Saccharification of Biomass Using Whole Solid-State Fermentation Medium to Avoid Additional Separation Steps

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The enzymatic hydrolysis of steam-exploded sugarcane bagasse (SESB) was investigated using enzymatic extracts (EE) and whole fermentation media (WM), produced in-house, from Aspergillus niger 3T5B8 and Trichoderma reesei Rut-C30 cultivated on wheat bran under solid-state fermentation (SSF). A detailed and quantitative comparison of the different hydrolysis conditions tested was carried out using the Chrastil approach for modeling enzymatic reactions by fitting the experimental data of total reducing sugar (TRS) released according to hydrolysis time. Conversion of SESB using A. niger enzymatic complex were up to 3.2-fold higher (in terms of TRS) than T. reesei at similar enzyme loadings, which could be correlated to the higher β-glucosidase levels (up to 35-fold higher) of A. niger enzymatic complex. Conversion yields after 72 h exceeded 40% in terms of TRS when the WM was supplemented with a low dosage of a commercial enzyme preparation. When the combination of WM (from either T. reesei or A. niger) and commercial cellulase was used, the dosage of the commercial enzyme could be reduced by half, while still providing a hydrolysis that was up to 36% more efficient. Furthermore, SESB hydrolysis using either EE or WM resulted in similar yields, indicating that the enzyme extraction/filtration steps could be eliminated from the overall process. This procedure is highly advantageous in terms of reduced enzyme and process costs, and also avoids the generation of unnecessary effluent streams. Thus, the enzymatic conversion of SESB using the WM from SSF is cost-effective and compatible with the biorefinery concept.
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Introduction

Enzymatic conversion of the polysaccharides present in lignocellulosic biomass will be a key technology in future biorefineries. However, in order to make this process economically feasible, it is necessary to improve the efficiency of enzyme production, since the cost of the enzymatic cocktails significantly influences the viability of the overall process of biomass bioconversion into fuels and other chemicals. To this end, recent studies have aimed at increasing the efficiency of enzyme production by identifying novel microbial strains[1–3] and more efficient fermentation techniques,[4–6] as well as strategies to recycle the enzymes.[7–9] Onsite production of enzymes is another strategy that could be used to reduce costs, since there is less need to stabilize the enzyme preparations, which avoids the expenses associated with transport and long-term storage.[10–12]

To break down plant cell wall components, an enzymatic cocktail containing cellulases, hemicellulases, and other accessory enzymes is required in order to achieve efficient hydrolysis. This type of enzymatic complex is produced by a wide variety of microorganisms (bacteria and fungi); however, the aerobic fungi especially are known for their high growth and protein secretion rates.[13] Most commercial cellulase preparations are produced using filamentous fungi of the genera *Trichoderma* and *Aspergillus*. The *Trichoderma reesei* fungus is known to be highly cellulolytic and is an important source of

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industrial cellulases, xylanases, and other cell wall-degrading enzymes. However, enzyme-prospecting research continues to identify opportunities to enhance the activity of *T. reesei* enzyme preparations by supplementation with enzymatic diversity from other microbes.[2] In the field of biotechnology, the fungus *Aspergillus niger* has been shown to be a highly efficient enzyme producer, and comparison of the genome sequences of *T. reesei* and *A. niger* has shown that *A. niger* is more versatile in terms of the range of encoded cellulases, hemicellulases, and esterases.[14]

The use of solid-state fermentation (SSF) for industrial enzyme production has received increasing attention over the past 20 years due to the inherent advantages of this cultivation system.[15,16] Many studies have described the use of SSF to produce enzymes involved in biomass deconstruction.[16] SSF is particularly advantageous for enzyme production by filamentous fungi, since it simulates the natural habitat of these microorganisms. From the environmental perspective, the benefit of SSF is related to the use of agro-industrial residues as solid substrate, acting as sources of both carbon and energy.[17]

In traditional SSF processes, after the cultivation period the enzymes synthesized by the microorganisms are extracted from the solid substrate by conventional solid–liquid extraction, with the final products after filtration being a liquid supernatant containing the enzymes of interest, which can be further concentrated, together with a solid residue. A potential way of avoiding production of this solid residue is to use the whole fermentation medium, containing the enzymes, mycelia, and the residual solid substrate, for the saccharification of lignocellulosic biomass such as sugarcane bagasse. A similar process configuration has been reported, using the whole submerged fermentation (SmF) broth of *T. reesei* to improve the performance of a simultaneous saccharification and fermentation process.[18] Whole SmF broth produced using *T. reesei* and *T. atrovide* has also been employed for the hydrolysis of pretreated spruce,[19] and whole SmF broth of *T. reesei* ZU-02 has been used for the hydrolysis of corn cob.[19,20] In addition, Sorensen et al.[11] demonstrated the use of whole SSF cultivation medium for the hydrolysis of wet oxidized wheat straw.

In Brazil, the production of first generation bioethanol was developed using sugarcane as feedstock, and the country is now one of the world's most competitive producers of bioethanol.[21] The production of second generation bioethanol from sugarcane bagasse is an important sustainable means of increasing yields. For such application, developments on sugarcane bagasse pretreatment using steam explosion,[22–24] hydrothermal,[25] ionic liquids,[26–28] dilute acid,[29,30] lime,[31] organosolv,[32] and chemithermomechanical processing[33] have been reported. Nevertheless, steam explosion has been considered one of the preferred technologies for sugarcane bagasse pretreatment.[34] For the enzymatic hydrolysis of sugarcane bagasse, configurations using either commercial or on-site produced enzyme preparations have been described.[35–37] However, to the best of our knowledge, there have been no studies concerning the enzymatic hydrolysis of sugarcane bagasse using the whole SSF cultivation medium, with evaluation of the contribution of mycelia-bound enzymes to the process. Elimination of the SSF extraction/filtration steps during enzyme production would be highly advantageous in terms of reduced process costs, and would also avoid the production of unnecessary effluent streams, hence being compatible with the biorefinery concept.

The present work investigates the enzymatic hydrolysis of steam-exploded sugarcane bagasse (SESB) using the enzyme extracts and whole fermentation media of *Aspergillus niger* 3T5B8 and *Trichoderma reesei* Rut-C30 cultivated on wheat bran under selected SSF conditions. The hydrolysis yields were compared with those obtained using a commercial cellulase preparation as well as combinations of different enzyme sources. To achieve a more detailed and quantitative comparison of the different hydrolysis conditions tested, the Chrastil approach for modeling enzymatic reactions was used to fit the experimental data of total reducing sugar released according to hydrolysis time.

Materials and Methods

Microorganisms

The microorganisms used in this study were a mutant strain of *Aspergillus niger* (*A. niger* 3T5B8) from the Embrapa Food Technology Collection (Rio de Janeiro, Brazil) and a strain of *Trichoderma reesei* (*T. reesei* Rut-C30) from the American Type Culture Collection. Stock cultures were stored at 4°C on potato dextrose agar (PDA) slants. The cultures were revitalized and maintained on PDA slants at 32°C for 5 days prior to inoculation.

Lignocellulosic materials

Solid-state fermentation (SSF) cultivations were carried out using wheat bran (Claro Agricopecuária, São Carlos, Brazil) as solid substrate. The enzymatic hydrolysis experiments employed steam-exploded sugarcane bagasse (SESB) kindly provided by CTBE (Campinas, Brazil). The composition of the SESB used in all hydrolysis experiments, in terms of cellulose, hemicellulose, and lignin, was 51.7% ± 0.6%, 8.9% ± 0.1%, and 34.3% ± 0.3%, respectively.[10]

Selection of conditions for SSF cultivations

Cultivations for the selection of SSF process conditions were carried out in 250 mL Erlenmeyer flasks for 72 h, using wheat bran as solid substrate. The solid medium was sterilized by autoclaving at 121°C for 20 min before inoculation. A spore suspension volume corresponding to 10^7 conidia per g of dry solid medium was inoculated into the solid medium by gently stirring with a glass rod until a uniform mixture was obtained. The strategy adopted for selection of SSF conditions was to evaluate each variable individually, and then select the best value for incorporation in the next variable selection step. Initially, cultivations were carried out at 35°C with different moisture levels (50, 60, 70, and 80%), adjusted using a nutrient solution.[38] After selection of the most favorable moisture content, different temperatures (28, 30, 32, 35, and 37°C) were evaluated using selected initial moisture contents of 80% for the *A. niger* strain and 60% for the *T. reesei* strain. After a 72 h cultivation period, the enzymes were extracted and analyzed as described in Enzyme Extraction and Analytical Measurements sections, respectively. The mean values obtained for each condition were analyzed statistically using Origin (v. 8.0) software.

Temporal profiles of enzymatic production under the selected SSF conditions

In the kinetic study, SSF cultivations were carried out in 250 mL Erlenmeyer flasks for 120 h, with samples (whole flasks) being withdrawn at 24-h intervals. The SSF conditions selected for *A. niger* were an initial wheat bran

moisture content of 80% and a temperature of 28°C, while for *T. reesei* an initial moisture content of 60% and 35°C were selected. In the hydrolysis experiments, cultivations were carried out under these same conditions, but for a period of 48 h. After the cultivation periods, the enzymes were extracted and analyzed as described in Enzyme Extraction and Analytical Measurements sections, respectively. The whole fermentation media were also used in the hydrolysis experiments, as described in Enzymatic Hydrolysis section. All cultivation experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

Enzyme extraction

After the cultivation period, the enzymes were extracted by adding a sufficient volume of 0.05 mol L^{-1} sodium citrate buffer, at pH 4.8, to achieve a solid/liquid ratio of 1:10 (w/v). The suspension was stirred at 120 rpm for 30 min at room temperature, and the crude enzymatic solution was recovered by filtration followed by centrifugation at 10,000*g* at 4°C for 20 min. The enzymatic extracts were stored at −18°C prior to the analyses. In the hydrolysis experiments, the crude enzymatic extract was mixed with the solid material to be hydrolyzed, immediately after the extraction/filtration step.

Enzymatic hydrolysis

The enzymatic hydrolysis experiments were carried out in 500 mL Erlenmeyer flasks containing 5 g of SESB and 100 mL of sodium citrate buffer at pH 4.8. The suspension was initially acclimatized at 50°C for 4 h with 200 rpm agitation. Subsequently, 5 g of the previously fermented wheat bran SSF material (as described in Temporal Profiles of Enzymatic Production Under the Selected SSF Conditions section) was added, and the mixture was incubated at 50°C for 72 h with agitation at 200 rpm. Samples were removed at 0, 6, 12, 24, 48, and 72 h for quantification of the glucose and total reducing sugar released. When the hydrolysis was conducted with crude enzymatic extract (EE) instead of the whole fermentation medium (WM), only 50 mL of sodium citrate buffer (pH 4.8) was added to the SESB for acclimatization, after which the final volume was completed to 100 mL by adding 50 mL of the EE from the *A. niger* or *T. reesei* SSF cultivations. All hydrolysis experiments were carried out using a 5% (w/v) solids loading, in terms of SESB. In addition, a commercial enzyme preparation (Cellic Cetec2®, kindly donated by Novozymes A/S, Denmark) was used either alone or in different combinations with the other enzyme sources, for comparative purposes and to evaluate the effect of synergism between the SSF medium and the enzymatic extract on the hydrolysis of SESB. The commercial cellulase preparation was diluted sufficiently to achieve an enzymatic activity (in terms of FPU/mL) equivalent to that of the crude fungal enzyme extracts. In all experiments, sodium azide (0.1%, w/v) was added in order to prevent fungal development during the hydrolysis step. All hydrolysis experiments were carried out in triplicate, and the data were calculated as means ± standard deviations. The mean values obtained for each condition were analyzed statistically using the Origin software.

Kinetic modeling of the enzymatic hydrolysis

The Chrastil approach for modeling enzymatic reactions was used to fit the experimental data of reducing sugar release according to hydrolysis time.[39] This model is described by,

$$P = P_\infty (1 - e^{(-kE_0 t)})^n \quad (1)$$

where P and P_∞ are the products that diffused at time t and at equilibrium, respectively, k is a rate constant proportional to the diffusion coefficient, E_0 is the initial enzyme concentration, and n is a constant related to the diffusion resistance that depends on the sterical structure of the system. For the present system, P is expressed in grams of TRS (total reducing sugar) per liter, P_∞ is the potential (theoretical) total reducing sugar that could be obtained from the cellulose and hemicellulose in the SESB (33.6 g L^{-1}), and E_0 is the initial enzyme loading of the hydrolysis (FPU/L). The model parameters for the different hydrolysis conditions were obtained by fitting Eq. 1 to the SESB enzymatic hydrolysis experimental data by nonlinear regression using the Origin software.

Analytical measurements

The activities of FPase and endoglucanase were determined according to the procedure recommended by the IUPAC Commission on Biotechnology, with modifications.[1] The activity of xylanase was measured according to the methodology described by Bailey and Poutanen.[40] Here, one unit of activity corresponds to 1 μmol of reducing sugar released per minute per mL, under the reaction conditions. Quantification of the reducing groups employed the dinitrosalicylic acid (DNS) method.[41] The β-glucosidase activity was determined using cellobiose (Sigma, St. Louis, USA) as substrate and quantifying the sugars released using an enzymatic kit for glucose measurement (Doles, Goiânia, Brazil). The results were expressed as activity units per mass of initial dry solid substrate (IU g^{-1}). In the hydrolysis experiments, glucose and total reducing sugar were measured using the enzymatic kit for glucose measurement and the DNS method, respectively. Total protein was measured using the Bradford method.[42]

Results and Discussion

Effect of substrate initial moisture content on enzyme production under SSF

Enzyme production by the fungi was characterized for cultivations performed under different SSF process conditions in order to identify a condition that offered high enzymatic production and that could be used in the hydrolysis experiments. First, the influence of substrate initial moisture content on enzyme production was investigated by cultivating the fungi for 72 h at 35°C using different initial moisture contents (50, 60, 70, and 80%) of the wheat bran substrate. Comparisons of the influence of moisture content on the production of xylanase, endoglucanase, β-glucosidase, and FPase by *A. niger* 3T5B8 and *T. reseei* Rut-C30 are presented in Table 1.

In general, there was a positive effect of moisture content on enzyme production by *A. niger* 3T5B8 (Table 1). While the activities of xylanase, endoglucanase, and FPase were higher when an initial moisture content of 80% was used, the production of β-glucosidase was favored by a moisture content of 50%. Nevertheless, statistical analysis using the Tukey comparison test ($P < 0.05$) indicated that the population means of the activities obtained using higher moisture contents were not significantly different. This is a valuable finding in terms of bioprocess development, since it implies

Table 1. Influence of Initial Substrate Moisture Content on Enzymatic Production by *A. niger* 3T5B8 and *T. reseei* Rut-C30 Cultivated Under SSF

Initial Moisture (%)	Xylanase (IUg^{-1})	Endoglucanase (IUg^{-1})	β-glucosidase (IUg^{-1})	FPase (IUg^{-1})
***A. niger* 3T5B8**				
50	801.1 ± 18.6[a]	176.7 ± 15.6[b]	**72.5 ± 1.7[a]**	0.39 ± 0.03[a]
60	811.4 ± 57.1[a]	179.9 ± 1.3[b]	55.6 ± 12.4[a]	0.43 ± 0.06[a]
70	823.6 ± 10.6[a]	201.4 ± 11.4[a,b]	54.2 ± 0.5[a]	0.44 ± 0.02[a]
80	**836.7 ± 15.9[a]**	**226.3 ± 3.2[a]**	52.0 ± 8.8[a]	**0.51 ± 0.04[a]**
***T. reesei* Rut-C30**				
50	**575.6 ± 65.5[a]**	109.6 ± 15.7[a]	**2.5 ± 0.4[a]**	0.49 ± 0.06[a]
60	378.9 ± 43.2[b]	**146.5 ± 24.5[a]**	1.8 ± 0.1[b,c]	**0.59 ± 0.1[a]**
70	216.7 ± 1.7[c]	43.4 ± 4.0[b]	1.4 ± 0.02[c]	0.28 ± 0.04[b]
80	155.7 ± 5.1[c]	35.1 ± 2.1[b]	0.8 ± 0.1[d]	0.23 ± 0.02[b]

Means with different letters are significantly different (Tukey's test, $P < 0.05$).

Table 2. Influence of Temperature on Enzymatic Production by *A. niger* 3T5B8 and *T. reseei* Rut-C30 Cultivated Under SSF

Temperature (°C)	Xylanase (IUg^{-1})	Endoglucanase (IUg^{-1})	β-glucosidase (IUg^{-1})	FPase (IUg^{-1})
***A. niger* 3T5B8**				
28	**2393.6 ± 35.8[a]**	202.7 ± 45.3[b]	28.5 ± 0.09[b]	0.69 ± 0.006[a,b,c]
30	2204.0 ± 144.7[a]	241.4 ± 3.6[b]	53.1 ± 0.03[a]	**0.83 ± 0.02[a]**
32	1494.6 ± 112.8[b]	253.1 ± 17.8[b]	**58.1 ± 0.26[a]**	0.77 ± 0.05[a,b]
35	836.7 ± 15.9[c]	260.5 ± 47.7[b]	54.2 ± 0.18[a]	0.56 ± 0.06[b,c]
37	704.4 ± 49.1[c]	**332.7 ± 18.6[a]**	54.5 ± 0.03[a]	0.51 ± 0.03[c]
***T. reesei* Rut-C30**				
28	**620.2 ± 56.3[a]**	40.4 ± 5.5[d]	0.9 ± 0.4[b,c]	0.35 ± 0.1[b]
30	520.8 ± 69.3[a,b]	69.8 ± 5.6[c]	1.0 ± 0.1[b]	0.40 ± 0.03[b]
32	440.1 ± 27.0[b,c]	102.1 ± 6.6[b]	1.5 ± 0.03[a]	0.54 ± 0.3[a]
35	381.9 ± 9.4[c]	**152.3 ± 4.0[a]**	**1.8 ± 0.03[a]**	**0.63 ± 0.2[a]**
37	246.8 ± 35.6[d]	80.3 ± 10.8[c]	0.6 ± 0.1[c]	0.38 ± 0.03[b]

Means with different letters are significantly different (Tukey's test, $P < 0.05$).

that cellulase and xylanase biosynthesis by *A. niger* 3T5B8 was not very sensitive to variations in substrate initial moisture content. Among the various operational parameters that affect SSF process efficiency, moisture content is one of the most important. If it is too high, the void spaces in the solids are filled with water, resulting in oxygen limitation. At the other extreme, if the moisture content is too low, the growth of the microorganism will be hindered.[17] Consequently, identification of the optimal moisture content for each solid substrate is crucial for the promotion of favorable growing conditions, and hence for satisfactory metabolite production. However, the optimal moisture content value depends on both the type of solid substrate and the microorganism used.[17] It is also important to note that the moisture content can vary during the course of the cultivation period, since microorganism growth and respiration result in heat release. The discovery of a strain that is not very sensitive to variations in substrate initial moisture content, as appears to be the case for *A. niger* 3T5B8, can greatly assist process development. The *T. reesei* strain seemed to be more sensitive to variations in the substrate moisture content (Table 1). Enzyme production by *T. reesei* was generally higher when the moisture content was low (50 or 60%), and decreased significantly at higher moisture levels. On the basis of these results, initial substrate moisture contents of 80 and 60% were selected in subsequent studies to evaluate the effect of temperature on enzymatic production by *A. niger* and *T. reesei*, respectively.

The effect of the initial substrate moisture content on the production of cellulase and xylanase enzymes by *Aspergillus* and *Trichoderma* strains cultivated under SSF has been described previously. Delabona et al.[43] used a strain of *A. niger* (P47C3) isolated from the Amazon Forest to evaluate the effect of moisture content on endoglucanase production, and found that a reduction of the moisture content of wheat bran from 70 to 50% (w/w) resulted in a 2.5-fold improvement in endoglucanase production. Pal and Khanum[44] compared xylanase production by *A. niger* cultivated under SSF for six moisture levels ranging from 55 to 80%. The highest production of xylanase was obtained for a moisture content of 70%, and either low or high initial moisture content significantly decreased enzyme production. Singhania et al.[45] investigated the influence of process parameters on the production of cellulase by *T. reesei* Rut-C30 grown on wheat bran under SSF, and found similar effects of the initial moisture content of the medium on enzyme production.

Effect of temperature on enzyme production under SSF

Temperature is another important variable that affects microbial growth under SSF, hence influencing product formation. SSF cultivations were carried out at temperatures of 28, 30, 32, 35, and 37°C, using wheat bran with fixed initial moisture contents of 80% for *A. niger* and 60% for *T. reesei,* as selected previously. Comparisons of the influence of temperature on xylanase, endoglucanase, β-glucosidase, and FPase production by *A. niger* 3T5B8 and *T. reseei* Rut-C30 are presented in Table 2.

The effect of temperature on enzymatic production by *A. niger* varied for each of the enzymes analyzed (Table 2). For xylanase, a higher temperature had a significant negative effect, since production decreased from 2396.3 IU g^{-1} at 28°C to 704.4 IU g^{-1} at 37°C, a 3.4-fold reduction. Conversely, endoglucanase production was favored by an increase in temperature, with the highest value (332.7 IU g^{-1}) achieved at 37°C. The highest production of β-glucosidase was achieved at 32°C, while maximum FPase activity was observed at 30°C. In the case of *T. reesei*, there was greater production of cellulases

(endoglucanase, β-glucosidase, and FPase) at 35°C, while xylanase production was higher at 28°C.

The influence of temperature on cellulase production has been reported for other *Aspergillus* strains cultivated under SSF. Jecu[46] studied the effect of temperatures between 25 and 37°C on endoglucanase production by *A. niger*, and found that the optimum for endoglucanase production was in the range 28–34°C. Singhania et al.[45] investigated the effect of temperature on cellulase production by *T. reesei* Rut C-30 cultivated on wheat bran under SSF, and also found that the incubation temperature positively influenced the production of cellulase by this strain.

Similar to the effect of substrate moisture content, the characterization of each particular microorganism in terms of the influence of temperature on the kinetics of growth and product formation is essential for SSF bioprocess development. Based on the results, SSF cultivation using an initial moisture content of 80% and a temperature of 28°C was used to evaluate the kinetic profile of enzymatic production by *A. niger* during a cultivation period of 120 h, while an initial moisture content of 60% and a temperature of 35°C were selected for *T. reesei*.

Temporal profiles of enzyme production under the selected SSF conditions

The temporal profiles of enzyme production over a 120-h period of SSF cultivation under the selected conditions are illustrated in Figures 1a,b for *A. niger* 3T5B8 and *T. reesei* Rut-C30, respectively. For both fungi, peak production of the majority of the enzymes occurred between 48 and 96 h of cultivation. However, the time of maximum enzymatic production varied for each enzyme, as well as between the two fungal strains, with the exception of xylanase, which presented a maximum value after 48 h of cultivation for both *A. niger* (3382.5 IU g^{-1}) and *T. reesei* (768.4 IU g^{-1}). Here, it is interesting to note the large difference between the amounts of xylanase enzymes synthesized by these two fungi, with *A. niger* secreting 4.4-fold more xylanase than *T. reesei,* under the conditions used. In terms of FPase, the activity values were similar for both fungi, although the maximum value was achieved after 48 h for *T. reesei* (0.76 IU g^{-1}) and only after 72 h for *A. niger* (0.64 IU g^{-1}). The endoglucanase maxima differed in terms of both time and magnitude, since the amount secreted by *A. niger* after 96 h (369.3 IU g^{-1}) was 2.3-fold higher than the amount secreted by *T. reesei* after 72 h (158.2 IU g^{-1}). The synthesis of β-glucosidase also differed between the fungi in terms of both the time at which maximum production occurred and the amount produced. However, the difference between the amounts of enzyme secreted was much larger; *T. reesei* secreted a maximum of 1.6 IU g^{-1} of β-glucosidase after 120 h of cultivation, while the production of β-glucosidase by *A. niger* was almost 35-fold higher, with 55 IU g^{-1} achieved after 96 h of cultivation.

The superiority of *A. niger* strains for β-glucosidase production, compared to *Trichoderma* strains, was expected, but the magnitude of the difference was unforeseen. *Trichoderma reesei* is one of the most productive cellulolytic fungi, and the *T. reesei* Rut-C30 strain is one of the most studied and best characterized.[47] Nonetheless, the amount of β-glucosidase secreted by *Trichoderma reesei* is not sufficient for efficient biomass conversion,[13] because the complete conversion of cellulose into glucose is dependent on the

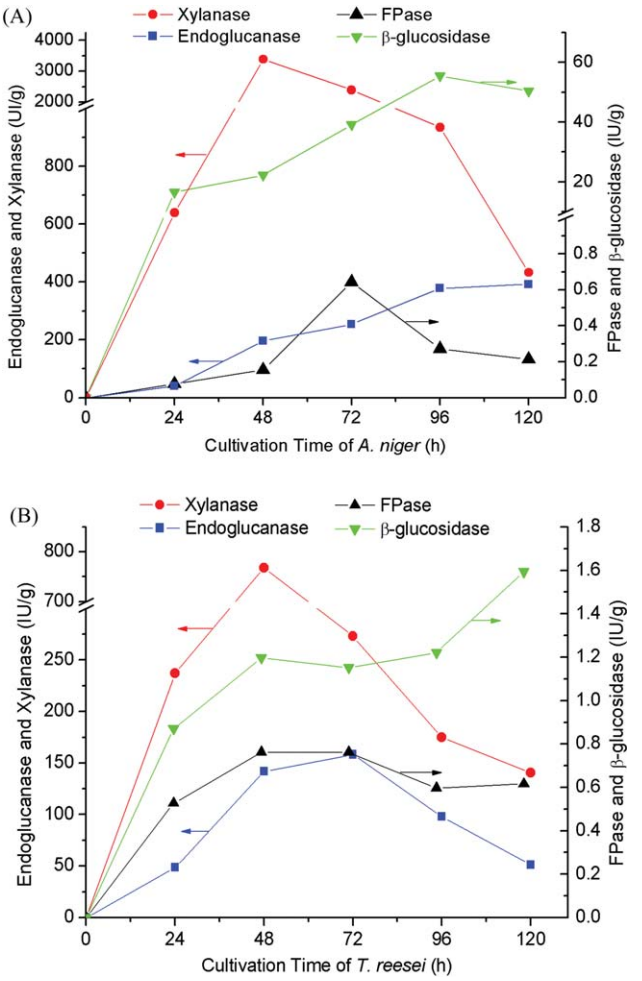

Figure 1. Kinetic profiles of enzyme production over a period of 120 h under SSF for (A) *A. niger* 3T5B8 and (B) *T. reesei* Rut-C30.

amount of active β-glucosidase. In addition, since β-glucosidase activity reduces the inhibitory effects of cellobiose, which is the end-product of exo- and endoglucanase activities, a suitable proportion of each of these enzymes is crucial for an efficient hydrolysis.[13] The importance of large amounts of β-glucosidase on cellulose hydrolysis can also be correlated to the significant impact of the inhibition and deactivation of β-glucosidase by the phenolic compounds generated during biomass pretreatment.[48] Besides the quantitative difference observed here between β-glucosidase from *A. niger* and *T. reesei*, it has been previously reported that β-glucosidase from *T. reesei* is more susceptible to inhibition as well as deactivation by phenolic compounds than β-glucosidase from *A. niger*.[49,50] Thus, the combination of *T. reesei* and *A. niger* enzymatic extracts may provide an enzyme mixture which is likely more resistant to inhibition and deactivation than enzyme derived from *T. reesei* alone. β-glucosidase production by *Aspergillus* strains such as *A. niger* has often been used to supplement commercial enzymatic cocktails.[13,47]

Overall, these results demonstrate the considerable potential of the *A. niger* 3T5B8 strain for the production of glycosyl hydrolases, since it showed a significantly higher enzymatic biosynthesis capacity when compared to the well-established cellulolytic strain *T. reesei* Rut-C30. Based on the results, the SESB hydrolysis experiments were conducted

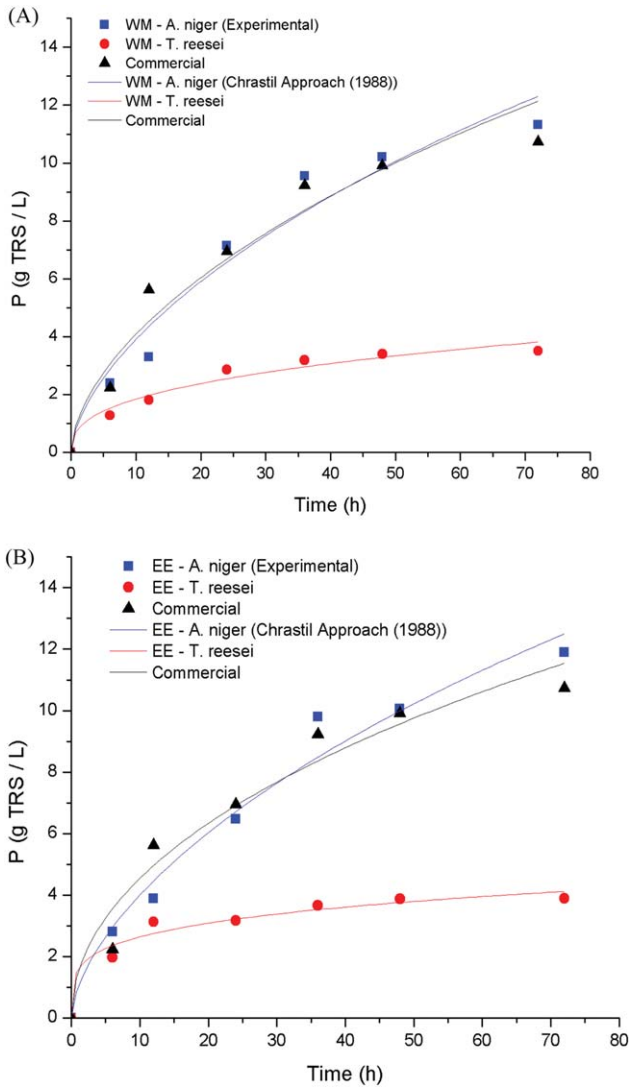

Figure 2. Temporal profiles of the concentrations of total reducing sugar released during the hydrolysis of pretreated sugarcane bagasse (SESB) using (A) the whole fermentation media (WM) from *A. niger* 3T5B8 and *T. reesei* Rut-C30, and the commercial preparation, and (B) the enzymatic extracts (EE) and the commercial preparation.

using 48-h cultivations of both fungi under the selected SSF conditions (28°C with an initial moisture content of 80% for *A. niger*, and 35°C with an initial moisture content of 60% for *T. reesei*).

Temporal profiles and modeling of the enzymatic hydrolysis step

The catalytic efficiencies of the enzymes produced by *A. niger* 3T5B8 and *T. reesei* Rut-C30 cultivated under SSF were evaluated during the hydrolysis of pretreated sugarcane bagasse (SESB). Two different experimental configurations were employed: (1) The whole fermentation media (WM) obtained after 48 h of cultivation under the previously selected conditions was mixed with the SESB, without separating the enzymes from the fungal mycelia; (2) The SESB was mixed with the enzymatic extract (EE) obtained from the extraction and filtration procedures that followed the SSF cultivations carried out under the same selected conditions.

Table 3. Total Enzymatic Activities of *A. niger* 3T5B8 and *T. reesei* Rut-C-30 Extracts, and a Commercial Preparation, Used for SESB Hydrolysis

Enzymatic Activity (IU)	*A. niger* 3T5B8	*T. reesei* Rut C-30	Commercial
Xylanase	16900	3840	1820
Endoglucanase	979	710	1730
β-glucosidase	110	6.0	468
FPase	0.75	3.9	4.7

In addition, experiments were conducted in order to evaluate the synergistic effect of different combinations of enzyme sources, and the possible use of the enzymes produced under SSF for supplementation of commercial enzyme cocktails. These experiments employed the enzymes present in the WM and EE from either *A. niger* or *T. reesei* in different combinations with a low dosage of a commercial enzyme preparation. The aim of these trials was to determine whether it was possible to reduce the quantity of commercial enzymes by using enzymes produced in-house, and whether the WM or the EE was preferable in terms of hydrolysis efficiency when used in combination with the commercial preparation.

Figure 2 presents the temporal profiles of total reducing sugar (TRS) released during the hydrolysis of SESB using the *A. niger* 3T5B8 or *T. reesei* Rut-C30 enzymes present in the whole fermentation media (WM) (Figure 2a) and in the enzymatic extracts (EE) (Figure 2b). For comparison, the graphs also include the SESB hydrolysis results obtained using the commercial preparation. Table 3 lists the total enzymatic activities of the EE obtained from both fungi under the selected cultivation conditions, as well as the total enzymatic activity of the commercial preparation. The latter was used at a similar dosage (in terms of FPase activity) as the enzymatic cocktails produced by *T. reesei*.

From Figure 2, it can be seen that the amount of TRS increased with time, and that the effect was much more pronounced when the *A. niger* enzymes were used, compared to those from *T. reesei*. The superior performance of the *A. niger* enzymes was observed using both the whole fermentation media (WM) (Figure 2a) as well as the extracts (EE) obtained from the cultivations (Figure 2b). It is also interesting to note that the enzymes from *A. niger* performed as well as the commercial preparation during the SESB hydrolysis, while for both fungi, the results obtained using either the whole fermentation media or the extract were similar. The concentrations of TRS released after 72 h were 11.3 and 11.9 g L^{-1} for the *A. niger* WM and EE, respectively, while the corresponding values for *T. reesei* were 3.5 and 3.9 g L^{-1}, respectively.

When the commercial preparation was supplemented with the WM from either *T. reesei* or *A. niger*, the dosage of the commercial enzyme could be reduced by half, and still maintain a hydrolysis efficiency that was up to 36% more efficient in terms of total reducing sugar released (Figures 3a,b). Use of the commercial enzyme alone resulted in a TRS release of 10.7 g L^{-1}, while 13.5 and 14.6 g L^{-1} of TRS were released using half the dosage of the commercial enzyme plus the WM from *A. niger* or *T. reesei*, respectively.

To better understand these results and provide a more detailed and quantitative comparison of the different hydrolysis conditions, the Chrastil approach for modeling enzymatic reactions,[39] as described by Eq. 1, was used to fit the experimental data of reducing sugar released according to hydrolysis time. The Chrastil approach was chosen because

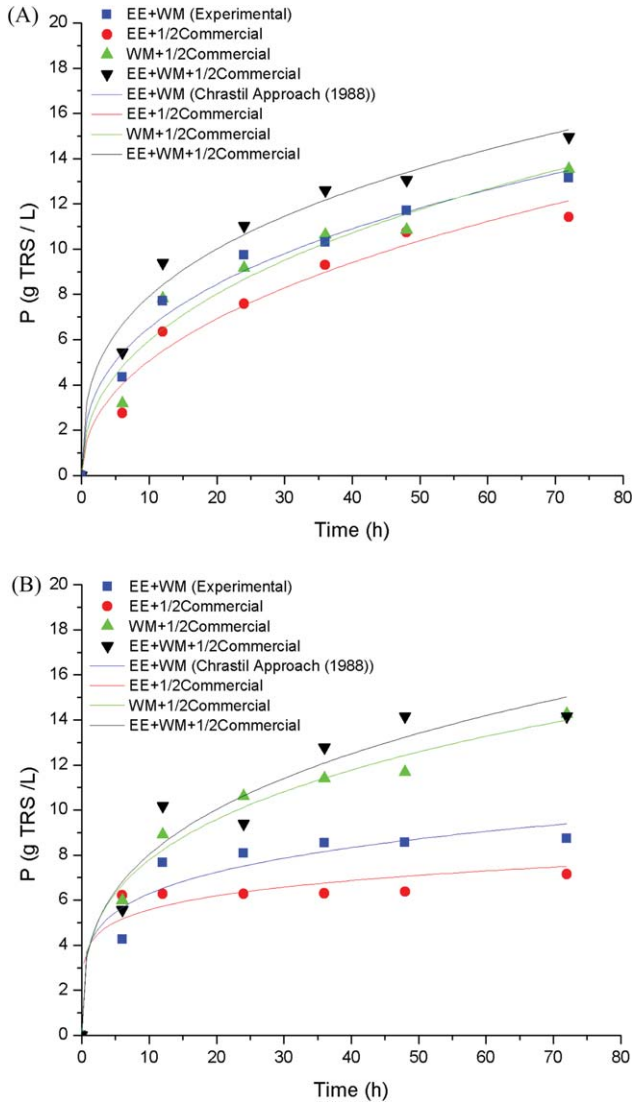

Figure 3. Temporal profiles of the concentrations of total reducing sugar released during the hydrolysis of pretreated sugarcane bagasse (SESB) using combinations of the whole fermentation media (WM), enzymatic extracts (EE), and the commercial enzyme preparation, for (A) *A. niger* 3T5B8 and (B) *T. reesei* Rut-C30.

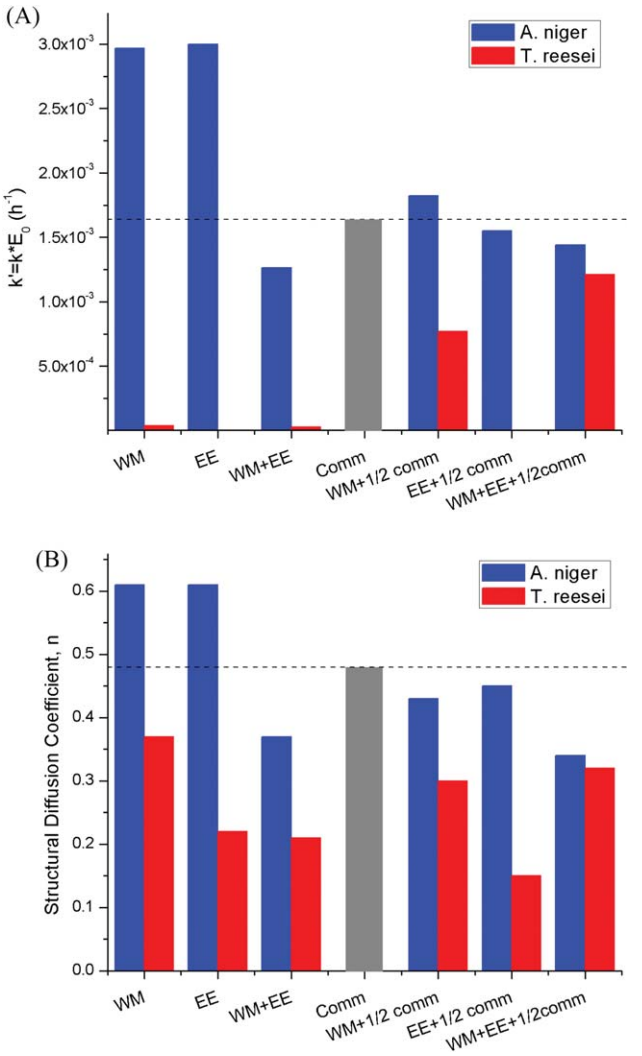

Figure 4. Values of the Chrastil model parameters, obtained by non-linear regression, for SESB hydrolyses using different enzyme sources. (A) specific velocity, k', of the system; (B) steric diffusion coefficient, n.

it employs all the temporal data, instead of initial velocity, and is suitable for the analysis of heterogeneous structures, especially when the enzymatic reaction is diffusion limited.[39] As highlighted by Carrillo et al.,[51] who used the Chrastil approach to evaluate the effect of alkali pretreatment on the hydrolysis of wheat straw cellulose, the kinetic and diffusion constants (k and n) obtained can provide important quantitative kinetic and structural information concerning the catalytic properties and overall sterical structure of the system, respectively. The semi-mechanistic model of Eq. 1 can be useful for understanding changes in the dynamics of the system during the reaction, especially when modeling long-term batch-type experiments that use highly heterogeneous substrates, such as the one used here. However, it is important to note that this model does not take into account inhibition effects, enzyme deactivation, or a decrease in substrate reactivity.

It can be seen from Eq. 1 that the parameters k and E_0 are linearly dependent. A simultaneous regression for these parameters would therefore produce a result that had little meaning, even though a good fit to the data might be achieved. Hence, in the present case E_0 was treated as a fixed parameter during the regression, and was assumed to be a measure of FPase activity (FPU/L). The FPase activities were the values shown in Table 3, divided by 0.1 L (the total reaction volume). To estimate E_0 for the runs using the whole medium (WM), it was assumed that ~20% of active enzymes were lost during extraction from the cultivation medium, as reported by Pirota et al.,[52] who analyzed successive SSF enzyme extractions under similar conditions. The activity values were summed in cases where the enzymes were derived from different sources. A better evaluation of the overall hydrolytic efficiency for each condition tested was achieved using the parameter k', which is the product of k and E_0. Figure 4 presents the values of k' (Figure 4a) and n (Figure 4b) for each fitting of the hydrolysis data obtained using the various enzymes sources (WM and EE from *A. niger* or *T. reesei*, and the commercial preparation), alone or in combination. All the regressions resulted in coefficients of multiple determination (R^2) $\geq$0.95.

From Figure 4a, it can be observed that the values of k' were much lower for *T. reesei,* except when the WM was

used in combination with the commercial enzyme, indicative of the poor SESB hydrolysis capacity of the *T. reesei* enzymes. The higher values of k' for the *A. niger* enzymes were indicative of better catalytic properties. This result was surprising, considering the lower E_0 value of the *A. niger* enzymes (measured in terms of FPase activity), compared to both the *T. reesei* enzymes and the commercial enzyme preparation (5.2-fold less activity in terms of FPase). A possible explanation for the superior performance of the *A. niger* enzymatic cocktail could be its higher xylanase activity (4.4-fold higher than for *T. reesei*, and 9.3-fold higher than the commercial preparation), as well as the higher β-glucosidase activity, compared to *T. reesei* (Table 3). The ratios between the activities of β-glucosidase and FPase were 145.7 IU/FPU for *A. niger,* 99.6 IU/FPU for the commercial enzyme, and only 1.54 IU/FPU for *T. reesei*, a value 100-fold lower than the ratio obtained for *A. niger*. According to Kovacs et al.,[19] even values of around 15–18 IU/FPU could be considered high. The importance of xylanase activity in the enzymatic cocktail is that it facilitates hydrolysis by increasing the accessibility of cellulose microfibrils to cellulose.[53] Another possible explanation for the superior performance of the *A. niger* enzymatic cocktail could be related to the characteristics of the enzymes in terms of their degree of inhibition or deactivation caused by the phenolic compounds present in the SESB. Ximenes et al.[50] reported that β-glucosidase from *A. niger* was the most resistant to inhibition and deactivation, requiring about 5-fold and 10-fold higher concentrations, respectively, for the same levels of inhibition or deactivation observed for enzymes from *T. reesei*. In addition, it is also possible that other accessory enzymes that were not quantified here could have participated in the hydrolysis. The recent discovery of the important role of lytic polysaccharide monooxygenases (LPMO) and other accessory proteins in enhancing the degradation of cellulose has resulted in the inclusion of a new category in the CAZy database, called "auxiliary activities" (AA), which integrates a group of catalytic modules involved in plant cell wall degradation.[54]

The values of the parameter n also reflected the superior hydrolytic capacity of the *A. niger* enzymes (Figure 4b). This dimensionless parameter is related to diffusion within the steric structure, and reflects the apparent order of the reaction. When the diffusion resistance is low, n tends toward 1, and the reaction shows first order kinetics. If the system is strongly limited by diffusion resistance, the value of n is small.[51] All reactions that are diffusion-limited show $n < 1$. The differences between the values of the structural diffusion resistance coefficient n indicated that diffusion limitations during SESB hydrolysis were lower for the *A. niger* enzymatic cocktail, compared to the enzymes from either *T. reesei* or the commercial preparation. However, the significantly lower activity of β-glucosidase in the preparations from *T. reesei* (Table 3) could result in cellobiose accumulation and enzymatic inhibition, which might explain the inferior hydrolysis performance. As this model does not consider inhibition effects, the values of the parameters k' and n obtained using the *T. reesei* enzymes alone should be considered with care, since they were probably influenced by product inhibition as well as by the diffusive characteristics of the product-substrate system.

Overall, the values of k' and n are very consistent with the experimental results obtained for the SESB hydrolysis. The higher values obtained for k' and n using the enzymes from *A. niger* are in agreement with the superior performance during SESB hydrolysis, compared to the *T. reesei* enzymes. Even though description of the kinetics of enzymatic hydrolysis of insoluble materials can be a very complex process, use of the Chrastil approach for modeling the enzymatic hydrolysis of SESB proved to be very useful in terms of generation of quantitative parameters that enabled a more detailed comparison of the kinetic characteristics of the different enzyme sources. The k and n values obtained here are of the same order of magnitude as those reported by Carrillo et al.[51] for alkali-treated wheat straw samples, even though the biomass, enzymes, and process conditions were different. This indicates that the Chrastil approach can be a valuable tool for comparing the performance of different procedures during the enzymatic hydrolysis of vegetal biomass.

From a practical point of view, another comparison of the SESB hydrolysis performance using *A. niger* and *T. reesei* enzymes can be carried out by analyzing the enzyme loadings used in terms of the total amount of enzyme protein added into the reactor system as mg protein/g glucan (i.e., the cellulose fraction of SESB). For the hydrolysis experiments using the enzymes from either *A. niger* or *T. reesei* alone, the enzyme loadings in terms of total protein were very similar (8.2 mg protein/g glucan for *A. niger* and 10.9 mg protein/g glucan for *T. reesei*). Therefore, it can be inferred that the superior performance of the *A. niger* enzymes on SESB hydrolysis is probably related to the higher specific activity of different enzymes such as xylanases (6-folds higher) and β-glucosidases (25-folds higher) in comparison to *T. reesei* enzymes. It is also worth to mention that these enzyme loading values are very compatible with the current level being reported in the literature for lignocellulosic biomass hydrolysis.[48]

Steam-exploded sugarcane bagasse (SESB) hydrolysis in terms of total conversion

An analysis was undertaken of the total sugars released (in terms of both glucose and total reducing sugar) at the end of the SESB hydrolysis. Figure 5 summarizes the conversion percentages achieved after 72 h of SESB hydrolysis using the *A. niger* (Figure 5a) and *T. reesei* (Figure 5b) enzymes from either the extracts (EE) or the whole fermentation media (WM), compared to use of the commercial enzyme cocktail as well as combinations of the different enzyme sources.

Use of the enzymatic complexes from *A. niger* (Figure 5a) for SESB hydrolysis resulted in similar biomass conversion yields for EE and WM, in terms of both glucose (18.5 and 16.7% of the theoretical yields for EE and WM, respectively) and total reducing sugar (33.6 and 35.3% for EE and WM, respectively). The values were not significantly different (Tukey's test, $P < 0.05$). It is important to note the good correlation between the release of glucose and total reducing sugar, which could be an indication that the β-glucosidase activity levels were sufficient for the formation of monomeric sugars. In addition, use of the enzymatic complex from *A. niger* resulted in conversion yields similar to those achieved using the commercial enzyme preparation (14.0 and 31.9% of the theoretical yields in terms of glucose and TRS). This is an interesting finding, especially considering that the dosage of enzymes (in terms of FPase activity) in the commercial cocktail was up to 6.2-fold higher than in the EE from *A. niger*.

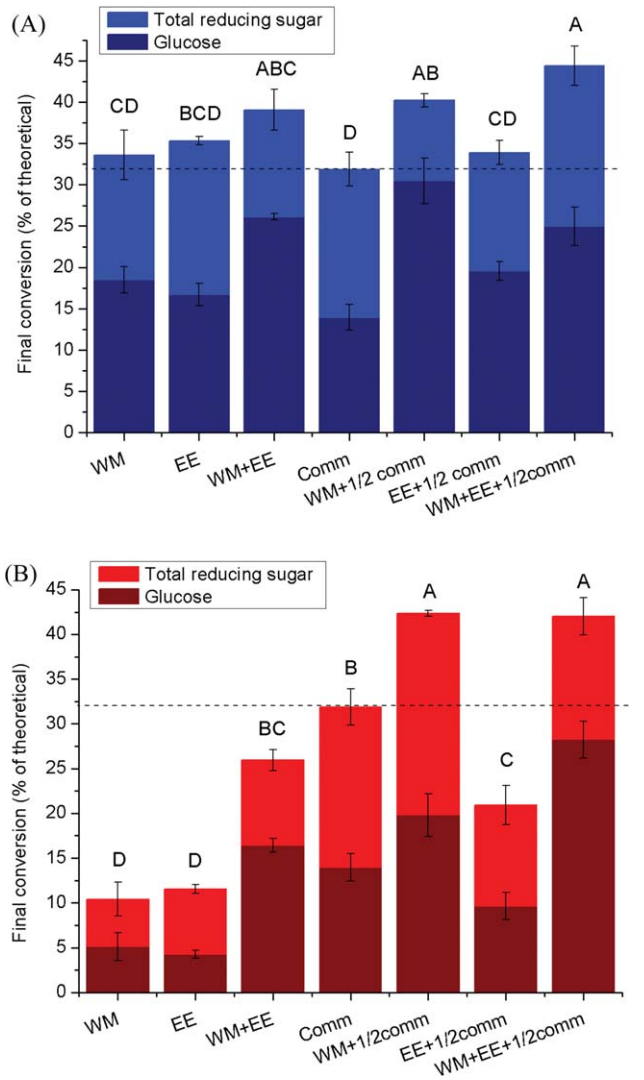

Figure 5. Kinetic profiles of enzyme production over a period of 120 h under SSF for (A) *A. niger* 3T5B8 and (B) *T. reesei* Rut-C30.

For the hydrolyses carried out using different combinations of *A. niger* enzymes (EE and WM) and a low dosage of commercial enzymes (Figure 5a), it is interesting to note that higher conversion yields were achieved when the whole medium was present. Considering that the sugars released from the wheat bran in the whole medium were already subtracted, since it was used as one of the controls, it is possible that enzymes that remained adsorbed to the fermented medium (mycelium-bound enzymes) were active during the saccharification step, hence improving SESB conversion. In this set of experiments (Figure 5a), higher conversion values (25.0% in terms of glucose and 44.4% in terms of TRS) were achieved using a combination of *A. niger* enzymes from the EE and WM, together with a low dosage of commercial enzymes. Nevertheless, the yields were not statistically different from those obtained using a combination of WM and EE enzymes from *A. niger* cultivated under SSF, or using the WM enzymes supplemented with a low dosage of the commercial enzymes.

For SESB hydrolysis using the enzymatic complex from *T. reesei* (Figure 5b), the trends were similar to those observed for *A. niger*. However, the conversion yields were substantially lower than those achieved with either the *A. niger* enzymes or the commercial enzymes. Similar conversion values were obtained for the *T. reesei* EE and WM, in terms of both glucose (4.3 and 5.1% of the theoretical yields for the EE and the WM, respectively) and total reducing sugar (11.6 and 10.4%, respectively). Nevertheless, higher conversion values (28.2% of the theoretical yield in terms of glucose and 42.0% in terms of TRS) were achieved using a combination of *T. reesei* enzymes from the WM and a low dosage of commercial enzymes. Further addition of *T. reesei* EE to the combination of the WM and a low dosage of commercial enzymes resulted in similar conversion yields. Kovacs et al.[19] also observed an improvement in hydrolytic capacity when the whole fermentation broths were used instead of the supernatants from *T. reesei* and *T. atroviride* cultivations under submerged fermentation. The improvement in the hydrolysis of pretreated spruce was attributed to the presence of mycelium-bound enzymes. Higher performance using the whole broth of *T. reesei* instead of the filtrate was also reported by Schell et al.[18] for a simultaneous saccharification and fermentation process, where higher ethanol yields were achieved using the whole broth. The present findings demonstrate that the use of in-house WM enzymes from SSF, combined with a low dosage of commercial cellulases, may be of potential interest for biotechnological processes involving the conversion of sugarcane bagasse into fuels and chemicals.

In summary, for both fungi, the enzymatic extract (EE) and the whole fermentation medium (WM) provided similar SESB hydrolysis yields, in terms of both glucose and total reducing sugar, giving a clear indication that the SSF enzyme extraction step could be eliminated. It is therefore possible to use a lignocellulosic agricultural waste for enzyme production under SSF, and to use it again during the saccharification step, eliminating the enzyme extraction/filtration steps. This procedure is highly advantageous in terms of reduced enzyme and process costs, and also avoids the generation of unnecessary effluent streams. Moreover, the proposed enzyme production process, with in-house enzyme production under SSF, followed by application of the whole fermentation medium in the biomass saccharification step to generate the sugars needed for the production of biofuel and other chemicals, offers a potential alternative for sustainable industrial biomass conversion within the biorefinery concept.

Comparison of the enzymatic conversion yields obtained using the enzymes from the two fungi revealed the superiority of the *A. niger* 3T5B8 enzymatic complex, which provided conversion values that were up to 3.2-fold higher (in terms of TRS) than those obtained using *T. reesei* Rut-C30 at very similar enzyme loadings (8.2 mg protein/g glucan for *A. niger* and 10.9 mg protein/g glucan for *T. reesei*). However, it is important to note that with supplementation of the *T. reesei* enzymes present in the WM it was possible to achieve conversion values that were similar to those achieved using *A. niger* enzymes. Although much work remains to scale up the proposed procedure, the conversion yields are very promising, especially given the relatively low enzyme dosages used in the hydrolyses. The findings highlight the potential of in-house SSF enzyme production, followed by application of the whole medium in the saccharification step, as a process configuration worthy of further investigation.

Conclusions

The *A. niger* 3T5B8 strain cultivated under SSF was able to produce an enzymatic cocktail that was more efficient for

hydrolysis of SESB, compared to the enzymes from either *T. reesei* Rut-C30 or a commercial preparation at a similar FPase activity dosage. Furthermore, SESB hydrolysis using either extract supernatant or whole fermentation medium resulted in similar yields in terms of glucose and total reducing sugar, giving a clear indication that the enzyme extraction/filtration steps in SSF could be eliminated. The proposed procedure is cost-effective and avoids unnecessary generation of effluents. The enzymatic conversion of SESB using whole SSF fermentation media is a potential alternative process configuration that conforms to the biorefinery concept.

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