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Enzymatic Hydrolysis of Sugarcane Bagasse Using Enzyme Extract and Whole Solid-state Fermentation Medium of Two Newly Isolated Strains of *Aspergillus Oryzae*

Rosangela D.P.B. Pirota [a,b], Priscila S. Delabona [b,c], Cristiane S. Farinas*[a,b]

[a]Embrapa Instrumentação, Rua XV de Novembro, 1452, 13560-970, São Carlos, SP, Brazil
[b]Programa de Pós-Graduação em Biotecnologia, Universidade Federal de São Carlos, 13565-905, São Carlos, SP, Brazil
[c]Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Rua Giuseppe Maximo Scolfaro 10000, Pólo II de Alta Tecnologia, 13083-970, Campinas, SP, Brazil
cristiane.farinas@embrapa.br

Enzymatic conversion of lignocellulosic biomass into fuels and chemicals will be a key technology in the future. 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. In this sense, the use of solid-state fermentation (SSF) is particularly advantageous for enzyme production. Here, a comparative study on the enzymatic hydrolysis of steam-exploded sugarcane bagasse (SESB) using enzymatic extract (EE) and whole solid-state fermentation medium (WM) of two newly isolated strains of *Aspergillus oryzae* (P6B2 and P27C3A) from the Amazon Rainforest was carried out. The biomass conversion using WM from *A. oryzae* P6B2 was more efficient when compared with the EE, while for *A. oryzae* P27C3A the conversion yields were similar. The WM from P27C3A supplemented with a low dosage of commercial enzyme resulted in a final conversion of 45% of the theoretical value. Furthermore, the combination of the enzymes from both strains, allowed up to 5 folds improvement in SESB conversion. These results showed that the in-house enzyme production by wild-type *A. oryzae* strains cultivated under SSF can be very advantageous for the enzymatic hydrolysis of biomass and, thus, it can be considered as a potential biotechnological configuration for the production of biofuels.

1. Introduction

The cost of the enzymatic cocktails significantly influences the viability of the overall process of biomass conversion into fuels and other chemicals. To address this issue, recent studies have aimed at increasing the efficiency of biomass-degrading enzyme production by identifying novel microbial strains (Delabona et al., 2012b; King et al., 2011) and more efficient fermentation techniques (Cunha et al., 2012; Jourdier et al., 2012; Lan et al., 2013; Pirota et al., 2013a). In this context, on-site production of enzymes has been considered as a potential strategy to reduce costs (Delabona et al., 2012a; Kovacs et al., 2009a; Sorensen et al., 2011). Most commercial cellulase preparations are produced using filamentous fungi of the genera *Trichoderma* and *Aspergillus*. Among the *Aspergillus* genera, *A. niger* along with *A. oryzae* are the two most important fungi worldwide for biotechnological applications (Hu et al., 2011). Nevertheless, recent findings on the genomics of *A. oryzae* have revealed that it is highly enriched with genes involved in biomass degradation (Kobayashi et al., 2007). Moreover, enzyme-prospecting research continues to identify opportunities to enhance the activity of enzyme preparations by supplementation with enzymatic diversity from different microbes (King et al., 2011).
The use of solid-state fermentation (SSF) for industrial enzyme production is particularly advantageous for enzyme production by filamentous fungi and enables the use of agro-industrial residues as solid substrate, acting as sources of both carbon and energy (Singhania et al., 2009). In a previous report we showed that by using the whole fermentation medium from SSF, containing the enzymes, mycelia, and the residual solid substrate, for the saccharification of lignocellulosic biomass it was possible to eliminate additional separation steps, thus contributing to cost reduction (Pirota et al., 2013b).

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The aim of the present study was to expand the validation of the proposed SSF configuration by investigating the enzymatic hydrolysis of steam-exploded sugarcane bagasse (SESB) using the enzyme extracts (EE) and whole fermentation media (WM) of two newly isolated strains of *Aspergillus oryzae* (P6B2 and P27C3) from the Amazon Forest, cultivated on wheat bran under selected SSF. The hydrolysis yields of SESB were compared with those obtained using a commercial cellulase preparation as well as combinations of different enzyme sources, with evaluation of the contribution of mycelia-bound enzymes to the process.

2. Materials and Methods

2.1 Microorganisms

The microorganisms used in this study were two wild-type strains of *Aspergillus oryzae* (P6B2 and P27C3A) isolated from the Amazon Rain forest (Delabona et al., 2012b), both from the Embrapa Food Technology Collection (Rio de Janeiro, Brazil). The cultures were revitalized and maintained on PDA slants at 32 °C for 5 days prior to inoculation.

2.2 Lignocellulosic materials

Solid-state fermentation (SSF) cultivations were carried out using wheat bran (Claro Agropecuária, 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 (Delabona et al., 2012a).

2.3 SSF cultivations for enzyme production

SSF cultivations were carried out in 250 mL Erlenmeyer flasks 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. Substrate moisture level was adjusted using a nutrient solution (Mandels and Sternberg, 1976). Cultivations were carried out at different moisture levels, temperatures and periods, according to previous selected conditions for each strain, as follows: *A. oryzae* P6B2 (80% moisture, 28°C for 24 h) and *A. oryzae* P27C3A (70% moisture, 28°C for 48 h) (Pirota et al., 2013a). 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 x g at 4 °C for 20 min. Alternatively, the whole fermentation media containing the enzymes, mycelia, and the residual solid substrate were used in the hydrolysis experiments, as described in Section 2.4. All cultivation experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

2.4 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 ºC for 4 h with 200 rpm agitation. Subsequently, 5 g of the previously fermented wheat bran SSF material (as described in Section 2.3) 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. oryzae* P6B2 or *A. oryzae* P27C3A 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. The commercial cellulase preparation was diluted sufficiently to achieve an enzymatic activity equivalent to that of the crude fungal enzyme extracts (0.05 FPU/mL). 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.

2.5 Analytical measurements

The activities of FPase and endoglucanase were determined according to the procedure recommended by the IUPAC Commission on Biotechnology, with modifications (Delabona et al., 2012b). The activity of xylanase was measured according to the methodology described by Bailey and Poutanen (1989). 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 (Miller, 1959). 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). In the hydrolysis experiments, glucose and total reducing sugar were measured using the enzymatic kit for glucose measurement and the DNS method, respectively.

3. Results and Discussion

3.1 Enzyme production under SSF

Enzyme production in terms of endoglucanase, β-glucosidase, FPase and xylanase activity by the fungi *A. oryzae* P6B2 and *A. oryzae* P27C3A cultivated under SSF is presented in Table 1. The endoglucanase and β-glucosidase activities produced by both *A. oryzae* strains were very similar, while the FPase and xylanase activity varied. The strain of *A. oryzae* P6B2 produced 3.3 folds more xylanase enzymes than *A. oryzae* P27C3A, whereas in terms of FPase activity, *A. oryzae* P27C3A activity was 2-fold higher than *A. oryzae* P6B2. It is interesting to note that even though these two wild-type strains of filamentous fungi belong to the same genus and specie and were both isolated from the same Amazon Forest region, their characteristics were rather distinct. The strain *A. oryzae* P6B2 was more efficient on xylanases production, while *A. oryzae* P27C3A strain was more efficient on cellulases production (Pirota et al., 2013a, Pirota et al., 2014).

Table 1: Enzymatic activities obtained from the cultivation of different fungi under SSF selected condition.

Enzymatic activity (IU/gds)	*A. oryzae* P6B2	*A. oryzae* P27C3A
Endoglucanase	115.8 ± 5.3	113.4 ± 0.7
β-glucosidase	2.7 ±0.10	2.0 ± 0.2
FPase	0.13 ± 0.01	0.25 ± 0.07
Xylanase	1658.1 ± 98.7	507.9 ± 14.0

Brijwani et al. (2010) using an *A. oryzae* strain and a mixture of wheat bran and soybean (4:1) as substrate in SSF obtained a production of endoglucanase, FPase, β-glucosidase and xylanase of 68.4, 6.7, 9.5 and 512.16 IU/gds, respectively. Yamane et al. (2002) reported the production of xylanase by *A. oryzae* cultivated under SSF in the order of 120 IU/gds. Overall, our results demonstrate the considerable potential of the *A. oryzae* P6B2 and *A. oryzae* P27C3A strains for the production of glycosyl hydrolases, since it showed a significantly high enzymatic biosynthesis capacity when compared to the literature.
Based on these results, the SESB hydrolysis experiments were conducted using the enzymatic extracts (EE) and whole fermentation medium (WM) from the cultivation of both *A. oryzae* strains, either separately or in combination between them and between a commercial preparation.

3.2 Enzymatic hydrolysis step

The catalytic efficiencies of the enzymes produced by *A. oryzae* P6B2 and *A. oryzae* P27C3A strains cultivated under SSF were evaluated during the hydrolysis of pretreated sugarcane bagasse (SESB). Different experimental configurations were employed: (1) The whole fermentation media (WM) obtained after SSF cultivation 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. In addition, experiments were conducted in order to evaluate the synergistic effect of different combinations of enzyme sources, such as the combination of *A. oryzae* P6B2 and *A. oryzae* P27C3A enzymes and the supplementation of commercial enzyme cocktails. These latter experiments employed the enzymes present in the WM and EE from either *A. oryzae* P6B2 or *A. oryzae* P27C3A in different combinations with a low dosage of a commercial enzyme preparation.
Figure 1 presents the temporal profiles of total reducing sugar (TRS) released during the hydrolysis of SESB using the *A. oryzae* P6B2 and *A. oryzae* P27C3A enzymes present in the whole fermentation media

(WM) or in the enzymatic extracts (EE). From Figure 1, it can be observed that the amount of TRS increased with time, and that the effect was much more pronounced when the *A. oryzae* P6B2 enzymes from the WM were used, compared to those from EE. The concentrations of TRS released after 72 h was 4.7 g/L for *A. oryzae* P6B2 WM, while the for EE was 2.2 g/L. A different pattern was observed using the enzymes from *A. oryzae* P27C3A, where the amount of TRS released during SESB hydrolysis was very similar using either WM or EE sources of enzymes (2.5 and 3.1 g/L, respectively). This result indicates that enzymes from the *A. oryzae* P6B2 that remained adsorbed to the fermented medium (mycelium-bound enzymes) were active during the saccharification step, hence improving SESB conversion.

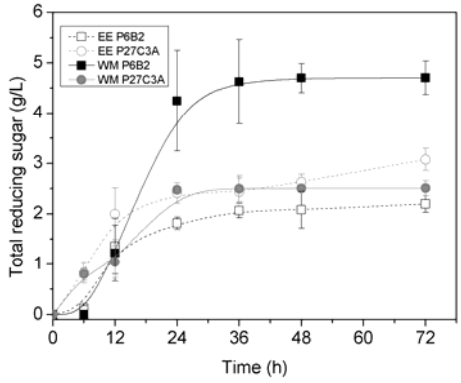

Figure 1: Temporal profiles of the concentrations of total reducing sugar released during the hydrolysis of pretreated sugarcane bagasse (SESB) using the whole fermentation media (WM) and the enzymatic extracts (EE) from A. oryzae P6B2 and A. oryzae P27C3A cultivated under SSF.

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 2 summarizes the conversion percentages achieved after 72 h of SESB hydrolysis using the *A. oryzae* P6B2 (Figure 2a) and *A. oryzae* P27C3A (Figure 2b) 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. oryzae* P6B2 (Figure 2a) for SESB hydrolysis resulted in very distinct biomass conversion yields for EE and WM, in terms of both glucose (0.9 and 4.0% of the theoretical yields for EE and WM, respectively) and total reducing sugar (6.5 and 14.0% for EE and WM, respectively). The values were significantly different (Tukey's test, $p<0.05$). For the hydrolyses carried out using different combinations of *A. oryzae* P6B2 enzymes (EE and WM) and a low dosage of commercial enzymes, it is interesting to note that higher conversion yields were achieved when the whole medium was present. As pointed before, such behavior could be related with the enzymes from *A. oryzae* P6B2 that remained adsorbed to the fermented medium (mycelium-bound enzymes). Nevertheless, the best result for SESB conversion (34.5% of the theoretical value in terms of TRS) was achieved by using a combination of enzymes from *A. oryzae* P6B2 (WM) and *A. oryzae* P27C3A (EE). The combination of the enzymes from both strains, allowed up to 5 folds improvement in SESB conversion. On the other hand, use of the enzymatic complexes from *A. oryzae* P27C3A (Figure 2b) for SESB hydrolysis resulted in similar biomass conversion yields for EE and WM, in terms of both glucose (2.9 and 3.1% of the theoretical yields for EE and WM, respectively) and total reducing sugar (9.1 and 7.5% for EE and WM, respectively). The values were not significantly different (Tukey's test, $p<0.05$). In this set of experiments (Figure 2b), higher conversion values (20.2% in terms of glucose and 44.8% in terms of TRS) were achieved using a combination of *A. oryzae* P27C3A enzymes from the WM together with a low dosage of commercial enzymes.

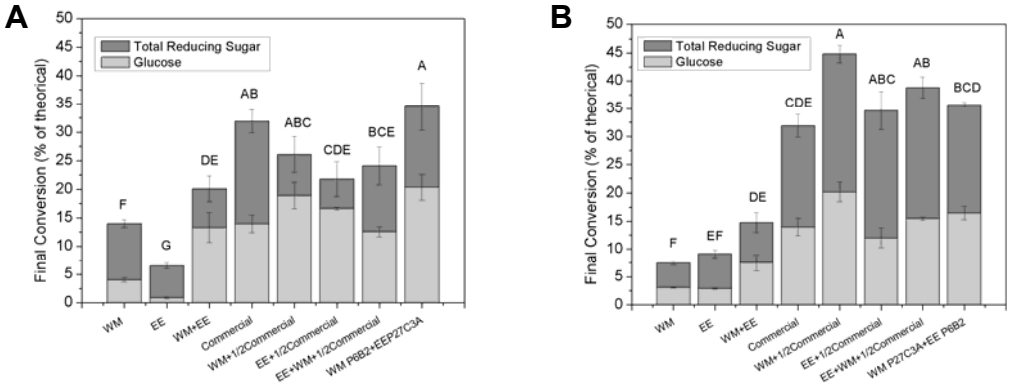

Figure 2: Conversion of the pretreated sugarcane bagasse (SESB) after 72 h of hydrolysis using enzymatic extracts (EE), whole fermentation media (WM), commercial enzymes, and different combinations, for (A) A. oryzae P6B2 and (B) A. oryzae P27C3A. Means with different letters are significantly different (Tukey's test, p<0.05).

Kovacs et al. (2009b) 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. (1990) for a simultaneous saccharification and fermentation process, where higher ethanol yields were achieved using the whole broth.

Our results showed that, for both fungi, the use of whole fermentation medium (WM) instead of the enzymatic extract (EE) provided similar or higher 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, thus corroborating our previous results (Pirota et al., 2013b). 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. Moreover, the present findings demonstrate that the use of in-house enzymes from wild-type strains isolated from the Amazon rainforest and cultivated under SSF may be of potential interest for biotechnological processes involving the conversion of biomass into fuels and chemicals.

4. Conclusions

The wild-type *A. oryzae* strains cultivated under SSF were able to produce an enzymatic cocktail that was highly efficient for hydrolysis of SESB. 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 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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