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Cellulases produced by the endophytic fungus *Pycnoporus sanguineus* (L.) Murrill

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Cellulases are enzymes that act on the hydrolysis of cellulosic substrates and comprise a complex capable of hydrolyzing cellulose and producing glucose. This conversion can be performed by an enzymatic complex found in secretions of such microorganisms as fungi and bacteria. The challenge is to transform this conversion into a quicker and more cost-effective process. The objective of this study was to evaluate the activity of the cellulase produced by the fungus *Pycnoporus sanguineus* (L.) Murrill, strains D1-FB, D3-FB, D5-FB and D10-FB, isolated from *Baccharis dracunculifolia* D. C. (*Asteraceae*), in the production of cellulolytic enzymes. This study was conducted using sugarcane bagasse enriched with carboxymethylcellulose at 1% as a substrate. The material was kept in an incubator at a temperature ranging from 25 to 45°C for 43 days, with the cellulase activity being quantified every seven days. The indirect quantification method was employed to quantify reducing sugars that were determined by reaction with Domain Name System (DNS). After evaluation, it was observed that the fungus strain *P. sanguineus* (L.) (D10-FB) presented the highest cellulase activity, with values of 16.32±2.65 IU/g of fermented substrate after 29 days of fermentation, using sugarcane bagasse as the substrate, at a temperature of 30°C and pH 5.5.

Key words: Cellulase, sugarcane bagasse, cellulose, *Pycnoporus* sp.

INTRODUCTION

Among naturally occurring materials, cellulose is the most abundant biopolymer in the world (Bayer and Lamed, 1992) and can be hydrolyzed by acids into glucose. Microbial degradation of cellulose is total and specific and has encouraged the use of cellulolytic fermentation processes by man. In nature, these

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processes represent the greatest source of carbon for soil (Sakumaran et al., 2009; Sanchez, 2009).

The hydrolysis of cellulose by cellulases results in the final product of glucose. Because these are proteins, however, they cannot easily penetrate the lignin barrier of plant cells and, as such, the difficult access of these enzymes to the cellulose fibers is the main problem in triggering of the degradation process (Thiemann et al., 1980). The hydrolysis of cellulose can occur through physical, chemical, and enzymatic processes. Enzymes produced by fungi stand out among the enzymatic processes (Castro and Junior, 2010; Onofre et al., 2014).

In recent years, there has been growing interest in the use of agricultural waste to produce renewable fuels such as cellulosic ethanol. In addition to the large-scale production of ethanol from sugarcane in Brazil, the production of alcohol derived from lignocellulose is also a viable and sustainable alternative, especially in the context of an impending world energy crisis (Sakumaran et al., 2009; Sun, 2009; Mussatto et al., 2010; Shrestha et al., 2010; Bansal et al., 2012).

Currently, the surplus of sugarcane bagasse available for the production of second-generation ethanol lies between 7 and 10% of the total bagasse generated in Brazil. In the harvest of 2010/2011, the total production of sugarcane bagasse was estimated at 163 million tons. The surplus bagasse could reach as high as 50% after the proper optimization of the combustion system of independent distilleries (Balat and Balat, 2008; Bressan Filho, 2011; Onofre et al., 2014).

In addition to sugarcane bagasse, other substrates have been used to obtain large quantities of cellulolytic enzymes from microorganisms (Vitti, 1988). Agricultural waste can be properly milled or crushed to serve as a source of nutrients, mostly carbon in submerged fermentation. Among this waste, however, sugarcane bagasse has been the most widely employed because of the quantity of enzymes and protein enrichment produced by it. The bioconversion of bagasse has been proven to be potentially economically advantageous in such cases as the production of enzymes (Pandey et al., 2000; Pandey et al., 2005; Goncalves et al., 2008; Sakumaran et al., 2009; Arantes and Milagres, 2009; Limayem and Riker, 2012; Onofre et al., 2014).

The fungi that decompose cellulosic substances occur in the soil, colonizing plants, their roots and residues, and playing an important part in the recycling of nutrients. Microorganisms have a high capacity for multiplication; they can adapt to various nutritional media, and synthesize a variety of chemical substances such as enzymes (Andreaus, 2002; Goncalves et al., 2008).

The enzymes produced by fungi are biological catalysts consisting of protein molecules produced by living cells. These biocatalysts have high catalytic activity and specific selectivity on the substrate (Said and Pietro, 2004; Onofre et al., 2014).

Among microorganisms, fungi stand out in the production of enzymes of industrial interest. They are eukaryotic, uni- or multicellular, heterotrophic, chemoorganotrophic, and aerobic or microaerophilic organisms. Some have cell walls composed of chitin and cellulose. With respect to nutrition, fungi absorb food, accumulate glycogen as reserve substance, and occur in diverse life forms ranging from saprobionts, commensal organisms, and symbiotes to parasites (Teixeira et al., 1999; Limayem and Riker, 2012).

The fungus *Pycnoporus sanguineus* (L.) Murrill stands out for having applications ranging from popular medicine to alternative plant disease control methods, with a potential for inducing resistance when the extract of its basidiocarps is used (Viecelli et al., 2009). This fungus is a basidiomycete and an efficient producer of a polyphenol oxidade that acts on a variety of aromatic hydrogen donors called laccases. It has been successfully used in the fermentation of agro-industrial waste, the decolorization of Kraft effluent, and in various dyes (Valeriano et al., 2007).

Despite the limited number of publications on this fungus, there is a growing trend to employ it in biotechnological processes. The most recent report on the use of this fungus was in the fermentation of agro-industrial waste, with good results being obtained regarding the growth of the fungus, highlighting the cellulolytic, proteolytic, lipolytic, and amylolitic compounds (Bononi and Grandi, 1999; Ferraz, 2004; Garcia, 2006).

It is therefore imperative that more research be done on this fungus to obtain new products that can be applied in productive activity, especially the enzymes, which can be applied to various fields of agro-industry.

It is in this context that this study evaluated the capacity of the endophytic fungus *P. sanguineus* (L.) Murrill, isolated from *Baccharis dracunculifolia* D.C. (*Asteraceae*), to produce cellulolytic enzymes.

MATERIALS AND METHODS

Study location

The experiments were carried out in the Chemistry and Microbiology Laboratories of the institutions União de Ensino do Sudoeste do Paraná - UNISEP - Francisco Beltrão, Paraná, Brazil and Universidade Comunitária da Região de Chapeco - UNOCHAPECÓ – Chapecó, Santa Catarina, Brazil.

Microorganisms

The strains of the endophytic fungus *P. sanguineus* (L.) Murrill D1-FB, D3-FB, D5-FB, and D10-FB, isolated from *B. dracunculifolia* D.C. (Asteraceae), kept in the mycology collection of the Microbiology Laboratory of the Paranaense University - UNIPAR - Campus Francisco Beltran - PR. All the collections were authorized by IBAMA (Brazilian Institute for the Enviroment) under protocol number 13.234-2, August 1st 2006.

Determination of cellulolytic activity

Sugarcane bagasse, washed successively in running water to

completely remove the sugars, was used as the basic support to determine cellulolytic activity. The bagasse was dried in a drying oven until a constant weight was obtained. It was subsequently ground in a mill, packed, and stored in a dry place for further testing (Onofre et al., 2014).

Fermentation in the supplemented medium

A semi-solid culture medium consisting of 10 g of dehydrated sugarcane bagasse supplemented with 0.5 g/L carboxymethylcellulose, 1 g/L glucose, 3 g/L $NaNO_3$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 10 mg/L $FeSO_4.7H_2O$, 5.0 mg/L $ZnSO_4$, and 3 g/L 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, after which the first analysis of enzyme activity was carried out. Further analyses were then carried out every three days until 43 days of incubation were completed (Onofre et al., 2014).

Fermentation in the non-supplemented medium

A semi-solid culture medium containing 10 g of dried and ground sugarcane bagasse was used. 0.5% carboxymethylcellulose and 0.1% glucose in 120 mL of distilled water was added to it. The medium was then 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 seven days until day 43 of fermentation. As the first step to 22 days, after that every seven days.

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 3,000 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 (Moura et al., 2012; Santos et al., 2012; Onofre et al., 2014).

Determination of reducing sugars

Reducing sugars were determined by reaction with 3,5-dinitrosalicylic acid (DNS) (Onofre et al., 2012, 2014). In a basic medium and at high temperatures, 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 releases 1 µmol of reducing sugar, expressed as glucose, *per* minute, under test conditions (Alazard and Raimbault, 1981; Onofre et al., 2011; Onofre et al., 2014).

pH optimization

The enzymatic activity of the strains 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.

Effect of temperature

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

Statistical analysis

Results were submitted to variance analysis using the statistical analysis system assistant (Silva and Azevedo 2002). The comparison of means was done by the Tukey test at 5% probability.

RESULTS AND DISCUSSION

After obtaining the data for the production of cellulases by the four endophytic strains of *P. sanguineus*, this information was grouped together and entered into Tables 1, 2, 3, and 4, and in Figures 1 and 2. The values contained in Table 1 are linked to the enzymatic volumes produced by strains at different pH values.

Among the physical parameters, the pH of the growth medium played an important role by inducing morphological changes in the organism and also in the enzyme secretion. The change in pH observed during the growth of an organism also affected the stability of the product in the medium (Pandey, 2003; Sales et al., 2010).

The pH of the cultures varied because of reactions that occur during the metabolic activities of the microorganism. When organic acids such as acetic or lactic acid are secreted, they cause a decrease in pH. However, the consumption of these acids, when they are present in the medium, causes an increase in pH. The nitrogen source used may also cause variations in pH. Ammonia salts, for example, usually decrease pH during cell growth because of the formation of hydrogen ions during the consumption of ammonia (Doelle et al., 2002).

The performed tests revealed that the greatest production of the cellulolytic complex occurred in the four studied strains when the pH was 5.5. Decreases occurred at pH levels below 4.0, 6.0, and 6.5.

The solid state fermentation (SSF) processes are exothermic, as a lot of heat is released in direct proportion to the metabolic activity of the microorganism. In filamentous fungi, the temperature has a direct influence on the germination of spores, growth, and product formation. Temperature is a critical factor in practically all SSF processes because of the accumulation of generated metabolic heat (Pinto, 2003).

Sato and Sudo (1999) reported that the temperature affects both the growth and the production of enzymes, making an efficient removal of heat necessary. In this sense, Bianchi et al. (2001) add that, because of the metabolic activities of microorganisms and depending on the substrate layer, a large amount of heat can be

Table 1. Behavior of the four endophytic strains of *Pycnoporus sanguineus* in the production of cellulases under the influence of various pH values.

Isolates	pH assessed				
	4.0	5.0	5.5	6.0	6.5
D1-FB	2.20±0.22^{bB}*	3.21±0.40^{bB}	11.44±1.02^{aA}	4.49±0.15^{aB}	3.45±1.04^{aB}
D3-FB	4.12±0.13^{aB}	3.42±0.72^{bB}	7.33±1.33^{bA}	3.47±0.24^{aB}	2.14±0.13^{aB}
D5-FB	5.53±1.09^{aB}	6.38±1.04^{aB}	9.52±1.65^{aA}	3.74±0.28^{aC}	3.63±0.21^{aC}
D10-FB	5.59±0.05^{aB}	4.42±0.54^{bB}	10.40±1.78^{aA}	3.85±0.33^{aC}	2.22±0.32^{aC}

*Means followed by lowercase letters vertically and capital letters horizontally do not differ from one another for the level of 5% by the Tukey Test.

Table 2. Behavior of the four endophytic strains of *Pycnoporus sanguineus* in the production of cellulases under the influence of various temperatures at pH 5.5.

Isolates	Temperature evaluated (pH 5.5)				
	25	30	35	40	45
D1-FB	0.90±0.23^{bC}*	10.03±0.20^{bA}	9.31±0.69^{aA}	6.33±1.01^{aB}	1.55±0.33^{aC}
D3-FB	1.03±0.20^{bB}	12.25±1.78^{bA}	8.25±0.35^{aA}	5.43±0.78^{aB}	2.22±0.31^{aB}
D5-FB	1.22±0.45^{bC}	13.42±2.87^{aA}	9.88±0.93^{aA}	5.23±0.86^{aB}	3.67±0.23^{aC}
D10-FB	3.21±0.37^{aB}	9.21±2.09^{aA}	7.44±0.08^{aA}	6.21±0.68^{aA}	3.34±0.19^{aB}

*Means followed by lowercase letters vertically and capital letters horizontally do not differ from one another for the level of 5% by the Tukey Test.

produced during the SSF process. Because temperature directly affects the germination of spores, the growth and sporulation of microorganisms, and product formation, the produced heat should be immediately dissipated if increases in temperature are not to affect the process.

The data in Table 2 reveal that 30°C is the optimum temperature for the growth and production of celulolytic enzymes for the four endophytic strains of *P. sanguineus* studied. For temperatures below and above 30°C, one can observe that the enzymatic volumes decrease for all the strains under study. With the data obtained for optimum pH and temperature, it was possible to continue the study using a temperature of 30°C and a pH of 5.5.

It should be stressed that temperatures close to 30°C comprise a range of temperatures in which mesophilic microorganisms develop. These data are in line with the concepts of Buswell et al. (1998), who showed that 30°C is an ideal temperature for the production of the celulolytic enzymatic complex by fungi of the genus *Pycnoporus.* This behavior is consistent with those found by Jan and Chen (2003), Dalsenter et al. (2005), Mitchell et al. (2006), Gomes et al. (2007), Carvalho et al. (2008), and Taneda et al. (2012).

Regarding the optimal pH for the production of cellulolytic enzymes, Camassola et al. (2004) stated that the largest production of these enzymes by *Penicillium echinulatum* occurs at pH 5.5. Alam et al. (2008) optimized the cellulase production process by *P. sanguineus* through the bioconversion of the liquid state of domestic sewage sludge and also found maximum activity at pH 5.5. This same behavior was observed by Qin et al. (2008), Zhang et al. (2012), Bendig and Weuster-Botz (2012), and Puglisi et al. (2012).

An analysis of the data in Table 3 and Figure 1 revealed that the four evaluated strains presented the same behavior in the production of the cellulolytic complex, with the production of enzymes starting at the 22nd day of fermentation and reaching maximum production values at the 29th day of the process. From then on, a decline occurred in the volumes of produced enzymes.

The highest production occurred at the 29th day, with values of 14.33±2.56; 12.73±2.34; 13.73±1.86; and 16.32±2.65 IU/g of substrate for D1-FB; D3-FB; D5-FB; and D10-FB, respectively, with no significant differences in the Tukey test at the level of 5%.

When the behavior of the four strains of *P. sanguineus* shown in Tables 3 and 4, subjected to growth in a non-supplemented culture medium is considered, one can observe that the volumes of enzymes produced over the 43 days of fermentation were lower than the volumes produced by the four strains grown in a medium supplemented with salts.

The enzymatic volumes produced at 22 days were 1.58±0.76, 6.42±1.23, 9.50±2.20, and 7.65±1.95 for the strains D1-FB, D3-FB, D5-FB, and D10-FB, respectively. These enzymatic indices differ at a significance level of 5%, as the strain D1-FB produced volumes that were lower than the other strains under study.

It should be pointed out that these volumes are lower than the volumes produced by the same strains at the same time but in the presence of supplements based on

Table 3. Cellulase activity (IU/g of substrate) produced by endophytic strains of *Pycnoporus sanguineus* in a supplemented medium.

Isolates	22	29	36	43
D1-FB	1.58±0.76^{bC}*	14.33±2.56^{aA}	7.99±0.47^{bB}	3.14±1.45^{aB}
D3-FB	6.42±1.23^{aB}	12.73±2.34^{aA}	5.93±0.78^{cC}	2.21±0.36^{aC}
D5-FB	9.50±2.20^{aB}	13.73±1.86^{aA}	6.56±1.58^{aC}	3.06±0.82^{aD}
D10-FB	7.65±1.95^{aB}	16.32±2.65^{aA}	4.82±0.74^{cC}	2.12±0.89^{aC}

*Means followed by lowercase letters vertically and capital letters horizontally do not differ from one another for the level of 5% by the Tukey Test.

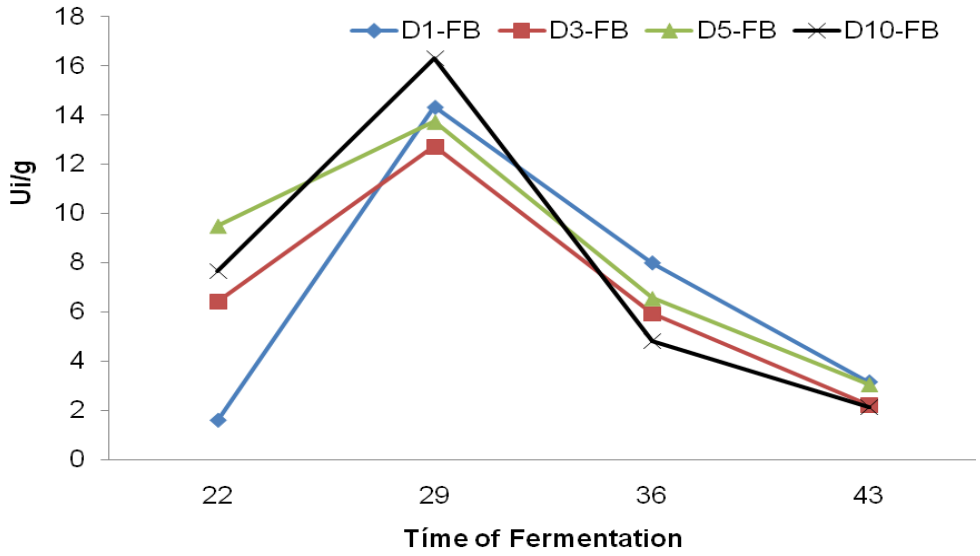

Figure 1. Behavior of the four endophytic strains of *Pycnoporus sanguineus* in the production of cellulases in a supplemented medium.

salts. The data in this study can be compared with data obtained by Aguiar (2008), who used the same culture conditions in 30 days of fermentation and observed that the fungus *P. sanguineus* produced 16.21 IU/g of the cellulolytic complex in a supplemented medium and 5.76 IU/g in a non-supplemented medium.

One can see similar data at 29 days when the endophytic strain FB4 produced 16.32 IU/g in a medium supplemented with salts and 7.93 IU/g in a medium that was not supplemented with salts. Basso et al. (2010), on the other hand, evaluated the cellulolytic activity of strains of *P. sanguineus* isolated from decomposing sugarcane bagasse and wood and observed values of 12.58 IU/g of non-supplemented substrate at a temperature of 28°C at 35 days. Once again, the data are similar to those found in this study, but when the behavior in the non-supplemented medium is considered, the values obtained in this study are lower, having reached 7.93 IU/g at 29 days (Table 4 and Figure 2).

The data obtained in this study are also similar to those obtained by Rifat et al. (2003) and Falkoski et al. (2012), who evaluated the crude enzymatic extract produced by *P. sanguineus* and observed that this fungus produces xylanases, cellulases, mananases, α-galactosidase, α-arabinofuranosidase, and pectinase in a culture medium, using a substrate based on sugarcane bagasse, at the same temperature and pH.

With respect to the substrate based on sugarcane bagasse supplemented with glucose, one can see by its having been present in both systems that it does not interfere with the production of cellulase. These results are in line with those of Rifat et al. (2003) and Castro (2006). They compared the production of endoglucanases and β-glucosidase by *P. sanguineus* and *Humicola grisea* when grown on insoluble (cellulignin from sugarcane bagasse and avicel) and soluble (carboxymethylcellulose and cellobiose) substrates.

They observed slower kinetics in the production of enzymes when insoluble sources were used, especially those of lignocellulosic origin, as a result of an initial period of acclimation of cells to this raw material. Regarding carboxymethylcellulose and cellobiose,

Table 4. Cellulase activity (IU/g of substrate) produced by endophytic strains of *Pycnoporus sanguineus* in a non-supplemented medium.

Isolates	22	29	36	43
D1-FB	1.79±0.23^{aC}*	10.85±0.34^{aA}	9.18±1.25^{aA}	3.24±0.65^{aB}
D3-FB	2.27±0.46^{aC}	8.02±1.45^{aA}	5.02±0.89^{bB}	3.95±0.79^{aB}
D5-FB	1.69±0.32^{aB}	9.88±0.79^{aA}	7.04±1.02^{bA}	3.67±0.54^{aB}
D10-FB	1.43±0.78^{bC}	7.93±0.68^{aA}	5.56±0.45^{bB}	2.83±0.34^{aC}

*Means followed by lowercase letters vertically and capital letters horizontally do not differ from one another for the level of 5% by the Tukey Test.

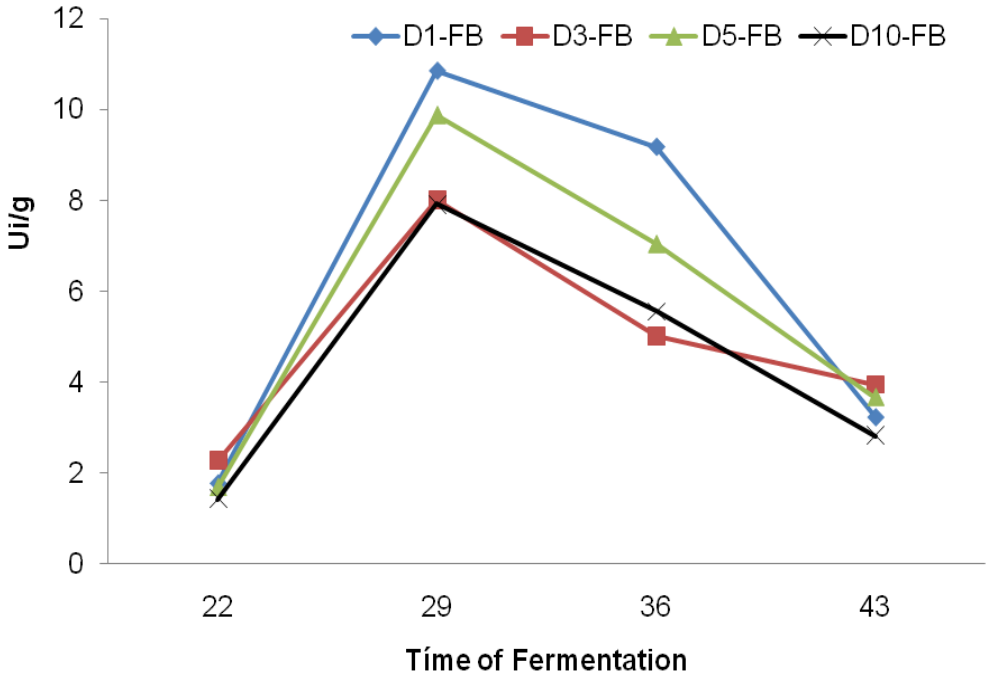

Figure 2. Behavior of the four endophytic strains of *Pycnoporus sanguineus* in the production of cellulases in a non-supplemented medium.

however, the times corresponding to the enzyme production were anticipated by approximately 50 h. It could therefore be suggested that the synthesis of the enzymes of the cellulolytic complex is not directly related to the carbon source used for induction, as cellulases were produced even when the strains were grown in the presence of cellobiose as a substrate, a synthesis that does not fit in the constitutive character.

The results obtained here were also in line with those reported by Rodríguez-Zú-iga et al. (2011). Working with *Aspergillus niger,* they found that the largest volumes of cellulases were obtained in a medium supplemented by salts, denominated as the modified Mandels and Weber medium (Mandels and Reese, 1960) with the addition of the inducer carboxymethylcellulose. Under these conditions, the activities of total cellulase and endoglucanase were 0.4 and 21.0 IU/g of substrate, respectively. These values represent increases of 2.6 and 4 times their respective enzymatic activities when compared to the medium without salt supplementation. This same behavior was observed by Buswell et al. (1998), Camassola et al. (2004), Spier (2005), Zhang et al. (2006), Chandra et al. (2007), Sanchez (2009), Ahamed and Vermette (2010), and Gao et al. (2011).

The results presented here suggest that the fungus *P. sanguineus* produces a cellulolytic complex with suitable characteristics for application in the saccharification of biomass and additional studies should be conducted to maximize the production of cellulases and hemicellulases.

Conclusions

The obtained results permit us to conclude that the endophytic fungus *P. sanguineus* isolated from *B.*

dracunculifolia, strains D1-FB; D3-FB; D5-FB and D10-FB are producers of cellulases in mediums based on sugarcane bagasse, whether supplemented or not, at pH 5.5 and a temperature of 30°C.

We can also conclude that the medium supplemented with salts proved to be a more appropriate medium to induce the production of cellulases at 29 days of fermentation and that the endophytic strain D10-FB of *P. sanguineus* stood out, producing 16.32±2.65 IU/gram of fermented substrate.

Conflicts of Interest

The author(s) have not declared any conflict of interest.

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