

# Production of Cellulolytic Enzymes by *Aspergillus flavus* Using Solid State Fermentation Based on Sugarcane Bagasse

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**Abstract** Cellulases are enzymes of great industrial interest, which are used in the food, pharmaceuticals, cosmetics, detergents and textile industries. Applications include the bleaching of pulp in the paper industry, the production of dissolved pulp, waste water treatment and recycling of waste paper. Studies have been carried out regarding the ability of microorganisms to produce enzymes, using available and affordable substrates. The aim of this study was to evaluate the ability of the endophytic fungus *Aspergillus flavus*, strain (D2-FB) to produce cellulase. The studies were carried out using a substrate of sugarcane bagasse supplemented with 1% cellobiose and carboxymethylcellulose. The material was kept in an oven at 27   C for 69 days and the enzymes were measured every 7 days. To quantify the enzymes, the DNS method was adopted. The results showed that the highest production occurred at 32 days, with a production of  $33.15 \pm 7.96$  U/g of substrate. After this period, the enzyme production decreased gradually up to  $3.06 \pm 0.53$  U/g. Based on these results, it can be concluded that the endophytic fungus *A. flavus*, strain (D2-FB), is a producer of cellulases.

**Keywords** Enzymes, Bioprocesses, Biotechnology, Fermentation

## 1. Introduction

Enzymes are biological catalysts, consisting of protein molecules produced by living cells. These biocatalysts have a high catalytic activity and specific selectivity for the substrate[1].

Cellulases are enzymes that are capable of breaking cellulose's glycosidic bonds, resulting in the release of oligosaccharides, cellobiose and glucose[2]. These hydrolytic enzymes are used in the food, pharmaceuticals, cosmetics, detergents and textile industries. Their main applications include the bleaching of pulp in the paper industry, the production of dissolved pulp, waste water treatment and recycling of paper residues. Enzyme technology is currently one of the most promising new technology fields for the synthesis of valuable compounds.

Industrial processes that employ enzymes in biotransformation processes have a lower environmental impact and concomitantly lower consumption of energy, since they are biodegradable and highly specific, which reduces undesirable effects[3,4].

A wide variety of cellulase-producing microorganisms are present in nature, and they are capable of degrading natural cellulose. Cotton and filter paper, among others, are used as inducing substrates for the production of exo-glycosidases and for measuring the activity of the total cellulolytic complex[5]. Among the microorganisms, fungi are noteworthy for producing enzymes of industrial interest. Fungi are uni or multicellular eukaryotic organisms, which are heterotrophic, chemoorganotrophic, aerobic or microaerophilic; some have cell walls composed of chitin and cellulose[6].

The genus *Aspergillus* sp. is the most common filamentous fungus, and is one of the most thoroughly studied. The species of this genus are distributed worldwide and are present on the surface, in air and water, both in plant organisms and in animals, and are associated with the deterioration of plant materials and food. Many species of *Aspergillus* are used to obtain enzymes, in chemical biosynthesis and transformation of compounds[7].

Agroindustrial wastes and low-cost cellulosic biomass can be used to produce cellulases, which not only greatly reduces the cost of production of these enzymes but also may result in a yield similar to that obtained with other carbon sources as well as contributing to the environment. Several agro-industrial residues can be used as a substrate, such as orange bagasse, wheat and rice bran, soybean, apple pulp,

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coffee pulp, sugar cane bagasse, passion fruit peel, pineapple bagasse and cashew apple pulp[2].

Bagasse from sugarcane has been used as a substrate for growing large numbers of microorganisms, including bacteria, yeasts and filamentous fungi. However, fungi are the most commonly used, due to the amount of enzymes and proteic enrichment that they produce. The bagasse has been used as a substrate for the production of cellulases and xylanases by various *Aspergillus* species, including *A. niger* and *A. phoenicis*[8,9].

The objective of this study was to evaluate the ability of the endophytic fungus *Aspergillus flavus*, strain (D2-FB), isolated from *Baccharis dracunculifolia* D.C (Asteraceae) to produce cellulases.

## 2. Materials and Methods

### 2.1. Studied Microorganism

This study used the endophytic fungus *Aspergillus flavus*, strain (D2-FB), isolated from *Baccharis dracunculifolia* D.C. (Asteraceae), isolated in the period 2008 to 2009 and kept in the fungi collection of the Microbiology Laboratory of the Universidade Paranaense - Unipar - Unidade Universitária de Francisco Beltrão - PR.

### 2.2. Determination of the Cellulolytic Activity

For the determination of cellulolytic activity, the study used a basic support of sugarcane bagasse that was successively rinsed in water for complete removal of sugars. The rinsed pulp was dried in an oven with air circulation at 65 °C for 24 hours, and was then packed in a polyethylene bag and stored in a dry environment.

### 2.3. Fermentation in Erlenmeyer Flasks

1% cellobiose and 1% carboxymethylcellulose were added to the sugarcane bagasse substrate to induce the production of cellulases and as the medium's initial carbon sources. This mixture was inoculated with a suspension of 5g of the fungus, previously grown on a rice medium. It was then homogenized in an Erlenmeyer flask and incubated at 28°C for 69 days.

### 2.4. Analysis of the 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 7.0 buffer. This suspension was stirred continuously for 30 minutes. It was then filtered to remove solids to yield a clear extract used for pH measurement. The extract was centrifuged at 3000 rpm for 15 minutes and the supernatant was considered an enzyme source to determine reducing sugar 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 the cellulolytic enzymes complex.

### 2.4. pH

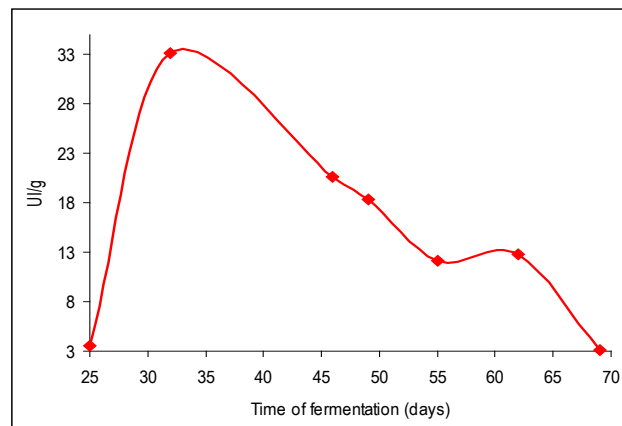
pH was measured on a suspension obtained after homogenization of 5g of ferment in 50 ml of distilled water, which was continuously stirred for 30 minutes.

### 2.5. Dosage of Reducing Sugars

Reducing sugars were determined by the reaction with 3,5-dinitrosalicylic "DNS"[10]. In an alkaline medium and at elevated temperature, the 3,5-dinitrosalicylic turns into 3-amino-5-nitrosalicylic. It develops a yellowish coffee color that absorbs at 540 nm. One unit of cellulases was defined as the quantity of released enzyme capable of acting on the substrate and releasing one  $\mu\text{mol}$  of reducing sugar (expressed as glucose) per minute under the test conditions [11].

## 3. Results and Discussion

The data obtained from the fermentation process using a substrate of sugarcane bagasse supplemented with 1% carboxymethylcellulose and cellobiose, inoculated with the endophytic fungus *Aspergillus flavus*, isolated from *Baccharis dracunculifolia* D. C. (Asteraceae), are shown Fig. 1.



**Figure 1.** Behavior of the endophytic fungus *Aspergillus flavus*, strain (D2-FB), in the production of cellulolytic complex in solid fermentation at a temperature of 28°C and pH of 6.54

The data analysis showed that the cellulase production, measured over 69 days, was  $3.50 \pm 0.40$ ;  $33.15 \pm 7.96$ ;  $20.63 \pm 6.54$ ;  $18.35 \pm 9.22$ ;  $12.11 \pm 5.46$ ;  $12.85 \pm 4.87$  enzyme units for each gram of fermented substrate for the times of 25, 32, 46, 49, 55, 62 and 69 days of fermentation, respectively.

Thus, it can be seen that the period of greatest expression of the cellulase enzyme occurred at 32 days of fermentation; after this period the expression rate gradually decreased, stabilizing at 55 and 62 days, and decreasing again at 69 days.

After 32 days of fermentation there was a decrease in the volume of the produced cellulase enzyme, which indicates genetic repression of this enzyme. The phenomenon of gene

suppression is due to the formation of a cascade of signals initiated within the cell by the presence of the signal molecule (glucose). This activates the repressor molecule, which binds in the promoter region of the cellulase genes, blocking the transcription and expression of these genes [12-16].

According to Soccol [11], pH monitoring is an important variable in the optimal conditions of enzymatic activity, because the fungus has a limited ability to grow under extreme conditions of acidity and alkalinity. In this study, the pH of the medium remained stable (between 6.2 and 7.7). The temperature is also a very important variable, because it interferes with the optimal conditions for activity of an enzyme [17]; in this study the initial temperature was 28°C.

Pandey [18] who studied the enzyme  $\alpha$ -amylase at temperatures between 28 and 37°C, achieved excellent results. In the same year, the same author obtained highest yields of  $\alpha$ -amylase with *Aspergillus niger* at a temperature that ranged from 28-30°C, which coincides with the fermentation process conditions used in this study.

Various substrates are used in order to obtain large amounts of cellulolytic enzymes by microorganisms [19,20]. This study used a substrate of sugarcane bagasse supplemented with 1% carboxymethylcellulose and cellobiose, which favored the production of cellulases. Likewise, [21] found that using a mixed culture of *Aspergillus fumigatus* and *Aspergillus ellipticus*, developed in sugarcane bagasse pre-treated with 2% calcium hydroxide solution, favored the production of cellulase, after 8 days of fermentation.

In a study by Menezes *et al.* [22], the cellulolytic activity of fungal strains, such as *Aspergillus niger*, grown in sugarcane bagasse was higher than those grown only in carboxymethylcellulose and filter paper. The authors suggested that these strains produce the exoglucanase fraction, since the bagasse is an *in natura* cellulose and had not received any chemical treatment, which would require the action of the pre-hydrolytic fraction of exoglucanase before being hydrolyzed by the other endoglucanase and  $\beta$ -glucosidase fractions.

The results of this study were higher than those obtained by Zúñiga [23], which evaluated the production of cellulases by *Aspergillus niger* grown in solid state fermentation on supplemented sugarcane bagasse. They obtained 0.59 U/g substrate using the same cultivation conditions. This difference can be explained by the fact that they used different species and the medium had different chemical and physical factors, which would cause the fungi to behave differently.

Milagres *et al.* [24], studied submerged fermentation in a medium based on hemicellulose hydrolysate from sugarcane bagasse using *Thermoascus aurantiacus* and obtained enzyme activities of 4.2 and 3.3 U/g substrate for endoglucanase and exoglucanase, respectively. These values are also lower than the maximum activity obtained in this study.

Umikalsen [25], who worked with a strain of *Chaetomium globosum* in delignified palm fiber, obtained similar results to those obtained in our study. Their activities of total cellulase, endoglucanase and  $\beta$ -glucosidase were 1.4, 30.8 and 9.8 U/g of substrate, respectively. The similarity of these results reinforces the idea that even though filamentous fungi may belong to a different genus, they have the same behavior when cultivated in specific media and conditions.

## 4. Conclusions

It can be concluded that the endophytic fungus *Aspergillus flavus*, strain (D2-FB) is able to produce enzymes of the cellulolytic complex. Therefore, its use in processes to obtain enzymes and produce energy sources such as glucose and cellobiose is extremely important. Sugarcane bagasse, used as substrate, was able to induce the expression of genes responsible for production of cellulases by the endophytic fungus *Aspergillus flavus*, strain (D2-FB), given that significant amounts of enzymes were obtained. The fermentation time that led to the highest production of the enzyme was 32 days, with a yield of  $33.15 \pm 7.96$  enzyme units/gram of substrate, at pH 6.82 and a temperature of 28°C.

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## Conflict of Interest

The authors declare no conflict of interest.

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