Pretreatment and hydrolysis of cellulosic agricultural wastes with a cellulase-
producing \textit{Streptomyces} for bioethanol production

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ABSTRACT

Production of reducing sugar by hydrolysis of corncob material with \textit{Streptomyces} sp. cellulase and ethanol fermentation of cellulosic hydrolysate was investigated. Cultures of \textit{Streptomyces} sp. T3-1 improved reducing sugar yields with the production of CMCase, Avicelase and \(\beta\)-glucosidase activity of 3.8, 3.9 and 3.8 IU/ml, respectively. CMCase, Avicelase, and \(\beta\)-glucosidase produced by the \textit{Streptomyces} sp. T3-1 favored the conversion of cellulose to glucose. It was recognized that the synergistic interaction of endoglucanase, exoglucanase and \(\beta\)-glucosidase resulted in efficient hydrolysis of cellulosic substrate. After 5 d of incubation, the overall reducing sugar yield reached 53.1 g/100g dried substrate. Further fermentation of cellulosic hydrolysate containing 40.5 g/l glucose was performed using \textit{Saccharomyces cerevisiae} BCRC 21812, 14.6 g/l biomass and 24.6 g/l ethanol was obtained within 3 d. The results have significant implications and future applications regarding to production of fuel ethanol from agricultural cellulosic waste.

Keywords: Corncob; Cellulosic hydrolysates; \textit{Streptomyces} sp.; Reducing sugar; Bioethanol

INTRODUCTION

Cellulosic biomass, which has been predicted to be the alternative raw material for bioethanol production, is an ideal source of energy because it is both renewable and available in large quantities throughout the world. Conversion of cellulosic biomass to glucose and other fermentable sugars has been considered in the last few decades, which is an attractive route for ethanol production [1]. However, the process for the production of bioethanol from cellulosic materials, which requires pretreatments such as liquefaction and saccharification, is more complicated than its production from sugar or starch-based ones. Over the past few decades, for the production of bioethanol from cellulosic materials, the focus of previous researchers has been on the enzymatic hydrolysis of cellulosic biomass to fermentable sugar. Nevertheless, there are still several technical and economical impediments with regards to the development of commercial processes that utilize cellulosic biomass.

Many different types of agricultural wastes, such as rice straws, wheat straws, and corncobs, have been proposed for enzymatic hydrolysis of cellulosic biomass and ethanol production. Corncob consists of polymers of mainly two types of sugars: glucose and xylose. Both sugars can be obtained, in monomeric forms, with high yield rates from the break-down of polysaccharide chains using steam pretreatment and subsequent enzymatic hydrolysis [2]. The monomeric glucose can then be fermented to ethanol with the help of \textit{S. cerevisiae}. Furthermore, appropriate pretreatment is a key step for the effective utilization of cellulosic biomass, due to its recalcitrant nature. Since the cost of raw materials contributes substantially to the cost of ethanol production [3], a lower raw material price, together with a high ethanol yield and efficient enzymes, will decrease the production cost significantly. Accordingly, several different pretreatment methods have been used to facilitate the enzymatic hydrolysis of cellulosic material [4] and it can be concluded from these methods that an efficient process for obtaining reducing sugars from cellulosic material is to use proper combination of chemical and physical pretreatments, followed by enzymatic hydrolysis.

In our previous work, the production of the thermostable cellulase by \textit{Streptomyces} sp. T3-1 has successfully demonstrated in a lab-scale fermenter [5]. In this study, the effect of prehydrolysis on reducing sugar concentrations was investigated using acid and autoclaving pretreated corncob. The hydrolysis efficiency towards corncob with cellulase-producing strain \textit{Streptomyces} sp. T3-1 was examined. The hydrolysis efficiency of commercial and microbial cellulase was compared following the prehydrolysis treatment. Further fermentation of the hydrolysate for ethanol production was also investigated using \textit{S. cerevisiae} BCRC 21812.

MATERIALS & METHODS

\textit{Microorganisms and cultivation}
The cellulase-gene cloning transformant Streptomyces sp. T3-1 was used for hydrolysis of cellulosic materials. The microorganism was grown in culture medium based on that of Mandels and Reese [6]. Initial pH of the medium was adjusted to 6.5 prior to sterilization at 121°C for 20 min. The strain of S. cerevisiae BCRC 21812 was used for ethanol fermentation and maintained in medium containing 2% (w/v) Dextrose, 1% (w/v) Peptone and 0.5% (w/v) Yeast extract and incubated at 25°C for 48 h.

**Cellulosic material and pretreatment**
The corncob materials were oven-dried at 50°C for more than 24 h, grounded into particles of about 2-10 mesh in diameter using a stainless-steel grinder, and stored in pill vials at room temperature. Acid pretreatment was performed with 0.25 or 0.5 % (v/v) sulfuric acid for 10-30 min at a solid-to-liquid ratio of 1:10. The mixture was filtered and then the filtrate was further hydrolyzed by autoclaving at 121°C for 15, 30, and 60 min, respectively. After the pretreatment, the cellulosic residue was soaked in distilled water and incubated in water bath at 50°C for 30 min, and then filtered. The filtrate collected from the acid and autoclave pretreatment were used to determine the reducing sugar contents.

After the cellulosic substrate was autoclaved for 60 min, prehydrolysis with the commercial enzyme mixture was performed at 50°C. A commercial cellulase mixture, 1.5 ml (1,000 IU/ml) Cellulase supplemented with 0.52 ml (250 IU/ml) Novozyme 188, was used to hydrolyze the cellulosic residue. Enzymatic hydrolysis of corncob cellulosic residue was performed in 500 ml serum bottles, containing 10 g solid substrate and commercial enzyme mixtures by adding 0.1 M sodium acetate buffer (pH 6.0) to make a solution of 500 ml in volume. The serum bottles were incubated in a water bath at 40°C and the reaction mixtures were sampled periodically and filtered through 0.45 μm Millipore membrane. The total reducing sugar, and contents of glucose, xylose and cellobiose of the filtrate was then determined by HPLC.

**Hydrolysis of cellulosic residue by Streptomyces sp. T3-1 cultures**
Corncob substrate was mixed with 0.1 M sodium acetate solution at a solid-to-liquid ratio of 1:10. The mixture was then pretreated by autoclaving at 121°C for 60 min and the pyrolysates were filtered with Whatman No. 4 filter paper. The filtrate substituting CMC was added to the culture medium of Mandels and Reese, and then autoclaved for 15 min. Ten percent (v/v) of Streptomyces sp. T3-1 at inocula of 10^8/ml was inoculated into the culture medium and incubated at 37°C in an orbital shaker with speed of 150 rpm. Samples were withdrawn periodically from the cultures, and the filtrate was analyzed for cellulase activity and the concentrations of total reducing sugar, glucose, xylose and cellobiose.

**Ethanol fermentation of cellulosic hydrolysate**
Inoculum was prepared by transferring 5% (v/v) of cells of S. cerevisiae BCRC 21812 into the fermentation medium. Each ml of inocula contained 10^8 yeast cells. The fermentation medium for ethanol production was (%, w/v): cellulosic hydrolysate, 4; peptone 0.5; yeast extract 0.25 at pH 6.0. After inoculation the cultures were shaken at 150 rpm for 2 d and 100 rpm thereafter and incubated at 25°C. Samples were regularly collected and filtered through 0.45 μm Millipore membrane. Glucose, xylose, cellobiose and ethanol were analyzed on an HPLC system.

**Analysis methods**
All enzyme activities were determined by incubating the crude enzyme solution with its respective substrate which was dissolved in sodium phosphate buffer (100 mM, pH 7.0) at 50°C using the modified methods by Ghose [7]. One international unit (IU) of enzyme activity was defined as the amount of enzyme that forms 1 μmol glucose/min.

The dry material content of the samples was determined by drying samples for 24 h in an oven at 110°C. Samples were withdrawn from the fermentation broth and the yeast biomass was determined by measuring cell optical density recorded at 600 nm with a spectrophotometer. The reducing sugars liberated by these reactions were measured using the 3, 5-dinitrosalicylic acid (DNS) method [8], with glucose as standard. Total reducing sugar was calculated as g/g dried substrate (DS).

Glucose, xylose, cellobiose and ethanol were analyzed by HPLC with a cation exchanger Sugarpak column (300 x 6.5 mm, Waters, MA, USA). Secondary de-ionized water was used as the mobile phase at a flow rate of 0.5 ml/min. The injection volume was 20 μl and the column temperature was maintained at 90°C. All samples were filtered through a 0.22 μm filter before subjecting to HPLC analysis. The eluate was detected by a refractive index detector at 50°C.
RESULTS & DISCUSSION

Acid and autoclave pretreatment of cellulosic residue

The effects of pretreatments were investigated first. The results are shown in Fig. 1. By treating with 0.25 or 0.5% sulfuric acid for 20 min, approximately 27.1 g/100 g DS of reducing sugar was produced (Fig. 1a). No significant difference was found between the substrates treated with 0.25 and 0.5% sulfuric acid. Although acid pretreatment helps the hydrolysis of cellulosic material, however, it has been reported that after acid treatment some by-products, e.g., furfural and 5-hydroxyfurfural, will be produced where these degradation compounds are known to inhibit the fermentation step [9]. To avoid formation of the by-products in the hydrolysis process, an alternative pretreatment method without acid addition should be performed. In Fig. 1b, after being autoclaved at 121°C for 60 min, highest yields of reducing sugars were observed (58.1 g/100g DS and higher), compared to the yields of autoclaving for only 15-30 min. This implies that most of the cellulosic components in the corncob were converted to reducing sugar after treatment with heating by autoclaving. Therefore, the hydrolysis efficiency of the pyrolysate by prolonged autoclaving time to 60 min can be increased.

Hydrolysis of cellulosic substrate with commercial enzyme for reducing sugar production

After being autoclaved, the cellulosic substrate was hydrolyzed with the commercial enzyme mixture. The reducing sugar yield was only 17.0 g/100 g DS after the commercial enzyme mixture was reacted for 48 h, whereas the glucose, xylose and cellobiose concentrations reached 7.5, 10.9, 0.7 g/l, respectively (data not shown). Generally, concentrations of reducing sugar, glucose, xylose and cellobiose reached their respective maxima and stayed unchanged after 2 h of enzymatic hydrolysis. However, the amounts of resulting reducing sugar were slightly higher compared to those of acid and autoclave pretreatment process. During the course of hydrolysis, activities of CMCase, Avicelase and β-glucosidase were maintained at 1.7, 1.0 and 1.3 IU/ml, respectively (data not shown). Since the total cellulase activity of the commercial enzyme mixture was only 3.3 IU/ml, the reducing sugar produced was comparably lower.

Cellulases and reducing sugar production from Streptomyces sp. T3-1 cultures

Time course of a typical batch cultivation of Streptomyces sp. T 3-1 is shown in Figure 3. Biomass concentration reached a maximum value of 8.0 g/l on the fifth day, where the yield of total reducing sugar was 53.1 g/100 g DS (Fig. 3a). In addition, the production of CMCase, Avicelase, and β-glucosidase was associated with the growth of Streptomyces sp. T 3-1. Maximum activities of CMCase, Avicelase and β-glucosidase were 3.8, 3.9 and 3.8 IU ml/1, respectively (Fig. 3b). Glucose, xylose and cellobiose produced during hydrolysis were analyzed by HPLC at the interval of 1 d (Fig. 3c). The synergistic interaction of endo- and exo- glucanase was observed, which resulted in efficient hydrolysis of cellulosic substrate. The β-1,4-
endoglucanase and β-1,4-exoglucanase produced from *Streptomyces* sp. T3-1 hydrolyzed cellulose chains and resulted in formation of cellobiose, which could be further cleaved into glucose by β-glucosidase. In addition, a high amount of glucose (40.5 g/l) and a comparably lower amount of cellobiose (3.0 g/l) were found, indicating satisfactory activities of β-1,4-endoglucanase, β-1,4-exoglucanase and β-glucosidase in *Streptomyces* sp. T3-1 cellulase.

**Figure 2.** Time courses of (a) biomass, pH and concentration of reducing sugar, (b) cellulase activity, and (c) sugar concentration, with the culture of *Streptomyces* sp. T3-1 in the autoclaved hydrolysate medium. Values of enzyme activity represent the average of three measurements ± standard deviation.

**Comparison of hydrolysis methods**

Fig. 3 shows the comparison of the highest yields obtained from various hydrolysis methods investigated, i.e., pretreatment by autoclaving followed by commercial enzymatic or *Streptomyces* sp. hydrolysis (Sample 1 and 2), as well as pretreatment with acid, and autoclaving followed by commercial enzymatic hydrolysis (Sample 3). For Sample 1, 17.0 g of total reducing sugar from 100 grams of cellulosic substrate was produced after the corn cob was treated with autoclave at 121°C for 60min, and then hydrolyzed by the commercial cellulase, without acid pretreatment. For Sample 2, pretreatment by autoclaving with *Streptomyces* sp. hydrolysis (without acid pretreatment either) resulted in significantly higher amount of both total reducing sugar yields (53.1 g/100 g DS and 40.5 g/l of glucose). The amount of reducing sugar from Sample 1 was approximately only 32% of the total reducing sugar yielded from Sample 2. The results suggest that samples treated with the combination of autoclaving and hydrolysis by *Streptomyces* sp. is highly effective for the subsequent ethanol fermentation with complete conversion of monosaccharides in the mixture. These results also demonstrate that cellulase activity produced by *Streptomyces* sp. cultures has the potential to improve the hydrolysis efficiency of cellulosic materials markedly without acid pretreatment.

As for Sample 3, 86.7 g/100 g DS (on the same dried weight basis) was obtained from the pretreatment of 0.5 % (v/v) sulfuric acid and autoclaving, followed by the commercial enzymatic hydrolysis. The effect of hydrolysis by the commercial enzyme was relatively marginal compared to the effect of the pretreatment of acid and autoclave. Similar results were found for the biomass samples processed with the pretreatment of sulfuric acid and autoclaving, followed by the hydrolysis of *Streptomyces* (data not shown). Though the treatments of Sample 3 resulted in highest yield of reducing sugars, it has been reported that the acid pretreatment had negative effects on the yield of ethanol of the following fermentation step [10]. To avoid the effects of the acid pretreatment mentioned above, it would require additional cost for extra pH adjustment of...
the biomass solution and raise the overall production cost as a consequence. We have continued our research to investigate the usage of cellulase hydrolysis without acid pre-treatment.

**Figure 3.** Comparison of yields of total reducing sugars produced from different hydrolysis methods at various stages of respective pretreatments. Sample 1 and 2 were the hydrolysate obtained from pretreating with autoclave followed by hydrolyzing with commercial cellulase or *Streptomyces* sp., respectively. Sample 3 was pretreated with acid, autoclave followed by commercial enzymatic hydrolysis. Values represent the average of three measurements ± standard deviation.

**Fermentation of cellulosic hydrolysate for bioethanol production**

Due to the concern that high concentration of glucose in the hydrolysate would inhibit the growth of yeast, a high concentration of glucose (40 g/l) was firstly used for the investigation during the course of ethanol fermentation by *S. cerevisiae*. Fig. 4 shows the time course of ethanol production using the hydrolysate as the substrate. For the hydrolysate medium, the pH decreased slowly and remained above 5.4 throughout the initial 5 d of the fermentation and then decreased rapidly from 5.4 to 4.7 after 6 d of cultivation (Fig. 4a). In addition, after the initial inoculation of yeast cells, the microbial biomass began to increase, reached the maximal value (14.6 g/l) after 48 h of incubation, and then remained steady thereafter. The pH stayed steadily around 5.0 during the entire fermentation process and did not affect the growth of yeast cells.

In the fermentation using the hydrolysate, the glucose concentration decreased rapidly from 40.0 to 9.1 g/l after 2 d (Fig. 4b). However, 12.1 g/l xylose and 0.6 g/l cellobiose were detected after 2 d, an indication that they were not utilized by yeast cells during fermentation. Results indicated that *S. cerevisiae* BCRC 21812 could readily ferment glucose in hydrolysate to ethanol but hardly metabolize xylose. The ethanol production rate in the early phase of the culture was relatively slow but rapidly increased after 1 d. Fermentation was completed after 2 d, where ethanol reached a maximal concentration of 24.6 g/l with approximately complete depletion of glucose. This indicates that the consumption of glucose by yeast cells was virtually in sync with the ethanol production. In addition, the ethanol yield was 0.67 g ethanol/g glucose, which is substantially higher than the ethanol yield (0.45 g ethanol/g glucose) reported by Yu and Zhang [11]. The results suggest that *S. cerevisiae* can grow well in the medium and can convert glucose to ethanol completely.

**CONCLUSION**

In this study, the hydrolysis efficiency of corncob-based cellulosic material can be substantial improved with the technology we developed by culturing of the cellulase-producing *Streptomyces* sp. T3-1. The amount of total reducing sugars and concentration of glucose in the hydrolysate increased significantly as a result. In search of an economic way for producing ethanol from cellulosic substrate, the microbial hydrolysis shown in our study has been demonstrated to be not only an excellent alternative to commercial cellulase hydrolysis, but also a feasible and economical process for the future scale-up production of bioethanol.
Figure 4. Time courses of (a) biomass and pH, and (b) concentration of sugars and ethanol, changes during the fermentation process with S. cerevisiae in the hydrolysate medium.

REFERENCES