillus *velezensis* BCRC 17467.

Studies indicate that this methodology is employed for the selection of cellulase-producing microorganisms. Saini and Yadav (2017) screened 371 isolates from different samples, where 124 isolates exhibited hydrolysis zones, with 84 of them showing indices ranging from 1.0 to 4.0. The halos formed around the colonies indicate the production of extracellular enzymes [12]. Balla *et al*. (2022) isolated 398 strains, of which 6.5% of the isolates showed indicators of CMCase activity with enzymatic indices between 0.34 and 5.2.

The strains with the best enzymatic indices, *B. velezensis* BCRC 17467, *B. velezensis* AP03, and *Bacillus amyloliquefaciens*, were selected for FPase activity evaluation. The values obtained for FPase production were 0.066 UI mL^{-1} for *B. amyloliquefaciens* and *B. velezensis* AP03, and 0.068 UI mL^{-1} for B. velezensis BCRC 17467. Microbial growth was observed after the incubation period, reaching 1.45 x 10^7 CFU mL^{-1} for *B. amyloliquefaciens*, 3.00 x 10^7 CFU mL^{-1} for *B. velezensis* AP03, and 3.75 x 10^7 CFU mL^{-1} for *B. velezensis* BCRC 17467.

Production of cellulases through the selected strains was observed; however, with low activities after 96 h of incubation. The low quantification of FPase activity can be attributed to the incubation time related to enzyme excretion, given that carboxymethyl cellulose (CMC) was used as the carbon source, inducing the production of cellulase enzymes. Zhang *et al.* (2018) observed that the use of CMC indicated a significant effect on cellulase production by the strain *B. velezensis* ZY-1-1, and also noted that cellulase activities were higher before the onset of the stationary phase, between 22-23 hours of incubation, reaching 3.59 U mL^{-1}, and then fluctuated in the stationary phase. Another study with *Bacillus* sp. pointed to a time of 20 h for maximum Fpase production, and when temperature (55°C) and pH (6.0) optimization were carried out, Fpase production increased to 15.4 U mL^{-1} with *B. subtilis* HUB-1-047. After this maximum production in 20 h, the enzyme concentration decreased [8].

4 CONCLUSION

This study indicates cellulase production, highlighting the need for process optimization to increase cellulase yield through the evaluation of various production variables such as temperature, pH, incubation time, and agitation.

REFERENCES

1 Singhania, R. R.; Ruiz, H. A.; Awasthi, M. K.; Dong, C. Di; Chen, C. W.; Patel, A. K. Challenges in Cellulase Bioprocess for Biofuel Applications. *Renewable and Sustainable Energy Reviews* **2021**, *151*. https://doi.org/10.1016/j.rser.2021.111622.

2 Singh, A.; Rodríguez Jasso, R. M.; Gonzalez-Gloria, K. D.; Rosales, M.; Belmares Cerda, R.; Aguilar, C. N.; Singhania, R. R.; Ruiz, H. A. The Enzyme Biorefinery Platform for Advanced Biofuels Production. *Bioresour Technol Rep* **2019**, *7*, 100257. https://doi.org/10.1016/j.biteb.2019.100257.
3 Saini, A.; Aggarwal, N. K.; Yadav, A. Isolation and Screening of Cellulose Hydrolyzing Bacteria from Different Ecological Niches. *Bioengineering and Bioscience* **2017**, *5* (1), 7–13. https://doi.org/10.13189/bb.2017.050102.
4 Ramamoorthy, N. K.; T R, S.; Sahadevan, R. Assessment of Fed-Batch Strategies for Enhanced Cellulase Production from a Waste Lignocellulosic Mixture. *Biochem Eng J* **2019**, *152* (May). https://doi.org/10.1016/j.bej.2019.107387.
5 Pramanik, S. K.; Mahmud, S.; Paul, G. K.; Jabin, T.; Naher, K.; Uddin, M. S.; Zaman, S.; Saleh, M. A. Fermentation Optimization of Cellulase Production from Sugarcane Bagasse by Bacillus Pseudomycoides and Molecular Modeling Study of Cellulase. *Curr Res Microb Sci* **2021**, *2* (August 2020), 100013. https://doi.org/10.1016/j.crmicr.2020.100013.
6 Li, F.; Xie, Y.; Gao, X.; Shan, M.; Sun, C.; Niu, Y. D.; Shan, A. Screening of Cellulose Degradation Bacteria from Min Pigs and Optimization of Its Cellulase Production. *Electronic Journal of Biotechnology* **2020**, *48*, 29–35. https://doi.org/10.1016/j.ejbt.2020.09.001.
7 Ejaz, U.; Muhammad, S.; Ali, F. I.; Hashmi, I. A.; Sohail, M. Methyltrioctylammonium Chloride Mediated Removal of Lignin from Sugarcane Bagasse for Themostable Cellulase Production. *Int J Biol Macromol* **2019**, *140*, 1064–1072. https://doi.org/10.1016/j.ijbiomac.2019.08.206.
8 Li, H.; Zhang, M.; Zhang, Y.; Xu, X.; Zhao, Y.; Jiang, X.; Zhang, R.; Gui, Z. Characterization of Cellulose-Degrading Bacteria Isolated from Silkworm Excrement and Optimization of Its Cellulase Production. *Polymers (Basel)* **2023**, *15* (20), 4142. https://doi.org/10.3390/polym15204142.
9 Banerjee, S.; Maiti, T. K.; Roy, R. N. Production, Purification, and Characterization of Cellulase from Acinetobacter Junii GAC 16.2, a Novel Cellulolytic Gut Isolate of Gryllotalpa Africana, and Its Effects on Cotton Fiber and Sawdust. *Ann Microbiol* **2020**, *70* (1), 28. https://doi.org/10.1186/s13213-020-01569-6.
10 Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. **1959**.
11 Inforsato, F. J.; Luiz, A.; Porto, M. *Atividade Enzimática de Celulases Pelo Método DNS de Fungos Isolados de Sementes Em Germinação*; 2016.
12 Masi, C.; Tebiso, A.; Selva Kumar, K. V. Isolation and Characterization of Potential Multiple Extracellular Enzyme-Producing Bacteria from Waste Dumping Area in Addis Ababa. *Heliyon* **2023**, *9* (2), e12645. https://doi.org/10.1016/j.heliyon.2022.e12645.

ACKNOWLEDGEMENTS

Thank you for the scholarship from the Human Resources Program of the Brazilian Agency for Petroleum, Natural Gas and Biofuels – PRH/ANP through the Human Resources Training Program for Oil and Biofuels Processing (PRH 52.1). This study was financed in part by the Coordination for the Improvement of Higher Education Personnel – Brazil (CAPES) – Financial Code 001.