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Saccharification of Lignocellulosic Materials by the Cellulases of *Bacillus subtilis*

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ABSTRACT

Cellulases produced by *Bacillus (B.) subtilis* were used for the saccharification of wheat straw, rice straw and bagasse. Pretreatment of these substrates with 2% NaOH was found to be more effective for increasing the saccharification. The saccharification rates of 33.0, 25.5 and 35.5% were obtained with 2% NaOH pretreated wheat straw, rice straw and bagasse, respectively. The saccharification of wheat straw was 33, 26 and 16.9% after 20 h at 50ºC when 4, 6 and 10% substrate was used, respectively. The per cent conversion reduced with increasing substrate concentration. Same trend in the hydrolysis rates of pretreated rice straw and bagasse were observed when used at increased concentrations. The per cent saccharification of these substrates decreased when the concentration was increased to 10%. It was observed that the per cent saccharification of these substrates increased with increasing enzyme concentration.

Key Words: Saccharification; Cellulase; *Bacillus subtilis*

INTRODUCTION

Structure of lignocellulosic (LC) in the cell wall resembles that of reinforced concrete pillar with cellulose fibres being the metal rods and lignin the matrix cement. Biodegradation of native LC is very slow and yields not more than 20% of reducing sugars. To increase the susceptibility of cellulosic materials, structural modifications by means of various schemes are essentially based on the economics of the process. These pretreatments are broadly classified as physical, chemical and biological treatments according to their principle mode of action on the substrate. Detroy *et al*. (1980) used commercial cellulase preparation @ 1.0 IU g^{-1} substrate, which hydrolysed EDA treated wheat straw (4%) to 70% glucose yield. Further modified by disc-milling EDA treatment yielded 83% of glucose. Chahal (1985) using cellulase produced in solid state fermentation by a mutant QMY-1 developed from *T. reesei* QM9414 resulted in 99.75 g sugars L^{-1} from 100 g of delignified wheat straw. Very little cellobiose accumulated in the hydrolysate up to 20 h; whereas, its level further decreased after 96 h. The hydrolysates analysed by HPLC contained in g L^{-1}; cellobiose 3, glucose 68, xylose 26.7 and arabinose 1.7. McCrae *et al*. (1989) found enzyme productivity values higher in the mutant strain of *P. pinophilum* (NTG 111/6) than those normally recorded for *T. reesei* mutant C-30. The cellulase (7 FP U mL^{-1}) was very effective in hydrolysing 10% solka floc to 8.7% sugar solution. Dekker and Wallis (1983) increased the saccharification of 10% bagasse from 50 to 80% in 24 h when ß-glucosidase from *A. niger* was added to the *T. reesei* cellulase (20 FP U g^{-1} substrate) in the ratio (1:1) of FPU to ß-glucosidase. This paper reports the saccharification of LC materials by the cellulases produced by locally isolated *Bacillus subtilis.*

MATERIALS AND METHODS

Enzyme production. *Bacillus subtilis* was grown in minimal medium containing cellobiose as carbon source as described by Akhtar *et al*. (1996). The fermentation was carried out at 50ºC for 10 h and culture supernatant was used as extracellular enzyme source.

Enzyme assay. Carboxymethyl cellulase activity (CMCase) was determined by incubating 0.5 mL of diluted culture supernatant with 0.5 mL of 1% CMC in 0.05M mcllvaine buffer pH 7.0 at 60ºC for 10 min. The reducing sugars thus released were estimated as glucose equivalent by the method described by Ghose (1987). One unit of enzyme activity was defined as the amount of enzyme that release one micro mole of reducing sugars equivalent to glucose per minute under the assay conditions.

Pre–treatment of LC substrates. LC substrates like wheat straw, rice straw and bagasse were milled to 20 mesh size. Pretreatment of these LC substrates were carried out with different concentrations of NaOH from 1 to 4% in 1:20 w/v ratio. Pretreatment were carried out at room temperature for 4 h. Similarly, pretreatment with NaOH was also carried out by autoclaving at 121ºC for 1 h. After pretreatment, the materials were washed to neutralization and dried at 50ºC in an oven to obtain a constant weight.

Effect of incubation time on hydrolysis The saccharification was carried out in screw caped bottles containing 4% (w/v) substrate in 0.05 M Mcllvaine buffer (pH 7) with 20U of enzyme activity. The reaction mixture was incubated at 50ºC for various time periods in orbital shaker. At specific time interval, aliquots (0.5 mL) were removed and the amount of reducing sugars was estimated up to 20 h and per cent saccharification was determined following the method of Mandels and Sternberg (1976).

Effect of substrate concentration on hydrolysis. Reaction mixture containing 20U CMCase activity in 25 mL of 0.05 M Mcllvaine buffer (pH 7.0) and different amounts of wheat straw, rice straw and bagasse (1.0, 1.5 and 2.5 g) were added and incubated at 50ºC for different time periods. Aliquots (0.5 mL) were withdrawn, diluted and reducing sugars were determined by DNS method (Ghose, 1987).

Effect of enzyme concentration on hydrolysis. One gram of pretreated substrates in 25 mL of Mcllvaine buffer (pH 7.0) were incubated with different enzyme concentrations (10-40U) at 50ºC for different time periods. Aliquots (0.5 mL) were removed and analysed for total reducing sugars released by DNS method.

RESULTS AND DISCUSSION

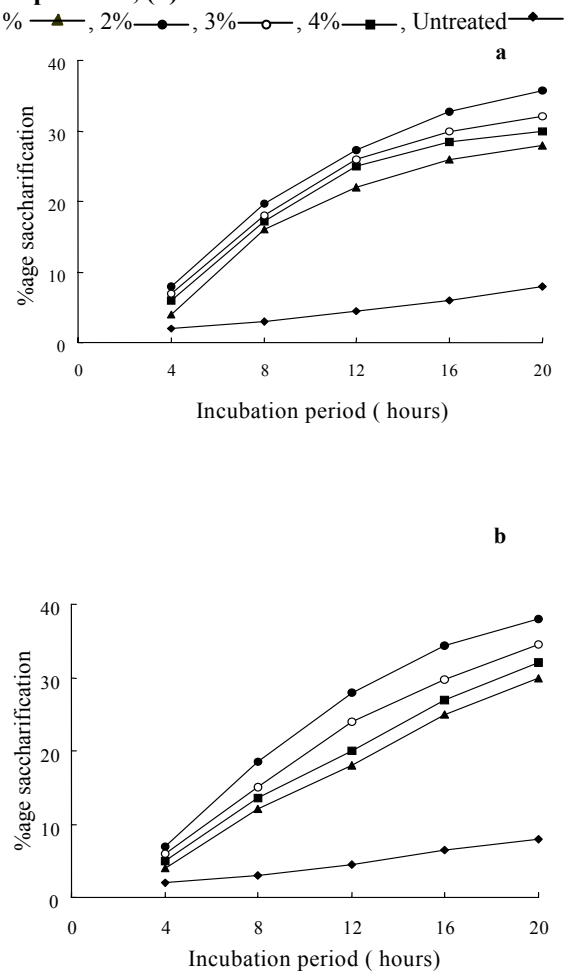
Effect of pre–treatment on saccharification. Wheat straw, rice straw and bagasse were separately pretreated with (1-4% w/v) NaOH for 4 h. Results of these pretreatments on wheat straw using cellulases of *B. subtilis* have been shown in Fig. 1a. It can be seen that saccharification level has been increased to 33 in comparison to 4% saccharification of untreated wheat straw when the substrate was pretreated with 2% NaOH for four hours at room temperature. However, when the substrate treated with 1, 3 and 4% NaOH was used, there was a slight decrease in saccharification rate under some conditions. It was noted that 35% saccharification was obtained when wheat straw autoclaved with 2% NaOH at 15 psi for 1 h. However, per cent saccharification decreased to 20, 25 and 28% when the substrate was autoclaved with 1, 3 and 4% NaOH, respectively (Fig. 1b).

Table I. Effect of substrate concentration on the saccharification of pretreated LC materials

Substrate %)	Reducing sugars (mg mL^{-1})	Saccharification(%)
Wheat straw		
4	14.4	33
6	17.4	26
10	18.8	16.9
Rice straw		
4	11.3	25.5
6	16.4	20.9
10	18.5	17.4
Bagasse		
4	15.7	35.5
6	19.8	29.7
10	20.8	18.7

The effect of pretreatment on rice straw and bagasse with different NaOH concentrations (1-4%) at room temperature and autoclaving at 121ºC for one hour are shown in Fig. 2 and 3. It can be seen that the trend in the saccharification rates of these substrates is similar to the trend in the wheat straw. The observed saccharification rates of rice straw and bagasse pretreated with 2% NaOH

Fig. 1. Effect of NaOH concentration on pretreatment of wheat straw by the cellulases of *Bacillus subtilis* (a) Pretreated at room temperature, (b) Pretreated at 121ºC

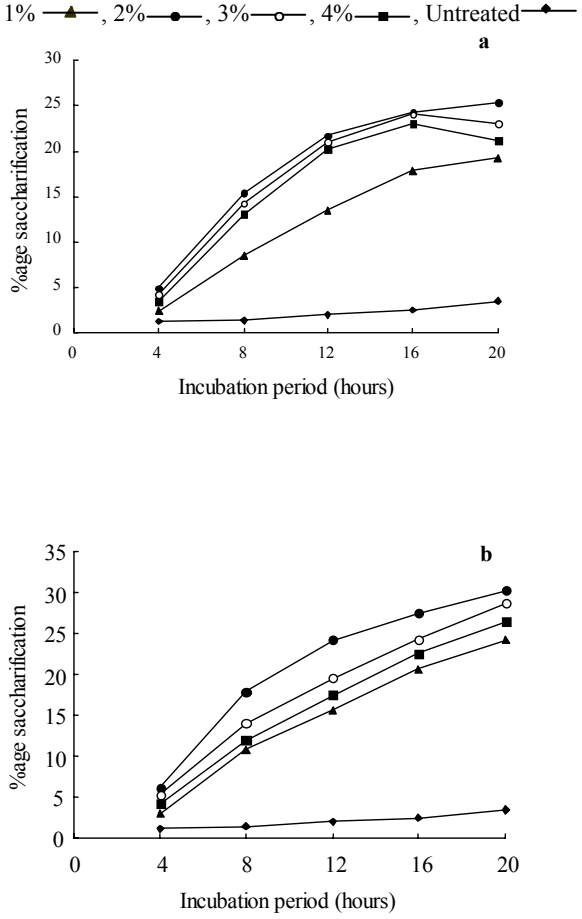


are found to be 25.5 and 35.5, respectively. It has been observed that although enzymatic saccharification was higher for autoclaved fibers but soaking of rice straw with 2% NaOH at room temperature for 4 h was more favourable with regard to carbohydrate recovery.

Table II. Effect of enzyme concentration on the hydrolysis of LC substrates

Substrate (%)	Cellulase activity (U)	Reducing sugars mg ml^{-1}	Saccharification (%)
Wheat straw	10	8.7	19.6
	15	10.4	23.4
	20	14.6	33.0
	40	15.6	35.1
Rice straw	10	8.5	16.3
	15	10.3	21.3
	20	11.3	25.5
	40	15.6	27.2
Bagasse	10	9.8	20.0
	15	12.9	29.0
	20	15.7	35.5
	40	18.5	37.3

Fig. 2. Effect of NaOH concentration on pretreatment of rice straw by the cellulases of *Bacillus subtilis* (a) Pretreated at room temperature, (b) Pretreated at 121ºC



It is concluded that alkali treatment at room temperature is simple and most appropriate for enzymic saccharification of LC substrate to remove acetyl and phenolic substances facilitating splitting of linkages between carbohydrates and lignin components.

Effect of substrate concentration on saccharification. The effect of different amounts of pretreated substrates on saccharification has been studied by adding different concentrations of the substrates (4-10%) with fixed amount of enzyme. It is evident from Table I that per cent saccharification of wheat straw increased to 33% after 20 h with 4% pretreated wheat straw. It is concluded that the per cent hydrolysis increased up to 20 h of incubation time and after that no increase was noted irrespective of the substrate concentration. The amount of total reducing sugar produced (Table I), however, increased with increasing substrate concentration and 18.8 mg mL^{-1} sugars were released when 10% wheat straw was used in the reaction mixture. The enzymatic hydrolysis of alkali treated rice straw with using at a concentration of 4% initially proceeded rapidly and increased to 25.5% after 20 h of incubation at 50ºC.

Similar decreasing trend in hydrolysis of alkali treated rice straw was observed when used at a concentration of 6 and 10% (Table I). The reducing sugar increased from 11.3 to 18.5 mg mL^{-1} when substrate concentration increased from 4 to 10%. Similarly, the effect of substrate concentration on saccharification of bagasse was investigated using different concentration (4 to 10%) in the reaction mixture; 4.7 fold increase in saccharification was observed when hydrolysis of 4% bagasse was carried out at 50ºC. Low saccharification at 3 and 4% NaOH concentration indicated that some carbohydartes may be solubilized during pretreatment. The reduction in saccharification may not be due to the lack of the substrate and can be due to the other reason like transfer action of insoluble cellulose into less accessible form. However, saccharification of the substrate was observed with autoclaving in the presence of 2% NaOH for one hour at 121ºC. Similar results have been reported earlier indicating that 0.1g NaOH g^{-1} wheat straw at room temperature delignities to a level of 8.6% increasing the accessibility to four times (Ghorpuray *et al.,* 1983; Latif & Malik, 1988).

Effect of enzyme concentration on saccharification. The result of the effect of cellulase concentration on the degree of hydrolysis of alkali treated wheat straw, presented in Table II, shows an increase in saccharification rates of substrate with increasing cellulase concentration. It was found that increase in enzyme concentration from 10 to 20U in the reaction mixture has resulted in increase of 20 to 33% after 20 h of incubation. However, when enzyme concentration was doubled (from 20 to 40U), there was a little increase in the hydrolysis rate. Similarly, in the case of alkali treated rice straw 1.3 fold increase in the saccharification was observed when enzyme concentration was increased from 10 to 20 U in the reaction mixture. However, further doubling the enzyme concentrations (20 to 40U), the increase in hydrolysis rate was not so significant and this may be due to hydrodynamic instability, improper mixing and suspension of slurry as reported by Lee *et al.* (1982, 1983). In a similar way, the effect of enzyme concentration on the saccharification rate of bagasse was studied and it was found that when enzyme concentration was increased from 10 to 20U, the saccharification rate increased from 20 to 35%. Further increase in the enzyme concentration i.e. 20 to 40U did not give significant increase in the hydrolysis rate confirming the findings of Araujo and Souza (1986). Concluding that increase in enzyme concentration may increase by processing cost and suggested the recovery of the enzymes for reuse.

Effect of residual substrate. The accessibility of the residual substrate (wheat straw, rice straw and bagasse), was determined by adding fresh enzyme (20U) to the residual substrates. After 20 h, the solids were separated

Fig. 3. Effect of NaOH concentration on pretreatment of bagasse by the cellulases of *Bacillus subtilis* (a) Pretreated at room temperature, (b) Pretreated at 121ºC

1% —▲—, 2%—●—, 3%—○—, 4%—■—, Untreated—◆—

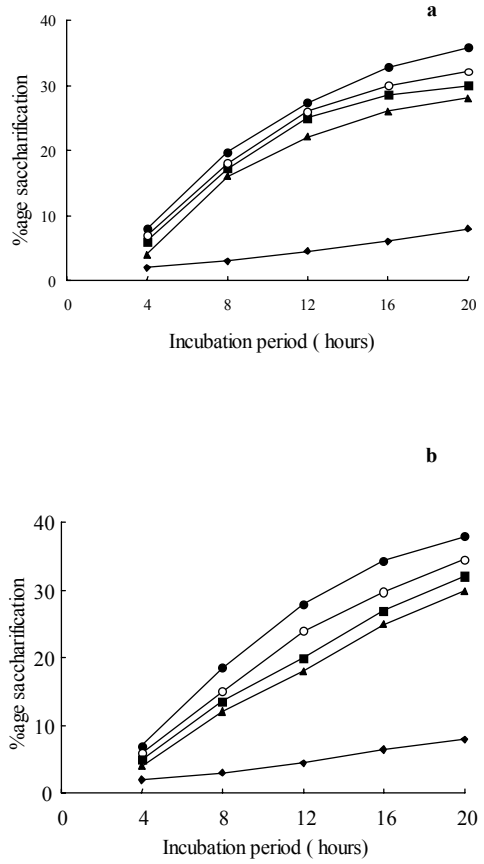
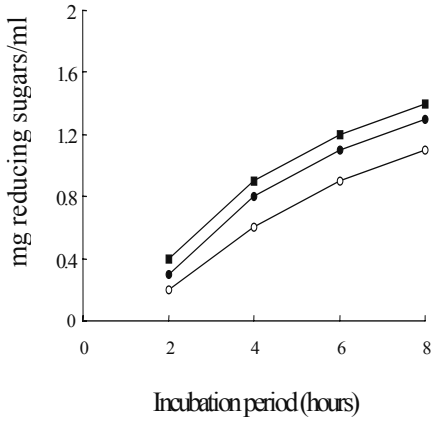


Fig. 4. Effect of cellulase on the partially hydrolysed substrates after 20 hours of reaction. The remaining substrate was washed and added 20 U of fresh cellulase activity

wheat straw —○—, rice straw—●—, bagasse—■—



from the reaction by centrifugation and washed. The residual substrate was resuspended in 25 mL Mcllvaine buffer pH 7.0 containing 20U of fresh enzyme and incubated for further 4 h (Fig. 4). It was observed that there was no appreciable increase in hydrolysis of the substrate indicating that the accessible cellulose fractions have been exhausted in the first hydrolysis step. These findings support the work of Howell (1978) who suggested that decline in the hydrolysis rate in the later part of sacchrification is due to the deactivation of enzyme by the formation of enzyme substrate complex.

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