

Production of Cellulases by the Endophytic Fungus *Fusarium oxysporum*

Sideney Becker Onofre^{1,*}, Shaiana Paula Mattiello², Gécica Caroline da Silva², Deizi Groth¹, Ivair Malagi²

¹Department of Biological Sciences and Pharmacy, FAED - Faculty of Educational of Dois Vizinhos - União de Ensino do Sudoeste do Paraná – UNISEP - Av. Presidente Kennedy, 2601 –Bairro Nossa Senhora Aparecida - Dois Vizinhos, Paraná, Brazil

²Microbiology Laboratory, Department of Biological Sciences and Health, Paranaense University – UNIPAR, Unit of Francisco Beltrão, Av. Julio Assis Cavalheiro, 2000, Bairro Centro, Francisco Beltrão, Paraná, Brazil

Abstract Cellulose has enormous potential as a renewable energy source and the application of cellulases in the conversion of cellulosic biomass may provide great economical benefits; in addition, cellulases can be used in a wide range of industrial applications. Given the biotechnological importance of cellulases, the aim of this study was to evaluate the production of cellulases by endophytic fungi of the genus *Fusarium oxysporum* isolated from *Baccharis dracunculifolia* D.C. (Asteraceae). The studies were conducted using a basic substrate of sugarcane bagasse that was pretreated for complete removal of the sugar content. The material was dried at 28°C for 96 days and quantified every seven days after 25 days of fermentation. To quantify the enzymes, the indirect spectrophotometric method was used, with DNS reagent. The results showed that the greatest peak of enzyme production was at 55 days of fermentation, with a yield of 55.21 ± 10.54 IU/g of fermented substrate, at pH 5.96. Thus, it can be concluded that the fungus *Fusarium oxysporum* is a producer of cellulase enzymes.

Keywords Enzymes, Bioprocesses, Biotechnology, Biomass, Fermentation

1. Introduction

The term cellulosic or lignocellulosic material is used to describe the major constituents of most plants, i.e., cellulose, hemicellulose and lignin, the composition of which not only depends on the type of plant, but also on growth conditions [1].

Cellulose is the most abundant organic polymer on Earth and the main component of plant biomass. It is found in pure form, as in cotton, but is often found associated with hemicellulose and lignin in the cell wall [2]. Cellulose has a relatively simple structure, comprising of D-glucose monomers linked by glycosidic bonds β -1, 4.

Hydrolase enzymes are widely used in various industries. Cellulases and amylases are enzymes from the most economically important group of carbohydrases. The high consumption of cellulases may be explained by their wide use in the food industry, in the process of extraction of vegetable oils, in the maceration of fruit and clarification of juices, as well as in the manufacture of beer and wine; they are also used in the formulation of animal feeds [3].

Filamentous fungi are the most widely used type of fungi in industry for the production of cellulases, especially those

of the genera *Aspergillus*, *Trichoderma*, *Hemicella*, *Penicillium*, *Fusarium* and *Phanerochaete* [4]. Currently, cellulases are the third most industrially produced enzymes worldwide because of their applications, for example in cotton processing, paper recycling, the extraction of juices, as enzymatic detergents and as animal food additives [2,5].

The identification of the activity of enzymes in microorganisms and the studies related to optimizing their production are of great industrial interest. The isolation of samples with different production potentials of enzymes may represent alternatives for industry. Furthermore, the isolation of fungi from natural environments may result in lineages with greater production potential and that are more adapted to the environment [6].

Biotechnological processes have gained a prominent place in worldwide technological development, and have economic and operational features that have more advantages than conventional chemical processes [7]. Annually, 5.4×10^8 tons of cane sugar are processed in the world; on average, one ton of sugar cane generates 280 kg of bagasse [8].

Approximately 50% of the bagasse is used in power generation in power plants, while the remainder is stored. Given the importance and the increased production of sugar cane bagasse as industrial waste, there is growing interest in developing methods of producing biofuels and chemical products that offer economic and environmental advantages.

Many trials have been carried out to degrade this complex

* Corresponding author:

becker@unisep.edu.br (Sideney Becker Onofre)

Published online at <http://journal.sapub.org/microbiology>

Copyright © 2013 Scientific & Academic Publishing. All Rights Reserved

material into simple, fermentable sugars for ethanol production. The degradation of cellulose by cellulases is a very well studied process and the role of these enzymes is affected by porosity and crystallinity of cellulose as well as the lignin and hemicellulose content of biomass[9].

Endophytic microorganisms were first discussed at the beginning of the nineteenth century, but the first person who distinguished between them and plant pathogens was Bary in 1866. Generally, all plants have endophytic microorganisms. A single plant may contain several, including fungi and bacteria. There may be quite frequent species in a particular host, which are called the dominant species, in contrast to other more rare ones, which are called the secondary species[10].

According to Martin[11], the endophytic fungi of the genus *Fusarium* are distributed worldwide and are found in the soil or associated with species of plants; they decompose organic matter in the soil and can cause many diseases in different plant species.

The plant species *Baccharis dracunculifolia* D.C. (Asteraceae) has biological and ecological importance for the isolation of epiphytic and endophytic microorganisms, due to its adaptability to the diverse biomes of the Americas, particularly South America. It is represented by more than 500 species distributed mainly in Brazil, Argentina, Colombia, Chile and Mexico, and occupies the higher regions.

The high concentration of species in Brazil and the Andes indicates that one of these areas is the probable center of origin of this genus. They are usually shrubs, and in Brazil are commonly called *vassoura* or *vassourinha* (which translates as broom, little broom) and measure 0.5 to 4.0 meters in height[12].

2. Materials and Methods

2.1. Microorganisms

This study used the strain D3-FB of the endophytic fungus *Fusarium oxysporum*, isolated from *Baccharis dracunculifolia* D.C. (Asteraceae) in the period 2008 to 2009 and stored in the mycology collection of the Microbiology Laboratory of the Paranaense University-UNIPAR - University of Francisco Beltran - PR. All the collections were authorized by IBAMA (Brazilian Institute for the Environment) under protocol number 13.234-2, August 1st 2006.

2.2. Determination of Cellulolytic Activity

The cellulolytic activity was measured using a basic support of sugarcane bagasse that had been rinsed successively in running water for complete removal of sugars. The washed bagasse was dried in a fan oven at 65 °C for 24 hours, and then was packed in polyethylene bags and stored in dry conditions.

2.3. Fermentation in Erlenmeyer Flasks

Cellobiose (1%) and carboxymethylcellulose (1%) were added to the sugar cane bagasse substrate to induce the production of cellulases and also as initial carbon sources of the medium. This mixture was inoculated with a suspension of 5 g of the fungus inoculums, which had been previously grown on a rice culture. It was then homogenized in an Erlenmeyer flask and incubated at 28 °C for 69 days.

2.4. Analysis of Fermented Substrate

Aliquots of five grams of the medium were collected every 7 days and mixed with 50 ml of distilled water in the presence of a 7.0 buffer. This suspension was continuously agitated for 30 minutes. It was then filtered to remove solids to yield a clear extract used for pH determination. The extract was centrifuged at 3000 rpm for 15 minutes and the supernatant was considered as an enzyme source to determine the reducing sugars via the indirect spectrophotometric method. The indirect spectrophotometric method was used to determine enzyme activity, based on the release of glucose molecules by the action of cellulolytic enzyme complex.

2.5. pH

The pH was measured on a suspension obtained after homogenization of 5 grams of ferment in 50 mL of distilled water, which was continuously agitated for 30 minutes.

2.6. Measurement of Reducing Sugars

The reducing sugars were measured by the reaction with 3,5-dinitrosalicylic "DNS"[13]. In an alkaline medium and at elevated temperature, 3,5-dinitrosalicylic turns into 3-amino-5-nitrosalicylic. It acquires a yellowish coffee color that absorbs at 540 nm. One unit of cellulases was defined as the amount of released enzyme capable of acting on the substrate and releasing 1 μmol of reducing sugar (expressed as glucose) per minute.

2.7. Statistical Analysis

The statistical analysis was carried out using the program Statistica, version 5.0. Analyses of variance were in line with ANOVA standards. The significant differences between means were determined using the Tukey's test. All activities were triplicated.

3. Results and Discussion

The data concerning the behavior of the endophytic fungus *Fusarium oxysporum* are shown in Fig 1.

Table 1 shows that the yields of cellulases during 69 days of fermentation were 21.25 ± 3.45 ; 43.5 ± 12.65 ; 48.32 ± 10.35 ; 50.85 ± 13.05 ; 55.21 ± 10.54 ; 45.02 ± 8.90 and 15.12 ± 5.40 enzyme units for each gram of fermented substrate for the observation periods of 25, 32, 46, 49, 55, 62 and 69 days of fermentation, respectively. The data show that the period of greatest yield of the enzymatic complex was at 55 days of fermentation with a production of 55.21 ± 10.54 IU/g, at pH

5.96.

Figure 1 shows that at the start of the fermentation process, when there is availability of cellobiose and carboxymethylcellulose, these supplements acted as inducers for the metabolism of the fungus *Fusarium oxysporum*. They therefore initiated the production of enzymatic complex, which reacted with the cellulolytic components, thus degrading the cellulose and releasing glucose.

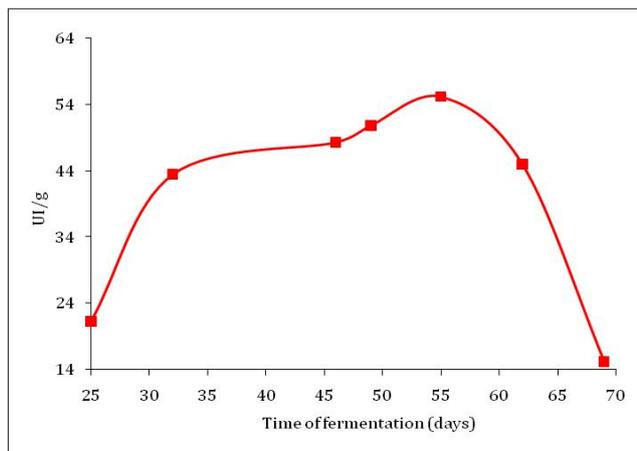


Figure 1. Behavior of the endophytic fungus *Fusarium oxysporum* as a producer of the cellulolytic complex in semi solid state fermentation at a temperature of 28°C and initial pH of 5.74

The data obtained in this study are similar to those obtained by Paschoalatti[14], who studied the *in vivo* and *in vitro* production of cellulase enzymes by the fungi *Sclerotinia* sp., *Rhizoctonia solani*, *Fusarium* sp., *Penicillium* sp. and *Pythium ultimum*; this similarity suggests that these enzymes play an important role, since cellulose is the major component of plant cell walls and all of them were able to degrade cellulose.

The results obtained in this study, are in line with Braga *et al.*[15], who reported that the fungus *Fusarium* sp. grew in medium containing cellulose as the sole carbon source: evidence of its ability to synthesize cellulolytic enzymes. All of the 33 strains tested were capable of degrading the cellulose-based substrate in semisolid fermentation based on sugar cane bagasse.

Shihata, Abdou and Galal[16] assessed the production of cellulase enzyme, and reported that *F. moniliforme*, *F. oxysporum* and *F. solani*, isolated from pea, are producers of pectinase and cellulase enzymes both *in vitro* and *in vivo*. In the study by Bueno *et al.*[17], it was observed that the isolates of *F. solani* of the passion fruit plant, produce a greater variety of extracellular enzymes, including amylases, lipases, cellulases, proteases, laccases and catalases and that the amounts of enzymes produced were different in each of the isolates.

This behavior justifies what Aguiar and Menezes[18] reported in a study evaluating the total cellulase activity obtained with sugar cane bagasse inoculated with *Aspergillus niger*: they found a yield of 25 IU/gram of fermented substrate after 168 hours fermentation. Thus, the data from different fermentation conditions show that the

fungus *Aspergillus niger* has the ability to secrete higher quantities of cellulase than the fungus *Fusarium oxysporum*, which was used in this study, in a shorter period of time.

It is important to highlight that the species of microorganism and the temperature are important variables, as they may directly interfere in the production of enzymes. In this study, the temperature was maintained at 28°C.

The monitoring of pH is also important, because according to Soccol[13], the fungus has a capacity for growth, albeit limited, under extreme conditions of acidity and alkalinity. These features are extremely important for fermentation processes, because they show that under these conditions the vast majority of the bacteria responsible for the contamination of the fermentation processes are inhibited.

4. Conclusions

From the present study, one can conclude that the fungus *Fusarium oxysporum* is a producer of cellulolytic enzymes, and may thus be used in biotechnological processes to obtain these enzymes or to produce glucose. The sugarcane bagasse was effective at inducing *Fusarium oxysporum* to produce the cellulase enzymes. The highest level of enzyme production was observed at 55 days of fermentation at a constant temperature of 28°C, with an enzymatic production of 55.21 ± 10.54 IU/g of fermented substrate, at pH 5.96.

ACKNOWLEDGEMENTS

We would like to thank IBAMA - Brazilian Institute for the Environment, for the license for collection of biological material and the União de Ensino do Sudoeste do Paraná, for financing this project.

Conflict of Interest

The authors declare no commercial or financial conflict of interest.

REFERENCES

- [1] B. Barl, C.G. Biliaderis, D.M. Murray and A.W. Macgregor, "Combined chemical and enzymatic treatments of corn husk lignocellulosics". *J. Sci. Food Agric.* Vol.56, No.2, pp.195-214, 1991.
- [2] L.R. Lynd, P.L. Weimer and W.H. Van-Zyl, "Pretorius, I. S. Microbial Cellulose Utilization: Fundamentals and Biotechnology". *Microbiology Molecular Biology Reviews.* Vol.66, |No.3, pp.506-577, 2002.
- [3] E.P.S. Bon, F. Gírio and N.Jr. Pereira, "Enzimas na produção de etanol". *In: E.P.S. Bom, M.A. Ferrara and M.L. Corvo. Enzimas em biotecnologia: produção, aplicações e mercado.*

- Rio de Janeiro: Interciência, pp.253-269, 2008.
- [4] R.R. Singhanía, R.K. Sukumaran, A.K. Patel, C. Larroche and A. Pandey, "Advancement and comparative profiles in the production Technologies using solid-state and submerged fermentation for microbial cellulases". *Enzyme and Microbial Technology*, Vol.46, pp.541-549, 2010.
- [5] X. Palomer, E.D. Puigjaner, M. Vendrell and I.L. Tous, "Study of the strawberry Cell endo- β -(1,4)-glucanase protein accumulation and characterization of its in vitro activity by heterologous expression in *Pichia pastoris*". *Plant Science*. Vol.167, pp.509-518, 2004.
- [6] S. Link and S.B. Onofre, (2010). "Microrganismos Epifíticos da Vassourinha – *Baccharis dracunculifolia* D.C. (Asteraceae)". *Revista em Agronegócios e Meio Ambiente*, Vol.3, No.1, pp.131-143, 2010.
- [7] G.M. Maciel, "Desenvolvimento de bioprocessos para produção de xilanases por fermentação no estado sólido utilizando bagaço de cana de açúcar e farelo de soja". 157p. Dissertação (Mestrado em Processos Biotecnológicos) – Universidade Federal do Paraná, Curitiba, Paraná – Brasil, 2006.
- [8] D.A. Cerqueira, G. Rodrigues and C.D. Meireles, "Optimization of sugarcane bagasse cellulose acetylation". *Carbohydrates Polymers*. Vol.69, pp.579-582, 2007.
- [9] M. Balat and H. Balat, "Progress in bioethanol processing. *Progress in Energy and Combustion Science*". Vol.12, pp.551-573, 2008.
- [10] J.L. Azevedo, "Microrganismos Endofíticos". Departamento de Biologia Geral, Universidade Federal de Goiás. Goiânia, GO, 1998.
- [11] M.K. Martins, "Variabilidade genética de isolados de *Fusarium* spp. e estudo da interação com a planta hospedeira". Tese de Doutorado, 186p. Universidade de São Paulo, Piracicaba, Brasil, 2005.
- [12] C. Cuzzi, S. Link, A. Vilani and S.B. Onofre, "Enzimas extracelulares produzidas por fungos endofíticos isolados de *Baccharis dracunculifolia* D.C (Asteraceae)". *Global Science and Technology*, Vol.4, No.2, pp.47-57, 2011.
- [13] C.R. Socol, "Physiologie et Métabolisme de *Rhizopus* en Culture Solide et Submergée en Relation Avec la Dégradation d'Amidon et la Production d'Acide L(+) Lactique". Thèse de Doctorat. 218p. Mention Génie enzymatique, Bioconversion et Microbiologie, Université de Technologie de Compiègne. Compiègne-France. 1992.
- [14] S.F. Paschoalatti, "Fitopatógenos: arsenal enzimático". In: Bergamin Filho, A.; Kimati, H.; Amorim, L. (Ed.) *Manual de Fitopatologia: princípios e conceitos*. São Paulo: Agronômica Ceres, Vol.1, No.19, pp.343-364, 1995.
- [15] R.M. Braga, G.S.F. Junior, V.R.O. Lopes, S.C. Martins, C. Miranda, "Avaliação da produção de celulases por cepas de *Fusarium* sp". XVII Simpósio Nacional de Bioprocessos. Natal/RN. 2009.
- [16] Z.A. Shihata, E.S. Abdou and A.A. Galal, "Production of pectolytic and cellulolytic enzymes by *Fusarium* species in diseased cowpea plants and in vitro". *Journal of Agricultural Sciences*, Vol.26, pp.339-344, 1995.
- [17] C.J. Bueno, I.H. Fischer, D.D. Rosa and E.L. Furtado, "Produção de enzimas extracelulares por *Fusarium solani* de maracujazeiro amarelo". *Tropical Plant Pathology*, Vol.34, No.5, pp.343-346, 2009.
- [18] C.L. Aguiar and T.J.B. Menezes, "Produção de celulases e xilanases por *Aspergillus niger* IZ9 usando fermentação submersa sobre bagaço de cana de açúcar". B. CEPPA. Vol.23, No.2, pp.23-28, 2000.