Appl Biochem Biotechnol (2010) 161:382–394
DOI 10.1007/s12010-009-8863-x

Production of Cellulase from Kraft Paper Mill Sludge by *Trichoderma Reesei* Rut C-30

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Received: 22 May 2009 / Accepted: 6 November 2009 /
Published online: 8 December 2009

Abstract Paper mill sludge is a solid waste material generated from pulping and papermaking operations. Because of high glucan content and its well-dispersed structure, paper mill sludges are well suited for bioconversion into value-added products. It also has high ash content originated from inorganic additives used in papermaking, which causes hindrance to bioconversion. In this study, paper mill sludges from Kraft process were de-ashed by a centrifugal cleaner and successive treatment by sulfuric acid and sodium hydroxide, and used as a substrate for cellulase production. The treated sludge was the only carbon source for cellulase production, and predominantly inorganic nutrients were used as the nitrogen source for this bioprocess. The cellulase enzyme produced from the de-ashed sludge exhibited cellulase activity of 8 filter paper unit (FPU)/mL, close to that obtainable from pure cellulosic substrates. The yield of cellulase enzyme was 307 FPU/g glucan of de-ashed sludge. Specific activity was 8.0 FPU/mg protein. In activity tests conducted against the corn stover and α-cellulose, the xylanse activity was found to be higher than that of a commercial cellulase. Relatively high xylan content in the sludge appears to have induced high xylanase production. Simultaneous saccharification and fermentation (SSF) was performed using partially de-ashed sludge as the feedstock for ethanol production using *Sacharomyces cerevisiae* and the cellulase produced in-house from the sludge. With 6% (*w*/*v*) glucan feed, ethanol yield of 72% of theoretical maximum and 24.4 g/L ethanol concentration were achieved. These results were identical to those of the SSF using commercial cellulases.

Keywords Cellulase production · Lignocellulosic substrates · Paper mill sludge · Ethanol production

Introduction

Cellulase enzyme is one of the major cost factors in production of cellulosic ethanol. For this reason, much of the recent research efforts on cellulosic ethanol have been devoted to cellulase

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enzymes. The major focus of the current research work is on cellulase-producing microorganisms targeting efficient production of cellulase enzymes and improving the characteristics of the enzymes on molecular level. Also related to cellulase enzyme is pretreatment research, the goal of which is to reduce the enzyme loading. These are important and meaningful tasks in the research of cellulosic ethanol. There is yet another important issue pertaining to cellulase production, namely, the feedstock. Judging from the available literature information or the lack of it, it appears that very little attention is being paid to this issue at the present time. Cellulases are currently produced commercially, but relatively in small scale because of its limited market. With the current market value of cellulase, the cost of feedstock is of minor concern. However, it will no longer be the case in the world of cellulosic ethanol, where low-cost feedstock is required for production of cellulase. In economic sense, it is as important as enzyme improvement or devising an efficient pretreatment.

With this understanding, we have chosen pulp mill sludge as feedstock for cellulase enzymes because of its zero/negative cost. The pulp and paper industry in the USA generates 4–5 million tons of sludges per year [1]. It is large enough quantity to meet the total cellulase enzyme need for the entire cellulosic ethanol industry in the USA for the foreseeable future. At the present time, most of it is disposed to landfill or incinerated, causing a significant financial burden to the industry. The paper mill sludges have characteristics suitable for conversion to value-added products. Especially, the Kraft paper mill sludges have much higher carbohydrate and lower lignin than natural lignocellulosic substrates [2–4]. The organic portion of the sludge has chemical composition very similar to that of commercial grade pulp. It is also important to note that the cost of the carbon source is a major cost item in the production of cellulases [5]. Physically, paper mill sludge is much smaller in size than commercial pulp, and has well-dispersed structure and high surface area. It has been proven to be highly efficient for bioconversion to ethanol [6]. These features make it possible to use it as a bioconversion feedstock without pretreatment, which is a significant economic benefit.

A number of low-cost lignocellulosic substrates have been investigated as a feedstock for cellulase production [7–12]. They include corn stover, wheat straw, bagasse, sawdust, and municipal solid wastes. These studies collectively indicate that these substrates even after substantial pretreatment are inferior to pure cellulosic materials as raw material for cellulase production, generally giving low cellulase titer. Literature information on using paper mill sludge as a source for cellulase production is scarce. Maheshwari [12] reported on paper mill waste as a substrate for cellulase production using mixed cultures of *Trichoderma reesei QM 9123* and *Aspergillus niger*, This study, however, did not give enough information to assess the properties of the cellulase produced from the sludge.

The main objective of this study was to see whether one can produce cellulases from paper mill sludges, and if so, assess the efficiency of enzyme production, determine the properties of the enzyme, and compare them with commercial cellulase where possible. It was also intended that the cellulase from paper mill sludge be evaluated subjecting it to hydrolysis of common feedstocks and saccharification and fermentation (SSF) of the pulp mill sludge.

Materials and Methods

Substrate Preparation

The paper mill sludge used in this work was primary sludge supplied by Boise Paper, Jackson, It was a waste discharged from the paper machine and the Kraft digester. The

primary sludge had the composition of: 44.5 wt.% glucan, 9.9 wt.% xylan, 8.7 wt.% lignin, 36.0 wt.% ash, and 2.0 wt.% unaccounted for (acetyl group and protein). The total ash content in the primary sludge was 36%, of which 26% is acid-soluble ash and the balance (10%) acid-insoluble ash. Based on the Boise papermaking process and the analysis data, acid-soluble ash is mainly $CaCO_3$, and acid-insoluble ash is mainly TiO_2 and clay. The collection was made from the wastewater treatment unit. The sludge slurry was separated by a centrifugal cleaner (Laboratory centrifuge LE, CEPA, Germany) into a fiber-rich portion which is carried upward by the current and an ash-rich portion which is carried downward by the current. The upward stream was returned three times to the centrifugal cleaner to get sludge 1, and returned the sludge 1 to the air Centrifugal cleaner three times to get sludge 2 to achieve high degree of mechanical de-ashing. It was further treated with H_2SO_4 until all of $CaCO_3$ in the ash of the sludge was transformed into $CaSO_4$, which was water-washed (sludge 3). The sludge was treated again with 1 N NaOH to bring the pH of the sludge became neutral (sludge 4).

Enzymes

Cellulase enzyme (Spezyme CP, Lot No. 301-00348-257), was a gift from Genecor-Danisco, Palo Alto, CA. The activity of Spezyme CP, as determined by NREL, was 59 filter paper unit (FPU)/mL. The activity of β-glucosidase (Novozyme-188, Novo Inc., Sigma Cat. No. C-6150) was 750 CBU/mL.

Microorganism

T. reesei Rut C-30 (ATCC 56765) was used in the study of cellulase production and *Saccharomyces cerevisiae* ATCC-200062 (NREL-D5A) was used for SSF. *T. reesei* was grown on potato dextrose agar (Difco Lab., Detroit) slants. It was grown for 5 days at 28 °C; the greenish conidia on the agar plate were harvested and suspended in sterile water. The spore suspension was used as inoculum to the media of cellulase production.

A seed culture of *S. cerevisiae D5A* for SSF was prepared by inoculating the Yeast extract Peptone Dextrose (YPD) flask with one loop of *S. cerevisiae D5A* on Petri dish. The yeast was incubated for 12 h in a rotary incubator shaker at 36 °C and 130 rpm. At the time of transfer to SSF flask, optical density (O.D.) of the inoculum was measured to keep a starting O.D. of 0.5.

Culture Media

Pre-cultivation was carried out in a 300-mL Erlenmeyer flask on medium containing: 2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4$, 0.005 g/L $FeSO_4$, 0.075 g/L $MnSO_4$, 0.0015 g/L $ZnSO_4$, 0.002 g/L $CoCL_2$, 0.3 g/L $CaCL_2$, 0.3 g/L urea, 0.015 g/L Tween 80, 0.1 g/L peptone, 5 g/L glucose, 10 g/L Solka Floc 200 (International Fiber Corporation). The composition of the production medium for cellulase was: 2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4$, 0.005 g/L $FeSO_4$, 0.075 g/L $MnSO_4$, 0.0015 g/L $ZnSO_4$, 0.002 g/L $CoCL_2$, 0.3 g/L $CaCL_2$, 0.3 g/L urea, 0.015 g/L Tween 80, 0.1 g/L peptone, 2.5 g/L solid sludge.

Cellulase Production

A pre-culture containing 100 mL of medium in a 300-mL Erlenmeyer flask was inoculated with 100 μL of spore suspension and incubated at 28 °C on a rotary shaker at 180 rpm. After 2 days

of pre-cultivation, a 10% inoculum from the pre-culture was added to the production medium to initiate the cellulase production. No external inducers were used in pre-culture or cellulase production stage. The enzyme production experiments were performed in a 3-L fermenter (BiofloIII 3000 Fermentor, New Brunswick Scientific, New Jersey, USA) with an operating volume of 2 L. Temperature, pH, agitation, aeration, and dissolved O_2 were controlled at fixed desired level as follows: 28 °C, pH 3.5 by addition of NH_4OH, dissolved oxygen at 25% of saturation by aeration and agitation revolutions per minute.

Enzyme Analysis

The filter paper activity was determined according to the method of the International Union of Pure and Applied Chemistry (IUPAC). Dinitrosalicylic acid (DNS) method [13] was used to estimate the reducing sugar released in 60 min from a mixture of 0.5 mL appropriately diluted enzyme solution, 1 mL 0.1 M acetate buffer (pH 4.8), and 50 mg of Whatman No.1 filter paper, incubated at 50 °C. After 60 min incubation, 3 mL of DNS reagent was added to the reaction mixture and boiled in a vigorously boiling water bath for 5 min. A 0.2 mL of reaction mixture was diluted by adding 2.5 mL of water, and the absorbance at 540 nm was measured. One filter paper unit was defined as the amount of enzyme that releases 1 μmol glucose per minute based on 2 mg glucose released in 60 min, according to the NREL procedure [14]. Activities were reported as FPU/mL. Carboxymethylcellulase (CMCase; endoglucanase) activity was determined by measuring the release of reducing sugars from 1% carboxymethylcellulose (Sigma) at 50 °C and pH 4.8 (0.1 M acetate buffer) for 10 min [15]. DNS method was also applied to this measurement as described above. One unit of activity is defined as the amount of enzyme that releases 1 μmol of glucose as reducing sugar equivalent per minute. Xylanase activity was determined by the reducing sugar released from a 1% birchwood xylan (Sigma) solution at 50 °C for 5 min [16]. One unit of activity is defined as the amount of enzyme that releases 1 μmol of xylose as reducing sugar equivalent per min. The xylose content was analyzed by HPLC equipped with BioRad Aminex HPX-87P column.

β-Glucosidase activity were determined by measuring the release of nitrophenol from 4-nitrophenyl-β-D-glucopyranoside (Sigma) for 15 min at 50 °C and pH 4.8 (0.05 M acetate buffer) [17]. The reaction was stopped by the addition of 1 M Na_2CO_3, and the absorbance was read at 410 nm from a nitrophenol standard curve. One unit of activity is defined as the amount of enzyme that releases 1 μmol of 4-nitrophenol per min.

Extracellular protein was assayed according to Bradford's method [18], using bovine serum albumin as the standard. The compositional analysis of sludge samples were conducted according to the NREL biomass analytical procedure [14]. The sugar contents in the hydrolyzates were analyzed by HPLC equipped with RI detector using BioRad Aminex HPX-87P column at 85 °C. The mobile phase was HPLC grade water, the flow rate was 0.55 mL/min. Acid-soluble lignin (weight percent) and ash content were determined by NREL standard procedures [14].

Enzymatic Hydrolysis

The enzymatic digestibility tests were carried out in 250-ml Erlenmeyer flask with total liquid volume of 100 ml according to the NREL Laboratory Chemical Analysis and Testing Standard Procedure [14]. The conditions of enzymatic digestibility tests were 50 °C and pH 4.8 (0.05 M sodium citrate buffer). Two different substrates, α-cellulose (Sigma C-8002, Lot 114 K0244) and pretreated corn stover (from NREL, treated with

1.4% H_2SO_4 and heated to 190 °C by direct steam injection for 10 min followed by explosive decompression at the NREL PDU) were tested for enzymatic hydrolysis, respectively. The dilute-acid-treated corn stover had the composition of: 59.7 wt.% glucan, 2.8 wt.% xylan, 1.2 wt.% other sugars, 26.9 wt.% Klason lignin, 1.9 wt.% aicd soluble lignin, 4.6 wt.% ash, and 2.3 wt.% acetyl. Sigma α-cellulose had a composition of 76 wt.% glucan, 20 wt.% xylan, 4 wt.% unaccounted for (lignin, acetyl group, ash, and protein). The initial glucan concentration was 3% or 6% (*w*/*v*). Enzyme loadings were: 5 and 15 FPU/g glucan. Spezyme CP (Genencor/Danisco) or the cellulase produced in this work from sludge was used with supplementation of 10 and 30 CBU/g glucan of β-glucosidase (Novozyme 188) for the respective cellulase loadings. Samples were taken periodically and analyzed for glucose, xylose, and cellobiose using HPLC. The glucan digestibility was defined as the percentage of actual glucose and cellobiose released over theoretical maximum.

Simultaneous Saccharification and Fermentation

SSF experiments were carried out at 36 °C in 250-mL Elenmeyer flasks according to NREL procedure [14]. Each SSF flask, with working volume of 100 mL, was loaded with 6% *w*/*v* glucan of untreated primary sludge, 1% yeast extract, and 2% peptone. The flasks were autoclaved and cooled. The flasks were then aseptically inoculated with 10 mL of yeast suspension which was grown for 12 h at 36 °C and 130 rpm in the YPD medium. The reaction was then initiated by addition of enzyme; Spezyme CP (Genencor, Lot No. 301-00348-257) or the cellulase produced in this work from sludge. The enzyme loading was 15 FPU of cellulase supplemented with 30 CBU of β–glucosidase/g glucan. Samples were taken aseptically over the course of 5 days and analyzed for ethanol by HPLC using a Biorad Aminex HPX-87H.

Results and Discussion

Composition of Paper Mill Sludge

Pulp and paper mills produce various types and amount of sludges depending on their raw material, process, and final product. The composition of sludges varies widely depending on the sources. The sludge used in this work was the primary sludge generated from a kraft paper mill. Table 1 presents the composition of the sludge. After two stages of mechanical de-ashing, the ash content was reduced to 11.4%.

Table 1 Composition of untreated and de-ashed kraft mill primary sludge.

Composition (%)	Untreated primary sludge	Sludge 1	Sludge 2
Glucan	44.5	52.4	63.2
Xylan	9.9	14.2	14.9
Lignin	8.1	6.3	6.2
Ash	36.0	21.8	11.4
Others	2.0	3.3	3.2

Production of Cellulase Enzyme from De-ashed Primary Sludge

The composition analysis of the primary sludge indicates that the lignin content is much lower than pretreated lignocellulosic feedstocks. The low lignin content of the sludge is highly desirable feature as a substrate for *T. reesei* that is known to show low viability when it is grown on a substrate containing high non-glucan impurities. On the other hand, high ash content in the sludge is a deterrent to the growth of this microorganism and a strong inhibitor to cellulase enzyme production. Inorganic residues accumulated from various stages of the papermaking process can give negative effects on the cellulase production. High ash content also causes additional problems in the bioconversion process in general even if it is to be used as a feedstock for other value-added products such as ethanol. It can reduce the enzyme efficiency and limit the solid loading which will lower the product concentration. In our initial test, use of untreated sludge as the carbon substrate, *T. reesei* did not show any growth in the fermentation medium described earlier. Obviously, ash and other components in the sludge greatly inhibit the cell growth, and most likely the cellulase production as well. In order to utilize it effectively, it was necessary to remove ash from the carbohydrates. To achieve it, primary paper sludge slurry was de-ashed in two stages as described previously (sludge 1 and sludge 2). The composition of sludge 1 is shown as in Table 1. The ash content of sludge 1 was decreased to 21.8%, but cellulase activity measured for the culture broth in 3-L fermenter was still poor (data not shown). The mycelium grew poorly in the sludge medium even after proper adjustment of the initial pH.

Sludge 1 was then further de-ashed by putting it to the centrifugal cleaner three times to get sludge 2, in which the ash content was reduced to 11.4% (Table 1). In the ensuing cellulase production experiment using sludge 2, 25-hr lag phase was observed before cellulase activity was detected. With initial substrate concentration of 2.5% (*w*/*v*) glucan of sludge 2, the maximum cellulase titer of 1.7 FPU/mL was achieved after 3 days (Fig. 1). Increase of the substrate concentration (sludge 2) from 2.5% to 3.5% glucan did not show any improvement of cellulase titer. Although sludge 2 had high glucan content and

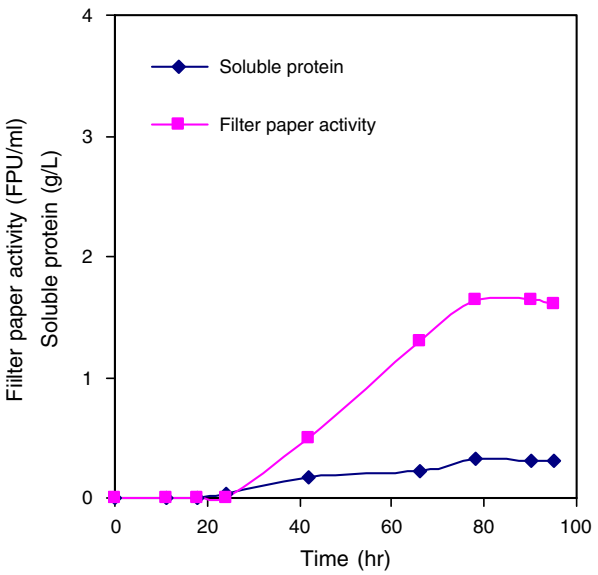

Fig. 1 Cellulase production by *T. reesei* using 2.5% glucan (*w*/*v*) water-washed sludge 2

relatively low ash and lignin, it was not effective in cellulase production. The ash content in sludge 2 somehow inhibited cellulase production. The ash contains mostly inorganic substances including $CaCO_3$, TiO2, and clay. They are water-insoluble and presumably inert. How they interfere with the microbial reaction is unclear. Interference with oxygen transfer into the microorganism may be one of the factors hampering cellulase production in *T. reesei*, which is a highly aerobic process.

Cellulase Production from De-ashed, Chemically Treated Primary Sludge

In an effort to enhance the cellulase production, further treatment of sludge 2 was attempted.

Sludge 2 was leached with 1 N sulfuric acid until most of $CaCO_3$ in the ash was converted to $CaSO_4$ by the reaction of: $H_2SO_4 + CaCO_3 \rightarrow CaSO_4 + H_2O + CO_2$

$CaSO_4$ has higher solubility in water than $CaCO_3$. The sludge was water-washed to remove $CaSO_4$ creating sludge 3. As shown in Table 2, the ash content in sludge 3 was reduced to 4.7%. Cellulase production from sludge 3 was substantially higher than the previous runs (Fig. 2). The cellulase production commenced within 24 h, indicating that the lag phase was shortened. Better substrate uptake and cell growth were also noticeable. The cellulase production was improved to attain the filter paper activity of 3.2 FPU/mL and volumetric productivity of 37.5 FPU/L h (over 96 h period).

Seeking further improvement of cellulase production sludge 3 was treated again with 1% (*w*/*v*) NaOH resulting sludge 4. By this treatment, the ash decreased to 3.8% and glucan and total carbohydrate was increased to 74.5% and 89.5%, resembling the composition of bleached kraft pulp. These successive treatments presumably have removed most of the inhibitory chemicals that are harmful to the cellulase production. In the ensuing experiment, sludge 4 was used at the level of 3.5% glucan (*w*/*v*) (Fig. 3). The strain grew well and produced cellulase titer much higher than the previous runs. The yield of cellulase enzyme from sludge 4 (named as AU-S4 enzyme, hereafter) and volumetric productivity reached 307 FPU/g of cellulose and 60.6 FPU/L h, respectively. Table 3 shows the activities of cellulolytic and hemicellulolytic enzymes in the fermentation broth. The cellulase titers, volumetric productivities of the sludge 4 run are summarized in Table 4.

As shown in Table 4, all aspects of cellulase enzyme production were far superior for sludge 4 to other sludges. Drastic difference between sludges 3 and 4 was surprising because composition-wise, there is little difference between them (Table 2). We have no clear explanation for it at this time. Sludge 3 may contain small amount of NaOH washable impurities that somehow strongly interfere with cell growth and cellulase production as increased cell mass and protein production was observed in fermentation with sludge 4. The titer of the cellulase produced from sludge 4 was 8.0 FPU/mL, which is comparable to

Table 2 Composition of primary sludge after centrifugal separation/acid leaching, and alkali leaching.

Composition (%)	Sludge3 (H_2SO_4 leaching)	Sludge 4 (H_2SO_4 and NaOH leaching)
Glucan	72.6	74.5
Xylan	15.8	15.0
Lignin	4.4	3.7
Ash	4.7	3.8
Others	2.1	2.5

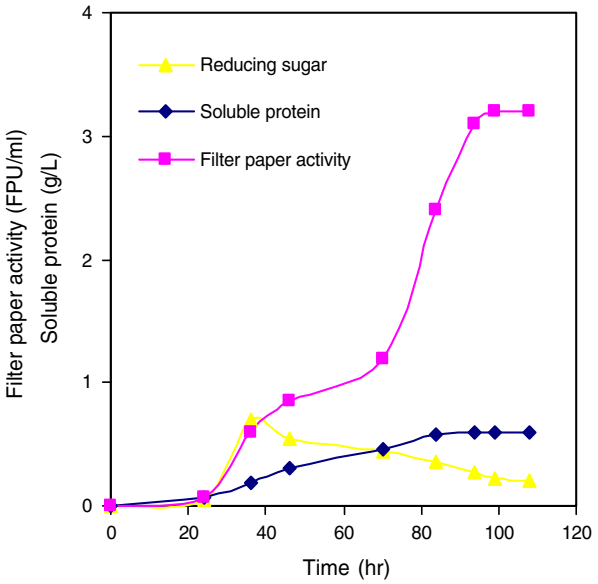

Fig. 2 Cellulase production by *T. reesei* using 2.5% (*w*/*v*) acid-leached sludge

those reported for cellulases produced from high-grade commercial cellulose including Solka floc [19, 20]. The specific activity of AU-S4 was 8.0 FPU/mg protein. Although the protein content of AU-S4 was measured without TCA precipitation, this value was much higher than reported specific activity of commercial cellulases. These numbers, however, do not necessarily reflect the molecular activities of the respective enzymes. It may have more to do with the fact that some of the commercial enzymes contain non-enzyme proteins

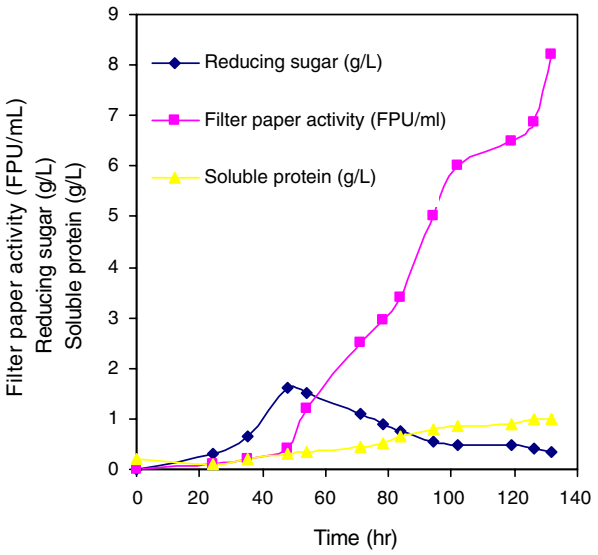

Fig. 3 Cellulase production by *T. reesei* using 3.5% (*w*/*v*) acid- and alkali-leached sludge

Table 3 Specific activities of cellulolytic and hemicellulolytic enzymes in the fermentation broth of sludge 4.

	Filter paper activity (FPU/mg)	CMCase activity (IU/mg)	β-Glucosidase (pNPG U/mg)	Xylannase (IU/mg)
Cellulase produced from sludge 4	8.0	4.4	0.5	170.2

mixed in as a nutrient during fermentation, whereas AU-S4 was produced using inorganic salt as the primary nitrogen source, therefore, background protein was very low in the broth. The titer and the specific activity of AU-S4 are high enough to be used without further concentration of the broth, if it is to be used on-site. Yield is also a very important cost factor in cellulase production. In production of AU-S4, the yield in terms of activity was 307 FPU/g cellulose. Translating it into practical terms, 1 ton of glucan in the sludge can produce cellulase enzyme that can hydrolyze 20 tons of cellulose in lignocellulosic feedstock (calculation basis, 15 FPU/g glucan). A typical pulp mill produces 100 dry MT of sludges/day, which is sufficient to support several commercial scale cellulosic ethanol plants.

Enzymatic Hydrolysis Test

Performance test was conducted for AU-S4 enzyme using two different substrates: corn stover pretreated by dilute-acid and Sigma α-cellulose. The same hydrolysis test was also done for Spezyme CP (Genencor, Lot No. 301-00348-257) for comparison purpose. Two different levels of glucan loadings (3% and 6% glucan, *w*/*v*) and two levels of enzyme loadings (5 FPU and 15 FPU/g glucan) were applied. The enzymes were supplemented with β-glucosidase (Novozyme-188) at the ratio of 2 CBU per FPU. The hydrolysis profiles for pretreated corn stover are shown in Fig. 4.

In side-by-side comparison of AU-S4 and Spezyme CP, the yield and the glucose profile were found to be almost identical at high enzyme loading of 15 FPU/g glucan. The yield with high glucan loading was lower than low glucan loading, a phenomenon commonly observed in enzymatic hydrolysis of cellulose. With low enzyme loading of 5 FPU/g glucan, Spezyme CP has shown slightly higher yield than AU-S4 (Fig. 4). The difference was more discernible (~3%) with 3% glucan loading (Fig. 4a) than 6% glucan loading (Fig. 4b). Since the enzymes were supplemented with β-glucosidase, the difference in gross activity indicates that there is a difference in endo- and exo-glucanase activities between the two enzyme products. In addition, the enzymatic hydrolysis test without addition of

Table 4 Cellulase activity and productivity of *T. reesei* Rut C-30 using de-ashed and chemically treated paper mill sludge as substrates.

Parameters	Substrate		
	Sludge 2	Sludge3	Sludge 4
Fermentation time (h)	78	96	132
Filter paper activity (FPU/mL)	1.6	3.2	8.0
Specific filter paper activity (FPU/mg protein)	4.8	5.8	8.0
Volumetric productivity (FPU/L h)	20.5	37.5	60.6
Yield (FPU/g cellulose)	101	176	307

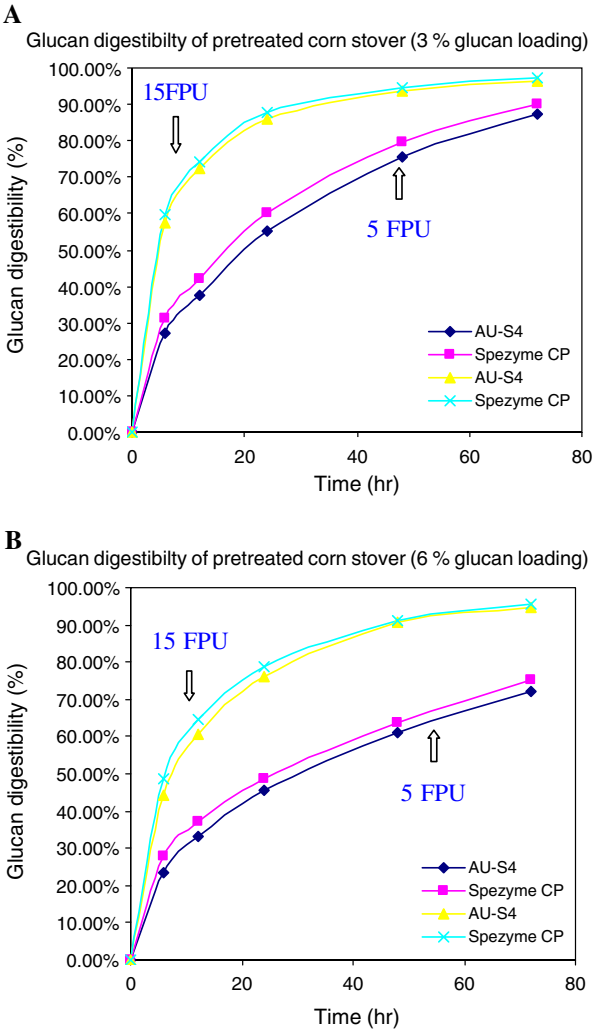

Fig. 4 Enzymatic hydrolysis of pretreated corn stover by AU-S4 and Spezyme CP

β-glucosidase showed Spezyme CP has slightly higher β-glucosidase activity than AU-S4 since more cellobiose accumulated in the process of hydrolysis with AU-S4. Without addition of β-glucosidase, Spezyme CP has shown higher yield than AU-S4 by 3% (data not shown).

The same comparison tests were repeated using α-cellulose as the substrate. The results were significantly different from the previous test as indicated in Fig. 5. In all four cases, high and low enzyme loading and glucan loading, AU-S4 has shown higher overall glucose yield than Spezyme CP. The difference is more discernible with high enzyme loading (15 FPU/g glucan). The 72-h yields were as follows: 85.82% (3% glucan loading) and 86.81% (6% glucan loading) for AU-S4 and 79.11% (3% glucan loading) and 78.53% (6% glucan loading) for Spezyme CP. Spezyme CP, however, has shown higher initial activity, especially with low enzyme loading (5 FPU/g glucan). The major difference between the

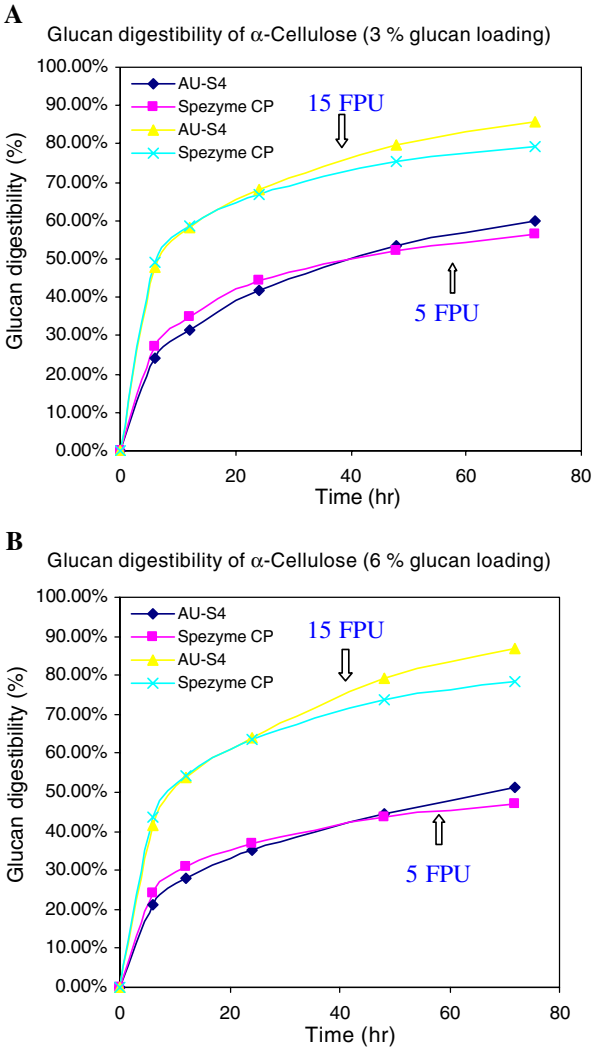

Fig. 5 Enzymatic hydrolysis of α-cellulose by AU enzyme and Spezyme CP

two substrates is in the xylan content; 2.8% in acid-treated corn stover vs. 20% xylan in α-cellulose. We speculate that the difference in yield has to do with the xylanase activity. We therefore extracted the xylan data from the same experiments to compare the xylanase activity of the two enzyme products. Xylan digestibility profiles for the two enzymes are shown in Fig. 6. The results confirm that AU-S4 has significantly higher xylanase activity than Spezyme CP. High xylanase activity of AU-S4 appears to be related with the fact that sludge 4 has relatively high xylan (15%) that may have acted as an effective inducer for xylanase production by *T. reesei*. It is well known that removal of hemicellulose increases the enzyme accessibility to cellulose, consequently increases the glucan digestibility [21]. These findings reaffirm that the high overall glucan yield observed with AU-S4 in hydrolysis of α-cellulose was due to its high xylanase activity.

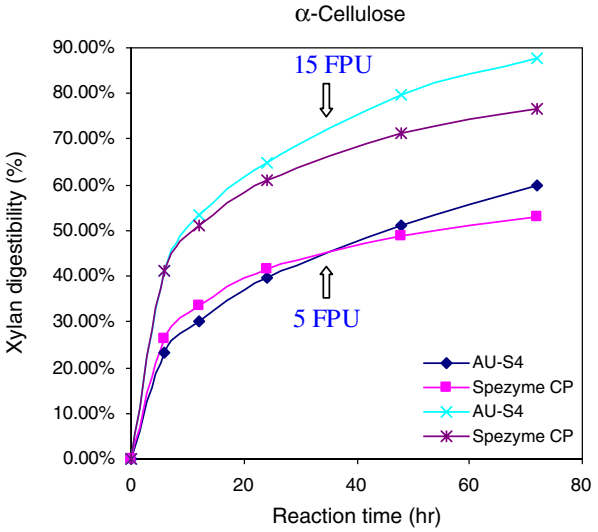

Fig. 6 Xylan digestibility of α-cellulose by AU enzyme and Spezyme CP

SSF Test

The AU-S4 was further tested for its performance by putting it through an SSF test using the enzyme and yeast (*S. cerevisiae*) simultaneously as described previously. In this test, partially de-ashed paper sludge was used. A comparison test was also made with Spezyme CP. The results of Fig. 7 indicate that the there is no significant difference between the two enzymes in their performance. For both enzymes, the 72-h ethanol yield based on glucan was 72% and the ethanol concentration was 24.4 g/L. These are acceptable performance indexes in ethanol production by batch SSF. Unlike cellulase production by *T. reesei*, ash in the sludge was not inhibitory to ethanol production by *S. cerevisiae*.

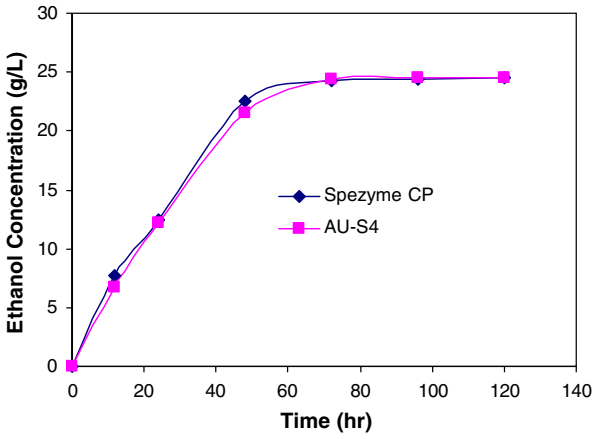

Fig. 7 Ethanol production from untreated primary sludge in SSF (*Saccharomyces cerevisiae,* ATCC-200062, 6% glucan loading)

Conclusion

Kraft paper mill sludge is a feedstock amenable for bioconversion to cellulase enzymes by *T. reesei* Rut C-30. For efficient production of cellulase, the ash content of the sludge needs to be reduced to less than 4%. One method to achieve this is centrifugal separation followed by successive chemical treatments by sulfuric acid and NaOH. The sludge thus treated and the cellulase enzyme produced from it are named as sludge 4 and AU-S4, respectively. *T. reesei* can produce cellulase in an efficient manner using sludge 4 as the only carbon source and inorganic salt as the primary nitrogen source. The cellulase production yield of 307 FPU/g glucan of sludge, and productivity of 60.6 FPU/L h were achievable from sludge 4. The titer of enzyme was 8.0 FPU/mL, which is comparable to those reported in the literature for cellulases produced from pure cellulosic substrates. The protein based specific activity of AU-S4 was much higher than reported specific activity of commercial cellulases. The main reason was that AU-S4 was produced using inorganic salt as the main nitrogen source; therefore, the amount of background protein was very low in the broth. On the basis of same FPU, AU-S4 has slightly lower glucanase activity, but higher xylanase activity than Spezyme CP. In the SSF test using partially de-ashed Kraft sludge as the substrate, no difference was found between AU-S4 and Spezyme CP in their performance, attaining identical ethanol profiles.

Acknowledgment The authors wish to acknowledge the financial support provided by Masada Oxynol, LLC, Alabama Center for Pulp and Bioresource Engineering, Laboratory of Biofuels and Biomaterials, Auburn University. They are grateful to Boise Paper, Jackson, AL for providing paper mill sludges and associated technical information, and to Genencor-Danisco, Palo Alto, CA for providing cellulase enzymes.

References

1. Ronneberg, D., & Naik, T. R. (2007). Available from: www1.eere.energy.gov/industry/forest/pdfs/res_solids_2007.pdf.
2. Duff, S. J. B., Moritz, J. W., & Anderson, K. L. (1994). *Canadian Journal of Chemical Engineering, 72*, 1013–1020.
3. Fan, Z. L., & Lynd, L. R. (2007). *Bioprocess and Biosystems Engineering, 30*, 27–34.
4. Fan, Z., & Lynd, L. R. (2006). *Bioprocess and Biosystems Engineering, 30*(1), 35–45.
5. Ruy, D., & Mandels, M. (1980). *Enzyme and Microbial Technology, 2*, 91–102.
6. Lynd, L. R. (1990). *Applied Biochemistry and Biotechnology, 24/25*, 695–719.
7. Chen, S., & Wayman, M. (1991). *Process Biochemistry, 26*, 93–100.
8. Shin, C. S., Lee, J. P., Lee, J. S., & Park, S. C. (2000). *Applied Biochemistry and Biotechnology, 84/86*, 237–245.
9. Juhasz, T., Szengyel, Z., Peczey, K., Aiika-Aho, M., & Viikari, L. (2005). *Process Biochemistry, 40*, 3519–3525.
10. Lo, C. M., Zhang, Q., Lee, P., & Ju, L. K. (2005). *Applied Biochemistry and Biotechnology, 121/124*, 561–573.
11. Bigelow, M., & Wyman, C. E. (2002). *Applied Biochemistry and Biotechnology, 98/100*, 921–934.
12. Maheshwari, D. K., Gohede, S., Paul, J., & Varma, A. (1994). *Carbohydrate Polymers, 23*, 161–163.
13. Miller, G. L. (1959). *Analytical Chemistry, 31*, 426–428.
14. NREL (2008). Laboratory Analytical Procedure (LAP), National Renewable Energy Laboratory,Golden, CO. Available from: www.nrel.gov/biomass/analytical_procedures.html.
15. Wood, T. M., & Bhat, K. M. (1988). *Methods in Enzymology, 160*, 87–112.
16. Bailey, M. J., Biely, P., & Poutanen, K. (1992). *Journal of Biotechnology, 23*, 257–270.
17. Hägerdal, B., Harris, H., & Pye, E. K. (1979). *Biotechnology and Bioengineering, 21*(3), 345–355.
18. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
19. Velkovska, S., Marten, M. R., & Ollis, D. F. (1997). *Journal of Biotechnology, 54*, 83–94.
20. Domingues, F. C., Queiroz, J. A., Cabral, J. M. C., & Fonseca, L. P. (2000). *Enzyme and Microbial Technology, 26*, 394–401.
21. Gupta, R., Kim, T. H., & Lee, Y. Y. (2008). *Applied Biochemistry and Biotechnology, 148*(1–3), 59–70.