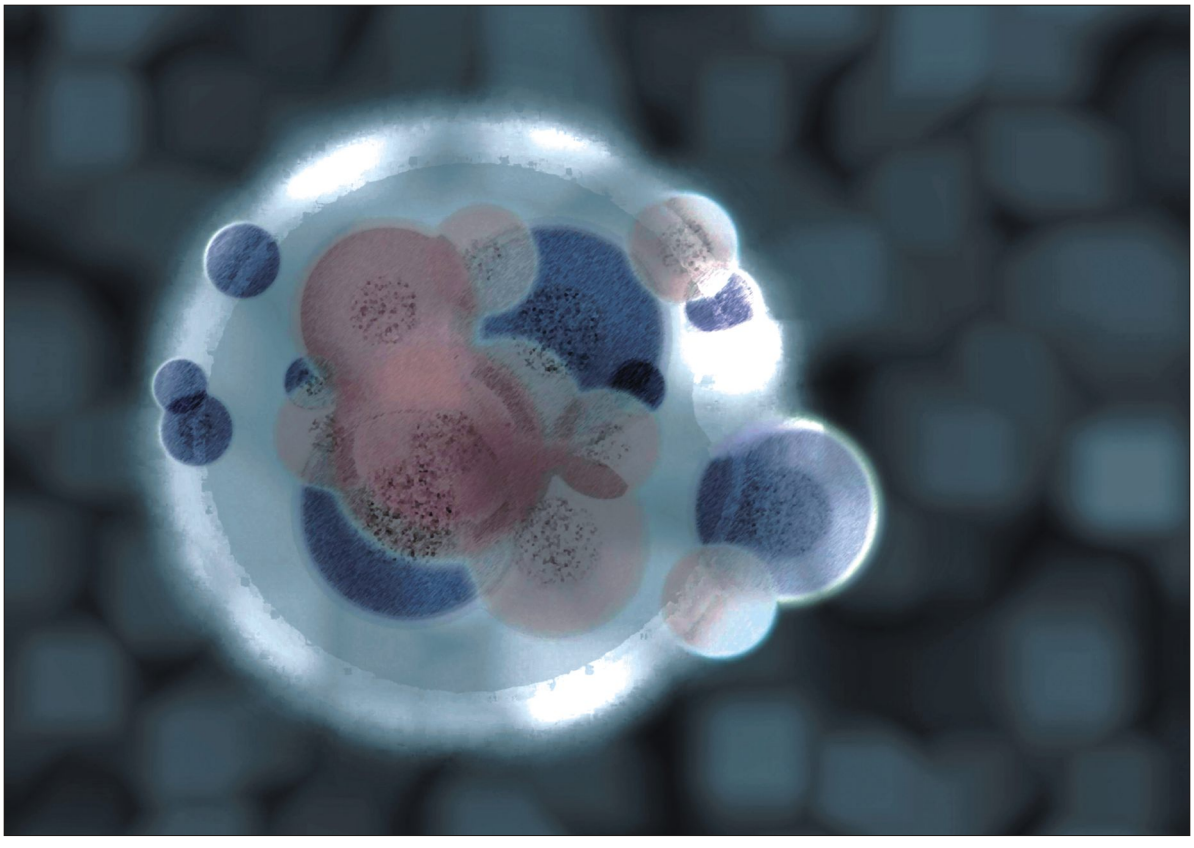

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Enzymatic Saccharification of Lignocellulosic Materials by the Xylanase of *Bacillus subtilis*

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Abstract: Pretreatment of rice straw, wheat straw and bagasse with 2% NaOH as compared to 2% H_3PO_4 was found to be more effective for increasing the saccharification of these substrates. 30.3, 28.0, 28.0% saccharification rates were obtained with 2% NaOH pretreated wheat straw, rice straw and bagasse, respectively. The percent hydrolysis of wheat straw was 30.0, 25.3 and 21.0 after 10 hours at 50°C when 4, 6 and 8% substrate was used, respectively. The percent conversion was reduced with increasing substrate concentration. Same trend in the results was seen when pretreated rice straw and bagasse were used at the increased concentratios, i.e, 4, 6 and 8%. Increasing the enzyme concentration from 10-40U in the reaction mixture containing wheat straw as a carbon source resulted in 16.5 to 37.5% increase in percent saccharification. Percentage saccharification of alkali treated rice straw and bagasse increased from 15.1 to 28.0 and 14.8 to 28.2%, respectively when amount of the enzyme was increased from 10U to 20U. On further increasing the enzyme concentration upto 40U, the degree of hydrolysis of rice straw and bagasse increased from 28.0 to 36.0 and 28.2 to 35.5%, respectively.

Key words: Saccharification, xylanase, *B. subtilis*

Introduction

Enzymatic hydrolysis is a preferable method for the conversion of lignocellulosic materials to sugars which could be used as a source of food, fuel and chemicals (John and Schmidt, 1988; Gibson and McCleary, 1987). Advantages favouring the enzymatic treatment over acid hydrolysis include less capital expense because acid resistant materials are not required, and also yields are higher with lesser impurities. The prospects seem good that for developing an enzymatic saccharification process of hemicellulose and the sugars thus obtained would find an eager market.

Transformation of hemicellulose into simple sugars depends largely on the physical and chemical pretreatment of lignocellulosic materials. The protective function of lignin consists both in a mechanical cover of cell walls and fibrillar bundles and in chemical binding to carbohydrates (Dekker, 1983). The initial mechanical grinding of lignocellulosic substrates causing an increase of surface area followed by further treatment with sodium hydroxide or phosphoric acid, etc., leads to merceration, causing a loosening of compact fibrous structure and swelling of lignin by breakdown of bonds with carbohydrates. So it is obvious that chemical and biological delignification would be required to achieve yields approaching commercial viability. McCarthy *et al*. (1985) reported increase in the amount of reducing sugars after mechanical pretreatment of wheat straw. About 20% of the xylose residues from corn shoot cell walls and 10% of those from bean shoot cell walls were solubilised by the xylanase purified from *T. pseudokoningii* (Baker *et al.,* 1977).

Here we report the saccharification of lignocellulosic substrates with the xylanase produced by locally isolated *Bacillus subtils.*

Materials and Methods

Enzyme production: *B. subtilis* was isolated from wheat straw compost and cultivated in medium having the composition in g/100 ml: ammonium sulphate 0.1, magnesium sulphate 0.02, dipotassium hydrogen phosphate 0.05, potassium dihydrogen phosphate 0.05, calcium chloride 0.01, yeast extract 0.2. Birchwood xylan, 0.5% (Sigma Chemical Co. USA) was used as a carbon source.

The fermentation medium containing a single colony of *B. subtilis* was incubated in a Gallenkamp Incubator Shaker at 50°C and 180 rpm for 10 hours and culture supernatant was used as a extracellular enzyme source.

Xylanase assay: To determine the xylanase activity, 0.5 ml of an appropriately diluted culture supernatant with 0.5 ml of 1% solubilised birchwood xylan (Sigma Chemical Co. USA) in 0.05M McIlvaine buffer, pH 6.0, was incubated at 60°C for 10 minutes. The reducing sugars liberated were estimated as xylose equivalents by DNS method (Ghose, 1987). One unit of the enzyme activity is defined as the amount of enzyme that released one micromole of reducing sugars equivalent to xylose per minute under the assay conditions.

Pretreatment of lignocellulosic materials: The substrates i.e. wheat straw, rice straw and bagasse were pretreated separately with 2% NaOH and H_3PO_4 in the ratio of 1:20 (w/v) for different periods (I, 2 and 4 hours) at room temperature. The treated samples were thoroughly washed with water in order to remove alkali or acid and then dried at 50°C in an oven to obtain a constant weight.

Saccharification of pretreated substrates: The reaction mixtures contained 1.0 g pretreated lignocellulosic substrates in 25 ml 0.05 M McIlvaine buffer (pH 6.0) and 20 U of xylanase activity. They were incubated at 50°C for 10 hours in screw capped bottles. Aliquots (0.5 ml) were removed and assayed for total reducing sugars released and percent saccharification was calculated.

Effect of substrate concentration on saccharification of hemicellulose: Assay mixtures containing 20U of xylanase activity, 25 ml of 0.05 M McIlvaine buffer (pH 6.0) and different amounts of rice straw, wheat straw and bagasse (1.0, 1.5 and 2.5 g), were incubated at 50°C for different time periods (2, 4, 6, 8, 10 and 12 hours). Aliquots (0.5 ml) were withdrawn after different time intervals, diluted and then assayed for total reducing sugars released and determined the percent saccharification.

Effect of enzyme concentration on saccharification of hemicellulose: Reaction mixtures containing 1.0 g of pretreated substrates, 0.05M McIlvaine buffer of pH 6.0 and different enzyme concentrations (10-40 U) in a final volume of 25 ml, were incubated at 50°C for 10 hours. Aliquots (0.5 ml) were removed and then assayed for total reducing sugars released.

Results and Discussion

Effect of pretreatment on saccharification: Rice straw, wheat straw and bagasse were separately pretreated with 2% NaOH and 2% phosphoric acid for 1, 2 and 4 hours. The results of the effects of pretreatment on three substrates using xylanase of *B. subtilis* has been presented in Table 1. It can be seen that saccharification levels has been enhanced to 10.3, 18.2 and 30.3% in comparison to 3.1% saccharification of untreated wheat straw. When wheat straw pretreated with 2% NaOH for 1, 2 and 4 hours, respectively. The percentage of saccharification observed with xylanase treated was 7.2, 14.3 and 24.0, respectively. Similarly 9.8, 14.5 and 28.0% saccharification has been obtained when rice straw was pretreated with 2% NaOH for 1, 2 and 4 hours, respectively during incubation with xylanase after 10 hours,where as under same conditions, only 3.5% saccharification occurred when untreated rice straw has been used as substrate as shown in Table 1. It is also shown that in comperison to the saccharification of untreated rice straw 1.94, 3.9, 5.8 fold increase in enzymatic saccharification occured when rice straw was pretreated with 2% phosphoric acid for 1, 2, 4 hours, respectively.It was also indicated in Table 1 that saccharification of bagasse when compared with wheat straw did not increase after two hours pretreatment. Similarly in comperison to untreated bagasse 1.95, 3.45 and 5.3 fold increase in percent hydrolysis of the substrate was obtained when bagasse was pretreated with 2% phosphoric acid for 1, 2 and 4 hours, respectively.

Effect of amount of substrate: It can be seen in Fig. 1 that percent saccharification of wheat straw obtained was 30.0, 25.3 and 21.0 after 10 hours of incubation period when 4, 6 and 8% wheat straw was used. It is concluded that the percent saccharification increased up to 10 hours of incubation time and after that time period no increase was noted in the saccharification irrespective of the substrate amount. It was also shown in Table 2 that the amount of reducing sugars formed increased with increasing the substrate concentration and 16.8 mg/ml sugars were released when 8% substrate was added in the reaction mixture.

The enzymatic hydrolysis of alkali treated rice straw, using at a concentration of 4% w/v, initially proceeded very rapidly upto 25.0% after 8 hours. Afterwards there was no significant increase in saccharification approaching 28.5% when reaction was carried out at 50°C for 12 hours as reported in Fig. 2. A similar trend in the increase in percent hydrolysis of alkali pretreated rice straw with the substrate concentration of 6 and 8 %was noted and reported in Fig. 2. It was found that the amount of reducing sugars released increased with increasing substrate concentration while the percent conversion was reduced. These results are in agreement with the findings of Dekker (1983) who reported that xylanase of *T. reesei* hydrolysed several hetroxylans. Varadi *et al.* (1971) has also reported similar observations in the saccharification of cellulose with cellulytic enzymes. It can be seen in Table 3

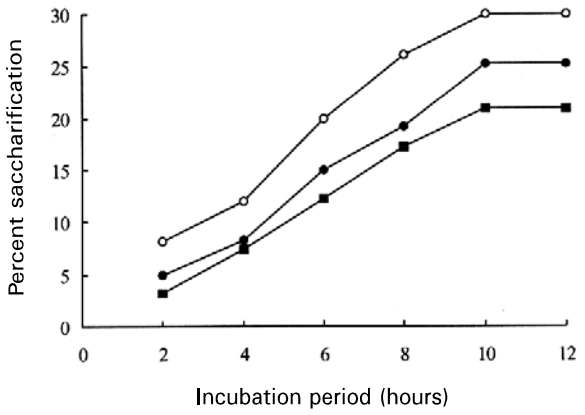

Fig. 1: Effect of substrate concentration on saccharification of wheat sraw. The amount of the substrate was 1g (—○—), 1.5g (—●—) and 2.0 g (—■—)

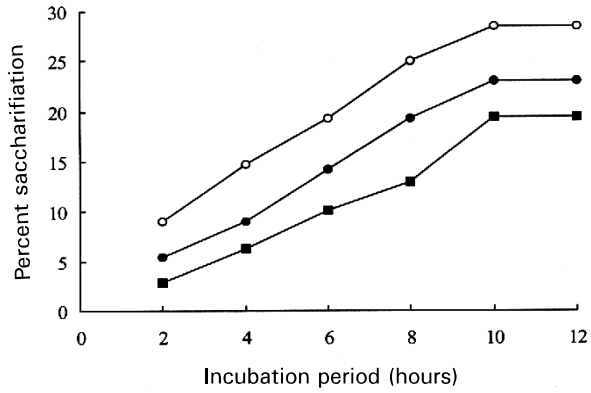

Fig. 2: Effect of substrate concentration on saccharification of rice straw. The amount of the substrate was 1g (—○—), 1.5g (—●—) and 2.0 g (—■—)

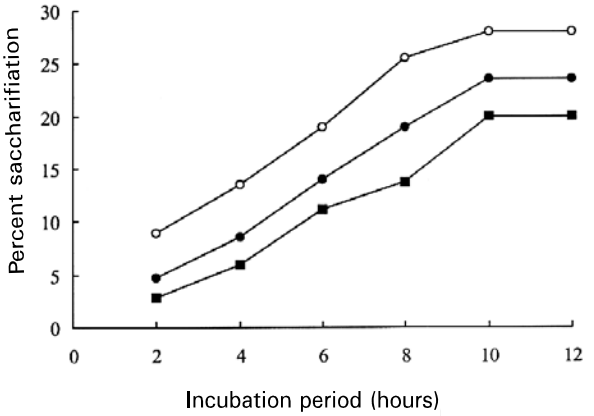

Fig. 3: Effect of substrate concentration on saccharification of bagasse. The amount of the substrate was 1g (—○—), 1.5g (—●—) and 2.0 g (—■—)

Table 1: Hydrolysis of pretreated and untreated lignocellulosic substrates by the xylanase of *B. subtilis* at 50°C for 10 hours. The assay mixture contained 1.0 g of the substrate suspended in 25 ml 0.05 M McIlvaine buffer (pH 6.0) and 20 U of xylanase activity

Substrate	Untreated	Percentage saccharification					
		Pretreatment with 2% NaOH			Pretreatment with 2% H_3PO_3		
		1 hr	2 hrs	4 hrs	1 hr	2 hrs	4 hrs
Wheat straw	3.1	10.3	18.2	30.3	7.2	14.3	24.0
Rice straw	3.5	9.8	14.5	28.0	6.8	13.7	20.3
Bagasse	4.2	12.7	28.0	28.0	8.2	14.5	22.2

Table 2: Hydrolysis of alkali treated wheat straw by *B. subtilis* xylanase at various concentrations of substrate after 10 hours incubation

Amount of substrate (g/25 ml)	Reducing sugars released (mg/ml)	%age hydrolysis
1.0	12.0	30.0
1.5	15.2	25.3
2.0	16.8	21.0

Table 3: Hydrolysis of alkali treated rice straw by *B. subtilis* xylanase at various concentrations of substrate after 10 hours incubation

Amount of substrate (g/25 ml)	Reducing sugars released (mg/ml)	%age hydrolysis
1.0	11.4	28.5
1.5	13.8	23.0
2.0	15.6	19.5

Table 4: Hydrolysis of alkali treated bagasse by *B. subtilis* xylanase at various concentrations of substrate after 10 hours incubation

Amount of substrate (g/25 ml)	Reducing sugars released (mg/ml)	%age hydrolysis
1.0	11.2	28.0
1.5	14.1	23.5
2.0	16.0	20.0

Table 5: Effect of enzyme concentration on saccharification of lignocellulosic substrates after 10 hours of incubation

Substrate	Substrate conc.% (w/v)	Enzyme activity (U)	Saccharification (%)
Wheat straw	4	10	16.5
	4	20	30.0
	4	40	37.5
Rice straw	4	10	15.1
	4	20	28.0
	4	40	36.0
Bagasse	4	10	14.8
	4	20	28.2
	4	40	35.5

that 11.4 mg/ml reducing sugars has been produced in 10 hours when 4% alkali treated rice straw was used in the assay mixture. The reducing sugars increased from 13.8 to 15.6 mg/ml when substrate concentration increased from 6 to 8%. It can be seen in Fig. 3 that 2.8 fold increase in percent hydrolysis was observed when saccharification of alkali treated bagasse was carried out for 8 hours at 50°C. It was also recorded that there was insignificant increase in degree of hydrolysis after 12 hours and with the increase in the substrate concentration the percent conversion was reduced. 23.5 and 20.0% saccharification levels were produced at the concentration of 6 and 8%, respectively in 12 hours hydrolysis time in comparison to 28.0% saccharification of 4% bagasse. As reported in Table 4 it is concluded that the amount of reducing sugars increased with increasing the amount of substrate in the reaction mixture after 10 hours of hydrolysis.

Effect of xylanase concentration: The results of the effect of xylanase concentration on the degree of hydrolysis of alkali treated wheat straw reported in Table 5 showed that the saccharification rates of substrate increased with increasing xylanase concentration. It was found that the increase in enzyme concentration from 10-20 U in the reaction mixture containing wheat straw as a carbon source resulted in 1.8 fold increase in percent saccharification. Further 1.25 fold increase was observed by further doubling the amount of xylanase, i.e., 20 to 40 U. 1.85 and 1.90 fold increase in saccharification of 1.0 g pretreated rice straw and bagasse occured, respectively with increasing the amount of xylanase from 10 to 20 U in 25 ml reaction mixture after 10 hours of incubation period. Further increase of 1.28 and 1.25 fold in saccharification was also noted when xylanase dose was further doubled i.e. from 20 to 40 U.

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