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Saccharification of Sugarcane Bagasse by Enzymatic Treatment for bioethanol production

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ABSTRACT

Aims: The escalating demands for traditional fossil fuels with unsecured deliverance and issues of climate change compel the researchers to develop alternative fuels like bioethanol. This study examines the prospect of biofuel production from high carbohydrate containing lignocellulosic material, e.g. sugarcane bagasse through biological means.
Methodology and Results: Cellulolytic enzymes were collected from the culture filtrate of thermotolerant *Trichoderma viride* grown on variously pre-treated sugarcane bagasse. CMCase and FPase enzyme activities were determined as a measure of suitable substrate pre-treatment and optimum condition for cellulolytic enzyme production. The highest CMCase and FPase activity was found to be 1.217 U/ml and 0.109 U/ml respectively under the production conditions of 200 rpm, pH 4.0 and 50 °C using steamed NaOH treated bagasse as substrate. SEM was carried out to compare and confirm the activity of cellulolytic enzymes on sugarcane bagasse. Saccharification of pre-treated bagasse was carried out with crude enzymes together using a two-factor experimental design. Under optimized conditions the pre-treated bagasse was saccharified up to 42.7 % in 24 h. The hydrolysate was concentrated by heating to suitable concentration and then used for fermentation by an indigenous isolate of *Saccharomyces cerevisiae*. With 50 and 80 % brix containing liquor the concentration of alcohol was 0.579 % and 1.15 % respectively.
Conclusion, significance and impact of study: This is the first report in Bangladesh for the production of cellulosic ethanol using local isolates. Though the rate of alcohol production was very low, a great impetus in this field can maximize the production thereby meet the demand for fuel in future.

Keywords: Bioethanol, Bagasse, CMCase, FPase, Scanning electron microscopy (SEM), Degree of saccharification (D_oS), *Trichoderma viride*, *Saccharomyces cerevisiae*

INTRODUCTION

Bioethanol can be planed from raw materials like starch, saccharine or cellulosics (Hill *et al*., 2006; Solomon *et. al.,* 2007). However the high conversion rate (1.5–30 %) of sunlight into biomass (Alexander, 1985) linked to high energy content (1.6 barrels of fuel oil energy per ton of sugarcane baggasse) have made sugarcane bagasse a suitable substrate of choice for ethanol production. Bangladesh produces more than 150,000 tons of sugar, 100,000 tons of molasses and 800,000 tons of bagasse per year (Banglapedia, 2006). Rapid growth of the sugarcane plants, climate and soil property making the material easily available and annually renewable. So it can act as a cheap substrate with constant supply as a substrate for bioconversion to fuel ethanol.

Cellulose, hemicellulose and lignin are the key biomass polymers found in sugarcane bagasse consisting about 50, 27.5 and 9.8% respectively. The rest 11.3 % are cell contents of sugarcane (Kewalramani *et. al.,* 1988). Structural features of cellulose such as the degree of crystallinity, the degree of polymerization, the degree of water swelling, and the surface area, limit accessibility of substrate to enzyme and have been demonstrated (Fan *et. al*., 1982) to affect the rate of enzymatic hydrolysis of cellulose. Pretreatment methods, which disrupt the highly-ordered cellulose structure and the lignin-carbohydrate complex, remove lignin, and increase the surface area accessible to enzymes, promote the hydrolysis, and increase the rate and extent of hydrolysis of cellulose in various lignocellulosic residues (Fan *et. al*., 1982). The enzymatic hydrolysis of cellulosic materials correlates with the level of cellulose crystallinity (Weimer and Weston, 1985) and complete enzymatic hydrolysis of the polysaccharides of lignocelluloses requires a concerted action of a complex array of hydrolases including cellulase, xylanase, pectinase, and other side-group cleavage enzymes (Broda *et. al.,* 1996).

Microbial cellulases are the most economic and available sources, because microorganisms can grow on inexpensive media such as agriculture and food industries by-products. The genus *Trichoderma,* filamentous ascomycetes are widely used in industrial applications because of high secretory capacity and inducible promoting characteristics (Mach and Zeilinger, 2003). *Saccharomyces cerevisiae* is well known yeast for its

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fermentation capacity (Charoenchai, 1998) and hence can be employed for alcohol production from various sugar containing materials. The conversion of lignocellulosic material into biofuels is complicated and not yet a commercial business, but the trends towards commercialization are evident (Hamelinck *et al*., 2003). The aim of this study was to consign an approach in Bangladesh in the production of alcohol from cheap lignocellulosic substrate like sugarcane bagasse, taking *Trichoderma viride* into account as a source of cellulolytic enzymes and *Saccharomyces cerevisiae* as an appliance for alcohol fermentation from saccharified liquor extracted from enzyme treated bagasse.

MATERIALS AND METHODS

Microorganisms

Trichoderma viride isolated from decomposing lignocellulosic materials was used for cellulolytic enzyme production using basal medium described by Copa-Patiño *et al*. (1993). The medium contained (per liter) 0.6 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.4 g K_2HPO_4, 0.74 g $CaCl_2.2H_2O$, 2.32 g $NH_4H_2PO_4$, and 1.0 g yeast extract, and 7.0 ml of trace salts solution (per 100 ml, 200 mg $CoCl.7H_2O$, 500 mg $FeSO_4.7H_2O$, 160 mg $MnSO_4.H_2O$, and 140 mg $ZnSO_4.7H_2O$) and Sugarcane bagasse 2.0 % (w/v) as a carbon source.

Pretreatment of substrate

A number of physical and chemical pretreatments of sugarcane bagasse were performed in an effort to improve the substrates for the extracellular enzyme production as well as for enzymatic saccharifications. The physical treatments included chopping, milling and boiling, while the chemical treatments were done using alkali, acid, peroxide or solvent. In later case with alkali treatment milled bagasse was kept at 85 °C for 1 h in 2 % NaOH (solid liquid ratio was 1:4). Acid treatment was done with H_2SO_4 where chopped bagasse was heated at 100 °C for 30 min with 10 % sulphuric acid solution (solid liquid ratio was 1:10) (Detroy *et al.,* 1981; Lynd *et al.*, 1987). For peroxide treatment, chopped bagasse was suspended in 5 % hydrogen peroxide (in 1:10 ratio) and kept in room temperature for 1 h. However for solvent treatment chopped bagasse was suspended in aqueous butanol (50 % v/v) in the presence of a 0.005 % aluminium chloride catalyst and heated at 121 °C for 1 h. In each chemical treatment the solid substrate was washed to neutral pH and dried in the oven at 70 °C until a constant weight. Both treated (NaOH, H_2SO_4, H_2O_2, butanol and boiling) and non-treated chopped bagasse were further passed with steam treatment (121 °C for 15 min in an autoclave). These substrates were used for enzyme production.

Fungal inoculum preparation

A suspension of 10^6–10^7 spores per ml (as determined by Neubauer counting chamber) was prepared by scraping conidiospores from agar slants into sterile saline water. Two milliliter of the suspension was pipetted into 50 ml of inoculum growth medium (Zhang and WeiMin, 2008) and incubated in an orbital shaking incubator at 30 °C and 200 rpm. After 36 h growth, the medium was used as the inoculum for enzyme production.

Culture conditions and enzyme production

The standard basal medium used for growth and enzyme production was described by Copa-Patiño *et al*. (1993). The initial pH was adjusted to 5.0. Cultures were conducted in 250 ml-Erlenmeyer flasks containing 100 ml basal medium and 2 % (w/v) sugarcane bagasse (pretreated) as a carbon source. The growth medium was sterilized at 121 °C for 15 min, then inoculated with 5 ml spore suspension (10^6–10^7 spores) and incubated at 30 °C on an orbital shaking incubator at 200 rpm for 3 days. After cultivation the culture filtrate was centrifuged at 10,000 rpm for 15 min using lab centrifuge. The clear supernatant fluid was used directly for the determination of the enzyme activities.

Chemical analysis

Reducing sugar in the wart and ferments was determined by dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose as standard. Both pretreated and native sugarcane bagasse was analyzed for their total carbohydrate content using a two-stage hydrolysis method essentially according to Wilke *et al.* (1981).

Enzyme assays

Filter paper activity (FPase) was determined essentially according to the IUPAC instructions (Ghose, 1987), and the liberated reducing sugars were estimated by the DNS method (Miller, 1959). FPase activity corresponds to 1 µ*M* of reducing sugars as glucose equivalents librated per min under the assay conditions. Carboxymethycellulase (CMCase) activity was estimated essentially according to IUPAC instructions (Ghose, 1987) using 1 % solution of carboxymethyl cellulose-sodium salt (in 0.05 M citrate buffer, pH 5.0) as substrate. One CMCase unit is the amount of enzyme necessary to produce 1 µM reducing sugar as glucose equivalents per min under the standard assay conditions. The assay was also carried out for different time intervals to find out the optimum time for enzyme activity.

Saccharification of sugarcane bagasse

Saccharificaiton experiment was performed in 100 ml Erlenmayer flasks with 200 mg (2 % dry wt) substrate and 10 ml enzyme solution in citrate buffer (0.05 M, pH 5.0). The reaction mixture was sealed and incubated at 50 °C under continuous agitation (150 rpm) for 48 h. Hydrolysates were transferred in screw-capped tubes, heated in a boiling water bath for 15 min and centrifuged to remove solid particles. The supernatant were used for analysis of released sugars. Saccharifcation study was

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carried out at various temperatures and also at various pHs to find out the best hydrolysis preparation. Sugar released was estimated by DNS method (Miller, 1959) and Degree of saccharifcation (D_oS) was calculated essentially described by Vallander & Eriksson (Vallander and Eriksson, 1985) using the following formula:

$$D_oS = \frac{c \times v \times f_1}{m \times f_2} \times 100$$

Where c is the sugar concentration in the hydrolysate estimated as total reducing sugars, in mg/ml; v is the liquid volume (ml) of the hydrolysates, f_1 is the factor (0.90 for hexoses) used to convert monosaccharide to polysaccharide due to water uptake during hydrolysis; m is the amount of initial substrate (mg) dry weight, and f_2 is the factor for the carbohydrate content of the substrate (total carbohydrate, mg/total substrate, mg).

Statistical optimization of saccharification conditions

The optimum temperature and pH for saccharification of sugarcane bagasse was finally determined using a central composite design. A two-factor experimental design was used; each factor was studied at three levels. The optima were calculated according to Retzlaff *et al*. (1975).

Sachharification kinetic studies

The saccharification experiments were carried out for 48 h and reducing sugar was estimated at various time intervals. Initially the reaction mixture contained 2 % of substrate that was incubated under optimized conditions. The degree of saccharification was plotted against time to evaluate time-dependent enzymatic hydrolysis of sugarcane bagasse.

Processing of the saccharified liquor for fermentation

Prior to fermentation the saccharified liquor was separated from the solid substrate. A refractometer was used to determine the percentage of sugar in that liquor. The liquid was boiled to reduce the volume until the sugar concentration reaches 80 % in the refractometer scale. The liquid was filtered to remove the impurities and used in the fermentation medium as substrate for ethanol production by yeast isolate.

Preparation of yeast inoculum for ethanol fermentation

One ml of yeast cell suspension (about 10^8 viable cells, determined using a Neubauer counting chamber) was inoculated to 100 ml MYGP broth in 500 ml-conical flask and incubated at 30 °C on a rotary shaker for 24 h. The standard concentration of the inoculum (1.5 x 10^8 cells/ml) was prepared by the properly diluting the suspension with sterile distilled water.

Fermentation process

Ethanol production was carried out in batch culture using various concentrations of the clarified liquor obtained from sugarcane bagasse. Malt extract (0.3 %), yeast extract (0.3 %) and peptone (0.5 %) was added to the liquor and final pH was set to 5.5. One milliliter of yeast inoculum was inoculated to 100 ml of the broth in 250 ml conical flask and was incubated at 30 °C for 72 h.

Estimation of ethanol

Ethanol produced in the fermentation medium was estimated by potassium dichromate oxidation method (Kumnuanta, 1983). Potassium dichromate (33.882 g/l), ferrous ammonium sulphate (135.5 g/l) and diphenylamine (0.5 g/100 ml concentrated H_2SO_4) solutions were used as reagent for estimation of ethanol concentration. The fermented sample was diluted ten times with distilled water. Ten millilitre of the diluted sample was distilled against $K_2Cr_2O_7$ (10 ml) containing concentrated H_2SO_4 (5-6 ml). Then distilled was titrated against freshly prepared ferrous ammonium sulphate solution with diphenylamine as an indicator. Appearance of green colour indicated the end point of the titration. Burette reading (amount ferrous ammonium sulphate) was recorded to calculate the amount (in percentage) of ethanol present in the sample.

RESULTS AND DISCUSSION

The development of a remunerative process to convert low value biomass like sugarcane bagasse to high value product like ethanol requires several key steps, especially cellulase production, be optimized (Eveleigh, 1987). The structural complexity are often easily degraded by xylanases, mannanases etc. which are present in some cellulase preparations, so that their presence may actually lead to increased production of reducing sugars and greater susceptibility of the residual cellulose (Tolan and Finn, 1987). Figure 1 shows various types of hydrolytic enzymes that were found in this experiment by growing *Trichoderma viride* on relevant media.

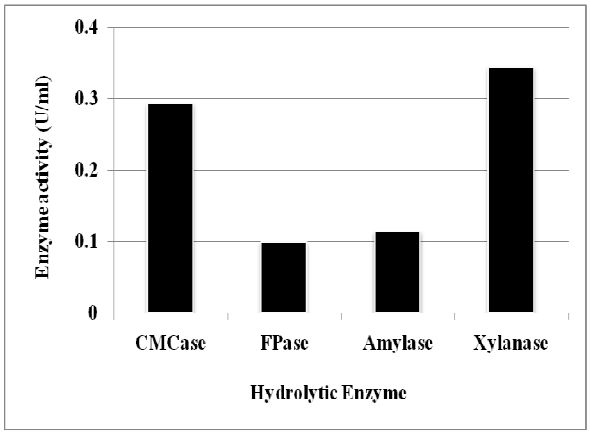

Figure 1: Production of various hydrolytic enzymes by *Trichoderma viride*

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Trichoderma viride was grown on both treated and untreated bagasse as substrate for cellulase enzyme production. With NaOH, H_2SO_4 or H_2O_2 treated bagasse an enhanced fungal attack on the substrate was noticed. NaOH is thought to have some saponification activity on bagasee (Fan *et al*., 1981), H_2SO_4 removes hemicellulose extensively while oxidizing agent i.e., H_2O_2 causes chemical oxidation of lignin and remove it (Fan *et al*., 1981). Chemical pretreatment with solvents like butanol was found to be inefficient. The accessibility of the chemically pretreated bagasse to the fungal attack was enhanced remarkably by additional steam treatment, as was also reported by Saddler *et al*. (1982). Chemical pretreatment with alkali (caustic soda) appeared to be most effective in enhancing the extent of subsequent enzymatic hydrolysis. Similar findings have been reported for alkali pretreatments of various lignocellulosic substrates (Fan *et al*., 1981). The highest CMCase activity (0.293 U/ml) was obtained from steamed NaOH treated (short piece) bagasse, followed by steamed H_2SO_4 substrate (0.285 U/ml) and steamed H_2O_2 treated substrate (0.283 U/ml). The highest FPase activity (0.099 U/ml) was found from steamed NaOH-treated (short piece) bagasse. It was followed by steamed H_2O_2 substrate (0.089 U/ml FPase) and steamed H_2SO_4 treated substrate (0.084 U/ml FPase). The enzyme activities were almost constant during storage at 4 °C refrigerator upto 2 weeks (Figure 2).

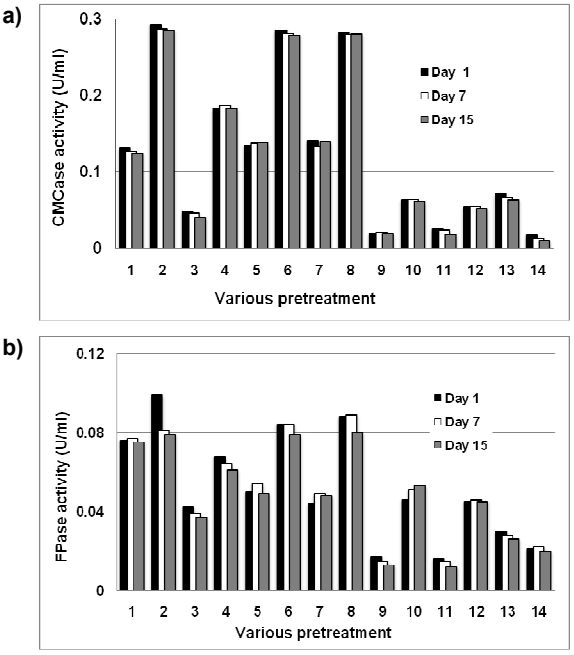

Figure 2: Effect of different pretreatments of sugarcane bagasse on extracellular (a) CMCase, (b) FPase production by *Trichoderma viride* in shake-flask culture (1. NaOH treated short pieces bagasse, 2. NaOH + steam treated short pieces bagasse, 3. NaOH treated ground bagasse, 4. NaOH + steam treated ground bagasse, 5. H_2SO_4 treated short pieces bagasse, 6. H_2SO_4 + steam treated short pieces bagasse, 7. H_2O_2 treated short pieces bagasse, 8. H_2O_2 + steam treated short pieces bagasse, 9. Butanol treated short pieces bagasse, 10. Butanol +steam treated short pieces bagasse, 11. Boiled short pieces bagasse,12. Boil + steam treated short pieces bagasse, 13. Short pieces bagasse, & 14. Steam treated short pieces bagasse)

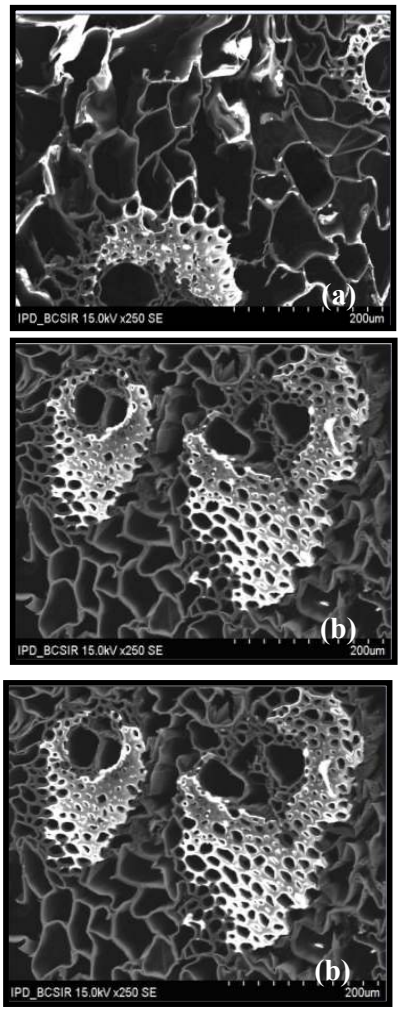

Figure 3: Scanning electron microscopy of sugarcane bagasse cross section- (a) untreated; (b) alkali-treated; (c) enzyme-treated bagasse

However, the cellulase yields of this study by *T. viride* were relatively inferior as compared to experiment of Thomas *et al.* (1981) and Acebal *et al.* (1986). The highest endoglucanase (CMCase) was found to be 1.217 U/ml in this study, while a 10-fold higher activity was reported by Acebal *et al*. (1986) with *T. reesei* QM 9414.

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Again, the FPase activity by *T. viride* in this study was 0.109 U/ml, which was considerably lower than that reported for *T. reesei* QM 9414 (0.34 U/ml) (Thomas *et al*., 1981). Biosynthesis of hydrolytic enzymes on lignocellulosics depends on multiple factors. Saccharification syrups contain not only glucose and cellobiose but also other sugars, like as xylose (Palmer, 1975). Cellulase is moderately inhibited by glucose and strongly inhibited by cellobiose (Mandels *et al*., 1978). For both sugars the extent of inhibition increases with increasing resistance of the cellulose. Cellobiase is competitively inhibited by glucose (Gong *et al*., 1977; Bissett *et al*., 1978).

Scanning Electron Microscopy (SEM) of untreated, alkali-treated and enzyme treated bagasse were taken and compared to find out what actually happens with NaOH treatment and what changes do crude enzyme introduces to sugarcane bagasse. All samples were coated with platinum and magnification of x250 was used. Figure 3(a) shows the electron micrograph of cross section of untreated bagasse. It shows large void and loose packing of the fibres. The wall of untreated fibres was intact. Figure 3(b) shows the SEM of alkali-treated fibre where the cell wall still looks normal, and no rupture or splitting was observed as a resulting of alkali treatment however, packing of the fibres was partially loosened. Figure 3(c) shows the cross section of enzyme treated bagasse where sponge-like structures of the bagasse is evident and the structure of the lignocellulosic biomass surface appears to be more fragile.

The extent of hydrolysis depended on the reaction temperature and maximum hydrolysis was achieved at 50 °C (Figure 4). Above and below of this temperature the degree of saccharification (D_oS) was reduced considerably. The D_oS of steamed alkali-treated chopped bagasse by the crude enzyme preparation from *T. viride* was 38.8 %, while the untreated bagasse was saccharified to 25.6 %. With the increasing temperature the D_oS of untreated and alkali-treated bagasse were reduced and at 70 °C the values were only 6 % and 8 % respectively. Increased susceptibility of lignocellulosics by alkali pretreatment might be due to removal of lignin seal and swelling of the substrates in an alkaline condition, which might increase accessibility of the substrate to enzymatic attack (Fan *et al.,* 1981).

The pretreated substrate was substantially hydrolyzed over a pH range between 4.0 and 6.0. A two-factor central composite design was employed to confirm the optimum pH and temperature for hydrolysis of treated bagasse by the enzyme of *T. viride*. Highest saccarification value was observed at pH 5.0 and 50 °C that corresponded to the D_oS value of 42.75 % (Figure 5).

Time course of enzymatic saccharification of alkali treated bagasse showed rapid initial increase of reducing sugar concentration (up to 8 h) and the rate of this increase was substantially reduced at later stages. The substrate was saccharified up to about 24.0 % within 8 h hydrolysis time and after 24 h, the degree of saccharification was increased to about 33.5 %. Further increase of the hydrolysis time from 24 h to 48 h, the subsequent increase of the extent of hydrolysis was only about 9.0 % (Figure 6).

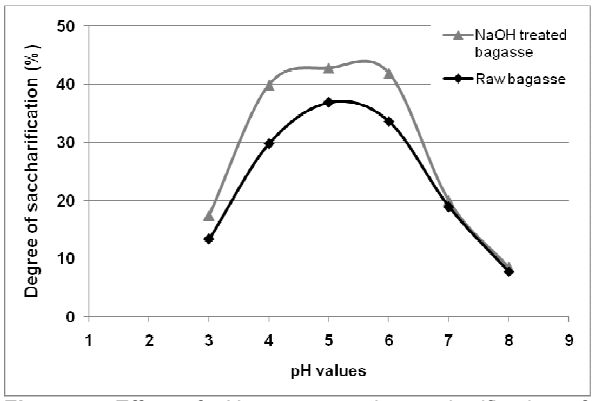

Figure 5: Effect of pH on enzymatic saccharification of sugarcane bagasse

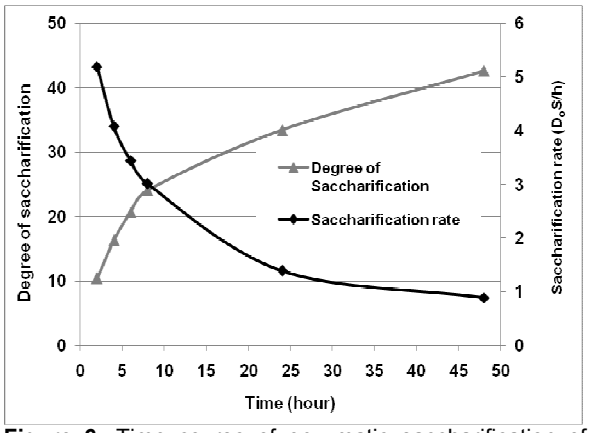

Figure 6: Time course of enzymatic saccharification of alkali pretreated bagasse by crude enzyme preparation of *Trichoderma viride*

The yeast *Saccharomyces* sp. is the choice of organisms due to its high ethanol production efficiency than others (Jones *et al*., 1981). The initial sugar concentration of the fermentation medium is an important factor since too much sugar results in lower ethanol production (Jones *et al*., 1981; Beuchat, 1983). The organism utilizes hexose sugars; however it is unable to utilize xylose (Jeffries, 1990). The yeast isolate used in this experiment was selected for higher alcohol productivity on pure glucose and molasses. But the organism was not very efficient in utilizing the saccharified liquor extracted from enzymatic hydrolysis of bagasse. With 80 % and 50 % brix containing liquor the concentration of alcohol was 1.15 % (v/v) and 0.579 % (v/v) respectively; those were very low as compared to the amount found by Tang *et al.* (2008)

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with *Saccharomyces cereviseae* strain KF-7. Since the saccharification study was carried out by enzymes extracted from fungal growth medium and later on the saccharified liquor was used for fermentation, some fungal proteins might be left on the preparation affecting the growth of fermenting yeast negatively. Again, though *S. cerevisiae* is one of the most prominent ethanol producing organisms using hexose sugars, it is unable to utilize xylose (Jeffries, 1990). As in this experiment a high xylanase activity was found in the crude enzyme extract, it can be said that a substantial amount of xylose might be present in the saccharified liquor which affected the rate of fermentation.

In recent years, engineered yeasts have been described efficiently fermenting xylose (Ohgre *et al.*, 2006; Brat *et al.*, 2009) and arabinose (Becker and Boles, 2003) and even both together (Karhumaa *et al.*, 2006). Besides *Saccharomyces cerevisiae*, microorganisms such as *Zymomonas mobilis* and *Escherichia coli* have been targeted through metabolic engineering for cellulosic ethanol production.

CONCLUSION

The choice of lignocellulosic materials like sugarcane bagasse for bioethanol production has become a recent interest (Sun and Cheng, 2002) however, in Bangladesh this is the first reported experimental work regarding cellulosic ethanol. This experiment was designed to evaluate the possibility of alcohol production from sugarcane bagasse using local fungal and yeast isolates. Our study suggests that *T. viride* is potentially useful for the production of various cellulolytic enzymes. Though, the amount of alcohol obtained was not satisfactory with the isolated *Saccharomyces* sp., additional work on purification of enzyme from hydrolysis media and separation of sugars from saccharified liqour for the preparation of fermentation media can enhance the production of alcohol. Further experiment may be designed using other cellulosic substances like rice straw, wheat straw, or switch grass as a substrate for bioethanol production. Besides, study can be planed to isolate yeast with improved fermentation capacity. This will ultimately uphold the promise of producing adequate amount of alcohol from given amount of substrate industrially thereby meet the demand for fuel in near future.

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