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Enzymatic hydrolysis and simultaneous saccharification and fermentation of steam-pretreated spruce using crude *Trichoderma reesei* and *Trichoderma atroviride* enzymes

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ABSTRACT

The aim of this study was to compare the performance of the enzymes produced by *Trichoderma reesei* Rut C30 and the good extracellular β-glucosidase-producing mutant *Trichoderma atroviride* TUB F-1663 to that of commercial preparations in the enzymatic hydrolysis and the simultaneous saccharification and fermentation (SSF) of steam-pretreated spruce (SPS).

The concentrated TUB F-1663 enzyme was found to be the most efficient in the hydrolysis of washed SPS at 50 g/L water-insoluble solids (WIS) in terms of the glucose produced (18.5 g/L), even in comparison with commercial cellulases (14.1–16.7 g/L). The enzyme preparations were studied at low enzyme loadings (5 FPU/g WIS) in SSF to produce ethanol from SPS. The enzyme supernatant and whole fermentation broth of *T. atroviride* as well as the whole broth of *T. reesei* proved to be as efficient in SSF as the commercial cellulase mixtures (ethanol yields of 61–76% of the theoretical were achieved), while low ethanol yields (<40%) were obtained with the β-glucosidase-deficient *T. reesei* supernatant.

Therefore, it seems, that instead of using commercial cellulases, the TUB F-1663 enzymes and the whole broth of Rut C30 may be produced on-site, using a process stream as carbon source, and employed directly in the biomass-to-bioethanol process.

1. Introduction

The cost and hydrolytic efficiency of enzymes used for the saccharification of pretreated lignocellulosic materials are critical factors in the biomass to ethanol process [1–3]. It is therefore important to develop more effective cellulolytic microorganisms and improve the hydrolytic properties of enzyme mixtures.

Trichoderma reesei mutants are most widely used to produce commercial cellulases for enzymatic hydrolysis [4–6]. However, the drawback of the enzyme cocktail produced by this species is the low level of extracellular β-glucosidase activity, which leads to the accumulation of cellobiose, i.e. incomplete degradation of the cellulose. Various methods of enhancing the hydrolytic potential of *T. reesei* enzyme mixtures include the genetically modification of *T. reesei* strains [7–10], the use of the whole culture broth of *T. reesei* instead of the fermentation supernatant [11–13], or the co-culture of *T. reesei* with a good β-glucosidase-producing fungus, e.g. *Aspergillus phoenicis* [14–16].

In the future, new strains with good filter paper activity (FPA) combined with enhanced extracellular β-glucosidase production may compete with *T. reesei* as sources of cellulases [17]. Some *Trichoderma atroviride* mutants have been found to be capable of secreting high levels of cellulases and β-glucosidase simultaneously on pretreated willow [18] and spruce [13]. The crude enzyme supernatants of *T. atroviride* hydrolyzed the cellulose completely, while the *T. reesei* supernatants and commercial enzyme preparations (e.g. Celluclast 1.5L) needed complementary β-glucosidase supplementation to be able to cleave cellobiose into glucose. Various *Penicillium* species [19–21] and *Trichoderma harzianum* E58 [22] have also been shown to secrete enzyme mixtures with similar or better hydrolytic potential than *T. reesei*. β-Glucosidase deficiency in enzyme complexes also limits the efficiency of the simultaneous saccharification and fermentation (SSF) process, since *Saccharomyces cerevisiae*, the preferred yeast for industrial ethanol production, does not ferment cellobiose to ethanol in its native form.

The production of ethanol from steam-pretreated spruce (SPS) has been widely studied with regard to pretreatment [23–26], enzymatic hydrolysis [27–31], fermentation [32–36] and process modeling [37,38]. In the present study, cellulolytic enzymes were

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produced in-house on SPS with two mutants, namely *T. reesei* Rut C30, the well-known hypercellulolytic strain, and *T. atroviride* TUB F-1663, our new, good β-glucosidase-producing isolate. The enzymes were mixed in different ratios in order to investigate whether a mixture of *T. reesei* and *T. atroviride* cellulase components had better hydrolytic capacity on washed SPS than the single enzyme supernatants. The hydrolytic potential of the in-house concentrated enzyme preparations was compared to that of commercial cellulases on the whole pretreated slurry, as well as on the washed pretreated material. Finally, the enzyme supernatants, the whole fermentation broths and the concentrated enzymes of *T. reesei* Rut C30 and *T. atroviride* TUB F-1663 were studied in SSF to produce ethanol from SPS, and the results were compared with those obtained with commercial cellulases. To the authors' knowledge, no research has so far been carried out on SSF using crude enzyme supernatants and whole fermentation broths produced in-house.

2. Materials and methods

2.1. Steam-pretreated spruce

Spruce was kindly provided by a sawmill in southern Sweden (Widtsköfle Sågwerk AB, Degeberga, Sweden). The material was steam pretreated and its composition was analyzed as described previously [13].

2.2. Enzymes and microorganisms

Celluclast 1.5L, a cellulase mixture produced by *T. reesei,* and Novozym 188, a β-glucosidase preparation from *Aspergillus niger*, were both kindly provided by Novozymes A/S (Bagsvaerd, Denmark). Accellerase™ 1000, a cellulase enzyme complex developed for lignocellulosic biomass hydrolysis, produced with a genetically modified strain derived from *T. reesei*, was kindly provided by Genencor, Danisco A/S (Copenhagen, Denmark). For simplicity, Celluclast 1.5L, Novozym 188 and Accellerase 1000 are referred to in the text as Celluclast, Novozym and Accellerase, respectively.

T. atroviride TUB F-1663 was obtained from the TUB collection (=Technical University of Budapest). Isolation and mutagenesis data for this strain have been presented in a previous study [18]. *T. reesei* Rut C30 was kindly donated by Prof. D. E. Eveleigh (Rutgers University, NJ, USA). For simplicity, *T. atroviride* TUB F-1663 and *T. reesei* Rut C30 are referred to in the text as TUB F-1663 and Rut C30, respectively. The freeze-dried cultures were revitalized and maintained on potato-dextrose-agar Petri plates at 30 °C. Properly sporulating cultures were used for inoculation. The Rut C30 and TUB F-1663 enzymes were produced on SPS as described previously [13]. The whole fermentation broths, the centrifuged enzyme supernatants and the concentrated (ultrafiltered) enzyme supernatants of Rut C30 and TUB F-1663 were used in enzymatic hydrolysis and SSF of SPS. The enzyme supernatants were concentrated using a Labscale TFF System and a Pellicon XL membrane with a 10-kDa cut-off (Millipore, Billerica, MA, USA). Prior to concentration the supernatants were filtered through a 0.2-μm nylon filter (Pall Corporation, New York, USA).

2.3. Enzyme assays

Total reducing sugars were determined colorimetrically using the dinitrosalicylic acid method [39]. The FPA was assayed according to Ghose [40]. The β-glucosidase activity was determined using Berghem's method [41] with slight modifications as described previously [13]. The FPA and β-glucosidase activities presented are the average of two separate measurements.

2.4. Enzymatic hydrolysis of SPS

When studying the hydrolytic efficiency of the crude *T. reesei* and *T. atroviride* enzyme mixtures, the soluble parts of SPS that contained inhibitors, sugars and degradation products of hemicellulose and lignin were removed by washing the slurry with distilled water. After filtration, the insoluble parts (mainly cellulose and lignin) of the steam-pretreated and washed spruce were subjected to enzymatic hydrolysis. The crude enzyme supernatants of Rut C30 and TUB F-1663 produced on SPS were mixed together at ratios of 0:1; 1:4; 2:3; 3:2; 4:1 and 1:0 (w:w). The FPA and β-glucosidase activity of each mixture were measured. The hydrolytic potential of the mixtures was investigated at an enzyme loading of 3 FPU/g WIS.

In order to compare the hydrolysis capacities of the various enzymes on washed and unwashed SPS, a 3:1 (w:w) mixture of Celluclast & Novozym, Accellerase, the concentrated enzyme supernatant of Rut C30 and that of TUB F-1663 were used at an enzyme loading of 5 FPU/g WIS. Hydrolysis was carried out in duplicate at 40 °C for 96 h in all cases. Both the washed and the unwashed materials were diluted with 0.1 M Na-acetate buffer (pH 4.8) to 50 g/L WIS in a total volume of 250 mL. Samples were withdrawn after 0, 3, 7, 24, 48, 72 and 96 h, and centrifuged at 3000 rpm for 5 min. The results presented are the mean values of two separate hydrolysis experiments.

2.5. Yeast cultivation

Baker's yeast, *S. cerevisiae* (Jästbolaget, Rotebro, Sweden), was first purified into single colonies by streaking on potato-dextrose-agar containing 100 μg/mL doxycycline. One-liter cotton-plugged Erlenmeyer flasks containing 200 mL sterile medium were inoculated with purified yeast from the agar plates. The composition of the medium was as follows (in g/L): glucose, 20; $(NH_4)_2SO_4$, 10; KH_2PO_4, 5; $MgSO_4{\cdot}7H_2O$, 1; yeast extract, 5, and (in mg/L): $CoCl_2{\cdot}6H_2O$, 2; $MnSO_4$, 1.6; $ZnSO_4{\cdot}7H_2O$, 3.45; and $FeSO_4{\cdot}7H_2O$, 5. The pH before inoculation was 5.2. Cultivation was performed on a shaker at 30 °C and 220 rpm for 24 h. The culture broth was centrifuged at 4000 rpm for 10 min, the supernatant was discarded and the DM of the harvested cells was determined before further use in SSF.

2.6. Simultaneous saccharification and fermentation

SSF was used to produce ethanol from SPS. The unwashed pretreated slurry was diluted with tap water to a final WIS concentration of 50 g/L and was then sterilized in an autoclave at 121 °C for 30 min. The nutrients, $(NH_4)_2HPO_4$, $MgSO_4{\cdot}7H_2O$ and yeast extract, were sterilized separately and added to the slurry to final concentrations of 0.5, 0.025 and 1.0 g/L, respectively.

In order to investigate whether the crude *T. reesei* and *T. atroviride* enzymes produced can be directly applied in SSF of SPS, the experiments were carried out in duplicate in 1-L Erlenmeyer flasks with a total working volume of 200 mL. The medium was inoculated with the centrifuged yeast suspension at 3.5 g/L dry yeast cells. The crude enzyme supernatants and whole fermentation broths of Rut C30 and TUB F-1663 were used at 10 g enzyme solution (supernatant or whole broth) per g WIS. The initial pH was set to 5 with 5% NaOH. SSF was carried out for 4 days at 35 °C, at 220 rpm. The flasks were covered with plastic film in order to provide semi-anaerobic conditions for the yeast. Samples were withdrawn after 0, 3, 8, 24, 48, 72 and 96 h, and centrifuged at 3000 rpm for 5 min. The mean values of two separate SSF experiments are presented.

To compare the in-house-produced enzymes with commercial cellulases, the SSF experiments were performed in 2-L fermentors (Infors AG, Bottmingen, Switzerland) with a total working volume of 1 L. The yeast suspension was added at a concentration of 2 g/L dry yeast cells. The performance of the concentrated enzyme supernatants of Rut C30 and TUB F-1663 was compared to those of the 3:1 mixture of Celluclast & Novozym and Accellerase at 5 FPU/g WIS. SSF was carried out in duplicate for 4 days at pH 5 ± 0.2 and 35 °C, and the pH was set with 5% NaOH. Samples were withdrawn after 0, 3, 5, 7, 24, 48, 72 and 96 h, and centrifuged at 3000 rpm for 5 min. The mean values of two separate SSF experiments are presented.

2.7. Analytical methods

All samples were filtered through 0.2-μm filters (MFS-13, Micro Filtration Systems, Dublin, CA, USA) and analyzed using an HPLC instrument (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (Shimadzu). Cellobiose, glucose, xylose, galactose, arabinose and mannose were separated using an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) at 80 °C, with deionized water as eluent at a flow rate of 0.5 mL/min, while ethanol, lactic acid, glycerol, acetic acid, hydroxyl-methyl-furfural and furfural were separated on an Aminex HPX-87H column (Bio-Rad) at 65 °C, with 5 mM H_2SO_4 as eluent at a flow rate of 0.5 mL/min.

3. Results and discussion

3.1. Steam pretreatment

Table 1 presents the composition of SPS. The total DM and WIS content of the material after pretreatment were 202 and 137 g/L, respectively. The two major insoluble components were glucan (54.1 w/w% of WIS) and lignin (42.3 w/w% of WIS), while the main soluble components were found to be glucose (22.3 g/L) and mannose (20.9 g/L). In addition to sugars, water-soluble inhibitors, such as acetic acid, lignin degradation products and sugar-derived by-products were also formed during pretreatment.

3.2. FPA and β-glucosidase activities of enzyme mixtures

In this study, low-β-glucosidase-containing cellulases (i.e. Celluclast and the supernatant of Rut C30) were mixed together with high-β-glucosidase-containing enzymes (i.e. Novozym and the supernatant of TUB F-1663) in different ratios in order to investigate the final FPA and β-glucosidase activities of the