

Cellulase Production by Endophytic Strains of *Trichoderma reesei* from *Baccharis dracunculifolia* D. C. (Asteraceae)

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Abstract

Cellulases are enzymes responsible for the degradation of cellulose, the major compound in plant cells. Cellulose is a polysaccharide composed of several glucose units linked together by chemical bonds. Cellulases, such as endoglucanases, beta-glucosidase and exoglucanases, break the chemical bonds between the glucose units. Fungi, including the endophytic species, can be great cellulase producers. This study aimed to evaluate cellulase production by four endophytic strains of *Trichoderma reesei* in semi-solid media containing sugarcane bagasse, supplemented or not with salts. Two fermentations were carried out for 43 days. Samples were taken every seven days to obtain production peaks. The enzymes were characterized by their optimum pH and temperature of activity and stability upon incubation in the presence of ions, pH and temperature variations. The results showed that the endophytic strains FB1, FB2, FB3 and FB4 of *Trichoderma reesei* produce cellulases in a sugarcane bagasse medium, supplemented or not with salts, at pH 5.5 and 30°C. The supplemented medium proved to be more appropriate to induce cellulase production after 29 days of fermentation, with FB4 having the best yield: 16.32 ± 2.65 IU/gram of fermented substrate.

Keywords

Substrate, Fermentation Processes, Enzymes, Cellulases

1. Introduction

A constant increase in agricultural activities has led to a growing need to find an application for agro-industrial residues, as they accumulate in the environment, changing their characteristics and losing their resources. Numerous technologies have been created for utilizing these materials, which became high value added products. Sugarcane bagasse, generated by sugar refineries and ethanol plants, is one of them [1] [2].

The study of industrial fermentation media for obtaining biotechnological products has received great attention in recent years. Several byproducts and raw materials from the food industry and agro-industry have been employed to obtain biotechnological products, such as enzymes, due to high availability and low commercial value [3] [4].

Enzyme production is often limited by the cost of the substrates used for microorganism cultivation. It is estimated that approximately 30% - 40% of the cost involved in the production of enzymes is due to the culture medium, making its optimization important to reduce costs [5].

Lignocellulosic residues are considered a great alternative source of energy due to their high availability in nature. They are frequently used in technological means of feeding, as protein for animal feed and in the paper industry [6].

Biodegradation of lignocellulosic materials can be employed in technological processes, besides corresponding to an important stage of the carbon cycle in nature. Enzymes produced by fungal decomposers of lignocellulosic materials have also been in demand: xylanases and laccases for pulp bleaching and cellulases for the enzymatic hydrolysis of cellulose in the production of second-generation ethanol [7].

Enzymatic hydrolysis of cellulose includes the synergistic activity of a cellulolytic complex, usually from fungi, consisting of endoglucanases, exoglucanases and β -D-glucosidase. However, such a technique has low productivity and high cost [8].

Cellulases form a complex able of acting on cellulosic materials, causing their hydrolysis. Cellulases are highly specific biocatalysts, which operate in synergy to release sugars. Glucose is the sugar with the highest industrial importance, as it can be converted into ethanol [6] [9] [10].

A wide variety of microorganisms have been used in fermentation processes with sugarcane bagasse as substrate, in order to obtain cellulases. The filamentous fungi are the most used, especially *Trichoderma reesei*, which is known for producing various extracellular enzyme systems involved in the hydrolysis of polysaccharides [11]-[14].

Trichoderma reesei is an endophytic filamentous fungus, which is distinguished by its ability to generate the cellulolytic enzyme complex [15]. It is found in various environments, such as soil and saprophytic, epiphytic and endophytic plants [16].

The search for new fungal species with potential to produce the cellulolytic complex justifies this work, which aims to evaluate cellulase production in four endophytic strains of *Trichoderma reesei* in a semi-solid medium containing sugarcane bagasse, supplemented or not with salts.

2. Materials and Methods

2.1. Microorganisms

Strains FB1, FB2, FB3 and FB4 of the endophytic fungus *Trichoderma reesei*, isolated from *Baccharis dracunculifolia* DC (Asteraceae), were used. These strains can potentially produce cellulases in a semi-solid medium containing sugarcane bagasse and supplemented with salts. They were isolated and kept in the mycology collection at União de Ensino do Sudoeste do Paraná (UNISEP), Francisco Beltrão Campus, Paraná, Brazil.

2.2. Determination of Cellulolytic Activity

Sugarcane bagasse, washed successively in running water to completely remove the sugars, was used as the basic support for determination of cellulolytic activity. The bagasse was dried in a drying oven until constant weight was obtained. It was subsequently ground in a mill, packaged and stored in a dry place for further testing.

2.3. Fermentation in Supplemented Medium

A semi-solid culture medium consisting of 10 g of dehydrated sugarcane bagasse, and supplemented with 0.5

g/L carboxymethylcellulose, 1 g/L glucose, 3 g/L NaNO₃, 1 g/L KH₂PO₄, 0.5 g/L MgSO₄, 0.5 g/L KCl, 10 mg/L FeSO₄·7H₂O, 5.0 mg/L ZnSO₄ and 3 g/L of urea was used to assess the cellulolytic activity. All medium components were added to 120 mL of distilled water and then autoclaved for 15 min. A total of 30 mL of a spore suspension ($\pm 10^8$ mL) was used for inoculation. The material was incubated at 28°C for 21 days, when the first analysis of enzyme activity occurred. Afterwards, further analyses were carried out every three days until completing 43 days of incubation.

2.4. Fermentation in Unsupplemented Medium

A semi-solid culture medium containing 10 g of dried and ground sugarcane bagasse was used. To it was added 0.5% carboxymethylcellulose and 0.1% glucose in 120 mL of distilled water. Afterwards, the medium was autoclaved for 15 min. A spore suspension ($\pm 10^8$ mL) was used for inoculation. A total of 30 mL of this inoculum suspension was added to each vial containing the culture medium. The material remained in an incubator at 30°C for 22 days, when the first enzyme quantification occurred. The cellulolytic activity was measured every three days until day 43 of fermentation.

2.5. Substrate Fermentation Analysis

Aliquots of 5 g of the medium of each strain were weighed and placed in Erlenmeyer flasks. A total of 50 mL of deionized sterile water was added to each flask, which were stirred for 30 min at 30°C. The solution was filtered to remove the solids and the pH was determined, after adding 2 mL of a 7.0 phosphate buffer. The extract was centrifuged at 3000 rpm for 15 min and the supernatant was the enzyme source to determine the reducing sugars by the indirect spectrophotometric method. This method was used to determine enzyme activity based on the release of glucose molecules by the action of the cellulolytic enzyme complex [16] [17].

2.6. Determination of Reducing Sugars

Reducing sugars were determined by reaction with 3,5-dinitrosalicylic acid (DNS) [18]. In a basic medium and at high temperature, this acid becomes 3-amino-5-nitrosalicylic acid, developing a red color and absorbing light at 540 nm. One unit of cellulase was defined as the amount of enzyme released on the substrate that liberate 1 μ mol of reducing sugar (expressed as glucose) per minute, under test conditions [19] [20].

2.7. pH Optimization

Strain enzymatic activity was evaluated under different pH values (4.0, 5.0, 5.5, 6.0 and 6.5). These tests were conducted to determine the optimum pH for subsequent assays.

2.8. Effect of Temperature

Tests were carried out at different temperatures (25°C, 30°C, 35°C, 40°C and 45°C) and under optimal pH conditions, to verify the optimum temperature for the production of the cellulolytic complex.

2.9. Statistical Analysis

Results were submitted to analysis of variance using the statistical analysis system Assisat [21]. The comparison of means was done by the Tukey's test at 5% probability.

3. Results and Discussion

Data on cellulase production by four endophytic strains of *T. reesei* are shown in **Tables 1-4**, and **Figure 1** and **Figure 2**. **Table 1** relates to enzymatic volumes produced by the strains in different pH values.

Among the physical parameters, the medium pH plays an important role because it induces morphological changes in the microorganism and interferes in enzyme secretion. The pH change observed during microorganism growth also affects product stability in the medium [22] [23].

The pH of a culture varies according to reactions that occur during the metabolic activities of the microorganism. The secretion of organic acids, such as acetic or lactic, causes a decrease in pH.

The use of a nitrogen source causes pH variation; for example, with ammonium salts, the pH usually decreases throughout cell growth, due to hydrogen ion formation during the consumption of ammonia [24].

The highest yields of cellulolytic complex were observed at pH 5.5 for the four tested strains, a decrease occurring at pH 4.0 and higher pH values (6.0 and 6.5).

Solid state fermentations (SSF) are exothermic. The large amounts of heat released are directly proportional to the metabolic activity of the microorganism. In filamentous fungi, the temperature directly influences spore germination, growth and product formation. The temperature is a critical factor in virtually all SSF, due to the accumulation of metabolic heat generated [25].

Table 1. Cellulase production by four endophytic strains of *Trichoderma reesei* in different pH values.

Strain	pH				
	4.0	5.0	5.5	6.0	6.5
FB1	1.22 ± 0.33cB*	4.22 ± 0.47aB	10.45 ± 1.65aA	3.45 ± 0.65aB	2.41 ± 1.66aB
FB2	3.12 ± 0.25bB	2.45 ± 0.78aB	6.34 ± 1.32aB	2.40 ± 0.34aB	1.11 ± 0.19aB
FB3	6.52 ± 1.28aA	5.33 ± 1.58aB	8.56 ± 1.87aA	3.78 ± 0.28aB	2.61 ± 0.36aB
FB4	4.57 ± 0.67bB	3.44 ± 0.74aB	9.44 ± 1.90aA	2.86 ± 0.43aC	1.20 ± 0.45aC

*Means followed by lowercase letters in the columns and uppercase letters horizontally do not differ at 5% significance level by the Tukey's test.

Table 2. Cellulase production by four endophytic strains of *Trichoderma reesei* in different temperatures and pH 5.5.

Strain	Temperature °C (pH-5.5)				
	25	30	35	40	45
FB1	0.98 ± 0.23bC*	9.43 ± 1.20aA	7.21 ± 1.67aA	5.32 ± 1.01aB	0.34 ± 0.20aC
FB2	1.23 ± 0.20bC	8.45 ± 1.78aA	6.22 ± 1.34aA	4.29 ± 0.78aB	1.01 ± 0.21aC
FB3	2.34 ± 0.45aC	10.32 ± 2.87aA	8.76 ± 1.99aA	4.55 ± 0.86aB	2.87 ± 0.23aC
FB4	3.43 ± 0.37aB	8.32 ± 2.09aA	6.54 ± 1.03aA	4.78 ± 0.68aB	2.56 ± 0.32aC

*Means followed by lowercase letters in the columns and uppercase letters horizontally do not differ at 5% significance level by the Tukey's test.

Table 3. Cellulase activity (U/g substrate) by endophytic strains of *Trichoderma reesei* in a supplemented medium.

Strain	22	29	36	43
FB1	1.58 ± 0.76cB*	14.33 ± 2.56aA	7.99 ± 0.47bB	3.14 ± 1.45aB
FB2	6.42 ± 1.23bB	12.73 ± 2.34aA	5.93 ± 0.78cC	2.21 ± 0.36aC
FB3	9.50 ± 2.20aB	13.73 ± 1.86aA	6.56 ± 1.58aC	3.06 ± 0.82aD
FB4	7.65 ± 1.95aB	16.32 ± 2.65aA	4.82 ± 0.74cC	2.12 ± 0.89aC

*Means followed by lowercase letters in the columns and uppercase letters horizontally do not differ at 5% significance level by the Tukey's test.

Table 4. Cellulase activity (U/g substrate) by endophytic strains of *Trichoderma reesei* in unsupplemented media.

Strain	22	29	36	43
FB1	1.79 ± 0.23aC*	10.85 ± 0.34aA	9.18 ± 1.25aA	3.24 ± 0.65aB
FB2	2.27 ± 0.46aC	8.02 ± 1.45aA	5.02 ± 0.89bB	3.95 ± 0.79aB
FB3	1.69 ± 0.32aC	9.88 ± 0.79aA	7.04 ± 1.02bA	3.67 ± 0.54aB
FB4	1.43 ± 0.78bC	7.93 ± 0.68aA	5.56 ± 0.45bB	2.83 ± 0.34aC

*Means followed by lowercase letters in the columns and uppercase letters horizontally do not differ at 5% significance level by the Tukey's test.

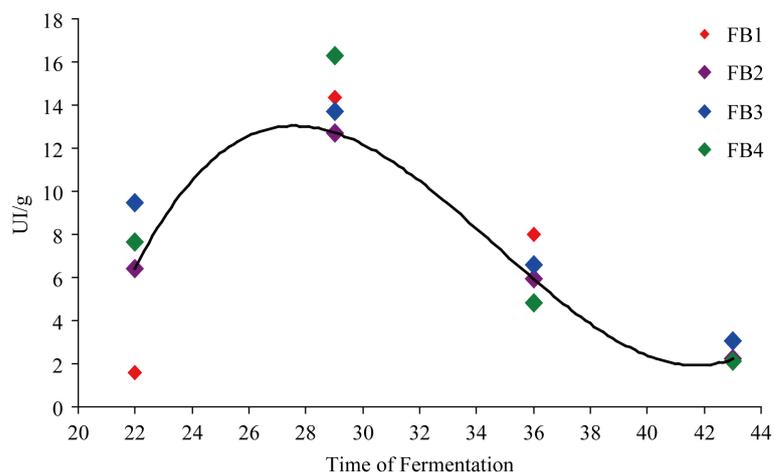


Figure 1. Cellulase production by four endophytic strains of *Trichoderma reesei* in supplemented media.

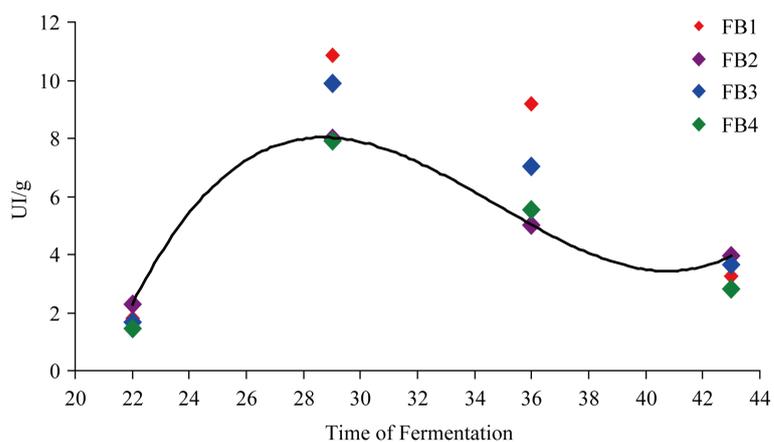


Figure 2. Cellulase production by four endophytic strains of *Trichoderma reesei* in unsupplemented media.

Sato and Sudo [26] reported that temperature affects both the growth and the production of enzymes, thus an efficient heat removal method is necessary. According to Bianchi *et al.* [27], a large amount of heat may be produced during SSF due to the metabolic activity of the microorganisms, and also depending on the substrate. The heat produced must be immediately dissipated to avoid hindering the process, because temperature directly affects spore germination, microorganism growth and sporulation, and product formation.

Table 2 shows that the optimum temperature for growth and production of cellulolytic enzymes for four endophytic strains of *Trichoderma reesei* is 30°C. Enzyme volumes decreased for all tested strains at temperatures below and above 30°C. Thus, with data referring to the optimum temperature and pH, it was possible to proceed to the other steps of this work.

It is noteworthy that 30°C is a suitable temperature for mesophilic bacteria to develop. This corroborates Buswell *et al.* [28], who reported 30°C as the optimum temperature for the production of cellulolytic enzyme complex by *Trichoderma* spp. This was also observed by Chen and Jan [29], Dalsenter *et al.* [30], Mitchell *et al.* [31], Gomes *et al.* [32], Carvalho *et al.* [33] and Taneda *et al.* [34].

According to Camassola *et al.* [35], 5.5 is the optimum pH for the production of cellulolytic enzymes by *Penicillium echinulatum*. Alam *et al.* [36] optimized the process of cellulase production by *Trichoderma reesei* through the bioconversion of the liquid sewage sludge, finding maximal activity at pH 5.5, thus corroborating Qin *et al.* [37], Zang *et al.* [38], Bendig and Weuster-Botz [39] and Puglisi *et al.* [40].

Table 3 and **Figure 1** show the same behavior for the four tested strains during the production of cellulolytic

complex, with enzyme production occurring from 22 days of fermentation to reach maximum yields at day 29. Afterwards, a decrease in enzyme production was observed.

At day 29, enzyme production was 14.33 ± 2.56 , 12.73 ± 2.34 , 13.73 ± 1.86 and 16.32 ± 2.65 IU/g of substrate for FB1, FB2, FB3 and FB4, respectively, showing no significant differences at 5% by the Tukey's test.

During the 42 days of fermentation, enzyme production in media unsupplemented with salts was lower when compared to the salt-supplemented media (Table 3).

Enzymatic production at 22 days was: 1.79 ± 0.23 , 2.27 ± 0.46 , 1.69 ± 0.32 and 1.43 ± 0.78 for FB1, FB2, FB3 and FB4, respectively. These enzymatic indices do not differ at 5% significance level.

These volumes are lower than those produced by the same strains at the same period of time, but in media supplemented with salts. It is noteworthy that FB2 and FB3 produced 6.42 ± 1.23 and 9.50 ± 2.20 , respectively, during the same time, values higher than those observed for unsupplemented media.

Trichoderma reesei produced 18 IU/g of cellulolytic complex in supplemented medium and 6.0 IU/g in unsupplemented medium, at 30 days of fermentation, corroborating Aguiar [1].

In 29 days, the strain FB4 produced 16.32 and 7.93 in media supplemented and unsupplemented with salts, respectively. Basso, Gallo and Basso [13] (2010), when evaluating the cellulolytic activity of *T. reesei* strains, isolated from sugarcane bagasse and decaying wood, observed values of 12.58 IU/g of unsupplemented substrate at 28°C in 35 days. Once more, the data are similar to those observed in this work, but considering the behavior in unsupplemented medium, the values obtained in this study are lower, reaching 7.93 IU/g in 29 days.

The data obtained in this study corroborate Juwaied *et al.* [10] on culture media optimization for the production of total cellulase, using different culture media. *T. reesei* yielded 2.6 IU/gram of substrate at pH 5.5 after 20 days in media containing sugarcane bagasse. This volume of produced enzyme is similar to that obtained at 22 days in this work, but much lower than the volumes obtained after 29 days of fermentation (up to 10.85 IU/g in unsupplemented media and 16.32 IU/g in supplemented media), thus showing potential to produce the cellulolytic complex.

No influence on enzymes production was observed by the sugarcane bagasse and glucose contained in the media; changes are related to the added salts. These results corroborate Castro [41], who compared the production of endoglucanase and β -glucosidase by *Trichoderma reesei* and *Humicola grisea*, when grown on insoluble (cellulignin from sugarcane bagasse and Avicel) and soluble substrates (carboxymethylcellulose and cellobiose). Slower kinetics of enzyme production were observed when the insoluble sources had been used, especially of lignocellulosic origin, due to an initial acclimation period of the cells to this raw material. Yet, when carboxymethylcellulose and cellobiose were considered, the times corresponding to the maximum enzyme production were anticipated in about 50 h. It has been concluded that there is no direct correlation with the carbon source used for induction in the synthesis of the cellulolytic complex enzymes by *T. reesei* and *Humicola grisea* var. *thermoidea*. Cellulases were also produced by strains grown on a substrate containing only cellobiose, and this synthesis had not a constitutive character.

The results obtained here also confirm Rodríguez-Zúñiga *et al.* [42] who studied *Aspergillus niger* and found that the largest amounts of cellulases were obtained in medium supplemented with salts, a modified Mandels and Weber medium, added of carboxymethylcellulose inductor. Under these conditions, cellulase and endoglucanase activities were 0.4 and 21.0 IU/g of substrate, respectively, values representing 2.6 and 4-fold increases in their enzyme activities compared to the unsupplemented medium. The same behavior has been observed by Buswell *et al.* [28], Camassola *et al.* [43], Spier [44], Zhang *et al.* [45], Chandra *et al.* [46], Sanchez [47], Ahamed and Vermette [48] and Gao *et al.* [49].

4. Conclusions

The endophytic strains FB1, FB2, FB3 and FB4 of *Trichoderma reesei* can produce cellulases in media containing sugarcane bagasse, supplemented and unsupplemented, at pH 5.5 and 30°C.

The medium supplemented with salts proved to be more appropriate to induce cellulase production after 29 days of fermentation, with the strain FB4 having the best yield: 16.32 ± 2.65 IU/gram of fermented substrate.

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Conflict of Interest

The authors declare no conflict of interest.

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