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Full Length Research Paper

Utilization of sugar beet pulp as a substrate for the fungal production of cellulase and bioethanol

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Microbial production of cellulase and bioethanol were studied using Sugar Beet Pulp (SBP) as a substrate by *Trichoderma reesei* and *Saccharomyces cerevisiae*, respectively. The subculture medium for cellulase production was a salt solution. Fungal cells were sub-cultured in an orbital shaker at 30°C for 4 to 6 days and then were used as an inoculum. Exponential cells were inoculated into a medium containing SBP. A maximum cellulase activity of 0.46 IU/ml of filter paper activity was obtained. Ethanol yield was higher in pretreated SBP than in non-treated SBP. After 24 h fermentation period, 0.11 g/L ethanol was produced from pretreated SBP, while non-treated SBP produced 0.05 g/L ethanol.

Key words: *Trichoderma reesei*, *Saccharomyces cerevisiae*, sugar beet pulp, cellulase, bioethanol.

INTRODUCTION

A significant decrease in fossil fuels during the last few years has turned the attention of societies to fuels obtaining from renewable resources. Constant increase in costs and environmental impact derived from the use of crude-based fuels during the recent years, have increased efforts for fuels production from renewable sources. Nowadays, the main renewable fuels are obtained from sugarcane, corn and cereal grains. The use of feedstocks reduces production costs since 40 - 70% of the total production cost is related to raw materials (Claasen et al., 1999).

Ethanol produced from biomass can be used as a transportation fuel in the future. Simple sugars, starchy or lignocellulosic materials have the potential to be used as substrates for ethanol production. Lignocellulosic materials constitute approximately 50% of all land produced biomass (Lee, 1997; Chum and Overend, 2001; Kim and Dale, 2004; Demirbas, 2005). Ligno-cellulosic residues are available in large quantities at low cost, thus they are suitable raw materials for fuel ethanol production (Parisi, 1989). By using waste products from forestry, agriculture and industry, the costs of the feedstocks may be reduced.

Lignocellulose is considered as an attractive feedstock for the production of fuel ethanol, because of its availability in large quantities at low cost (Cardona and Sanchez, 2007; Cheng et al., 2008) and for reducing competition with food but not necessarily with feed. The main components of lignocellulosic materials are cellulose, hemicelluloses and lignin. The hemicellulose fraction under mild conditions can be hydrolysed to monomeric sugars, xylose and glucose (Mussatto and Roberto, 2004). Sugar Beet Pulp (SBP) is a by-product of the sugar beet industry containing large amounts of cellulose (Wen et al., 1998).

Cellulose has the potential to be converted to useful materials, bio-based chemicals and energy. One possible approach to SBP lignocellulose utilization is to hydrolyze the materials into fermentable saccharides, which can then be converted into value-added products or bio-energy (Colberg, 1988). Commercial cellulase enzymes could be used to convert lignocellulose into reducing sugars (Wen et al., 2004). The cost of commercial cellulase enzyme is very high; therefore, this process is non-economical (Von Sivers and Zacchi, 1995). If cellulase could be produced directly using SBP by a microorganism and then be applied further to degrade the SBP cellulose, the cost of cellulase production would be significantly reduced. Cellulolytic enzymes might be produced by a number of bacteria and fungi.

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Cellulase production by the two fungi *Trichoderma viride* and *Trichoderma reesei* have been studied broadly (Domigues et al., 2000; Gadgil et al., 1995; Mandels and Weber, 1971; Montenecourt and Eveleigh, 1979; Velkovska et al., 1997). Various mutants of *Trichoderma* spp. have been developed to enhance the cellulase activity. The mutant of *T. reesei* RUT C30 has a high cellulase production level with the ability to grow on waste cellulosic materials (Domigues et al., 2000; Montenecourt and Eveleigh, 1979; Ju and Afolabi, 1999; Reczey et al., 1996).

Cellulolytic fungi can use cellulose as a primary carbone source. Pure cellulose such as cotton and Avicel are good cellulase inducers, but expensive and non-economical, consequently the use of less expensive substrates can decrease the production cost (Wen et al., 2005). Many cellulosic materials such as wastepaper (Ju and Afolabi, 1999), wood (Reczey et al., 1996; Duff and Murray, 1996), bagasse (Ogel et al., 2001; Rajoka and Malik, 1997), straw (Kalogerist et al., 2003; and Romero et al., 1999), corn cob (Xia and Cen, 1999), wheat bran (Smits et al., 1996), and fruit pomace (Haddadin et al., 2001) have been used as substrates for cellulase production.

Cara et al. (2008) used steam-explosion pretreated olive tree pruning for fuel ethanol production. Pretreatment conditions for the maximum ethanol yield included 1% sulphuric acid impregnation and steam explosion pretreatment at 230°C. In the process of ethanol production from lignocellulosic materials, enzymatic hydrolysis and fermentation can be performed separately or simultaneously. Zhao and Xia (2009) produced ethanol from an alkaline-pretreated corn Stover using a recombinant yeast strain of *Saccharomyces cerevisiae* ZU-10 and simultaneous saccharification and fermentation system.

There is a lack of investigation on the cellulase production from SBP cellulosics. The aims of the present work were to study the potential of using SBP for cellulolytic enzyme production by the fungus *T. reesei*, then cellulose hydrolysis and finally ethanol production from the released sugars by *S. cerevisiae*. These informations would be useful for the development of a cost-effective process for cellulase production and subsequent enzymatic hydrolysis of SBP lignocelluloses, and production of bioethanol.

MATERIALS AND METHODS

Biomass material

Fresh sugar beet pulp was collected from the Marvdasht sugar factory. This pulp was obtained from extracted cossettes after diffusion and press, and was without formaline. SBP was dried at 60°C for 16 h. Dry pulp was milled and reduced to 60 meshes for uniformity of particle size. Pulp powders were stored at 25°C and kept away from light and moisture. These pulp powders were used for physical and chemical analyses.

Analysis of biomass components

Chemical analysis of biomass (SBP) was performed. Fat content was determined by soxhlet method using chloroform and methanol as solvents (Wen et al., 1998). Protein content was determined by the microKjeldahl method with a conversion factor of 6.25 (Morris and Jacob, 1965). Moisture (AOAC, 2002) and ash (Membrillo et al., 2008) content were also determined for SBP.

Cellulose measurement of SBP

A biomass of 2 g was transferred into a 250 ml Erlenmeyer flask. One hundred ml of 96% ethyl alcohol and 50 ml of 65% nitric acid were added. The flask was put on a heater equipped with a condenser and heated for 1 h. After hydrolysis, flask contents were filtered. Once more, remaining cellulose on the filter paper was transferred into the flask. This step was repeated similarly to the previous stage. This process was repeated for the third time and then cellulose together with the filter paper were dried at 102°C. The cellulose content was calculated from the following equation (Oakley, 1984; Ritter and Fleck, 1924):

$$Cellulose\% = \frac{Cellulose\ dry\ weight}{Sample\ dry\ weight} \times 100 \quad (1)$$

Microorganisms and culture media

Fungal strains of *T. reesei* (PTCC 5142) and *S. cerevisiae* (PTCC 5052) were purchased from Persian type culture collection. These microorganisms were transferred into nutrient broth and incubated at 30°C for activation. The fungus of *T. reesei* was cultivated on Potato Dextrose Agar (PDA) plates and incubated at 30°C to form yellow colonies. Then, the fungi were maintained in PDA at 4°C. The salt solution was prepared in a 500 ml Erlenmeyer flask containing 100 ml of medium (Wen et al., 2005). A salt solution containing 2 ml/L tween-80, 1 g/L peptone, and 10 g/L glucose was used as a subculture medium (Table 1). The initial pH of the medium was adjusted to 4.7 then autoclaved at 121°C for 15 min. Fungal cells were sub-cultured in an orbital shaker (180 rpm) at 30°C for 4 to 6 days and were then used as inoculums into medium containing SBP as a substrate. In this study, SBP concentration was adjusted at 10 g/L (dry basis). The composition of this medium was similar to the subculture medium (Table 1), except that peptone was eliminated and glucose was replaced by SBP (Wen et al., 2005).

Enzyme activity

The activity of total cellulase Filter Paper Activity, (FPA) was determined according to the standard IUPAC procedure and expressed as an international unit (IU). Each unit of FPA activity was defined as the amount of enzyme which releases 1 µmol of glucose equivalents from Whatman No. 1 filter paper in 1 min (Ghose, 1987).

Acid pretreatment and enzymatic saccharification

In acid pretreatment, a biomass of 160 g was pretreated with 1% H_2SO_4 at 120°C for 1 h. After pretreatment, the biomass was separated into a solid residue and liquid. The solid residue was washed with water and then dried. After, 30 g of pretreated and non-treated SBP were mixed separately with 100 mL of nutrient medium and

Table 1. Medium composition for subculturing and cellulase production from the fungus *T. reesei* (Wen et al., 2005).

Components	Unit	Concentration	
		Subculture	Cellulase production
Salt solution			
KH_2PO_4	g/L	2.0	2.0
$CaCl_2.2H_2O$	g/L	0.4	0.4
$MgSO_4.7H_2O$	g/L	0.3	0.3
$(NH_4)_2SO_4$	g/L	1.4	1.4
Urea	g/L	0.3	0.3
Trace elements			
$FeSO_4.7H_2O$	mg/L	5.0	0.5
$MnSO_4.H_2O$	mg/L	1.6	1.6
$ZnSO_4.7H_2O$	mg/L	1.4	1.4
$CoCl_2$	mg/L	2.0	2.0
Tween-80	ml/L	2.0	2.0
Peptone	g/L	1.0	--
Glucose	g/L	10	--
Sugar beet pulp (DM) †	g/L	--	10

†Dry matter.

the pH of mixtures was adjusted to 5.5 with 50 mM sodium citrate buffer. Nutrient medium contained $(NH_4)_2HPO_4$ (10 g/L), $MgSO_4.7H_2O$ (0.05 g/L), and yeast extract (2 g/L) (Lee et al., 2008). Produced cellulase enzyme was added to the reaction mixture and the flasks were incubated at 50°C for 7 days to hydrolyze cellulose.

Determination and quantification of released sugars

To detect the kind of released sugars after enzymatic hydrolysis, Thin Layer Chromatography (TLC) was performed. A solvent consisting of acetate, pyridine and water (55:25:20 by volume, respectively) was poured into the tank and the lid was replaced. TLC plastic sheets pre-coated with silica gel (sheet dimensions: 20 × 20 cm^2 and layer thickness: 0.2 mm) were used. One drop of each sugar solution (containing 1% of raffinose, maltose, xylose, arabinose, fructose, galactose and glucose) and one drop of the unknown (pretreated and non-pretreated SBP after enzymatic hydrolysis) were applied to nine separated origins. After drying the spots, the sheet was placed into the tank covered with the lid. When running was finished, the sheet was removed from the tank and dried until it was free from the solvent. To detect the spots, freshly-prepared reagent (m-phenylene diamine, stannous chloride, acetic acid and ethanol) was sprayed on the sheet and placed in the oven at 110°C for 10 min (White and Robyt, 1988). The amount of released sugars from non-treated and pretreated SBP on which cellulase enzyme had influenced was determined using AOAC method (AOAC, 2002).

Fermentation and ethanol production

For fermentation, *S. cerevisiae* (PTCC, 5052) was cultured on YPD medium (yeast extract 1%, peptone 2%, and dextrose 2%) at 30°C, 180 rpm for 24 h (Lee et al., 2008). After enzymatic saccharification, the saccharified mixtures (pretreated and non-treated SBP) were autoclaved at 120°C for 15 min, and then *S. cerevisiae* was inoculated at a certain cell concentration (OD_{600} = 1). The flasks were incubated at 30°C in a shaker at 180 rpm. Samplings were performed after 6, 12, 24, 36 and 48 h, and were analyzed for assessing the ethanol yield.

Ethanol concentration was determined using gas chromatography (Varian, series 2800) equipped with a 1.5% OV 101, Chrom GHB column and Flame Ionization Detector (FID) with a flux of 40 ml/min and n-Butanol as an internal standard. The injector and detector temperature was maintained at 150°C. Carrier gas was nitrogen.

Statistical analysis

All data were statistically analyzed by independent and paired T tests, ANOVAs using the SPSS/PC software and the mean comparison was performed using DMRT ($p < 0.05$).

RESULTS AND DISCUSSION

Biomass characterization

The chemical characteristics of sugar beet pulp are given in Table 2. SBP contained 97.12% dry matter after drying 25.55% of which was cellulose, a suitable percentage for microbial cellulase production. The amounts of other ingredients of SBP consisting of fat, protein and ash indicate that in terms of nutrition, SBP is a relatively worthless material and the use of this biomass reduces the production costs.

Amount and types of sugars released from SBP after enzymatic saccharification

The results from TLC including the R_f values of unknown

Table 2. Major composition of sugar beet pulp[a].

Ingredients	± SD%
Fat	1.50 ± 0.70
Protein	5.95 ± 0.35
Moisture	2.88 ± 0.10
Ash	4.00 ± 0.50
Cellulose	25.55 ± 1.76

[a]Data is expressed as mean ± SD of three replicate samples.

samples and the comparison of the R_f value of each sugar revealed that the released sugar from pretreated SBP affected by cellulase enzyme was glucose only, but no sugar was detected from the SBP with no cellulase added. The amount of released sugars from pretreated SBP affected by cellulase enzyme was 3% against < 0.1% in SBP without cellulase.

Possibility of using SBP as a substrate for cellulase production

Sugar beet pulp has cellulosic components that can induce the production of cellulase when used as a carbon source for fungus growth. Among cellulolytic fungi, *T. reesei* and its mutants *T. reesei* RUT-C30 and *T. reesei* QM 9414 have been the most extensively studied. It was clear that the mutatnts improved the filter paper activity (Domigues et al., 2000; Gadgil et al., 1995; Ju and Afolabi, 1999; Reczey et al., 1996; Smits et al., 1996). In this study, *T. reesei* was grown in a medium containing SBP as a substrate. It was found that this fungus could produce cellulase in medium containing SBP (Figure 1). The pattern of cellulase production indicates that the cellulase activity increased during the first four to five days, reached the maximum on day 5 and then decreased at the end of cultivation.

The mutants of *T. reesei* produce higher cellulase activity. Cellulase production by the fungus *T. reesei* was studied using dairy manure as a substrate by Wen et al. (2005). Data showed that the mutant of *T. reesei* RUT-C30 had higher cellulase production than *T. reesei* QM 9414. The maximum cellulase production activity of 1.74 IU/ml of filter paper activity was obtained by *T. reesei* RUT-C30. Membrillo et al. (2008) used two strains of *Pleurotus ostreatus* (IE-8 and CP-50) for production of lignocellulolytic enzymes. Mycelia from these cultures were used as an inoculum for solid fermentation using sugar cane bagasse. Strain IE-8 produced the highest level of cellulase (0.18 IU/g dry wt on smallest particles of substrate).

Ahamed and Vermette (2008) investigated production of cellulase by co-culturing *T. reesei* and *Aspergillus niger* in a bioreactor to convert cellulose substrate into soluble sugars through a synergetic action of enzyme complex simultaneously produced by these two fungi. The results of mixed culture experiments exhibited a highly significant increase in production of filter paper activity (7.1 U mL^{-1}).

Sun et al. (2008) also used *T. reesei* Rut C-30 for production of cellulase. The use of alkaline-treated rice straw sticks and non-pretreated rice straw powder as the inducing substrates in batch culture could result in cellulase activities of 1.07 and 0.71 FPU/ml, respectively. Thus, the results of cellulase production and its activity in this research are comparable to values reported in the literature on the use of other cellulose sources as inducers. Despite using non-pretreated SBP, produced cellulase enzyme had a relatively high enzyme activity; this substrate is abundant in the waste of sugar factories, thus sugar beet pulp can be used as a suitable substrate for cellulase enzyme production.

Ethanol production from SBP

The concentration of ethanol increased by time until 24 h of fermentation. The maximum ethanol concentration was 0.11 and 0.05 g/L in pretreated and non-treated SBP at 24 h, respectively (Figure 2). After 24 h, ethanol concentration was slightly lower in both substrates. Pretreated SBP produced more ethanol than non-treated SBP, implying that pretreatment of SBP could increase bioethanol production. The amount of released sugars during enzymatic hydrolysis of pretreated SBP was more than non-treated SBP. The combination of hemicellulose and lignin provides a protective sheath around the cellulose, which must be modified or removed before efficient hydrolysis of cellulose can occur. Therefore, for cost-effective hydrolysis of cellulose, more advanced pretreatment technologies are required.

Lee et al. (2008) investigated the possibility of using waste mushroom logs as a biomass resource for alternative energy production. After 24 h fermentation, 12 g/L ethanol was produced in waste mushroom logs. Ethanol yield is more in comparison with our research because lignin was degraded by inoculation with *Lentinus edodes* and the activity of enzymes used for enzymatic hydrolysis (cellulase and β-glucosidase) was the highest.

Telli-Okur and Eken-Saracoglu (2008) evaluated ethanol production from sunflower seed hull hydrolysate using *Pichia stipitis* NRRL Y-7124. The hydrolysate prepared with 0.7 M H_2SO_4 at 90°C was fermented as a substrate in shaking bath at 30°C. *P. stipitis* has an advantage over *S. cerevisiae* in its ability to ferment xylose, glucose and cellobiose. With this condition, higher ethanol level 11 g/L was achieved.

Pretreatment of lignocellulosic material has a high effect on the ethanol production yield. Sugar Cane Bagasse (SCB) has proven to be a feasible raw material for fuel ethanol production due to its relative low lignin content and high production of sugars by appropriate

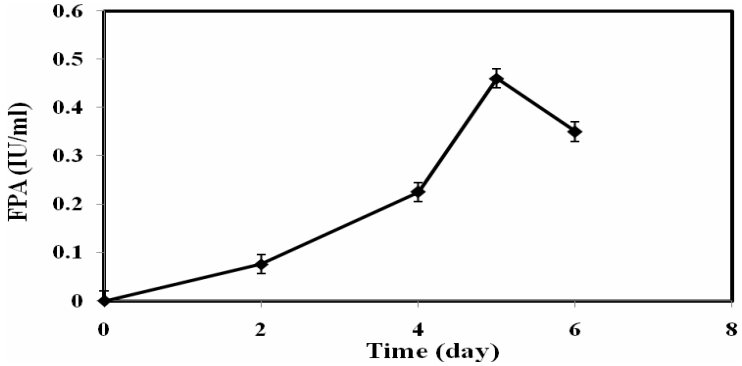

Figure 1. Cellulase production by *T. reesei* using SBP as a substrate. Time course of FPA in medium containing SBP. Data are means of three replicates and error bars show standard deviation.

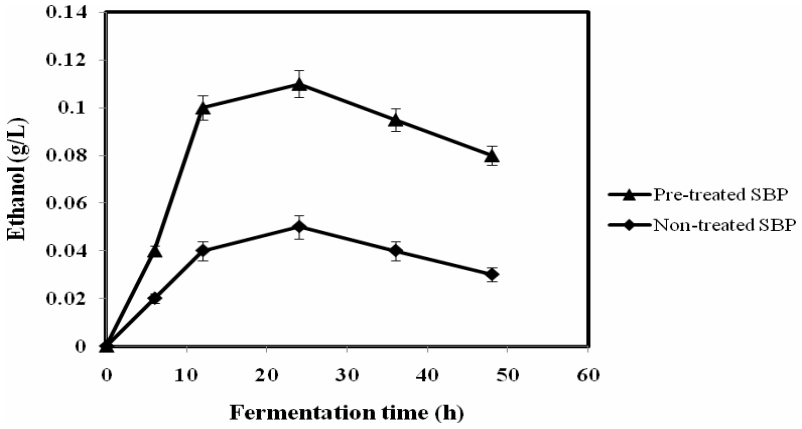

Figure 2. Ethanol production by *S. cerevisiae* from pretreated and non-treated SBP during fermentation. Data are means of three replicates and error bars show standard deviation.

pretreatments. Some of the more currently advances in fuel ethanol production using bagasse reported the alcohol yields up to 48% (w/w of reducing sugars).

Hernandez-Salas et al. (2009) pretreated the whole SCB and different fractions of it using dilute acid (HCl) and alkaline (NaOH). Selected hydrolysates were fermented with a non-recombinant strain of *S. cerevisiae* and the maximum alcohol yield by fermentation (32.6%) was obtained from the hydrolysates of sugarcane depithed bagasse.

Therefore, using commercial cellulase enzymes with high enzymatic activity, suitable pretreatment of biomass and application of fungi with broad ability in various sugars fermentation can increase ethanol production.

Conclusions

This study showed that SBP was a suitable substrate for cellulase production by *T. reesei*. With the optimal culture condition and SBP as a suitable source of cellulose content, filter paper activity achived 0.46 IU/ml, which is much higher than the results, obtained using other lignocellulosics residue, although, wild type of *T. reesei* with low cellulase activity was used in this study. Further work is needed to find out how to enhance cellulase activity. In addition, this study indicated that SBP (especially pretreated SBP) had the potential for development as an economical alternative energy source because of the easy conversion to fermentable sugars. Additionally, SBP is an inexpensive by-product from the sugar beet industry, therefore, the cost of ethanol production decreases. Finally, ethanol production was higher in pretreated SBP than from non-treated SBP. These results suggest that SBP is a potential biomass resource for cellulase and bioethanol production.

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