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Cellulase production by *Penicillium funiculosum* and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation

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ABSTRACT

This study aimed to produce a cellulase blend and to evaluate its application in a simultaneous saccharification and fermentation (SSF) process for second generation ethanol production from sugar cane bagasse. The sugar cane bagasse was subjected to pretreatments (diluted acid and alkaline), as for disorganizing the ligocellulosic complex, and making the cellulose component more amenable to enzymatic hydrolysis. The residual solid fraction was named sugar cane bagasse partially delignified cellulignin (PDC), and was used for enzyme production and ethanol fermentation. The enzyme production was performed in a bioreactor with two inoculum concentrations (5 and 10% v/v). The fermentation inoculated with higher inoculum size reduced the time for maximum enzyme production (from 72 to 48). The enzyme extract was concentrated using tangential ultrafiltration in hollow fiber membranes, and the produced cellulase blend was evaluated for its stability at 37 °C, operation temperature of the simultaneous SSF process, and at 50 °C, optimum temperature of cellulase blend activity. The cellulolytic preparation was stable for at least 300 h at both 37 °C and 50 °C. The ethanol production was carried out by PDC fed-batch SSF process, using the onsite cellulase blend. The feeding strategy circumvented the classic problems of diffusion limitations by diminishing the presence of a high solid:liquid ratio at any time, resulting in high ethanol concentration at the end of the process (100 g/L), which corresponded to a fermentation efficiency of 78% of the maximum obtainable theoretically. The experimental results led to the ratio of 380 L of ethanol per ton of sugar cane bagasse PDC.

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1. Introduction

The production of first-generation ethanol, such as from sugarcane in Brazil and from corn in US, is characterized by mature commercial markets and well understood technologies. In these countries, major producers, the ethanol production increased 4.5-fold between 2000 and 2010. Future targets and investment plans suggest strong growth will continue in the near future (RFA, 2012).

Many of the problems associated with first-generation ethanol can be addressed by its production from non-food crop feedstocks, which has been termed second-generation ethanol. Low-cost agricultural and forest residues, wood process wastes, and the organic fraction of municipal solid wastes can all be used as lignocellulosic feedstocks. Where these materials are available, it should be possible to produce ethanol and other chemicals with virtually no additional land requirements or impacts on food and fiber crop production (Sims et al., 2008). This second-generation ethanol production is under the spotlight with the hope it will soon become fully commercialized at the large production scale. However, as its technology of production is relatively immature, there is room for improvement for cost reductions, increase in ethanol yield and in enzyme efficiency as more knowledge is acquired.

The effectiveness of the fermentation of lignocellulosic materials presents two principal challenges. First, the crystalline structure of the cellulose, which is highly resistant to hydrolysis and the lignin–cellulose association, forms a physical barrier that hinders enzymatic access to the cellulose fibers. Additionally, cellulose acid hydrolysis requires the use of high temperatures and pressures, leading to the destruction of part of the carbohydrates, which are converted mainly to furanic compounds, and the generation of toxic substances by the partial degradation of lignin (Jacobsen and Wyman, 2000).

To make possible the use of lignocellulosic materials as feedstocks for the production of ethanol and other chemicals, according to the biochemical platform, it is necessary to separate their main components. For this separation, a pretreatment stage is essential, which aims at to disorganize the lignocellulosic matrix. The

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pretreatment can be realized through physical, physical–chemical, chemical or biological processes, and can be either associated or followed by hydrolysis procedures of the polysaccharides (hemicellulose and cellulose) in their respective monomeric units – pentoses and hexoses (Betancur and Pereira, 2010a,b).

For the cellulose component the enzymatic hydrolysis has been the tendency, since it takes place in moderate conditions of temperature and pressure, demanding low energy consumption, presents high specificity, and eliminates the generation of toxic substances, commonly found in the chemical hydrolysis. The conversion of cellulose into glucose requires the use of enzymatic pools, mainly composed of endo-glucanase, exo-glucanase and β-glucosidase, and accessory proteins, such as swolleins (Wood and Garica-Campayo, 1990; Henrissat, 1994; Lynd et al., 2002; Zhang and Lynd, 2004; Arantes and Saddler, 2010). They all have in common the ability of hydrolyzing the 1,4-β-glycosidic bond between the D-glucose molecules, but they differ in their starting point and substrate when hydrolyzing. Endoglucanases cleave the internal bindings of the cellulose fiber producing cellodextrins; exoglucanases act in the external region of the cellulose producing cellobiose; and β-glucosidases hydrolyse cellobiose and soluble oligosaccharides to glucose (Lynd et al., 2002).

These enzymes can be produced by several microorganisms. Studies in our laboratories have shown that the filamentous fungus *Penicillium funiculosum* has great potential for cellulases production, depicting a well-balanced amount of the main enzymatic activities (Castro et al., 2010; Maeda et al., 2010), particularly a higher content of β-glucosidase, a key-enzyme for second-generation ethanol production, when compare with commercial preparations. Furthermore, our group has been evaluating the performance of cellulase from *P. funiculosum* on enzymatic hydrolysis of pretreated sugarcane bagasse (Castro et al., 2010; Maeda et al., 2010, 2011). Maeda et al. (2011) used an enzymatic blend of this a strain of this filamentous fungus to hydrolyze pretreated sugarcane bagasse and reached a hydrolysis yield of 88% while the commercial blend (Multifect®) achieved a hydrolysis yield of 68% in the same experimental conditions.

This exceptional performance in cellulignin of sugar cane bagasse hydrolysis was ascribed to the fact that *P. funiculosum* is able to secrete a balanced cellulasic system (Rao et al., 1988; Castro et al., 2010). Jorgensen and Olsson (2006) reported that several species of *Penicillium* were shown to have the ability to produce a complete cellulasic system and a better ratio between FPase and β-glucosidase activities as compared to *Trichoderma reesei*.

According to Maeda et al. (2011) the FPase and β-glucosidase activities ratio in the crude extract of *P. funiculosum* is 1:3.8 and of Multifect® is 1:0.8. The high ratio of β-glucosidase activity to FPase is important in simultaneous saccharification and fermentation (SSF) process to avoid accumulation of the cellobiohidrolases inhibitor (cellobiose) in the reaction medium.

Nevertheless, for an efficient enzymatic hydrolysis of sugar cane bagasse, another pretreatment to remove lignin and thereafter improving the cellulose digestibility, i.e. making it more accessible to enzymes shows to be also necessary. According to Barcelos et al. (2012), the increase in NaOH concentrations up to 2% w/v resulted in a positive effect on enhancing cellulose hydrolysis efficiency. The alkaline pretreatment appeared to be essential since there was no improvement whatsoever in the enzymatic hydrolysis of untreated samples (sugar cane bagasse acid cellulignin) with increasing enzyme loads (25–150 FPU/g solids).

High cost of cellulase is one of the major hindrance to make the second-generation ethanol process commercialized. An alternative approach for reducing costs with cellulolytic enzymes is to obtain them by dedicated production (i.e. in plant production), eliminating costs with transportation, and developing better cellulase preparations for plant cell wall cellulose hydrolysis. Additionally, the dedicated production of these biocatalysts is within the context of biorefinery and circumvents their high import prices, providing a great motivation to develop the present work.

In the process of ethanol production from lignocellulosic biomass 4 steps are driven by biological processes. (1) The production of cellulase enzymes, (2) the hydrolysis of cellulosic material, (3) the fermentation of hexose sugars and (4) the fermentation of pentose sugars. The present work focuses the three first steps, with the conversion of cellulose to ethanol realized by the simultaneous saccharification and fermentation process (SSF), in order to avoid the classic inhibition of the enzymatic pool by the final products of its catalytic action. Thus, this investigation aims at the production of cellulase by a selected strain of *P. funiculosum* and the application of its producing enzymatic blend in SSF process for second generation ethanol production.

2. Materials and methods

2.1. Microorganism and media composition

The filamentous fungus *P. funiculosum* ATCC 11797 was maintained in potato dextrose agar medium (PDA). The mineral medium used for both pre-inoculum and production was composed of the following (in g/L): KH_2PO_4, 2.0; $CaCl_2{\cdot}2H_2O$, 0.4; $MgSO_4{\cdot}7H_2O$, 0.3; $FeSO_4{\cdot}7H_2O$, 5.0; $MnSO_4{\cdot}4H_2O$, 1.6; $ZnSO_4{\cdot}7H_2O$, 1.4 and $CoCl_2{\cdot}6H_2O$, 2.0 (Mandels and Weber, 1969). The nitrogen sources and concentrations used were those optimized by Maeda et al. (2010). Glucose (4 g/L) and sugar cane bagasse (15 g/L) after acid and alkaline pretreatment were added as carbon sources. The temperature and agitation were maintained at 30 °C and 200 rpm, respectively.

2.2. Sugar cane bagasse pretreatment

To enhance the microorganism accessibility to the substrate for cellulase and ethanol production a pretreatment was carried out with diluted sulfuric acid (1% v/v) with a solid:liquid ratio of 1:2 at 121 °C for 45 min, as reported by Betancur and Pereira (2010a,b). After sugar cane bagasse acid pretreatment, two fractions were obtained: a hemicellulose hydrolysate liquid phase and a solid fraction, composed mainly by cellulose and lignin, which was named as celluligin. These two fractions were separated by pressing filtration at 10 kgf/cm^2, and the cellulignin was even washed repeatedly with water until pH 5. The solid material was dried at 65 °C with forced air circulation overnight. The cellulignin was subjected to an alkaline pretreatment with sodium hydroxide solution (4% w/v) with a solid:liquid ratio of 1:20 at 121 °C for 30 min as established by Vásquez et al. (2007). The alkali pretreatment was performed in 6 L-conical flasks with a working volume of 4 L, containing the mixture of cellulignin/alkali solution. After thermal pretreatment, the solid fraction was separated with sieves of stainless steel and 0.5 mm mesh. The solid was washed with water to remove the residual lignin and the pH was adjusted to 5.0. The resulting solid material was called partially delignified cellulignin (PDC). For the determination of sugar cane bagasse composition and partially delignified cellulignin, a chemical hydrolysis was carried out with H_2SO_4 in two steps, according to National Renewable Energy Laboratory – NREL (Sluiter et al., 2008) and Ververis et al. (2007).

2.3. Cellulase production in an instrumented bioreactor

To obtain the pre-inoculum, 10^6 conidia/mL were inoculated in 1000 mL conical flasks containing 350 mL of culture medium and were incubated at 30 °C, 200 rpm for 72 h. Cellulase production was performed batchwise in an instrumented bioreactor (Biostat B, B. Braun Biotech International, Germany), which was inoculated

with 5 and 10% v/v of pre-inoculum. Pretreated sugar cane bagasse (20 g/L) was used as a carbon source for cellulase production. The nominal and working volumes were 10 and 7 L, respectively. The temperature, agitation and pH were maintained at 30 °C, 200–350 rpm and 5.0, respectively. Air was sparged at 0.5–1 vvm to provide dissolved oxygen concentrations within 20–40% of the saturated level. Samples were withdrawn every 12 h for quantification of FPase, Avicelase, CMCase and β-glucosidase activities, as described by Eveleigh et al. (2009), NREL (Adney and Baker, 1996) and Ghose (1987). Total protein content was measured using the Bio-Rad protein assay (Bio-Rad Laboratories), which is based on the Bradford method. BSA was used as standard. All experiments were performed in duplicate and the samples analyses were performed in triplicate.

2.4. Crude extract concentration

The crude extract was concentrated using a tangential filtration system with polysulfone hollow fibers membranes. First, a glass wool filtration was performed to separate the mycelium, and then, it was performed using a microfiltration cartridge with 0.2 μm porosity (CFP-2-E-4X2MA) followed by ultrafiltration using a membrane with a cut-off of 5 kDa (UFP-5-C-4X2MA). The micro and ultrafiltration was performed at room temperature, and the maximum pressure in the column inlet was 12 psi, using QuixStand QSM-03SP benchtop system, GE Healthcare. The enzymatic product obtained was coded by LADEBIO/BR (Laboratories of Bioprocess Development of the Federal University of Rio de Janeiro/Brazilian Oil Company-Petrobras) cellulase preparation.

The stability of the concentrated cellulase extract was measured at the temperatures that are used in the SSF process (37 °C and 50 °C). The cellulosic preparation was incubated at these temperatures, and samples were collected at regular time intervals to evaluate the cellulase activity.

2.5. Ethanol production from pretreated sugar cane bagasse using a simultaneous saccharification and fermentation process

The SSF process was performed in a Biostat B bioreactor (B. Braun Biotech Int.) with a nominal volume of 1.5 L, a working volume of 600 mL and a solid (g):liquid (mL) ratio of 200:420. The medium contained the necessary nutrients for the yeast *Saccharomyces cerevisiae*, and its composition was as follows (in g/L, including a 40 mL/L salt solution): urea, 1.25; KH_2PO_4, 1.10; and yeast extract, 2.00. The concentrated salt solution was composed of the following (in g/L): $MgSO_4 \cdot 7H_2O$, 12.5; $CaCl_2 \cdot 2H_2O$, 1.25; $FeSO_4 \cdot 7H_2O$, 0.9; $MnSO_4$, 0.19; $ZnSO_4 \cdot 7H_2O$, 0.3; $CuSO_4 \cdot 5H_2O$, 0.025; $CaCl_2 \cdot 6H_2O$, 0.025; $NaMoO_4 \cdot 2H_2O$, 0.035; H_3BO_3, 0.05; KI, 0.009; $Al_2(SO_4)_3$, 0.0125. Additionally, the medium pH was maintained with a solution of sodium citrate buffer (25 mM, pH 5.0).

The vessel containing PDC (100 g) and the medium containing the other components were sterilized separately at 110 °C during 20 min. They were gathered together and after cooling to 50 °C, the LADEBIO/BR cellulase preparation (40 mL) was added at a total enzyme load of 12.5 FPU/g of total PDC. The concentrated cellulase blend used in this study had activities of 63.1 U/mL FPase, 602.0 U/mL CMCase and 140.6 U/mL β-glucosidase.

First, a pre-hydrolysis was performed with 100 g of bagasse at 50 °C and pH 5.0 as well as agitation of 200 rpm for 12 h. After liquefaction, the temperature was decreased to 37 °C. The industrial yeast strain *S. cerevisiae* JP1 was inoculated (15 g/L), and the system was fed with 50 g of partially delignified cellulignin. At 24 h intervals, two more feeds with a load of 25 g each were supplied, totaling 200 g of solids.

The results were evaluated regarding yields in: (a) tons of pretreated sugar cane bagasse to produce cellulase used to hydrolyse 1 ton of pretreated sugar cane bagasse; (b) liters of ethanol produced per ton of sugar cane bagasse; (c) liters of ethanol produced per total ton of sugar cane bagasse that includes the biomass used to enzyme production; (d) percent of theoretical ethanol that was produced using the following equation:

$$\%\text{ of theoretical ethanol} = \frac{\text{ethanol produced (g/L)}}{\text{PDC (g/L)} \times 0.68 \times 1.11 \times 0.511} \times 100$$

where PDC: PDC concentration (g/L), 0.68: celulose content in the PDC (g/g), 1.11: factor considering water molecule joing to the anhydro glucose residue in cellulose, and 0.511: theoretical ethanol yield.

Table 1
Composition of sugar cane bagasse and partially delignified cellulignin after acid pre-treatment followed by pre-treatment with 4.0% of NaOH.

Components	Sugar cane bagasse (% w/w)	Partially delignified cellulignin (% w/w)
Cellulose	34.1 ± 1.2	68.0 ± 1.3
Hemicellulose	29.6 ± 1.4	12.2 ± 0.9
Lignin	19.4 ± 0.4	9.3 ± 0.6
Ash	7.9 ± 1.1	3.5 ± 0.4
Moisture	4.4 ± 0.1	4 ± 0.2

2.6. Analytical methods

The samples withdrawn during the SSF process were centrifuged at 10,000 × g for 15 min and the supernatant was collected for analysis. The concentrations of glucose, cellobiose and ethanol were measured using high-performance liquid chromatography (instrument manufactured by Waters) with a 2414 refractive index detector and an HPX87P column. MiliQ water was used as the mobile phase at a flow rate of 0.6 mL/min.

3. Results and discussion

Lignin represents one of the main obstacles to using lignocellulosic materials in biotechnological applications based on lignocellulosic biomass (Reyes et al., 1998). The purpose of pretreatment is to remove part of the lignin and hemicellulose, reduce cellulose crystallinity and enhance porosity (Sun and Cheng, 2002; Zhang and Lynd, 2004). Table 1 presents the experimentally determined composition of sugar cane bagasse and partially delignified cellulignin obtained in our study. The sum of all components (95.5 ± 4.3% and 97.0 ± 3.4% for sugar cane bagasse and cellulignin, respectively) was close to 100%, taking into account experimental error and extractives that were partially unaccounted for.

The results show that the pretreatments (acid and alkaline) were efficient in reducing the hemicellulose (from 29.6 to 12.2%) and lignin (from 19.4 to 9.3%) fractions, with a concomitant increase in the cellulose content of cellulignin from 34.1% to 68.0%.

The kinetic profile of cellulase production with different inoculum concentrations is shown in Fig. 1. The use of a higher inoculum size (10% v/v) resulted in increased enzyme titer and volumetric productivity. Percentage increases of 88%, 84% and 42% for FPase, Avicelase and CMCase activities, respectively, were achieved using the highest inoculum concentration. Regarding β-glucosidase activity, there was only a slight increase of 4% when the inoculum concentration was raised. In addition, a reduction in the time of enzyme production (from 72 to 60 h) was obtained by using a higher inoculum size, and, consequently, the volumetric productivity (Q_P) was increased (Table 2).

Likewise, the protein content was higher and the production was faster in the bioreactor that was inoculated with 10% v/v of pre-inoculum (Fig. 2). The increase in cellulase activity in the medium was concomitant with an increase in the production of

Table 2
Volumetric productivity (U/L h) of cellulases.

Fermentation time (h)	5% v/v of pre-inoculum			10% v/v of pre-inoculum		
	FPase	CMCase	β-Glucosidase	FPase	CMCase	β-Glucosidase
12	1.7	11.5	1.4	1.4	14.8	2.3
24	1.2	10.8	1.6	1.8	43.8	3.4
36	2.0	34.2	2.9	4.3	100.5	6.2
48	2.7	49.8	6.6	13.1	189.6	37.0
60	4.5	99.7	13.6	15.0	179.5	31.0
72	6.2	106.7	22.4	12.6	154.5	26.2
84	5.7	93.3	21.6	nd	nd	nd

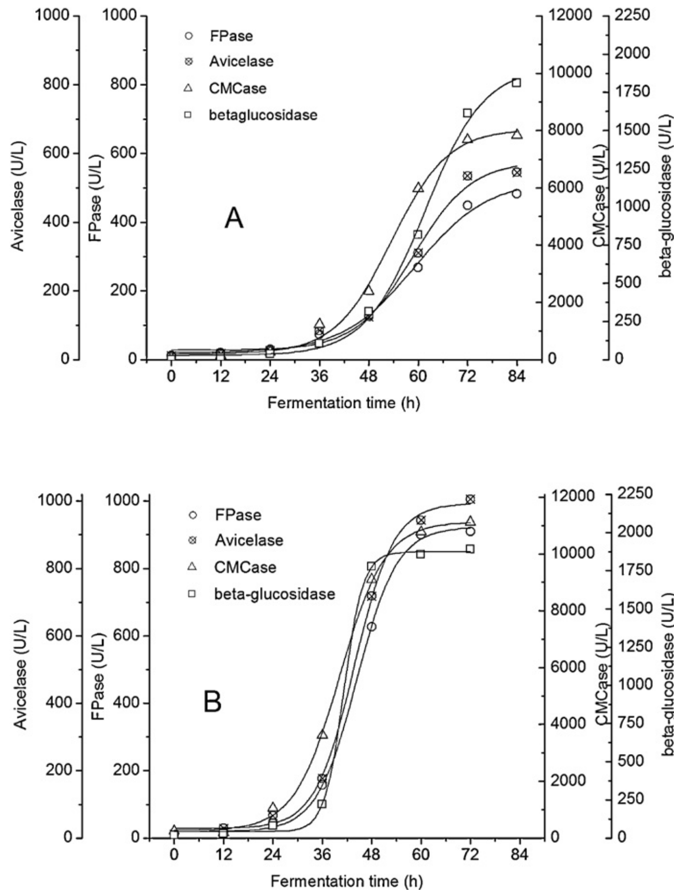

Fig. 1. Kinetic profiles of cellulase production in bioreactors with 5% v/v (A) and 10% v/v (B) of pre-inoculum.

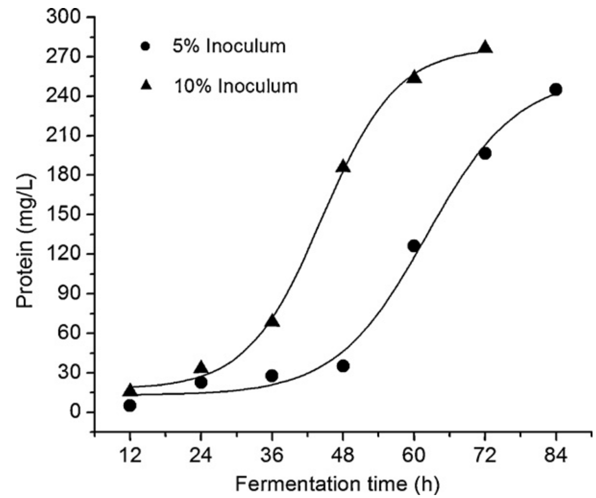

Fig. 2. Kinetic profiles of protein production in bioreactors with 5% v/v (A) and 10% v/v (B) of pre-inoculum.

protein that was secreted by the microorganism. The specific FPase and Avicelase activities remained roughly steady throughout the fermentation, which indicated that the exoglucanase enzyme production was associated with protein secretion (Fig. 3). However, the specific CMCase activity increased up to 36 h (with 10% v/v of inoculum) and 48 h (with 5% v/v of inoculum) and displayed an increased proportion of endoglucanase with respect to the total amount of proteins that were produced. After the period of maximum specific activity for CMCase, there was a decline that was triggered by the production of other proteins. The decrease in CMCase activity coincided with the increase of β-glucosidase specific activity.

The higher production of endoglucanase (estimated by CMCase activity) in the initial phase (36–48 h), the constant production of exoglucanase (estimated by Avicelase activity) throughout the fermentation period, and the greatest proportion of β-glucosidase after 36–48 h confirmed that there was a synergistic mode of action

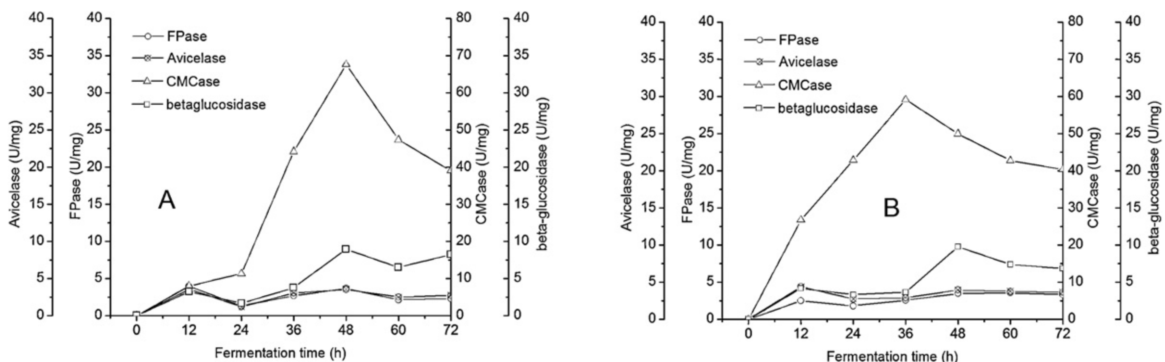

Fig. 3. Specific activities of cellulases produced by *P. funiculosum* in bioreactors with 5% v/v (A) and 10% v/v (B) of pre-inoculum.

of the cellulases, which act in two distinct phases: primary and secondary hydrolysis. The primary hydrolysis occurs on the surface of solid substrates by releasing soluble sugars with a degree of polymerization of 9–6 units, in the liquefaction stage, by the action of endoglucanases and release of cellobiose by the action of exoglucanase. Secondary hydrolysis, which occurs during the post-liquefaction stage, involves the hydrolysis of cellobiose to glucose by β-glucosidase (Zhang and Lynd, 2004). These differences in the proportions of cellulases that are produced may be due to factors such as chemical changes in the culture medium during fermentation (increase and/or decrease in the inducing substance) or by the physiological needs associated with cell growth.

The results obtained in the present study showed to be superior when compared with those reported by Castro et al. (2010), who produced cellulase from *P. funiculosum* using microcrystalline cellulose as substrate. After optimization of nitrogen and mineral sources, the activities reached by Castro et al. (2010) in shake flasks were as follows: 0.17 U/mL of FPase, 4.4–5.6 U/mL of CMCase and 1.3 U/mL of β-glucosidase), and the activities obtained in bioreactor were closer to these values. Furthermore, the production time herein reported is shorter (60–72 h) when compared to that reported by Castro et al. (2010) (120 h), resulting in a high productivity value of the cellulase production.

Castro et al. (2010) reported the production of cellulase by the same strain of *P. funiculosum* using sugar cane bagasse PDC as substrate, resulting in the following enzymatic activities (in U/mL): 0.25 of FPase; 1.80 of CMCase and 0.80 of β-glucosidase. The enzymatic activities were increased and the production time was reduced by Maeda et al. (2010), who defined different medium composition and reaction conditions by adopting the experimental design planning techniques.

3.1. Cellulase activity and stability of the concentrated extract

Regarding the cellulase activities in the concentrated extracts that were evaluated using the hollow fiber membrane tangential ultrafiltration system (Table 3), it was found that as the pore size diminishes, the losses are reduced. Membranes with a pore size of 10 kDa allowed the passage of molecules up to 30 kDa. Thus, the lower-molecular-weight cellulases were lost during the concentration process using this cut-off, depicting significant losses of 22% to FPase, 13.3% to CMCase and 5.6% to β-glucosidase activities.

To reduce the losses, a membrane with a smaller pore size (5 kDa) was used, which allowed recovery of almost all cellulase activity and only a 3% loss in the CMCase activity. Therefore, tangential ultrafiltration in the hollow fiber membrane with a cut-off of 5 kDa was ideal and was adopted for the LADEBIO/BR cellulase preparation.

Fig. 4 shows the enzyme activities for the incubation periods at 37 °C and 50 °C. The *P. funiculosum* enzymatic blend displayed good stability over 6 days of incubation considering the 3 evaluated activities. The high enzymatic preparation stability is of importance

Table 3
Cellulase activities (U/mL) at different steps of concentrations on hollow fiber membranes at cut-off of 5 kDa.

	Activities (U/mL)			Volume (mL)
	FPase	CMCase	β-Glucosidase	
Crude extract	1.135	10.252	2.260	6350
Microfiltrated	0.961	9.664	2.200	6300
Microfiltration residue	0.234	0.981	0.584	100
Concentrated extract	63.066	601.995	140.562	98
Permeate	0.030	0.194	0.003	6250
Theoretical activity	61.810	621.272	141.429	
Losses (%)	0.0	3.1	0.6	

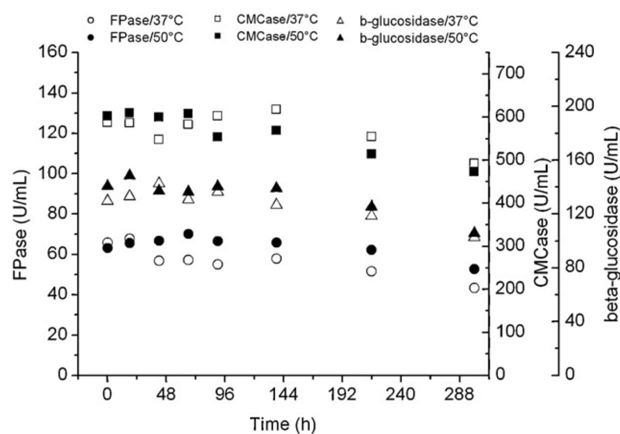

Fig. 4. Stability of the enzyme blend LADEBIO/BR incubated at 37 °C and 50 °C.

because its use in the SSF process requires good catalytic activity and stability at 50 °C (pre-hydrolysis) and at 37 °C (fermentation stage simultaneously to hydrolysis).

3.2. Simultaneous saccharification and fermentation processes using cellulase from P. funiculosum

Several authors (Rudolph et al., 2005; Vásquez et al., 2007) reported that during the SSF process, the ethanol concentration was limited by the concentration of solids due to diffusion limitations of mass transfer, thus leading to a decrease in the hydrolysis yield and an inhibition of cellulose hydrolysis. To solve this problem, a fed-batch SSF process was adopted. After pre-hydrolysis, the solids were fed at 24 h intervals such that the solid:liquid ratio remained low enough to minimize the classic problems that arise due to diffusion limitations.

Fig. 5 shows the profiles of ethanol production and glucose and cellobiose consumption in the fed-batch SSF. The vertical gray strip indicates the pre-hydrolysis stage (12 h), after which the SSF process starts. The solids were added at the times indicated by arrows. At the end of the pre-hydrolysis stage, the glucose concentration was 64 g/L, and the medium was liquefied. Inoculation using yeast was possible, which was accompanied by a solid load of partially delignified sugarcane bagasse cellulignin. The ethanol volumetric productivity (Q_P) diminished during the process. This behavior reflected a gradual reduction in the rate of product formation. According to Vásquez et al. (2007), enzymatic hydrolysis is the

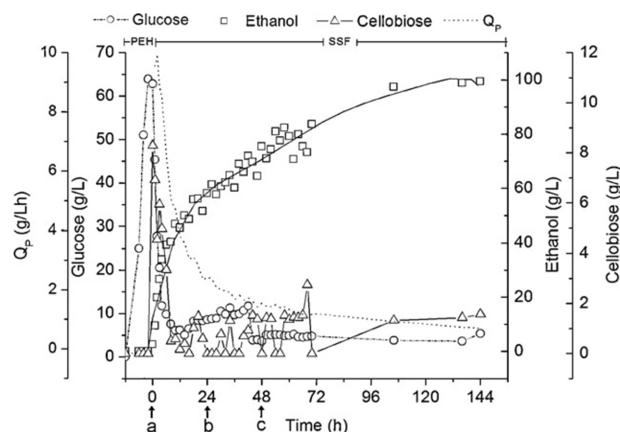

Fig. 5. Kinetic profile of ethanol production from partially delignified cellulignin using a *Penicillium funiculosum* enzymatic preparation (LADEBIO/BR) in the fed-batch mode of the SSF process. PEH: pre-enzymatic hydrolysis, SSF: simultaneous saccharification and fermentation; a, b and c: times of feeding.

limiting step of the SSF process, particularly when the medium to be fermented is inoculated with high cell concentrations, which causes the yeast to consume glucose at high rates. The volumetric productivity in the first 8 h of fermentation, the phase in which glucose is readily available, ranged from 5 to 9 g/L h (the same values obtained during conventional ethanol fermentations from sugarcane juice in Brazil, 5–8 g/L h). Therefore, the rate of ethanol production from cellulose became limited by the rate of hydrolysis beyond the first 8 h of fermentation, when glucose concentration was maintained at low levels.

According to Rudolf et al. (2005) and Sassner et al. (2006), the hydrolysis rate in many reported cases of SSF processes is the ruling rate that governs the SSF process. Olofsson et al. (2008) did not observe a strong positive correlation between cell concentration and ethanol production in processes inoculated with 1–2 g/L for typical conditions of SSF (10% solids and 30 FPU/g). Therefore, the inoculum size in the case of the present work (15 g/L) can be reduced in this process.

In addition to the great performance of the cellulase blend on enzymatic hydrolysis of sugarcane bagasse cellulignin, the high ethanol concentration that was achieved in this study signaled the possible use of this enzymatic blend for second generation ethanol production.

Based on the ethanol produced, it was possible to estimate that in the experimental conditions used, having added 200 g of PDC throughout the process, 81.9% of it was consumed. The remaining 18.1%, which were not utilized, may be due to the non accessible cellulose associated with residual lignin or with non productive enzyme linkages.

Concerning the fed-batch SSF, the results attained in this study were preeminent when compared with recent studies that have been reported in the literature (Zhu et al., 2010; Jorgensen et al., 2010; Ruiz et al., 2006; Santos et al., 2010; Zhang et al., 2009; Xu et al., 2010; Öhgren et al., 2007; Ballesteros et al., 2006; Linde et al., 2006). Most of the studies that employed the SSF process used low initial solid concentrations (less than or equal to 10%). In this study, high solid concentrations were used to ensure high product concentrations, as this is a *sine qua non* condition for the technical and economic feasibility of any fermentative process that primarily involves the production of a low value-high volume substance, which in this case was ethanol.

4. Conclusions

In addition to the good performance of the filamentous fungus *P. funiculosum* cellulase blend on sugar cane bagasse cellulignin (high stability, well balanced endoglucanase–exo glucanase and β-glucosidase activities, high glucose concentration during pre-enzymatic hydrolysis), the high ethanol concentration achieved in this study (100 g/L), during the simultaneous saccharification and fermentation, by an industrial strain of *S. cerevisiae* point to a possible use of this enzymatic product for the production of second generation ethanol at a productive scale.

Considering the experimental results herein obtained, involving the cellulase production, the use of the enzymatic blend in the simultaneous saccharification and fermentation for second generation ethanol production (only from the cellulose fraction), it was possible to do some estimates: an amount of 210 kg of sugar cane bagasse PDC is necessary for producing sufficient cellulase (12.5×10^6 FPU) to hydrolyse one ton of sugar cane bagasse PDC being intended for second generation ethanol production. Also, for a total solid concentration (PDC) of 33.3% w/v, an ethanol concentration of 100 g/L was obtained, which leads to the ratio of 380 L of ethanol/ton of sugar cane PDC.

When the ethanol production is evaluated taking into account the whole sugar cane bagasse, which yielded 350 kg of PDC, a ratio of 135 L/ton is attained. However, not considering enzyme recycling or immobilization, 17% of PDC should be used for enzyme production and the remaining 83% for ethanol production. Thus, in the experimental conditions used in the present work, 110 L of ethanol is estimated to be produced from each ton of sugar cane bagasse, considering only the cellulose component. Finally, thinking over the SSF process, the fermentation efficiency was 78% of the maximum obtainable theoretically.

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