STUDY OF CELLULASE PRODUCTION FROM GRAPE STALKS USING *PENICILLIUM* SP

Emmely V. S. Cabral[1], Hellen A. S. Silva[2], Nattanael C. Melo[2], Adna C. B. Sousa[3] & Andréa F. Almeida[4*]

[1]*Mestrado em Engenharia Química/Centro de Tecnologia/Programa de Pós-Graduação em Engenharia Química, Universidade Federal da Paraíba, João Pessoa, Brasil.*
[2]*Graduação em Biotecnologia/Centro de Biotecnologia, Universidade Federal da Paraíba, João Pessoa, Brasil.*
[3]*Departamento de Biologia Celular e Molecular/Centro de Biotecnologia, Universidade Federal da Paraíba, João Pessoa, Brasil.*
[4]*Departamento de Biotecnologia e Programa de Pós-Graduação em Engenharia Química, Universidade Federal da Paraíba, João Pessoa, Brasil.*
** Corresponding author's email andreafalm@cbiotec.ufpb.br*

ABSTRACT

This work aimed to verify the potential use of grape stalks as a substrate for the production of fungal cellulases by *Penicillium* sp. Cultures were carried out in Erlenmeyer flasks (125 mL) with 5 g of grape stalks with 70% humidity, grain size of 30 mesh and inoculum concentration 1,0x10^7 spores/g, incubated at 30 ºC for 10 days for enzyme production. Samples were taken daily for analysis of the enzymatic activity of englucanases (CMCase) and total cellulases (FPase). The results showed that the enzyme complex synthesized by the isolated *Penicillium* sp. at the inoculum concentration studied, it presented a maximum CMCase activity of 80.1 U/g and FPase activity of 506.4 U/g in 48 h of cultivation. Therefore, based on the results achieved, the potential for cellulase production when using grape stalks as a substrate under the conditions used becomes evident.

Keywords: Fungal enzymes. Low cost substrate. Solid state fermentation. Enzymatic activity. Agro-industrial waste.

1 INTRODUCTION

Wine growing in Brazil is booming, and the Northeast is no different, whose production is concentrated in the São Francisco Valley (Pernambuco and Bahia) representing 13.94% of the national area (IBGE, 2020 and DE MELLO and MACHADO, 2021). The increase in production in cultivated areas within non-traditional regions allows new opportunities for the national market, also enabling the expansion of the number of artisanal wineries. However, there is concern about the disposal of waste generated in the processing of grapes, around 25% of the quantity processed becomes waste, mainly skins, seeds and stalks.

Among grape processing residues, grape stalks can be considered an interesting substrate for industrial bioconversion processes, due to their high content of lignin (17-18%), cellulose (30-31%) and hemicellulose (21 %) (CUNHA, 2018) Substrates made up of cellulose and hemicellulose have potential for application in biotechnological processes to obtain products with higher added value, highlighting the production of biofuels, bioenergy and, mainly, microbial enzymes (PROZIL et al., 2013).

Enzymes represent one of the most explored microbial products in the biotechnology industry, being widely used in food processing and the production of cleaning products, as well as in the textile and pharmaceutical industries (MANGE, 2009). Among microbial enzymes, cellulases formed by an enzyme complex that act in the depolymerization of cellulose stand out through the synergistic action of endoglucanases and exoglucanases (SUBISSAY, 2022). Thus, solid state processes stand out in the production of microbial enzymes and in the reuse of waste, as they involve the use of a solid substrate with favorable humidity for the surface growth of microorganisms. From a biotechnological point of view, the heterotrophic characteristic and absorption-based nutrition of filamentous fungi make them outstanding agents for the production of extracellular enzymes in these processes (ALVES et al., 2020). In this context, the present work sought to evaluate grape stalks as a potential substrate for the production of cellulolytic enzymes by the action of the filamentous fungus *Penicillium* sp. in solid cultivation.

2 MATERIAL & METHODS

Substrate: The grape stalk with a particle size of 30 mesh was characterized physically and chemically in terms of moisture percentage, pH, apparent density, real density, porosity of the bed and total reducing sugar content, according to the methodologies of the Adolf Lutz Institute (1985).

Microorganism: The fungus *Penicillium* sp. was used. maintained in Agar-Sabouraud-Dextrose medium in Petri dishes incubated at 30 ºC for 15 days. After mycelial growth of the fungus, the spores were removed with the help of sterilized distilled water. The spore concentration was determined by counting in a Neubaeur chamber to obtain the volume of spore suspension with the desired concentration of 1,0x10^7 spores per gram of substrate.

Enzyme production: Enzyme production was carried out in Erlenmeyer flasks (125 mL) containing 5 g of dried and crushed grape stalks and humidity adjusted to 70%. Then, inoculation (1,0x10^7 spores/g) was carried out in each flask. To control production, one of the bottles had the inoculum concentration replaced by the equivalent amount of sterilized distilled water. All flasks were incubated at a controlled temperature at 30±1 ºC. To obtain the crude enzyme extract, a citrate buffer solution (pH 4.8) was used as the extracting solvent in a proportion of 10 mL for each gram of substrate, and the mixture was homogenized

on a shaking table at 300 rpm for 60 min. Then, the suspension was filtered through a Buchner funnel and qualitative filter paper using a vacuum pump. The collected filtrate was then stored in centrifuge tubes at 4 °C for further analysis.

Determination of total cellulase activity (FPase): Quantification of total cellulase activity was carried out according to an adaptation of the methodology proposed by OLIVEIRA JÚNIOR (2014), where the substrate used was qualitative filter paper (14 µm) cut into strips of 1 cm x 6 cm. The strips were placed in test tubes, where 1 mL of citrate buffer (pH 4.8) was added, followed by a thermostated bath for 1 min at 50 °C. Then, 0.5 mL of the obtained enzyme extract was added. The mixture was then incubated at 50 ºC for 60 min for enzymatic reaction.

At the end of the incubation, a 0.5 mL aliquot of the reaction mixture was removed and 0.5 mL of the DNS reagent was added and the mixture was placed in a thermostated bath at 100 ºC for 5 min. After the reaction, a 3 mL aliquot of distilled water was added. Once the sample was cooled and homogenized, it was read on a spectrophotometer at 540 nm to determine the reducing sugars obtained. The analyzes were carried out in triplicate, and the values obtained from the readings were compared with a standard glucose curve (1 mg/mL). One unit of total cellulase activity is equivalent to 1 µmol of glucose released per min, under the process conditions, expressed by U/g.

Determination of endoglucanase activity (CMCase): The determination of endoglucanase activity was also carried out according to an adaptation of the protocol proposed by OLIVEIRA JÚNIOR (2014), in which a solution of 2% sodium carboxymethylcellulose (CMC) in citrate buffer 0, 05 M (pH 4.8) was used as a specific substrate. In test tubes containing 0.25 mL of the obtained enzyme extract, 0.25 mL of 2% (w/v) CMC solution were added, and the mixture was incubated at 50 ºC for 30 min. After that, 0.5mL of DNS reagent was added and the tubes were taken to a thermostated bath for 5 min at 100 ºC. After reaction, a 3 mL aliquot of distilled water was added. Once the sample was cooled and homogenized, it was read on a spectrophotometer at 540 nm to determine the reducing sugars obtained. The analyzes were carried out in triplicate, and the values obtained from the readings were compared with a standard glucose curve (1 mg/mL). One unit of endoglucanase activity is equivalent to 1 µmol of glucose released per min, under the process conditions, expressed by U /g.

3 RESULTS & DISCUSSION

The results of the physicochemical characterization of the grape stalk regarding pH, moisture content, bed porosity and total reducing sugars are presented in Table 1.

Table 1 Physicochemical characterization of dried and crushed grape stalks

Parameter	Result
Moisture (%)	18.83
pH	4.22
Bed porosity	0.421
Total reducing sugars (g/L)	1.03

The characterized stalk presented a moisture content within the ideal parameters for solid state cultivation, and close to the values of 20% and 15.5% obtained by PROZIL (2013), YAHYA (2015) respectively. The small variation between these values is attributed to some factors such as the species of vine from which the stalk being analyzed comes from, as well as the producing regions, conservation status at the time of collection, as well as the pre-treatment processes of this material.

The material also presented a total reducing sugar concentration of 1.03 g/L. Evaluating the average composition of monosaccharides in the grape stalk, PROZIL (2013) identified a glucose content of 62.7% in the total mass of monosaccharides present in the residue. The origin of this glucose content, as stated by this author, is related to the 30.3% cellulose and 21% hemicellulose identified in his chemical analyzes of the composition of the grape stalk.

In Figure 1 it is possible to observe the values of the enzyme avitity analysis for the process with Penicillium sp. from the grape stalk, where there is greater enzymatic activity in 48 h of cultivation for both cellulases studied, with 80.1 U/g of total cellulase activity (FPase) and 506.4 U/g of endoglucanases (CMCase).

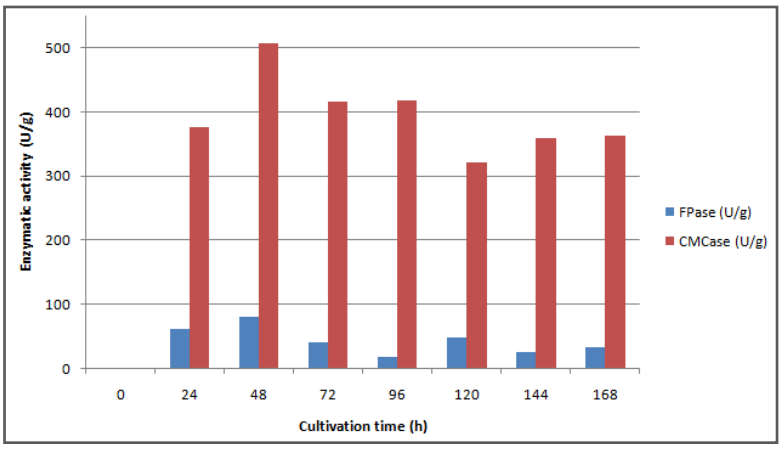

Figure 1 Enzymatic activity from grape stalks using *Penicillium* sp

ANDRADE (2022) used the same substrate, under the same humidity conditions, with the fungus Paecilomyces sp. and obtained an activity of 2.11 U/g of FPase and 7.48 U/g of CMCase after 240 h of cultivation. Using the isolated *Penicillium* sp. FSDE15 with a combination of wheat bran and corncob at 50% as substrate, SANTOS (2021) obtained maximum FPase and CMCase activities of 1.29 U/g and 21.11 U/g, respectively, both in 216 h of cultivation.

Therefore, from the comparison of these results, it is possible to see that the use of *Penicillium* sp. combined with grape stalks, under the cultivation conditions studied in this work, it favored the production of cellulolytic enzymes. The microorganism used and the humidity of the medium used are factors that can explain this performance, in addition to the low pH of the substrate, which is favorable to this type of process using filamentous fungi.

4 CONCLUSION

The fungus *Penicillium* sp proved capable of producing cellulolytic enzymes from grape stalks, verifying the potential of this residue as a substrate under the process conditions studied.

REFERENCES

1 IBGE. Cenário da vitivinicultura nacional. 2020. Disponível em: *https://agroinsight.com.br/12275-2/ Acesso em 09.06.2024.*

2 MELLO, L.M.R, MACHADO C.A.E. Vitivinicultura brasileira: panorama 2020. Comunicado Técnico 223. Bento Gonçalves-RS, 18p., 2021.

3 CUNHA N. Projeto de uma planta de tratamento anaeróbio de resíduos vínicos com aproveitamento energético do biogás gerado. Trabalho de Conclusão de Curso, Engenharia ambiental, Universidade de Caxias do Sul. Caxias do Sul, RS. 2018.

4 PROZIL S, MENDES J, EVTUGUIN D, CRUZ-LOPES L. Caracterização Química e Estrutural do Engaço da Uva e Avaliação do seu Potencial Como Matéria-Prima Lenhocelulosica. Millenium. 44. 23-40. 2013.

5 MANGE 2009. Revista de processos químicos/ SENAI: Departamento Regional de Goiás - v.3, n 5 (jan/jun 2009).

6 SUBISSAY, H. K. Determinação de temperatura ótima e pH ótimo das celulases produzidas por *Penicillium* sp. FSDE 15. Dissertação de mestrado, Universidade Federal da Paraíba. João Pessoa, 2022.

7 ALVES, M. F. et al. Produção de enzimas por fungos filamentosos. Fundamentos e atualidades em tecnologia e inspeção de alimentos, 2020.

8 YAHYA, M. A; AL-QODAH, Z.; NGAH, C. W. Z. Agricultural bio-waste materials as potential sustainable precursors used for activated carbon production: A review. Renewable and Sustainable Energy Reviews, v. 46, p. 218-235, 2015.

9 ANDRADE, E. S. P. Produção de Proteases e Celulases fúngicas a partir do engaço de uva. Dissertação de mestrado, Universidade Federal da Paraíba. João Pessoa, 2022.

10 SANTOS F.A. et al. Evaluation of the production of cellulases by *Penicillium* sp. FSDE15 using corncob and wheat bran as substrates. Bioresource Technology Reports, vol 14, 2021.

11 COÊLHO M. C. et al. Use of agroindustrial wastes for the production of cellulases by *Penicillium* sp. FSDE15. Journal of King Saud University - Science, vol 33, 2021.

ACKNOWLEDGEMENTS

The authors would like to thank the Research Support Foundation of the State of Paraíba (FAPESQ) for the financial support.