J Ind Microbiol Biotechnol (2014) 41:1191–1200
DOI 10.1007/s10295-014-1457-9

BIOENERGY/BIOFUELS/BIOCHEMICALS

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Production of cellulosic ethanol and enzyme from waste fiber sludge using SSF, recycling of hydrolytic enzymes and yeast, and recombinant cellulase-producing *Aspergillus niger*

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Received: 25 March 2014 / Accepted: 5 May 2014 / Published online: 27 May 2014

Abstract Bioethanol and enzymes were produced from fiber sludges through sequential microbial cultivations. After a first simultaneous saccharification and fermentation (SSF) with yeast, the bioethanol concentrations of sulfate and sulfite fiber sludges were 45.6 and 64.7 g/L, respectively. The second SSF, which included fresh fiber sludges and recycled yeast and enzymes from the first SSF, resulted in ethanol concentrations of 38.3 g/L for sulfate fiber sludge and 24.4 g/L for sulfite fiber sludge. *Aspergillus niger* carrying the endoglucanase-encoding *Cel7B* gene of *Trichoderma reesei* was grown in the spent fiber sludge hydrolysates. The cellulase activities obtained with spent hydrolysates of sulfate and sulfite fiber sludges were 2,700 and 2,900 nkat/mL, respectively. The high cellulase activities produced by using stillage and the significant ethanol concentrations produced in the second SSF suggest that onsite enzyme production and recycling of enzyme are realistic concepts that warrant further attention.

Keywords Biorefinery · Fiber sludge · Cellulosic ethanol · Enzymes · Cellulase

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Introduction

Utilization of renewable feedstock for production of liquid biofuels and high value added biorefinery products is gaining increasing attention. The potential of replacing fossil resources for production of fuels, materials and chemicals with renewable feedstocks is a promising opportunity. However, increasing demands for renewable feedstock in the future should encourage investigations of the possibility to exploit industrial residual streams in the production of commodities. Waste fiber sludge is a residual stream produced by lignocellulose-based biorefineries and pulp mills [13, 18]. Cellulose and hemicellulose are the main components of fiber sludges. However, the abundance and composition of fiber sludges vary greatly from mill to mill and depend on the process and the feedstock. The main utilization of fiber sludge in Europe is either land filling or incineration to generate energy. The high carbohydrate and low lignin content of fiber sludge could, however, make it suitable as a feedstock for the production of liquid biofuels, such as ethanol, in lignocellulose-based biorefineries [13]. It would be of even greater interest if the fiber sludge, at least partly, could be converted into high value added products.

In recent years, cellulolytic enzymes have gained increasing attention for the production of liquid biofuels from lignocellulosic feedstocks. Main benefits of using enzymes for hydrolysis of lignocellulose, include high sugar yields and reduced formation of fermentation inhibitors in the hydrolysates. The main drawback is that the cost of enzymes contributes significantly to the total process costs. The enzyme costs could tentatively be reduced by consolidated bioprocessing (CBP), in which the fermenting microorganism also produces enzyme, by onsite enzyme production using inexpensive growth medium based on the

lignocellulose degradation products, or by enzyme recycling [1, 4, 21].

Trichoderma reesei (*Hypocrea jecorina*) is an efficient producer of cellulases and hemicellulases [8]. However, enzyme production is governed by gene regulation, i.e. induction and repression, which in turn is dependent on the growth medium. Enzyme production using media based on lignocellulose degradation products may also be difficult considering inhibitory compounds frequently found in industrial residual streams, such as phenols and organic acids. Fungi of the *Aspergillus* genus, such as *Aspergillus niger,* are, however, known for their ability to grow oligotrophically [11, 17], i.e., in nutrient poor environments. They also display the ability to tolerate and consume inhibitory compounds found in lignocellulosic hydrolysates [1]. An additional benefit of *A. niger* is the ability to produce and secrete heterologous proteins in the range of grams per liter [4]. These characteristics and abilities could potentially make *A. niger* suitable for production of hydrolytic enzymes from industrial residual streams where the conditions are far from ideal for growth of many other microorganisms.

The objectives of this study were to investigate the possible use of fiber sludge for co-production of biofuel (by *Saccharomyces cerevisiae*) and enzymes (by recombinant *A. niger*) in sequential microbial cultivation processes (Fig. 1). The fermentation with *S. cerevisiae* was carried out as a simultaneous saccharification and fermentation (SSF) with a high dry weight content of fiber sludge. Furthermore, we also investigated the possibility to recycle a portion of the yeast and the enzymes used in the first SSF to a second SSF, in which fresh fiber sludge was added to the solid fraction from the first SSF (Fig. 1). The approach studied, sequential cultivation with *S. cerevisiae* and recombinant *A. niger*, combines several potential advantages, including (i) efficient utilization of most of the components of the fiber sludge with the (ii) simultaneous production of liquid biofuel and (iii) enzymes, which represent a high value added commodity, and finally (iv) reduction in the need for external supply of enzyme through onsite enzyme production and enzyme recycling. Recombinant enzyme was identified using peptide mass fingerprint spectra generated with the use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The results from the MALDI-MS analysis were verified through analysis of individual peptides by post-source decay tandem mass spectrometry (PSD-MS/MS). The present study differs from those of Cavka et al. [3] and Alriksson et al. [1] since the previous studies were focused on separate hydrolysis and fermentation (SHF) process mode rather than SSF. Furthermore, the current study explores recycling of yeast and enzyme, and the production of enzyme from both fiber sludge and spent fiber sludge hydrolysate.

Materials and methods

Raw material

Waste fiber sludge was collected from two different Swedish pulp mills, one using the sulfate process (kraft pulping) and one using the sulfite process. The fiber sludges were dried in an oven at 70 °C for 96 h prior to further characterization. The characterization and analysis of monosaccharides, lignin and ash content in the two fiber sludges were performed by MoRe Research (Örnsköldsvik, Sweden).

Simultaneous saccharification and fermentation

The sulfite and sulfate fiber sludges were enzymatically hydrolyzed without any prior thermochemical pretreatment for 24 h prior to the addition of yeast. In the initial step, 16.2 g of dried fiber sludge was mixed with citrate buffer (0.05 M, pH 5.2) in four 750-mL shake flasks. The initial dry-matter content was 10 % and the total content per flask was 150 g. The enzyme preparations used for hydrolysis were Celluclast 1.5 L and Novozyme 188 (Novozymes, Bagsvaerd, Denmark). The stated activities of Celluclast 1.5 L and Novozyme 188 were 700 endoglucanase units (EGU)/g and 250 cellobiase units (CBU)/g, respectively. Each of the enzyme preparations was added to an initial concentration of 2 % (w/w) of the total weight of the reaction mixtures in the flasks. The flasks were incubated with orbital shaking (Infors Ecotron, Infors AG, Bottmingen, Switzerland) at 45 °C and 90 rpm for 24 h. Following the 24 h of hydrolysis, each vessel was supplemented with 4 mL of nutrient solution [150 g/L yeast extract, 75 g/L $(NH_4)_2HPO_4$, 3.75 g/L $MgSO_4{\cdot}7H_2O$, 238.2 g/L $NaH_2PO_4{\cdot}2H_2O$]. Each flask was inoculated with 6 mL of suspended *S. cerevisiae* cells (Ethanol Red, Fermentis, Marcq-en-Baroeul, France) to a final concentration of 2 g/L (dry cell weight, DW). Pre-cultures were grown in 750-mL shake flasks containing YEPD medium (2 % yeast extract, 1 % peptone, 2 % D-glucose). The cells were harvested in the late exponential growth phase by centrifugation (1,500*g*, 5 min, 4 °C) and re-suspended in sterile water. The fermentation was performed at 30 °C with orbital shaking (90 rpm). During the first 24 h of the fermentation, a total amount of 25.9 g fiber sludge was added as four separate additions to the flask. The final dry weight content of fiber sludge in the flasks was 22.5 %. At the final addition of fiber sludge, 24 h after inoculation with yeast, another 1 % of each of the enzyme preparations was added together with 4 mL of nutrient solution (described above). The fermentation continued for another 24 h before it was terminated by centrifugation (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at 4 °C and 1,700*g* for 10 min. The liquid fraction was recovered and stored at −20 °C until further

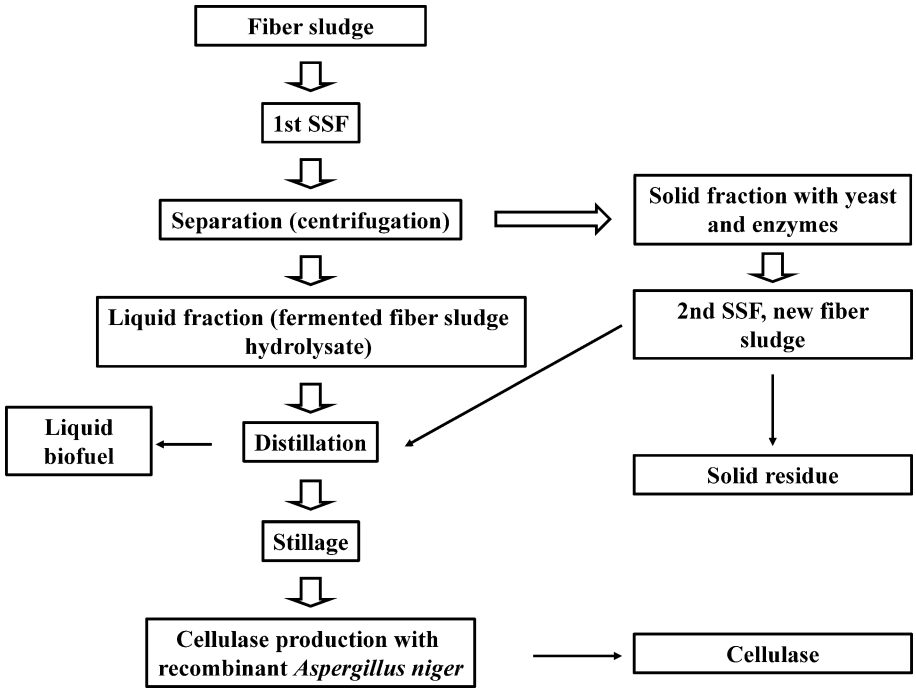

Fig. 1 Schematic view of the experimental approach and concept of recirculation of yeast and enzymes in a fiber sludge-to-ethanol process

use. The solid fractions of three of the four flasks for the sulfite and the sulfate sludges were dried in an oven at 80 °C for 48 h prior to weighing on an analytical scale. The remaining solid fraction of each type of fiber sludge was utilized in a subsequent recirculation experiment.

Recirculation of enzymes and yeast

Solid fractions of the sulfite and sulfate fiber sludges from the SSF experiment were re-suspended in 134.7 mL citrate buffer (0.05 M, pH 5.2) in two 1-L shake flasks. The re-suspended slurries were supplemented with 16.2 g fiber sludge and 3 mL of nutrient solution (as described above). The flasks were incubated at 30 °C with orbital shaking (90 rpm) for 72 h and samples taken every 24 h. The fermentations were terminated after 72 h by centrifugation at 1,700*g* and 4 °C for 10 min. The liquid fraction was recovered and the solid fraction was dried and weighed as previously described.

Distillation

Before distillation, the pH of the fermented hydrolysate was adjusted to 7.0 with 5 M NaOH to reduce the risk of sugar degradation. Distillation was performed using standard glassware and a magnetically stirred polyethylene glycol heating bath. The fermented fiber sludge hydrolysate was transferred to 500 mL round-bottom flasks and three drops of antifoam added to prevent excessive bubble formation during the distillation. The distillation was performed until all ethanol was recovered. The remaining fractions that were left in the round-bottom flasks after distillation, hereafter referred to as spent fiber sludge hydrolysates (SFSH), were filtered through 0.22-µm filters and stored at −20 °C until further use.

Aspergillus niger transformants

A recombinant *A. niger* D15 transformant expressing the *T. reesei* endoglucanase I (*egI*) gene was used for cellulase production (hereafter referred to as *A. niger* D15[*egI*]). The *egI* gene was cloned under transcriptional control of the constitutive glyceraldehyde-6-phosphate dehydrogenase (*gpd*) promoter from *A. niger* and the *gla*A terminator from *Aspergillus awamori* [12]. An *A. niger* D15 transformant carrying the expression cassette with the above-mentioned promoter and terminator, but without the *egI* gene was used as reference strain (hereafter referred to as *A. niger* D15[pGT]). The construction of the two transformants has previously been described by Rose and van Zyl [12].

Experiments with *A. niger* D15 cultivated on spent fiber sludge hydrolysates (SFSH)

Sixteen 100-mL Erlenmeyer flasks, labeled 1–16, were used in the experiments. Untreated waste fiber sludges suspended in citrate buffer (0.05 M, pH 6.5) were used as references in these experiments to determine if enzyme was produced from waste fiber sludge without prior hydrolysis and fermentation. Flasks 1–4 were filled

with 28.8 mL of spent fiber sludge hydrolysate originated from the sulfate fiber sludge flasks 5–8 were filled with 28.8 mL of spent fiber sludge hydrolysate originating from the sulfite fiber sludge. Flasks 9–12 contained 3.4 g of sulfate fiber sludge suspended in 26.7 mL citrate buffer (0.05 M, pH 6.5). Flasks 13–16 contained 2.1 g of sulfite fiber sludge suspended in 28.8 mL citrate buffer (0.05 M, pH 6.5). The amounts of fiber sludge that were used in the reference experiment were based on the amount of total organic carbon (TOC) which was found in the corresponding spent fiber sludge hydrolysates. All flasks were supplemented with 0.6 mL of nutrient solution (25 g/L $(NH_4)_2HPO_4$, 1.25 g/L $MgSO_4 \cdot 7H_2O$, 69 g/L $NaH_2PO_4 \cdot H_2O$, and 50 g/L yeast extract), and 0.05 mL of trace element solution (0.22 g/L $ZnSO_4 \cdot 7H_2O$, 0.11 g/L H_3BO_3, 0.05 g/L $MnCl_2 \cdot 4H_2O$, 0.05 g/L $FeSO_4 \cdot 7H_2O$, 0.017 g/L $CoCl_2 \cdot 6H_2O$, 0.016 g/L $CuSO_4 \cdot 5H_2O$, 0.015 g/L $Na_2MoO_4 \cdot 2H_2O$ and 0.5 g/L EDTA). The pH of all media used in the experiments was adjusted to 6.5 prior to the cultivation by the addition of 1 M HCl. For preparation of inoculum, spores of *A. niger* D15[pGT] and *A. niger* D15[*egI*] were generated on separate spore plates (0.2 % neopeptone, 0.1 % yeast extract, 0.2 % casamino acids, 0.04 % $MgSO_4 \cdot 7H_2O$, 1 % glucose, and 1.8 % agar) and incubated at 30 °C for 96 h. The spores were harvested and resuspended in a solution of sodium chloride (9 g/L). Spores were counted manually using a microscope (Olympus CX31, Olympus, Tokyo, Japan) and a counting chamber (Bürker, Marienfeld, Lauda-Königshofen, Germany). All flasks were inoculated to a final concentration of 1×10^6 spores/mL medium. Flasks 1–2, 5–6, 9–10 and 13–14 were inoculated with *A. niger* D15[*egI*], while flasks 3–4, 7–8, 11–12 and 15–16 were inoculated with *A. niger* D15[pGT]. All flasks were sealed with cotton plugs and no further aeration was introduced during the experiments. The flasks were incubated for 11 days at 30 °C and 120 rpm in an orbital shaker (Infors Ecotron). The CMCase activity was measured on a daily basis during the course of the cultivation (described below).

Biomass measurement

The dry weight of the *A. niger* mycelium was measured after filtration through circular pieces of Miracloth (Calbiochem, EMD Biosciences, La Jolla, CA, USA) with an approximate diameter of 90 mm. The Miracloth was dried in an oven at 80 °C for 24 h prior to drying in a desiccator for 1 h. The Miracloth pieces were weighed on an analytical scale prior to use. After 11 days of cultivation, the volumes of all the *A. niger* cultures were measured and the cultures were then filtered with suction through the predried Miracloth. The Miracloth pieces with biomass were then washed with 50 mL of deionized water and dried at 80 °C for 72 h before weighing.

Enzyme activity

The CMCase and β-glucosidase activities were measured using a reducing sugar assay based on dinitrosalicylic acid (DNS) [2]. The substrates 1 % carboxymethyl cellulose and 1 % salicin (Sigma-Aldrich, Steinheim, Germany) were suspended in 0.05 M citrate buffer (pH 5.5). The assays were carried out in 15-mL glass test tubes, which were incubated in a water bath at 55 °C. All enzymatic activities are given in nkat/mL.

SDS-PAGE

Determinations of the size and the concentration of the recombinant protein were based on samples from two separate cultivations. Samples from day 11 were chosen for the measurements since these samples displayed high final CMCase activity as well as relatively low standard deviation. The samples were diluted sixfold with deionized water prior to separation by using 10 % SDS-PAGE gels. After electrophoresis, the gels were stained with Sypro Red (Molecular Probes, Eugene, OR, USA) according to the procedure described by Steinberg et al. [15]. The subsequent analyses of the gels were performed by using a laser-excited gel scanner equipped with a 610-nm emission filter (Typhoon 9400, GE Healthcare, Uppsala, Sweden). The concentration of recombinant endoglucanase I (Cel7B) was determined by running a concentration gradient of bovine serum albumin (Thermo Fisher Scientific, Waltham, MA, USA) as standard protein under the same conditions. Protein concentrations were determined by intensity comparison of the Sypro-Red-stained gels using the ImageJ software [5]. The protein concentrations were calculated as the mean value of four replicate measurements (i.e. four separate gels).

Protein identification

Identification and verification of the proteins separated by SDS-PAGE were carried out using MALDI-MS. The analysis was performed using two different samples exhibiting different molecular masses based on the SDS-PAGE analysis. Both these samples originated from supernatants of a single cultivation experiment with the *A. niger* D15[*egI*] transformant and SFSH medium. The SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 prior to in-gel digestion using sequencing grade trypsin (Promega Co., Fitchburg, WI, USA). The mass lists obtained from peptide mass fingerprint spectra were used for database searches in

Table 1 Chemical analysis of waste fiber sludge content (g/kg)

	Glucose (g/kg)	Xylose (g/kg)	Arabinose (g/kg)	Galactose (g/kg)	Mannose (g/kg)	Lignin (g/kg)	Ash content (g/kg)
Sulfate fiber sludge	691	154	2.8	2.2	33.1	35	36
Sulfite fiber sludge	897	16.5	<0.2	0.8	27.3	8	17

the database of the best protein sequences of *T. reesei* of the Joint Genome Institute and the Uniprot database.

As a complementary approach, an analysis of individual peptides by PSD-MS/MS was performed. The acquired PSD-MS/MS spectra from the samples were used for database searches of matching peptides in the Uniprot database.

Chemical analyses

Analyses of monosaccharides were performed by MoRe Research using an ion-exchange chromatography system (IC) equipped with a CarboPac PA1 column and an ED40 Electrochemical Detector (Dionex, Sunnyvale, CA, USA). The oligosaccharide content in the SFSH was determined using dilute sulfuric acid hydrolysis (2.5 % sulfuric acid, 60 min at 120 °C). Detection of released monosaccharides was performed using IC-PAD (MoRe Research). Samples for analysis of organic acids were performed by MoRe Research using an Ultimate 3000 HPLC system (Dionex) equipped with an Aminex HPX–87H (Bio-Rad Laboratories, Hercules, CA, USA). Total organic carbon analysis was also performed by MoRe Research. Ethanol measurements were performed using high-performance liquid chromatography (HPLC). A Shodex SH-1011 column (6 µm, 8 × 300 mm) (Showa Denko, Kawasaki, Japan) was used in a YoungLin YL9100 series system (YoungLin, Anyang, Korea) equipped with a YL9170 series refractive index (RI) detector. Elution was performed with isocratic flow of a 0.01 M aqueous solution of H_2SO_4.

Results

The average dry weight content of the fiber sludges after drying was approximately 95 %. The sulfate fiber sludge contained 69 % glucan, 15 % xylan, and 3.5 % lignin (Table 1). The main component of the sulfite fiber sludge was glucan (90 %). The contents of lignin and xylan were 0.8 and 2 %, respectively.

The ethanol production in the SSF experiment with sulfite fiber sludge was faster than for the experiment with sulfate fiber sludge (Fig. 2). The final concentration of ethanol was 64.7 g/L in the sulfite fiber sludge experiment as compared to 45.6 g/L in the sulfate fiber sludge experiment (Fig. 2). The second SSF, which was undertaken using

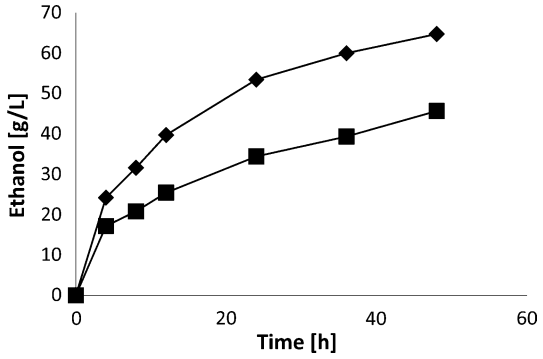

Fig. 2 Ethanol production during the first simultaneous saccharification and fermentation (SSF) of waste fiber sludge. The data indicate: *filled diamond* sulfite fiber sludge, *filled square* sulfate fiber sludge. Every data point is calculated as the mean value of two fermentations. The *error bars* indicate standard deviations

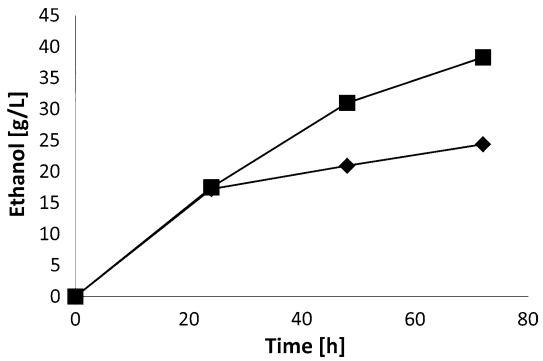

Fig. 3 Ethanol production during the second simultaneous saccharification and fermentation (SSF) of waste fiber sludge. The data indicate: *filled diamond* sulfite fiber sludge, *filled square* sulfate fiber sludge. Every data point is calculated as the mean value of two fermentations. The *error bars* indicate standard deviations (minor deviations may be difficult to discern)

recycled yeast and enzymes from the first SSF and fresh fiber sludge, yielded ethanol concentrations of 38.3 g/L for the sulfate fiber sludge and 24.4 g/L for the sulfite fiber sludge after 72 h of fermentation (Fig. 3). The average ethanol production for the two sequential SSFs was rather similar, with an average ethanol production of 42.0 g/L for the sulfate sludge and 44.6 g/L for the sulfite sludge (Table 2). The average ethanol yields per batch of enzymes and yeast were also rather similar for the two fiber sludges when the

Table 2 Ethanol yields

	Ethanol (g/L)[a]		Yield (g/g)[b]		Yield (g/g)[c]	
	Sulfite	Sulfate	Sulfite	Sulfate	Sulfite	Sulfate
SSF 1	64.7	45.6	0.31	0.22	0.34	0.31
SSF 2	24.7	38.3	N.D	N.D	N.D	N.D
SSF 1 and 2	44.7	42.0	0.21	0.20	0.24	0.29

[a] The table shows the values obtained after 48 h (SSF 1), 72 h (SSF 2), and average ethanol concentrations for SSF 1 and 2

[b] g EtOH/g added fiber sludge

[c] g EtOH/g added glucan

yield is calculated on the total amount of glucan that was added to the fermentations (Table 2).

Measurements of the cellulase activity (the CMCase activity) in the liquid fractions from the SSF recirculation experiments showed that 40 and 32 % of the initial activity was present in the sulfate and sulfite fiber sludge hydrolysates, respectively. The corresponding values for β-glucosidase activity levels for the sulfate and sulfite fiber sludge hydrolysates were 56 and 52 %, respectively. The results indicate that at least 60–68 % of the cellulase activity and at least 44–48 % of the β-glucosidase activity was recirculated. The fraction of enzyme activity reported as recirculated may be an underestimate due to loss of some of the activity during the extended reaction time at elevated temperature. Volume measurements of the liquid fraction showed that approximately 50 % of the liquid added during the first part of the experiment was adsorbed by the residual fibers after centrifugation.

The monosaccharide contents of the SFSH obtained after distillation were rather different for the two fiber sludge stillages. The sulfate SFSH contained 5.2 g/L xylose and 0.2 g/L glucose (Table 3), while the sulfite SFSH contained low concentrations of monosaccharides, only 0.2 g/L xylose and 0.1 g/L glucose (Table 3). The analysis of organic acids after distillation showed that the sulfate SFSH contained 0.6 g/L acetic acid, 0.1 g/L formic acid and <0.1 g/L levulinic acid while the sulfite SFSH contained 1.3 g/L acetic acid and the same amounts of formic and levulinic acid as the sulfate SFSH (Table 3). HMF and furfural were not detected. The TOC content and the oligosaccharide content of the two stillages differed significantly. The sulfate stillage contained as much as 38 g/L TOC with 22 g/L oligosaccharides, whereas the sulfite stillage contained 25 g/L TOC and 4 g/L oligosaccharides.

Cultivation of *A. niger* transformants in SFSH medium resulted in a high density of fungal pellets. No signs of inhibition of the growth of the fungus were noticed. Growth was also detected on the fiber sludge references, which contained untreated and unhydrolyzed fiber sludge suspended in citrate buffer. After 11 days of cultivation, the analyses of organic acids and monosaccharides indicated that these were consumed completely by *A. niger* D15[*egI*] (Table 3). Further chemical analysis showed that approximately 60 % the TOC was consumed by *A. niger* D15[*egI*] in the sulfite stillage, while around 45 % was consumed in the sulfate stillage. About 80 % the oligosaccharides in the sulfate SFSH were consumed, while the corresponding figure for the sulfite SFSH was only 15 %. The CMCase activity in cultures of *A. niger* D15[*egI*] in SFSH media increased for 11 days for the sulfate SFSH, and for 7 days for the sulfite SFSH. The highest activity reached in the sulfate SFSH was 2,700 nkat/mL and in the sulfite SFSH 2,900 nkat/mL (Fig. 4). The highest specific activity, 2,100 nkat/mg, was noted for the Cel7B produced in the cultivations with *A. niger* D15[*egI*] grown on sulfate SFSH. A similar Cel7B specific activity, 1,900 nkat/mg, was reached in the cultivations with *A. niger* D15[*egI*] grown on sulfite SFSH (Table 4).

Cultivations with unhydrolyzed dried fiber sludge in citrate buffer were performed as a comparison with the cultivations performed with fiber sludge stillage. In these experiments, the *A. niger* D15[*egI*] transformants, which were grown on fiber sludge in citrate buffer, did not display any activity. Fungal growth was however detected in the flasks containing unhydrolyzed fiber sludge. This would indicate that *A. niger* has the ability to utilize unhydrolyzed fiber sludge as a substrate. The cellulose fibers present in the media may be the reason for the lack of activity in these cultures, as the expressed enzymes are probably bound to the cellulose fibers of the fiber sludge. *A. niger* D15[pGT] did not produce any detectable CMCase activity in either of the cultivations where SFSH was used as growth medium or in the cultivations where fiber sludge was used as reference medium. The volumetric CMCase activity, the cellulase protein concentrations, the specific CMCase activity, and the biomass production in the experiments with *A. niger* D15[*egI*] and *A. niger* D15[pGT] are presented in Table 4. The highest biomass production was reached for *A. niger* D15[*egI*] and *A. niger* D15[pGT] grown on sulfate SFSH, where the fungal cultures reached the biomass concentrations of 19.2 and 18.8 g/L (DW), respectively (Table 4). The *A. niger* D15[*egI*] and *A. niger* D15[pGT] grown on sulfite SFSH reached a biomass concentration of 11.0–12.1 g/L (DW), almost half of the biomass which was reached on the sulfate SFSH. No determination of biomass yield was performed on the reference cultivations with fiber sludge due to difficulty to separate the fungal mycelium from the fiber sludge. In contrast to the biomass formation in the two SFSH experiments, the highest cellulase protein concentration was noted in the experiment where *A. niger* D15[*egI*] was grown on sulfite SFSH. This experiment resulted in a Cel7B protein concentration of 1.52 mg/mL after 11 days of cultivation. The cultures in which *A. niger*

Table 3 Concentrations (g/L) of monosaccharides, organic acids, total organic carbon (TOC) and oligosaccharides

	Glucose	Xylose	Arabinose	Galactose	Mannose	Acetic acid	Formic acid	Levulinic acid	TOC	Oligo-saccharides
Sulfate SFSH[a]	0.2	5.2	0.3	<0.1	0.1	0.6	0.1	<0.1	38	22
Sulfate SFSH[b]	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	22	3.9
Sulfite SFSH[c]	0.1	0.2	<0.1	<0.1	<0.1	1.3	0.1	<0.1	25	4
Sulfite SFSH[d]	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	9.4	3.4

[a] Sulfate SFSH prior to cultivation of *A. niger* D15[*egI*]

[b] Sulfate SFSH after cultivation of *A. niger* D15[*egI*]

[c] Sulfite SFSH prior to cultivation of *A. niger* D15[*egI*]

[d] Sulfite SFSH after cultivation of *A. niger* D15[*egI*]

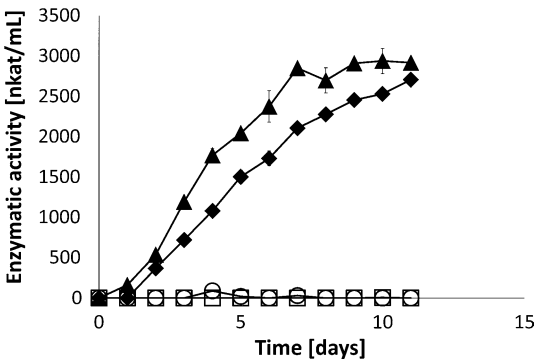

Fig. 4 CMCase activity for *A. niger* transformants growing on spent fiber sludge hydrolysates (SFSH). Mean values of samples from two separate cultures are shown. The data indicate: *filled triangle A. niger* D15[*egI*] and sulfite SFSH, *filled diamond A. niger* D15[*egI*] and sulfate SFSH; *open square A. niger* D15[pGT] and sulfite SFSH; *open circle A. niger* D15[pGT] and sulfate SFSH. *Error bars* indicate standard deviations

D15[*egI*] was grown on sulfate SFSH yielded a protein concentration of 1.34 mg/mL.

The analysis with SDS-PAGE indicated that Cel7B was the predominant protein in the supernatants from the experiments in which *A. niger* D15[*egI*] was grown on SFSH. The Cel7B protein from *A. niger* D15[*egI*] grown on SFSH had a molecular mass of about 76 kDa (Fig. 5). Because the theoretical molecular mass of the protein is 49 kDa, the analysis showed that the Cel7B protein possibly could be glycosylated. The SDS-PAGE analysis also revealed the presence of a protein with a molecular mass of approximately 48.6 kDa (Fig. 5), which may represent the non-glycosylated form of the Cel7B protein. This suggests that a major part of the recombinant Cel7B protein is glycosylated during the heterologous expression in *A. niger*.

Identification of the two protein species (Fig. 5) with molecular masses of 76 and 49 kDa were performed using MALDI-MS and PSD-MS/MS. Protein samples were taken from the cultivations on SFSH. The results of peptide mass fingerprinting and analysis of individual peptides of the two proteins that displayed different molecular mass (49 and 76 kDa) showed that both were identified as the Cel7B from *T. reesei*. The analysis of duplicate protein samples with the molecular mass of 76 kDa resulted in sequence coverage of around 27 % and Mascot software (Matrix Science, London, UK) scores of 82 and 94, using the MALDI-MS/MS fingerprinting analysis. Database searches in the Uniprot database using PSD-MS/MS spectra from duplicate samples of individual peptides for the protein sample

Table 4 Results from cultivations of *A. niger* transformants on spent fiber sludge hydrolysate

Transformant	Growth medium	Endoglucanase activity (nkat/mL)[a]	Cel7B protein concentration (mg/mL)[b]	Endoglucanase activity/Cel7B protein (nkat/mg)[c]	Biomass (DW g/L)[d]
A. niger D15[*egI*]	Sulfate SFSH	2,700 ± 100	1.3 ± 0.3	2,100	19.2 ± 0.8
A. niger D15[pGT]	Sulfate SFSH	NDTD	NDTD	–	18.8 ± 4.1
A. niger D15[*egI*]	Sulfite SFSH	2,900 ± 200	1.5 ± 0.2	1,900	11.0 ± 0.2
A. niger D15[pGT]	Sulfite SFSH	NDTD	NDTD	–	12.1 ± 0.3

NDTD not detected

[a] Mean values of activity measurements of samples from two separate cultures

[b] Mean values of samples from two separate cultures repeatedly analyzed on four SDS-PAGE gels

[c] Values are based on averages of analyses of samples from two separate cultures (after 11 days of cultivation)

[d] Mean values of two separate cultures after 11 days of cultivation

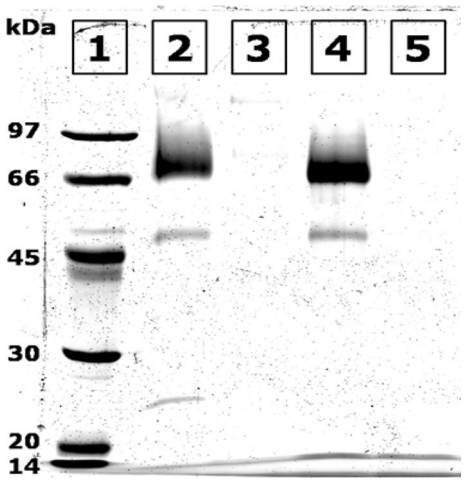

Fig. 5 SDS-PAGE of extracellular protein species in samples from *A. niger* cultures. The *lanes* show: *1* molecular size markers, *2* sample from *A. niger* D15[*egI*] grown in sulfate SFSH, *3 A. niger* D15[pGT] grown in sulfate SFSH, *4* sample from *A. niger* D15[*egI*] grown in sulfite SFSH, *5 A. niger* D15[pGT] grown in sulfite SFSH

with the molecular mass of 49 kDa resulted in peptide scores of 56 and 52 for the peptide LYLLDSDGEYVMLK of Cel7B from *T. reseei*. The identity scores for peptide spectra in these database searches were 43 indicating that the identifications were correct.

The protein concentrations and the specific activities reported in Table 4 are based on the concentrations of the 76 kDa protein, as determined with Sypro-Red-based analysis of samples analyzed with SDS-PAGE. This approach was used considering that the glycosylated form of the protein was predominant in the cultures.

Discussion

Production of cellulosic ethanol from fiber sludge has previously been studied in SHF as well as SSF. Marques et al. [7] performed both SHF and SSF on recycled paper sludge using *Pichia stipitis* as the fermenting microorganism. Yamashita et al. [20] showed that ethanol from paper sludge can be produced by using immobilized *Zymomonas mobilis*, an ethanol-producing bacteria, in an SSF configuration. Another attempt included SSF as well as simultaneous saccharification and co-fermentation (SSCF) of kraft paper mill sludge with *S. cerevisiae* and recombinant *E. coli* [6]. However, none of these studies indicate recirculation of enzymes or yeast. Alriksson et al. [1] presented the possibility to produce enzymes from pentoses, acids and other organic substances remaining in the stillage after fermentation with *S. cerevisiae* and distillation. Cavka et al. [3] have shown that waste fiber sludge can be used as raw material for the production of bioethanol and xylanase through sequential cultivations using yeast and a filamentous fungus. The first cultivation was based on the consumption of hexoses by *S. cerevisiae* and the second cultivation was based on the consumption of organic substances rejected by *S. cerevisiae* in the first cultivation step.

The SSF with fiber sludge resulted in different ethanol concentrations for the sulfite and sulfate fiber sludges. The difference in final ethanol concentrations can possibly indicate that the two fiber sludges differ with respect to enzymatic convertibility. The sulfite fiber sludge had a higher degree of release of sugar per unit of enzyme per hour than the sulfate fiber sludge. This might be due to the structure and composition of the fiber sludges. However, the slower release of monosaccharides from the sulfate fiber sludge is not a disadvantage if a second SSF, which uses yeast and enzymes from the first SSF, is performed.

Retention of water by recirculated fibers could explain why it was possible to recover a large part of the β-glucosidase activity even if the enzyme would not bind specifically to the residual fibers that were left at the end of the SSF experiment. With respect to the cellulases, the retention of liquid could contribute to the recirculation, but in this case the residual fibers offer a binding site for the enzymes and that would allow them to be recirculated to a higher degree than enzymes that do not bind to fibers. Recirculation is of essence when the economic aspects of biofuel production are taken into consideration, as enzyme cost is considered to be one of the major bottlenecks for commercialization of second generation biofuels [19]. Zhang et al. [21] estimated that ethanol production from corn stover at an annual production of 1.42 million kg would result in a cost of 15.21 USD/kg of ethanol. In the initial cost estimate, no recirculation of enzymes was included. However, if recovery of enzymes is included and reaches an efficiency of 50 % then the production cost per kg of ethanol would drop to 9.45 USD/kg. If 90 % enzyme recovery is achieved, the cost of the ethanol production would drop further to 4.96 USD/kg. This would indicate that recirculation of enzymes within a biofuel process based on lignocellulose is of crucial importance to the overall production cost. Furthermore, recirculation of yeast would also be important for the economy of the process [21]. In this study, we have showed that recirculation of both yeast and enzymes can be achieved in an SSF process. Our results also indicate that the composition and structure of the raw material may be of importance when recirculation is considered, hence the difference in efficiency between the first and the second SSF of sulfite and sulfate fiber sludges. The cultivation of *A. niger* resulted in high CMCase activities for both sulfite and sulfate SFSH. This was achieved despite different nutrient conditions in the two SFSH cultivations. The sulfite SFSH had low concentrations of monosaccharides and oligosaccharides

as compared to the sulfate SFSH. One distinct difference between the two SFSH cultivations regards formation of fungal biomass. It seems that *A. niger* has a preference for biomass formation when it is grown in a medium with easily accessible nutrients, while protein production is prioritized when the abundance of easily accessible nutrient is low.

It is known that heterologous expression of *T. reesei* proteins in *Aspergillus* may result in proteins with lower activity than those of the native host, possibly due to the different glycosylation patterns exhibited by the two organisms [16]. Variable glycosylation may also be due to medium composition [14]. Alriksson et al. [1] showed that *A. niger* D15[*egI*], the same transformant that was used in this study, does indeed produce a hyperglycosylated protein with the same size as the protein which was detected and quantified with SDS-PAGE analysis in this study.

Several different microorganisms have previously been used as hosts for production of recombinant endoglucanase I (Cel7B) from *T. reesei* and these include *S. cerevisiae* [12], *Y. lipolytica* [10], and *A. niger* [12]. The highest activity reached in our study, 2,900 nkat/mL, is about 25 times higher than obtained with *S. cerevisiae* and about eight times higher than obtained with *Y. lipolytica*. Rose and van Zyl [12] reached a similar activity as in this study when the *A. niger* transformant was grown on a nutrient-rich glucose-based medium. About half (1,400 nkat/mL) of the activity achieved in our study was reached when *A. niger* was grown on 20 % (v/v) molasses as medium. The results of this study clearly indicate that higher levels of enzyme activity as well as high concentrations of the Cel7B protein can be achieved with medium based mainly on lignocellulose degradation products that are discarded by *S. cerevisiae*. The endoglucanase activity levels reported in this study are also about two times higher than what was achieved with native *T. reesei* [9]. When taking all the data into consideration, the comparisons indicate that spent fiber sludge hydrolysate serves as an excellent growth medium for enzyme production with recombinant *A. niger*.

Conclusions

In conclusion, the results of this study indicate that sequential cultivations with yeast and filamentous fungi to produce ethanol and cellulase is a feasible process concept. The ability of *A. niger* to grow on inexpensive and nutrient-poor industrial residual streams and still produce impressive amounts of enzyme makes it to a very promising microorganism for future research on onsite enzyme production in biorefineries. The reference cultivations where fiber sludge was used as the carbon source were unsuccessful. The fungus was able to grow on and hydrolyze the fiber sludges, but the enzyme activity in the culture medium was not detectable. This indicates that *A. niger* does have the ability to grow on untreated fiber sludge, but the lack of activity suggests that the enzymes were probably bound to the cellulose of the fiber sludge and would thus be hard to separate from the growth medium. This would make the fiber sludge unsuitable as a carbon source for enzyme production. It would be of interest to optimize the cultivation conditions to shorten the time period needed to reach the enzyme activity levels achieved using stillage-based media. The high cellulase activities produced by using stillage and the significant ethanol concentrations produced in the second SSF suggest that on-site enzyme production and recycling of enzyme are realistic concepts that warrant further attention in the future.

Acknowledgments We thank the pulp mills for providing the fiber sludge. One of the authors (AC) was supported by SEKAB E-Technology AB (Örnsköldsvik, Sweden) through the Umeå University Industrial Graduate School. Mass spectrometry was performed by Dr. T. Kieselbach at the KBC Proteomics Core Facility of Umeå University and the Swedish University of Agricultural Sciences in Umeå. The research received financial support from The Kempe Foundations, The Knut and Alice Wallenberg Foundation, The Swedish Energy Agency, and Bio4Energy (http://www.bio4energy.se).

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