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Biofpr
Biofuels Bioproducts & Biorefining

Use of an inexpensive carbon source for the production of a cellulase enzyme complex from *Penicillium ucsense* S1M29 and enzymatic hydrolysis optimization

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Received October 4 2023; Revised January 3 2024; Accepted January 8 2024;
View online 21 February 2024 at Wiley Online Library (wileyonlinelibrary.com);
DOI: 10.1002/bbb.2595; *Biofuels, Bioprod. Bioref.* 18:1137–1151 (2024)

Abstract: The high cost of cellulolytic enzyme complexes (CECs) has been a significant impediment to the commercial production of bioproducts from lignocellulose biomass. This study aimed to develop a cost-effective CEC derived from *Penicillium ucsense* (former *Penicillium echinulatum*), utilizing diverse forms of pretreated sugarcane bagasse as the primary carbon/inductor source. Among the different pretreatments used, the hydrothermal pretreatment followed by NaOH delignification (BHD) produced higher FPase and xylanase activities (4.5 FPU mL^{-1} and 120 IU mL^{-1}) in bioreactor experiments at 20 g BHD L^{-1} initial concentration. A batch-mode assay conducted across a range of initial carbon source (5 to 60 g L^{-1}) confirmed the highest FPase activity (4.0 to 5.0 FPU mL^{-1} at 120 h), in the range of 20–40 g BHD L^{-1}. During these assays the agitation rate, controlled by dissolved O_2, tended to stabilize at lower levels, indicating substrate limitation. Conversely, higher initial carbon source concentrations led to an excess of glucose, likely triggering carbon catabolite repression and inhibiting cellulase production. This insight prompted the development of a controlled pulsed fed-batch strategy, resulting in FPase activity of 11 FPU mL^{-1} at 220 h using 90 g L^{-1} BHD controlled fed into the bioreactor. An enzymatic hydrolysis procedure using the generated CEC was also optimized using a central composite rotational design (CCRD). The optimized enzyme hydrolysis conditions achieved a reducing sugar concentration of 80.9 g L^{-1} in 48 h using 170 g L^{-1} of BHD as the substrate at a ratio of 15 FPU of enzyme substrate per g of BHD.

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A preliminary economic assessment demonstrated that, for a first- and second-generation (1G+2G) ethanol biorefinery, the cost contribution of enzymes would be about US$0.2/L of biofuel. In conclusion, an efficient and highly productive procedure was developed successfully for the production of a CEC. It was particularly effective for the enzymatic hydrolysis of pretreated sugarcane bagasse. © 2024 Society of Industrial Chemistry and John Wiley & Sons Ltd.

Supporting information may be found in the online version of this article.

Key words: *Penicillium echinulatum*; pulsed fed batch; low-cost carbon source; high celullase productivity; enzymatic hydrolysis optimization

Introduction

The production of biofuel, biopolymers and other bioproducts from lignocellulosic biomass material (LCB) has been claimed as one of the solutions for the transition economy to a nonfossil oil alternative. Lignocellulosic biomass material is a promising alternative to petroleum-based feedstocks for the production of valuable fuels, chemicals, and materials. Terrestrial plant biomass, in particular, is an abundant and renewable source of complex carbohydrates (CCs) such as cellulose, hemicellulose, starch, and other hydrocarbons (i.e. polysaccharides, lignin, and low molecular weight extractives).[1–4]

To ensure availability for the various microbial species utilized in bioprocessing, primarily converting carbonaceous compounds (CC) into valuable bioproducts, a cascade of unit operations must be implemented. Enzymatic hydrolysis of previously pretreated lignocellulosic biomass (LCB) plays a crucial role in this chain. The enzymatic hydrolysis step is carried out with the participation of a set of glycohydrolases known generically as cellulases, which are divided into two groups according to their site of action on cellulose: endoglucanases and exoglucanases. Endoglucanases (EC 3.3.1.4) randomly hydrolyze the internal regions of the cellulose molecule, with greater affinity for amorphous regions. This action results in the formation of cello-oligosaccharides with varying degrees of polymerization, with the consumption of a water molecule and the formation of a new reducing and nonreducing terminal. These are the enzymes responsible for initiating hydrolysis. These enzymes reduce the degree of polymerization of the cellulose molecule. The group of exoglucanases is made up of cellobiohydrolase and glucanhydrolase. Glucanhydrolase (EC 3.2.1.74) is little reported but it has an important cellulosic fiber hydrolysis role as it is capable of releasing glucose directly from the polymer. Cellobiohydrolase (EC 3.2.1.91) participates in the primary hydrolysis of cellulose fiber and is responsible for amorphogenesis, a phenomenon that involves a physical rupture of the substrate, leading to the destratification of the fibers, due to the increase in interstitial regions.

Cellobiohydrolase can further be divided into two types: I and II. Type I exoglucanases hydrolyze reducing terminals and type II hydrolyze nonreducing terminals. Cellobiohydrolases (EC 3.2.1.91) are inhibited by the product of their hydrolysis – the glucose dimer called cellobiose. Cellulose degradation is completed with the action of cellobiase (β-glucosidase – EC 3.2.1.21) to hydrolyze cellobiose to 2 mol of glucose. Cellobiase is inhibited by its hydrolysis product. The correct level of this enzyme in an enzyme preparation avoids the inhibition of cellobiohydrolases and the release of the product of interest, glucose, in the greatest possible quantity.[5,6]

The so-called cellulolytic enzymatic complex (CEC) is responsible for this step and it is produced mainly using submerged fermentation in large-scale well-controlled bioreactors with saprophytic filamentous fungi belonging to the genera *Trichoderma*, *Penicillium*, and *Aspergillus*. The CECs are produced during the growth of these filamentous fungi by induction, typically using lactose, sophorose, cellobiose, soluble oligomers of β, 1–4 glucose, or cellulose.[7]

The wild-type strain of *Penicillium echinulatum*, renamed as *Penicillium ucnenses*, was isolated from the digestive tract of the *Anobium punctatum* beetle larva in 1979 in Caxias do Sul, Brazil, and deposited in the culture collection of the Laboratory of Enzymes and Biomass (University of Caxias do Sul; Caxias do Sul, Brazil) under accession number 2HH. This wild-type strain was also deposited in the Centraalbureau voor Schimmelcultures (CBS) culture collection housed in the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands) under accession number CBS 146492 in 2020, and the Culture Collection of Filamentous Fungi (CCFF), housed in the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) under accession number IOC 4717 in 2022. Its first mutant strain was deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as *P. echinulatum* DSMZ 343443. The mutant strain S1M29

derived from DSMZ 343443 was obtained by mutagenesis induced by hydrogen peroxide and was deposited in the culture collection of the Laboratory of Enzymes and Biomass (University of Caxias do Sul; Caxias do Sul, Brazil) under accession number S1M29.[8,9]

In past years, secretome studies of either the wild strain and the mutant strain S1M29 revealed that the its enzymatic repertoire is geared mainly toward enzymes (from the cellulose complex (endogluganases, cellobiohydrolases and β-glucosidases). Glycoside hydrolase (GH) family members, important to biomass conversion strategies, were identified, including endoglucanases GH5, 7, 6, 12, 17 and 61, β-glycosidase GH3, xylanases GH10 and GH11, as well as debranching hemicellulases from GH4 3, GH62 and CE2, and pectinases from GH28.[10,11] It was also demonstrated that the level of expression of these protein are substrate-type dependent.[12]

Penicillium echinulatum has the ability to produce a complete set of cellulolytic enzymes with high specific activity against filter paper activity and the ability to hydrolyze pretreated sugarcane residue at a high yield, making it a strong candidate for the industrial production of cellulolytic enzyme complexes.[13,14] Some studies demonstrated that the mutant strain had considerable potential for the production of cellulolytic enzymes in submerged fed-batch fermentation using commercial cellulose (Cellufloc 200, Celuflok Indústria e Comércio, São Paulo, Brazil) as carbon/inductor source.[15]

However, high cost of carbon source, processing and formulation, and logistical cost of transportation has pointed to on-site enzyme production using lignocellulosic material as a carbon source as a means of minimizing its production cost.[16–20]

The aims of the present study were therefore as follows: (a) the selection of a low-cost pretreated sugarcane waste substrate for use as carbon/inductor source by *P. echinulatum* S1M29 aiming for onsite enzyme production; (b) the development of an intensified process for cellulolytic enzyme production in submerged fermentation using a low-cost substrate; (c) the assessment of the hydrolytic potential of the enzyme complex produced using standard pretreated sugarcane bagasse.

Materials and methods

Microorganism

The mutant strain *Penicillium echinulatum* S1M29[9] was provided by the University of Caxias do Sul (Rio Grande do Sul, Brazil). The filamentous fungus was kept on potato dextrose agar (PDA) plates at 5 °C and was subcultured every 2 months.

Preparation of the inoculum

Spores of *P. echinulatum* S1M29 grown on PDA plates for 7 days at 29 °C were scraped into 20 mL of a solution of Tween 80 (0.1% w/v), in a sterile environment, and were transferred to 500 mL Erlenmeyer flasks containing 200 mL of culture medium, followed by incubation on a rotary shaker at 29 °C, at 250 rpm, for 72 h. The culture medium was composed of 10 g L^{-1} commercial micropulverized cellulose Cellufloc 200 (CEL), 10 g L^{-1} glucose, 1 g L^{-1} peptone, 1 mL L^{-1} Tween 80, and 100 mL L^{-1} saline solution as described.[15] The culture media were sterilized by autoclaving and the pH was adjusted to 5.0. For the experiments, the prepared inoculum was transferred to shake flasks and bioreactors at a ratio of 10% (v/v).

General conditions of bioreactor experiments

Submerged fermentation bioreactor cultivation was carried out in a mechanically agitated and aerated bioreactor with a 3 L total volume (1.5 L working volume). The vessel with culture medium was previously sterilized by autoclaving at 121 °C for 30 min, cooled to 29 °C, and the pH was adjusted automatically and controlled at pH 6.0 with aqueous 3:1 NH_4OH solution and 1 mol L^{-1} H_2SO_4.

The dissolved oxygen concentration was set at 30% of air saturation and was controlled automatically using airflow rates up to 1.0 vvm and mechanical agitation at up to an agitation rate of 600 min^{-1}. All these experiments used the basic culture medium optimized to enhance FPase activity, described by Costa *et al.*[12] This culture medium was composed of 5 g L^{-1} soybean flour, 1.5 g L^{-1} yeast extract, 1 mL L^{-1} Tween 80, and 100 mL L^{-1} of salt solution with the following composition: 20 g L^{-1} KH_2PO_4; 14 g L^{-1} $(NH_4)_2SO_4$; 3 g L^{-1} urea; 3 g L^{-1} $MgSO_4{\cdot}7H_2O$; 3 g L^{-1} $CaCl_2$; 0.05 g L^{-1} $FeSO_4{\cdot}7H_2O$; 0.014 g L^{-1} $ZnSO_4{\cdot}7H_2O$; 0.016 g L^{-1} $MnSO_4{\cdot}H_2O$; 0.02 g L^{-1} $COCl_2$.

Effect of carbon source

The effect of type of carbon source on the enzyme (FPase, xylanase, and β-glucosidase) activity kinetics was determined in batch mode experiments performed with 20 g L^{-1} initial concentrations of the following carbon/inductor sources: Cellufloc 200™ (CEL), steam exploded sugarcane bagasse (BEX), steam exploded and hot NaOH delignified bagasse (BED) according to literature,[21] and hydrothermally pretreated and hot NaOH delignified bagasse (BHD), which consisted of heating by water vapor at 140 °C for 10 min followed by a delignification step with NaOH. The materials were dried at

room temperature, milled, sieved using a 2 mm screen (N° 10 mesh), and stored in plastic bags until used. Compositional analyses (considering cellulose, hemicellulose, lignin, and ash) were performed using standard methodologies.[21] The bioreactor conditions and culture medium were the same as those described in the previous section. The experiments were performed at least in triplicate and the results were reported as means and standard deviations.

Effect of BHD concentration

Batch bioreactor experiments were performed with increasing initial BHD concentrations (10, 20, 30, 40, and 60 g L^{-1}) in order to understand the effect of carbon source concentration on enzyme activity (FPase, xylanase, and β-glucosidase) and biomass concentration over time. The bioreactor conditions and culture medium employed were the same as described above.

Bioprocess intensification and CEC production

A pulsed fed-batch approach was used, as described below based on the results of the experiments mentioned in the previous section. After production, microorganism cells were separated by centrifugation (4000×*g*, 4 °C, for 15 min) and the supernatant collected was afterward concentrated ten times in Amicon Stirred Cell with a 10 kDa cutoff membrane.

Enzymatic hydrolysis optimization

Prepared CEC according to the previous section was used to assess the enzymatic hydrolysis optimization of BHD aimed the influence of BHD and the CEC concentrations on the level of concentration of reducing sugars (RS) obtained during hydrolysis. A statistical central composite rotational design (CCRD) was used for this purpose with the aid of Minitab 14 software (Minitab, LCC, State College, PA, USA). Pretreated sugarcane bagasse (BHD) was weighed and added to 250 mL Erlenmeyer flasks previously prepared with 50 mL of water. The pH was corrected to 5.00, using NaOH as necessary. The Erlenmeyer flasks were sterilized in an autoclave at 121 °C for 15 min. With the medium sterilized, the enzyme cellulolytic complex was added. The Erlenmeyer flasks were incubated on a rotary shaker at an agitation rate of 180 min^{-1} and a temperature of 50 °C. Samples were taken at hydrolysis times of 0, 24 and 48 h for subsequent analysis of the reducing sugar level.

Analytical methods

Aliquots of the fermentation medium were collected periodically, centrifuged at 10 000×*g*, 4 °C, for 15 min, and the supernatants were used for measurements of total protein, enzymatic activity, and pH. In each test, the activity was determined in triplicate. The total cellulolytic enzyme activity in the supernatant was determined by the filter paper activity (FPA) method, modified to reduce the scale of the reaction tenfold. β-Glucosidase activity was measured using *p*-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA) as substrate, at a concentration of 5 mol L^{-1} in 0.05 mol L^{-1} citrate buffer (pH 4.8). Hemicellulase activity was determined using beechwood xylan as substrate, and the concentration of reducing sugars was measured using dinitro salicylic acid reagent.

The BHD concentration was referred as the measured cellulose concentration according to the method described by Ahamed and Vermette.[22] Ten milliliters of the total culture were centrifuged (3000×*g* for 20 min) and the supernatant was carefully removed with a Pasteur pipette. The pellets were suspended in acetic acid–nitric acid reagent (3 mL: 150 mL of 80% acetic acid with 15 mL of pure nitric acid) and boiled for 30 min in a water bath. After cooling and centrifuging (3000×*g* for 20 min), the pellets were washed with distilled water (10 mL), and the residual cellulose was dried at 104 °C until constant weight.

The dry weight of the solids was determined by centrifuging the culture (20 mL; 9600×*g* for 20 min), washing the pellets three times with water (10 mL), and drying at 104 °C until constant weight. The dry cell biomass was calculated from the difference between the total dry weight of the solids (comprising mycelium and residual cellulose) and that of the residual BHD.

Results and discussion

The type of the pretreatment affects the cellulase production

The type of biomass pretreatment was found to have a strong influence on the amounts and types of cellulolytic enzymes secreted during the growth of the filamentous fungi in submerged fermentations. The results using batch experiments with 20 g L^{-1} of carbon source are presented in Fig. 1(a) and (b). In general, the FPase and xylanase activity obtained with the different substrates was in the following order: CEL > BHD > BED > BEX (Fig. 1(a) and (b)). The maximum FPase activity was obtained with the carbon source CEL, which reached about 4 FPU mL^{-1} at 100 h fermentation time, followed by BHD at same order of magnitude at approximately 120 h of fermentation time (Fig. 1(a)). Maximum xylanase activity was about 120–140 IU mL^{-1}, obtained for BHD and CEL and also at 120 h. The maximum

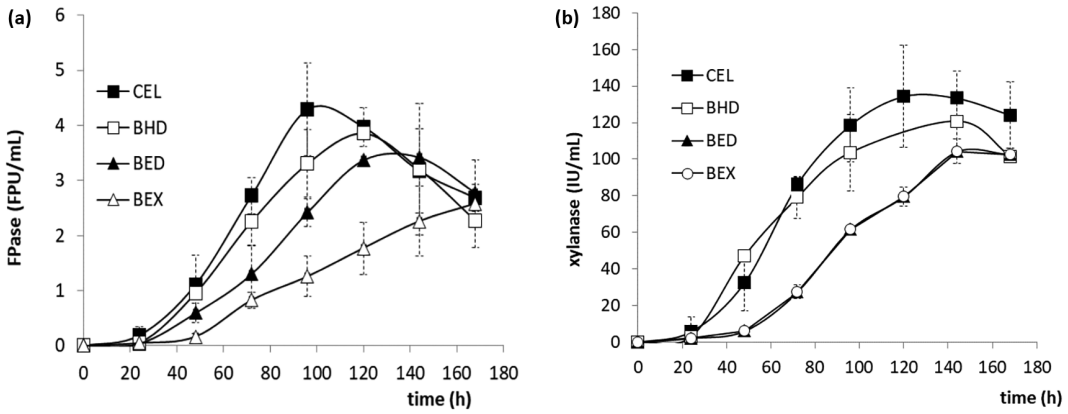

Figure 1. Time courses for (a) FPase and (b) xylanase enzyme activities obtained with *Penicillium echinulatum* S1M29 grown in bioreactor batch cultivations with 20 g L^{-1} of different carbon sources. BHD: delignified hydrothermally pretreated bagasse; BEX: steam exploded bagasse; BED: delignified steam exploded bagasse; CEL: commercial cellulose Cellufloc 200 (Celuflok Ind. Com., Brazil).

Table 1. Compositions of carbon/inductor sources for cellulase production using *Penicillium echinulatum* S1M29 in batch submerged fermentation.

Carbon source	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
SCB	43.1 ± 1.4	25.2 ± 1.9	22.9 ± 1.1	7.1 ± 1.5*
CEL	86.0 ± 0.5	14.9 ± 0.2	1.0 ± 0.1	0.2 ± 0.1
BHD	83.4 ± 0.2	2.2 ± 0.1	9.1 ± 0.1	5.8 ± 0.8
BED	74.0 ± 0.1	6.8 ± 0.1	16.2 ± 0.0	4.9 ± 0.1
BEX	57.1 ± 0.3	10.9 ± 0.1	27.6 ± 0.8	4.6 ± 0.1

Abbreviations: BED, steam exploded and hot NaOH delignified bagasse; BEX, steam exploded sugarcane bagasse; BHD, hydrothermally pretreated and hot NaOH delignified bagasse; CEL, Cellufloc 200; SCB, sugarcane bagasse.
Note: The SCB composition mean values were according to Rocha *et al*.[21]
*Ash + extractives.

β-glucosidase activity (data not shown) were always very low obtained at 140 h fermentation time at 0.8 IU mL^{-1} for the CEL substrate and below 0.4 IU mL^{-1} for BHD, BED or BEX.

It was previously demonstrated that the type of biomass pretreatment was found to have a strong influence on the amounts and types of cellulolytic enzymes secreted during the growth of the filamentous fungi in submerged fermentations,[5,23] and the present results corroborate those findings.

The present results clearly showed an inverse correspondence between the maximum enzyme activities obtained and the amount of lignin present in these carbon sources (1.0%, 9.1%, 16.2%, and 27.6% w/w, respectively) (Table 1), probably because lignin maintains cellulose less available to carry out its inductive activity in the cellulase production. Xu *et al.*[24] demonstrated pretreated corn stover samples with lower crystallinity were more easily hydrolyzed by enzyme and could get more final reducing sugar. Although measurements of the crystallinity of the materials have not been carried out, it is likely that BHD, sugarcane cane bagasse steam pretreated followed by NaOH lignin removal might contribute better performance of this material as compared with BED and BEX. The tested carbon sources also possessed different cellulose content, which was strongly correlated with their corresponding cellulases activity.

The derived mutant strain *P. echinulatum* S1M29 is considered to be a more efficient strain in terms of cellulase enzyme production according to Dillon *et al.*[9] It was demonstrated previously that for *P. echinulatum* DSMZ 343443 grown in submerged fermentation, sugarcane bagasse that had been steam exploded and then delignified with hot NaOH (BED) provided a better carbon source than *in natura*, hydrothermally treated, or acid pretreated bagasse. The process achieved a productivity of approximately 4.0 FPU mL^{-1} in 140 h (28 FPU L^{-1} h^{-1}) in fed-batch bioreactor cultivation, dispensing 46 g BED L^{-1} during this time interval.[5] In the present work, about 20 g BHD L^{-1} was sufficient to achieve ca. 4 FPU mL^{-1} in 120 h (33 FPU L^{-1} h^{-1}) (Fig. 1(a)). This demonstrated the superiority of *P. echinulatum* S1M29 over the DSMZ 343443 parent strain,

corroborating the findings of Dillon *et al.*[9] Nonetheless, there have been previous reports of the use of low-cost pretreated biomass as carbon source for the production of glycohydrolase using filamentous fungi other than *P. echinulatum*. Bigelow and Wyman[25] reported cellulase production of 1.2 FPU mL^{-1} by *Trichoderma reesei* RUT-C30 grown on bagasse pretreated with hot water. Kovács *et al.*[26] described the production of cellulase by a mutant strain of *Trichoderma atroviride*, using willow pretreated using steam (205 °C for 3 min, with 3% SO_2) as the carbon source in submerged fermentation, and achieved values of up to 0.7 FPU mL^{-1} for FPase and 8.0 IU mL^{-1} for β-glucosidase after 76 h of cultivation. Delabona *et al.*[27] reported FPase activity of 2.7 FPU mL^{-1} at 76 h in submerged bioreactor cultivation with the wild strain *T. harzianum* P49P11. The strain was previously cultivated in glycerol and grown in a culture medium containing 20 g L^{-1} of delignified steam-exploded sugarcane bagasse (BED). In comparison with these values reported in the literature, the present results for *P. echinulatum* S1M29 grown on a low-cost carbon source in batch cultivation were therefore superior in terms of FPase enzyme activity and productivity.

Xylanase activity is important because it is responsible for solubilizing the hemicellulose remaining in the pretreated lignocellulosic material, producing xylose, and providing access to the cellulolytic enzymes responsible for cellulose hydrolysis. The endo-1,4-β-xylanase enzyme cleaves the β-1,4 glycoside linkages between xylose residues in the xylan backbone. The action of xylanases and other accessory enzymes has been reported previously to improve cellulose conversion by removing hemicellulose and increasing the access of cellulase to the substrate.[28–30] In the present work, very high xylanase activity in the supernatant was achieved using either Cellufloc or BHD as substrate, with values exceeding 120 IU mL^{-1} in 100–120 h of fermentation (Fig. 1(b)).

The results broadly suggest that delignified hydrothermally pretreated sugarcane bagasse could be used as a low-cost substrate to produce enzymatic complexes, substituting for expensive cellulose sources, without appreciable loss of productivity, yield, or quality of the enzyme profile.

The influence of BHD concentration on growth and enzyme title

An obvious way to increase productivity and enzymatic activity is to use a higher initial concentration of the carbon source, which is assumed to be the limiting substrate. Batch experiments were therefore performed with *P. echinulatum* S1M29, grown using increasing initial BHD concentrations from 5 to 60 g L^{-1}, to identify any positive effects on glycoside hydrolase (FPase) activity. Figure 2 summarizes the results. For initial BHD concentrations up to 30 g L^{-1}, virtually all the carbon source was consumed within 140 h of fermentation time. At initial BHD concentrations of 40 and 60 g L^{-1}, the BHD consumption rate declined sharply at 100 h fermentation time and about 5 and 14.5 g L^{-1}, respectively, of carbon source remained unconsumed at the end of the experiment (Fig. 2(a)). On the other hand, the growth kinetics were very similar for the experiments using 5 to 40 g L^{-1} BHD, with a sharp increase in the cell mass concentration from the time of inoculation up to 72 h, followed by a sudden decrease after this point (Fig. 2(b)). The maximum cell concentration increased in line with the initial BHD concentration, although the increase was not proportional to the initial BHD concentration. Interestingly, a decrease in cell mass occurred even while the BHD concentration was still high, which was probably indicative of a nutrient limitation. Glucose and cellobiose, and probably other soluble cello-oligomers, are main products of the enzymatic hydrolysis of the cellulose component of BHD, performed by the secreted cellulolytic enzyme complex. As cell growth proceeds and the cell mass concentration increases, it therefore is likely that at a certain cell concentration the available glucose (or other assimilable carbohydrates) from BHD hydrolysis becomes scarce in the culture broth, leading to the cessation of cell growth, with cell death and/or sporulation, and consequently a decrease in cell mass over time. A different cell concentration profile was obtained in the experiment with an initial BHD concentration of 60 g L^{-1}, with no apparent substrate limitation and a cellulose concentration of 14.5 g L^{-1} at the end of the fermentation (Fig. 2(a)). In this experiment, the cell mass concentration did not decrease with time and reached a plateau of about 11 g L^{-1} (Fig. 2(b)).

The FPase activity obtained for *P. echinulatum* S1M29 grown on BHD as carbon/inductor source increased in line with the initial BHD concentration. There was an almost proportional relation between the peak enzyme activity and the initial BHD concentration in the range from 5 to 40 g L^{-1} (Fig. 2(c)). The temporal profiles of FPase activity were very similar for all these runs. It sharply increased when the cell mass growth began to leveled off and attained a maximum activity during the period of cessation or decrease of the cell mass growth. It was also noted that, at least up to the 30 g L^{-1} initial BHD concentration experiment, the FPase activity ceased to increase apparently due to substrate (cellulose) limitation (Fig. 2(a) and (c)).

On the other hand, in the case of the 60 g L^{-1} experiment, the carbon source was in excess and was not totally

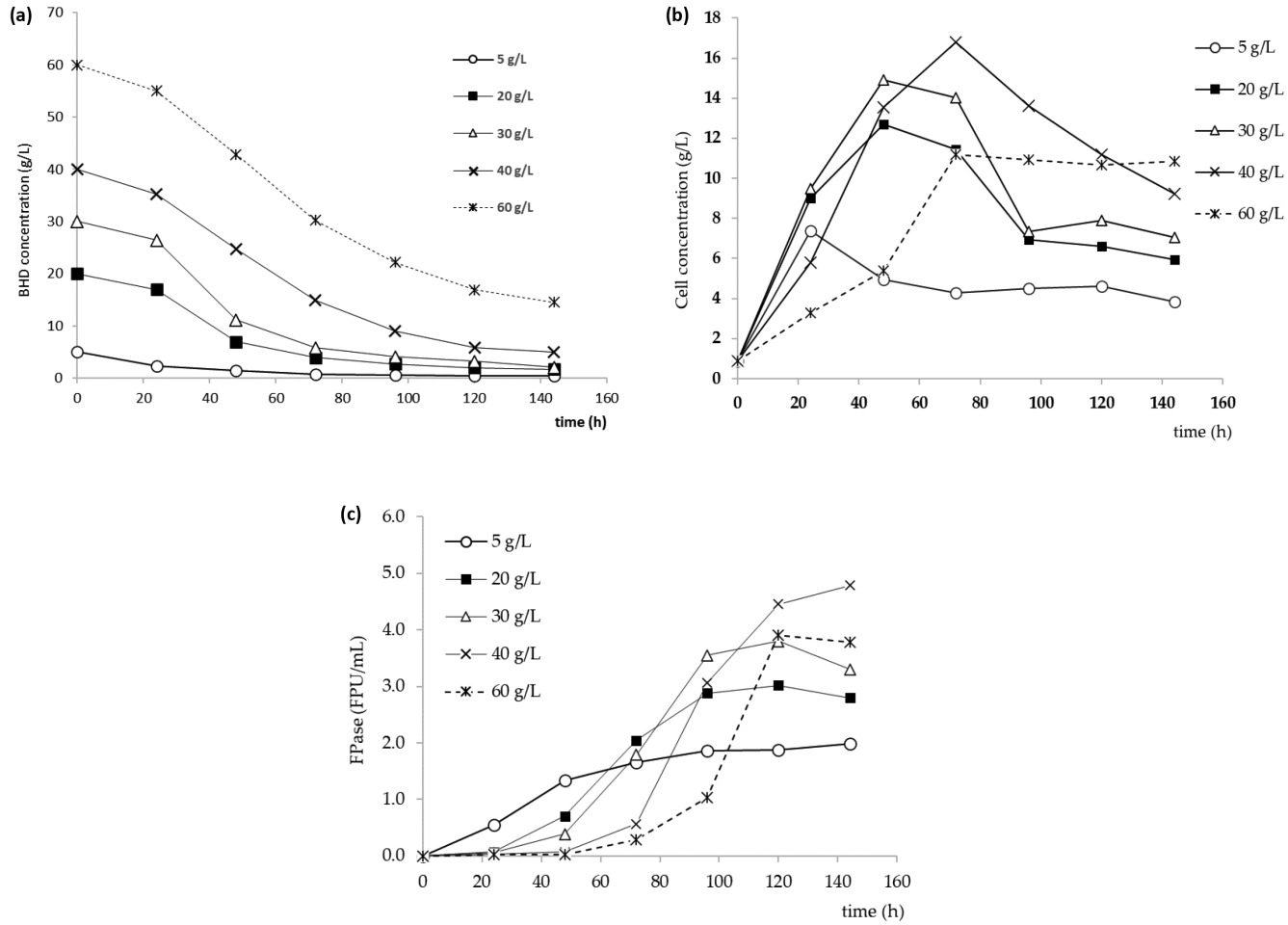

Figure 2. Time courses of (a) BHD consumption, (b) cell concentration, and (c) FPase activity for *Penicillium echinulatum* S1M29 in bioreactor batch mode cultivation with delignified hydrothermally pretreated bagasse (BHD) at different initial BHD concentrations.

consumed by the end of experiment (Fig. 2(a)), the cell mass concentration reached a plateau (Fig. 2(b)) and FPase activity production probably was jeopardized (Fig. 2(c)) by a substrate excess (Fig. 2(a)).

The effect of BHD concentration on the specific production rate of cellulase

The specific production rate, μ_p, is defined as the rate of production at a given fermentation time, dP/dt, associated with the cell concentration X at this fermentation time, given by $\mu_P = \frac{1}{X}\frac{dP}{dt}$. Figure 3 was constructed using data from Fig. 2. It shows the variation of the specific production rate of FPU activity (μFPase) according to the BHD concentration in the fermentation broth. Values of μFPase up to 5.7 FPU $g^{-1} h^{-1}$ were obtained when the BHD concentration was between 7 and 10 g L^{-1}. On the other hand, BHD above 30 g L^{-1} resulted in a very low specific production rate. As shown in (Fig. 3(b)), in this situation, an increase of available glucose in the culture medium was observed at about 60 h of fermentation time. However, the maximum observed values for RS concentration were below 1 g L^{-1}, and they were probably not high enough to trigger the carbon catabolite repression (CCR) mechanism *in P. echinulatum* S1M29, a common control mechanism in filamentous fungi during CEC biosynthesis.[31–33]

The influence of cellulose concentration in CEC production has been assessed using filamentous fungi. An initial cellulose concentration of up to 3% benefited cellulase production in *T. reesei* RUT C30, whereas a higher concentration decreased the enzyme production rate.[32] In recent investigations, utilizing a wild strain of *Trichoderma harzianum* P49P11, submerged batch cultivation experiments were carried out in a bioreactor. These studies unveiled a significant

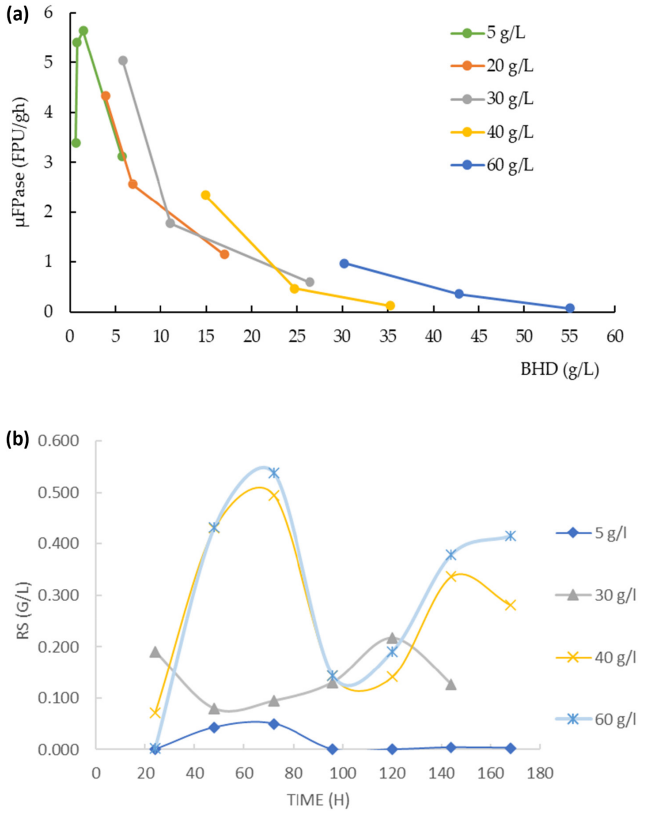

Figure 3. Cultivation of *Penicillium echinulatum* S1M29 in bioreactor batch mode cultivation with delignified hydrothermally pretreated bagasse (BHD) at initial concentrations of 5 to 60 g L^{-1}. (a) Relation between specific FPase production rate and BHD concentration. (b) Reducing sugar concentration during experiments.

augmentation in the activities of Filter Paper Activity (FPase), xylanase, and β-glucosidase within the culture medium as the initial concentration of delignified steam-exploded sugarcane bagasse, serving as the sole carbon source, was raised from 5 g L^{-1} to 30 g L^{-1}. However, surpassing the threshold value of 30 g L^{-1} pretreated sugarcane bagasse concentration jeopardized the enzyme production rate.[23] Recently Gelain *et al.*[34] proposed that an increase in the concentration of insoluble substrate in the bioreactor could hinder the mass transfer of substrates to the growing mycelial biomass. He also suggested that the removal of inhibitory products negatively influenced the biosynthesis of cellulolytic enzymes of filamentous fungi, and this might be the reason for the decreased of the enzyme specific rate production μFPase with BHD concentration (Fig. 3(a)).

Likewise, in the present work, BHD concentration below 1–2 g L^{-1} appeared to lead to substrate limitation and a decrease in μFPase (Fig. 3(a)).

Nevertheless, the maximum FPase activity was 4.5 FPU mL^{-1} at 120 h, achieved with an initial BHD concentration of 40 g L^{-1}. The temporal profiles of xylanase and *β*-glucosidase activity showed the same behavior with the maximum values occurring towards 40 g L^{-1}. Xylanase activity at BHD concentrations of 10, 20, 30, 40 and 60 g L^{-1} was, respectively, 75.6, 126.7, 132.4, 146.2, and 126.7 IU mL^{-1} at 120 h. *β*-glucosidase activity at BHD concentrations of 10, 20, 30, 40 and 60 g L^{-1} was, respectively, 0.96, 1.36, 1.46, 1.36, and 0.22 IU mL^{-1} at 120 h.

Bioprocess intensification based on the previous observations

In the previous batch experiments (Fig. 2), an unlimited amount of dissolved oxygen, above 30% air saturation, was maintained in the fermentation broth throughout cultivation. This was achieved using the bioreactor automatic cascade control to adjust the agitation rate from 150 to 600 min^{-1}, and the aeration rate up to 1.5 L air min^{-1} The dynamics of agitation rates during the substrate limitation phase were observed, and were characterized by BHD concentrations at levels below 10 g L^{-1}, typically occurring around the 100 h mark for assays utilizing initial concentrations of 20, 30, and 40 g L^{-1} (as illustrated in Fig. 2(b)). During this phase, the agitation rate exhibited a trend of stabilizing at lower levels, indicative of substrate limitation. Conversely, when higher initial concentrations of the carbon source were employed (as depicted in Fig. 3), it is plausible that an excess of available glucose was generated, triggering the phenomenon of carbon catabolite repression, thereby inhibiting cellulase biosynthesis. This might be true as, in a recent study, it was suggested that the transcription factor XlnR played a key role in positive regulation of the xylanolytic and cellulolytic systems in the wild-strain *P. echinulatun* 2H.[8]

The recognition of a plausible mechanism underlying carbon (glucose) catabolite repression in the mutant strain *P. echinulatum* S1M29, coupled with the apparent association of slower agitation rates with substrate limitation, therefore prompted the development of a pulsed fed-batch strategy informed by the observed fluctuations in this parameter. The data presented in Fig. 3 also suggested that maintaining a BHD concentration in the range of 5–10 g L^{-1} could be advantageous for enhancing μFPase (specific FPase productivity). In summary, a batch process commenced with an initial concentration of 10 g L^{-1} of BHD. Whenever there was evidence of substrate limitation, typically indicated by a decrease in agitation rate, the system received a supplementary infusion of 10 g of BHD (on a dry weight basis) per liter of fermentation broth. The consumption

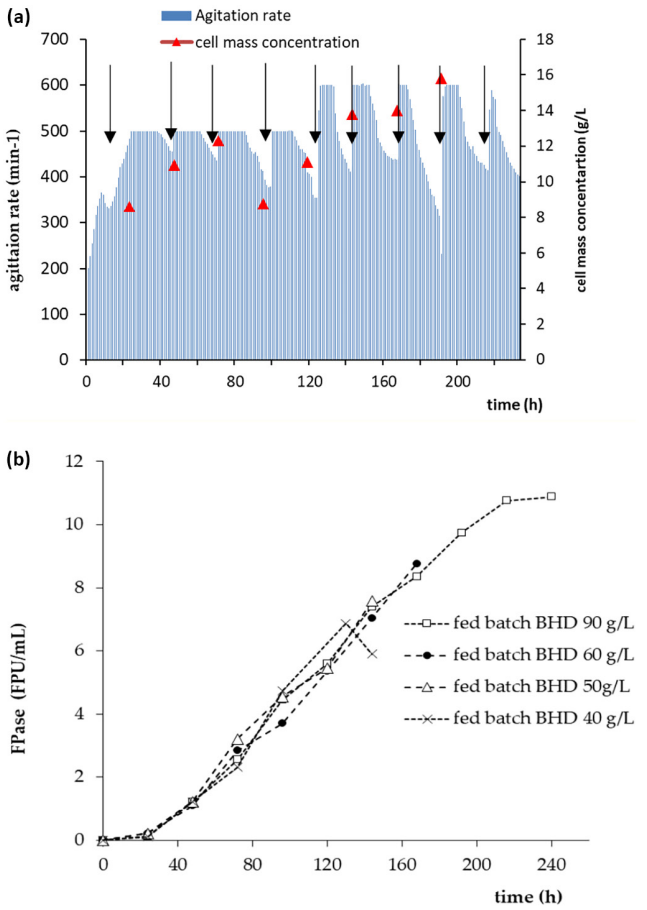

Figure 4. Time courses of (a) agitation rate and cell mass concentration for *Penicillium echinulatum* S1M29 in bioreactor pulsed fed-batch cultivation with 90 g L^{-1} of BHD; arrows indicate the BHD pulse according to the agitation rate decrease; (b) FPase activity for *P. echinulatum* S1M29 in bioreactor pulsed fed-batch cultivation with delignified hydrothermally pretreated bagasse (BHD) fed in different amounts.

profile of BHD at the 10 g L^{-1} concentration (Fig. 2(a)) suggested that this limitation would occur at approximately 48 h into the fermentation. Consequently, the first infusion of BHD was administered at this juncture. Subsequent BHD infusions were provided each time the agitation rate, which was maintained within a range of 150 to 600 min^{-1}, plateaued from its maximum value. Figure 4(a) is an example of how the procedure is realized. The arrows indicated the point of BHD fed every time the agitation rate decreased indicating a substrate limitation. The observed plateau along fermentation time soon after a pulse was typically the indicative of no limiting situation led to an increase of the FPase activity in the fermentation broth (Fig. 3(a)).

Figure 4(b) presents the FPase temporal profile of the pulsed fed-batch procedure carried out with total BHD additions of 40, 50, 60, and 90 g L^{-1}, using the protocol described above. As mentioned previously, the pulsed feeding with BHD was guided by decreases in the values of the variables, especially agitation rate, controlling the dissolved oxygen concentration of the fermentation broth above 30% air saturation. Figure 4(b) displays the agitation rate values during the fed-batch experiment with total BHD addition of 90 g L^{-1}, and the evolution of the cell mass concentration over time. At the end this experiment attained a final cellulase concentration of 11 FPU mL^{-1} at 220 h and FPase productivity of 50 FPU $L^{-1}h^{-1}$. The calculated cellulase production yield from the consumed carbon source was of about 120 FPU g^{-1} of BHD consumed.

The results obtained in these experiments were highly reproducible, demonstrating the reliability of the protocol. Unlike in the batch experiments (Fig. 2), the cell mass concentration did not stop growing in the proposed pulsed fed-batch protocol (Fig. 4(b)), and the FPase concentration increased steadily during the entire process (Fig. 4(a)). This indicated that use of the proposed protocol successfully overcame the effects of substrate limitation and glucose repression. In other recent work using a fed-batch procedure, maximum activity values of 8.3 FPU mL^{-1} and 57.6 mL FPU $L^{-1}h^{-1}$ were obtained using the same strain but with Cellufloc 200 as the carbon/inductor source.[15] However, the use of this commercial microcrystalline cellulose as inductor/carbon source implies a high-cost enzyme production process.[19] The present protocol represents an improvement over the earlier procedures. The cost of using commercial carbon source as Cellufloc is very high, and BHD appears to be well suited for use in onsite enzyme production in a second-generation biofuel plant, decreasing the production costs of enzyme complexes, as previously proposed.[5] Table 2 displays maximum xylanase and β-glucosidase activity obtained in the experiments using the *P. echinulatum* S1M29 pulsed fed-batch procedure with BHD as the main carbon source. Values of xylanase activity from 427 to 1050 IU mL^{-1} were observed for all the fed-batch experiments with BHD concentrations from 40 to 90 g L^{-1}.

Table 2. Maximum xylanase and β-glucosidase activities obtained in the pulsed fed-batch submerge cultivation of *Penicillium echinulatum* S1M29 using BHD as main carbon source.

BHD added (g L^{-1})	Xylanase (IU mL^{-1})	Time (h)	β-Glucosidase (IU mL^{-1})	Time (h)
40	460.2	144	0.40	144
50	427.2	144	0.72	144
60	446.7	168	0.81	168
90	1050.9	240	0.82	120

Table 3. Results of the Central Composite Rotational Design (CCRD) aiming to optimize reducing sugar concentration (RS) of the pretreated sugarcane bagasse (BHD) hydrolysis with the produced enzyme complex (CEC).

Experiment number	g BHD per 50 mL	mL CEC per 50 mL	RS concentration (g L^{-1})
1	5.00	0.625	40.87
2	5.00	0.625	34.42
3	2.50	0.250	11.19
4	7.50	0.250	18.51
5	8.53	0.625	55.90
6	5.00	0.625	33.72
7	2.50	1.000	21.68
8	5.00	0.095	7.53
9	5.00	1.553	55.49
10	7.50	1.000	57.66
11	1.46	0.625	15.38
12	5.00	0.625	34.96
13	5.00	0.625	37.91

Enzymatic hydrolysis CCRD optimization with *Penicillium echinulatum* CEC

A CCRD[35] was applied to evaluate the effects of BHD (P) and *P. echinulatum* enzymatic complex (E) concentration on enzymatic hydrolysis. An analysis was conducted at different levels for each variable resulting in an experimental design of 13 runs. The parameter reducing sugar concentration (RS) was the response variable, which generated a second-order polynomial response, Eqn (1), where E and P are respectively, the concentrations of CEC (mL/50 mL of water) and BHD (g/50 mL of water) used in the experiments (Table 3), and letters a to f are the adjusted coefficients of the terms of Eqn (1):

$$RS = a + b\,E + c\,P + d\,E^2 + e\,P^2 + f\,E\,P \quad (1)$$

Each term in the equation corresponds to a different effect on the reducing sugar concentration, RS: a is the intercept term, representing the baseline reducing sugar concentration when both E and P are at their lowest levels. b and c represent the linear effects of E and P, respectively. d and e represent the quadratic effects of E and P, respectively. These terms account for curvature in the response. f represents the interaction effect between E and P.

Table 3 consolidates the outcomes of CCRD experiments numbered 1 to 13, conducted using Minitab 14 statistical software. These experiments investigated the influence of two chosen variables, the quantity of BHD and the quantity of CEC, on the concentration of RS following a 48 h experimental period.

Experiments 5, 9 and 10 led to the best results in terms of reducing sugar (RS) concentration of around 55 g L^{-1}. The central points of CCRD, experiments 1, 2, 6, 12 and 13 presented a mean value of 36.38 g L^{-1} and a standard deviation of 2.97 g L^{-1}. An analysis of variance (ANOVA), performed with Minitab, is summarized in Table 4, choosing a confidence level of 95%. According to the ANOVA, the P-value for the proposed regression is $P = 0.00$ (<0.05), and for the lack-of-fit $P = 0.067$ (>0.05). If the P-value is less than or equal to the significance level, it may be concluded that the model explains variation in the response. If the P-value is larger than the significance level, the test does not detect any lack of fit.[35]

A quadratic model may therefore be proposed to explain the relationship of RS with the concentration of the variables BHD and CEC. Equation (2) is the adjusted quadratic model for the experiments.

$$RS = -8.294 + 3.368\,BHD + 36.602\,CEC + 7.643\,CEC\,BHD - 28.528\,CEC^2 - 0.311\,BHD^2 \quad (2)$$

Table 4. ANOVA for the experiments; the analysis was conducted using uncoded units (P-value significance level = 0.05).

Source	*df*	Seq^2	Adj^2	Adj MS	*F*	*P*
Regression	*5*	3321.57	3321.57	664.313	25.69	0.000
Linear	2	2989.92	72.46	36.231	1.40	0.308
Square	2	126.30	126.30	63.150	2.44	0.157
Interaction	1	205.35	205.35	205.349	7.94	0.026
Residual error	*7*	181.05	181.05	25.864		
Lack of fit	3	145.61	145.61	48.537	5.48	0.067
Pure error	4	35.43	35.43	8.859		
Total	*12*	3502.61				

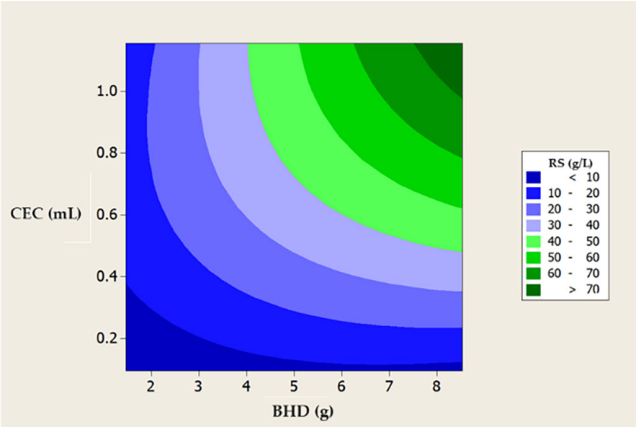

Figure 5. Contour plot for enzymatic hydrolysis of pretreated sugar cane bagasse (BHD) with cellulase enzyme complex produced by *Penicillium echinulatum* S1M29 (CEC). Enzymatic hydrolysis performed in 250 mL Erlenmeyer shaking flasks at 180 rpm, for 48 h, at 50 °C, with variable mass of BHD and volume of CEC in 50 mL of water (see Table 3).

Table 5. Summary of results of hydrolysis under optimized conditions.

Experiment number	g BHD per 50 mL	mL CEC per 50 mL	RS (g L^{-1})
1	8.53	1.15	87.25
2	8.53	1.15	73.51
3	8.53	1.15	82.13
Mean			80.96
Standard deviation			4.91

The high value $R-Sq$ (94.8%) and $R-Sq(adj)$ (91.1%) indicate an acceptable fit with the experimental data and low variability around the mean value. The significant terms in the model were those with P values less than 0.05, which indicate a 95% confidence level. Other authors demonstrated a confidence level of 80% for the study of enzymatic hydrolysis of BPT with commercial cellulase.[36] Figure 5 constructed with the aid of Minitab 14 software, represent the contour graph that relates the RS and the concentrations of pretreated sugarcane bagasse (BHD) and enzyme complex (CEC). The maximization of the enzymatic hydrolysis process performed with Minitab utilizing Eqn (2) indicated a maximum RS concentration of around 77.34 g L^{-1} achieved with BHD concentration of 170 g L^{-1} (8.53 g BHD per 50 mL) and CEC volume of 23 mL L^{-1} (1.15 mL CEC per 50 mL), complaining about 15 FPU per g of BHD. Based on these results the calculated glucose yield from the BHD cellulose content (Table 1) was 57.7 g RS per 100 g of utilized BHD.

The validation of this prediction was carried out by conducting enzymatic hydrolysis experiments at the specified values of BED and CEC concentrations, namely, a BED concentration of 170 g L^{-1} and a CEC of 23 mL L^{-1}. The results obtained from trials conducted in triplicate under these conditions are summarized in Table 5. The average value of the RS concentration obtained was 80.96 g L^{-1} with a standard deviation of 4.91 g L^{-1}, thus corroborating the optimization indicated by Eqn (2). The obtained results are comparable with reported pretreated bagasse hydrolysis performed with commercial cellulolytic enzyme complex, indicating the potential of the present results.[24,36,37,38]

Cost assessment of CEC in the case of an onsite first- and second-generation ethanol biorefinery

Onsite cellulase enzyme complex production (OSP) had been claimed to be a strategy to decrease the cost contribution of CEC to second-generation (2G) bioproducts.

As an example, we assessed the impact of CEC on ethanol production cost from sugarcane in a first- and second-generation (1G + 2G) ethanol biorefinery with OSP represented in Fig. 6. The OSP was used to supply an enzyme complex for 2G ethanol production integrated into 1G ethanol production from sugarcane with a standard milling capacity of 2 000 000 t $year^{-1}$ as proposed by Dias *et al.*[39] Briefly, the plant design of the enzyme OSP will use a train of fermenters up to 500 m^3 of volume, and will utilize surplus utilities (steam, water, electricity) from the 1G plant and a small part (up to 10% of total amount) of cellulosic pretreated sugarcane bagasse as the main carbon source.[22] The enzyme plant will run as a fed-batch process as described above and, at the end, the completely fermented broth will be sent to a holding tank to feed the enzymatic hydrolysis section, after removal of mycelia by filtration.

The economical assessment was based on the block diagram described in Figure 5. The technical coefficients used for the 1G+2G ethanol plant production is summarized in Table 6. The Table 7 presents the technical coefficients used for the on-site enzyme production (OSP) used in the 1G+2G ethanol production plant, and the calculated enzyme contribution cost for the described 1G+2G production plant (Table 7). Spreadsheets (Excel, Microsoft, Redmond, WA, USA) were built with technical coefficients based in the present data as described (Tables 6 and 7).

Supporting Information, Tables A and B summarize, respectively, the capital expenditure (CapEx) and operating expense (OpEx) values for a the OSP based on the results for the adopted fed-batch production (Fig. 4).

During enzymatic hydrolysis it was shown that the appropriate value of the ratio CEC/BHD of 23 mL L^{-1}

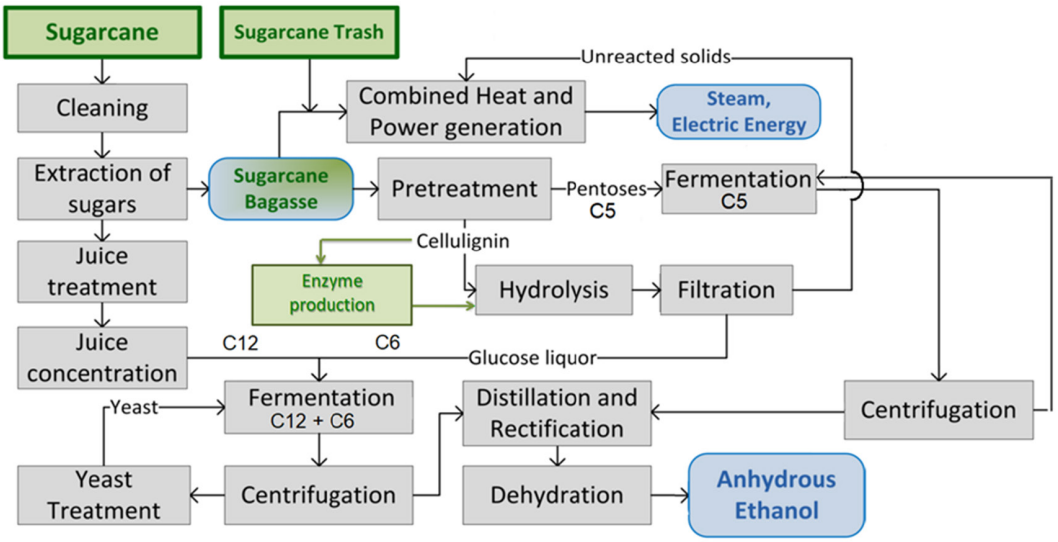

Figure 6. Block diagram of ethanol 1G + 2G biorefinery using OSP production.

Table 6. Technical coefficients for ethanol production in a 1G + 2G biorefinery.

Technical coefficients for ethanol production	Amount	Units
Sugarcane mill capacity	2 000 000	t year^{-1}
1G ethanol production from SCB	85	L t^{-1}
1G ethanol production	170 000 000	L year^{-1}
Bagasse sugarcane fraction	0.136	
Bagasse production (dry basis)	272 000	t year^{-1}
Bagasse surplus	0.85	
Bagasse available	231 200.00	t year^{-1}
BHD (pretreated sugarcane bagasse)	150 280.00	t year^{-1}
Cellulose fraction in BHD	0.83	
Hemicellulose fraction in SCB	0.25	
Lignin fraction in SCB	0.23	
Mass of cellulose	124 732	t year^{-1}
Mass of hemicellulose	57 800	t year^{-1}
Mass of lignin	53 176	t year^{-1}
C6 yield over BHD from enzimatic hydrolisys	65%	
C5 yield from pretreated SCB	80%	
Mass of C6 sugar from BHD enzymatic hydrolisys	80 576	t year^{-1}
Mass of C5 sugar from hemicellulose	46 240	t year^{-1}
Ethanol C6 + C5 yield over theoritical	80.00%	
Ethanol 2G total mass	51 842	t ethanol/t C6 + C5
Ethanol 2G total volume	65 706 543	L year^{-1}
Ethanol 1G + 2G total volume	**235 706 543**	**L year^{-1}**

Note: Bold indicates significant values.

Table 7. Technical coefficients for enzyme production in a 1G + 2G biorefinery.

Technical coefficients for enzyme production	Amount	Units
BHD production	150 280 000	kg year^{-1}
FPU/BHD ratio	14 950	FPU kg^{-1}
Aeration/agitation power	1	kW m^{-3}
Annual CEC demand	2 246 686 000 000	FPU year^{-1}
Enzyme yield recovery	0.90	
Carbon source to enzyme yield	120 000	FPU kg^{-1}
Fermentation time plus downtime	210	h
Enzyme concentration	11 000	FPU L^{-1}
Operational time (days/year)	7920	h year^{-1}
Enzyme productivity	52.4	FPU L^{-1} h^{-1}
Number of batch/year	38	Batches
Required FPU	2 496 317 777 778	FPU
Required broth volume	226 938	m^3 year^{-1}
Volume/batch	6017	m^3
Fermenter working volume	1000	m^3
Number of fermenters	6	Unities
FPU production/year	2 496 317 777 778	FPU year^{-1}
FPU/protein factor	2 000 000	FPU kg^{-1} protein
Protein production/year	1 248 159	kg year^{-1}
Ethanol production/year	235 706 543	L year^{-1}
Enzyme cost (US$/100 000FPU)	**0.59**	**US$/100 000 FPU**
Enzyme cost (US$/kg protein)	**11.85**	**US$/kg protein**
Enzyme cost/L of produced ethanol	**0.23**	**US$/L**

Note: Bold indicates significant values.

(1.15 mL CEC/50 mL) and 170 g BHD L^{-1} complaining about 15 FPU g^{-1} BHD. This would provide a glucose yield from the BHD cellulose content of about 57.7 g RS/100 g BHD and a RS concentration of 80 g L^{-1}. This liquor would be used to be fermented together with the sugarcane juice in the 1G + 2G ethanol biorefinery (Fig. 6).

Using the data from this evaluation, the cost contribution of the proposed OSP production is about US$0.23/L of ethanol. The present model indicated that an intensification of the process productivity up to 100 FPU L^{-1} h^{-1} and 150 FPU L^{-1} h^{-1} would bring the CEC contribution cost to, respectively, US$0.15 and US$0.13/L. Another possibility that might be explored is to reduce the CEC/pretreated bagasse ratio. The present model indicated that, with the present CEC productivity of about 50 FPU L^{-1} h^{-1}, reducing the ratio to 10 and 5 FPU g^{-1} BHD would decrease the CEC ethanol cost contribution to, respectively, US$0.16 and US$0.09/L of ethanol. Dos Reis *et al.*[17] used the same strain and in a fed-batch procedure attained 8.3 FPU mL^{-1} in 144 h but employing as carbon source Cellufloc 200, an expensive microcrystalline cellulose, suggesting a cost contribution of enzyme according to the present model greater than US$1.00/L.

The present calculated values of enzyme contribution cost of US$0.23/L of ethanol using BHD and the proposed procedure agreed with data from the literature. Klein-Marcuschamer *et al.*[17] calculated an enzyme contribution cost of between US$0.18 and 0.38/L ethanol.

Overall, the results pointed out that the proposed procedure using OSP, *P. echinulatum*, and pretreated sugarcane bagasse might provide a valuable platform to be used for the production of biofuels and other bio commodities such as biopolymers and biosolvents.

Conclusions

The research presented in this study has led to several important conclusions. The study successfully developed an efficient procedure for the production of a CEC. This is a significant achievement as a CEC is essential for the enzymatic hydrolysis of cellulose in biomass. Hydrothermal pretreatment of sugarcane bagasse at 140 °C, followed by extensive lignin removal, was found to be highly effective. This pretreatment method likely improved the accessibility of cellulose to enzymes, enhancing the overall process efficiency. The study achieved a remarkable productivity of 50 FPU L^{-1} h^{-1}, indicating that the CEC production process was highly efficient. High productivity is a crucial factor in making enzyme-based processes economically viable. The use of a pulsed fed-batch procedure controlled by agitation rate variation contributed to the high productivity achieved. This innovative approach to enzyme production could have broader implications for bioprocessing and biomanufacturing. The CEC produced was utilized effectively to hydrolyze pretreated sugarcane bagasse, resulting in an impressive 65% yield within 48 h. A preliminary economic assessment demonstrated that that for a 1G + 2G ethanol biorefinery, the cost contribution of enzymes would be about US$0.23/L of biofuel. This outcome demonstrates the potential of the CEC that was developed and the effectiveness of the entire process for biomass conversion. Further research and optimization of bioprocess in terms of productivity and the amount of CEC during the process will bring the cost of the contribution of co-enzymes to a more attractive value.

Acknowledgements

We thank CNPEM, Campinas, Brazil, and the Instituto de Pesquisa e Desenvolvimento of UNIVAP for the technical support for development of this work.

Funding information

This research was partially funded by CNPq, grant number 479599/2011-4.

Conflicts of interest

The authors declare no conflict of interest.

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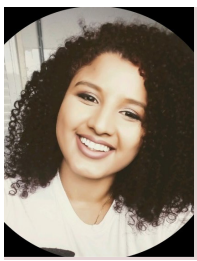

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