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Enzymatic Saccharification of Sugar Cane Bagasse by the Crude Enzyme from Indigenous Fungi

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Abstract

Sugar cane bagasse is a cheap lignocelluloses which has high carbohydrate content and, if properly pre-treated, could be converted to fermentable sugar. In this study various fungal isolates isolated from lignocellulosic waste were assayed for their CMCase, FPase and xylanase activity. Four potent plant cell wall-degrading fungi were identified as *Trichoderma* sp., *Aspergillus niger*, *Cladosporium* sp. and *Curvularia* sp. Among these isolates *Trichoderma* sp. produced the highest enzymatic activity in the culture filtrates containing steamed NaOH treated-bagasse. The yield of endo-*β*-glucanase *i.e.*, carboxymethylcellulase (CMCase), FPase and xylanase were 0.977, 0.110 and 9.280 U/ml. The highest degree of saccharification (D_oS) was also achieved by *Trichoderma* sp. which was 45.71%. *Trichoderma* sp. is a potential source of extracellular hydrolases, which can be used for enzymatic saccharification of bagasse to produce fermentable sugar for ethanol production.

Keywords: Bagasse; CMCase; FPase; Xylanase; Lignocellulose; Degree of saccharification; Graeco-Latin square design.

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1. Introduction

The increase in oil prices in recent years, imminent shortages of the classical energy sources and environmental concerns related to global warming has focused attention on renewable alternative fuels and on technology for producing fuel ethanol. There is much interest at present in the application of cellulolytic enzymes for the conversion of lignocellulosic residues in fermentable sugars for producing ethanol.

Sugarcane bagasse is regarded as a cheap substrate for fermentable sugar production and there is a constant supply generated within the sugarcane industry [1, 2]. In Bangladesh around 425,000 acres of land are under sugarcane and the annual production is about 7.5 million tons, of which only 2.28 million tons are used in sugar mills and the

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rest goes to molasses making. Bangladesh now produces about 150,000 tons of sugar, 100,000 tons of molasses and 800,000 tons of bagasse per year [3].

Sugar cane diverges from most species of the grass plants, by having a solid stem. Sugarcane bagasse composed of around 50% cellulose, 27.9% hemi-cellulose, 9.8% lignin and 11.3% cell content [4]. Structural properties of cellulose such as the degree of crystallinity, the degree of polymerization and the surface area, limit accessibility of substrate to enzyme and have been demonstrated [5] 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. The enzymatic hydrolysis of cellulosic materials correlates with the level of cellulose crystallinity [6] 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 [7].

Several cell-decomposing Microorganisms produce cellulases which are the most economic and available sources for fermentable sugar production from sugar cane bagasse, because these microorganisms can grow on inexpensive media. The genus *Trichoderma*, filamentous ascomycetes are widely used in industrial applications because of high secretary capacity and inducible promoting characteristics [8]. 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 [9, 10 and 11].

The conversion of lignocellulosic material into fermentable sugar is complicated and not yet a commercial business, but the trends towards commercialization are evident. [12]. Cellulose and hemicelluloses polysaccharides in bagasse are largely protected from attack by microorganism and their association of these polysaccharides with lignin, which acts as a barrier shielding the polysaccharides. The aim of this study was to isolate potent sugar cane bagasse saccharifying isolates from lignocellulosic waste and to optimize the saccharification condition statistically by Graeco-Latin Square Design [13] for commercialization of fermentable sugar production process in Bangladesh.

2. Materials and Methods

2.1. *Microorganisms*

The cellulolytic fungi used in this experiment were isolated from decomposing lignocellulosic waste. Fungal isolates such as *Trichoderma* sp., *Aspergillus niger*, *Cladosporium* sp. and *Curvularia* sp were used for cellulolytic enzyme production using basal medium described by Copa-Patiño [14]. 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 two percent (w/v) as a carbon source.

2.2. *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 productions as well as for enzymatic saccharification. The physical treatments included chopping, milling and boiling, while the chemical treatments were done using alkali and acid. In later case with alkali treatment milled bagasse was kept at 85^oC for one hour in two percent 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 minutes with 10% sulphuric acid solution (solid liquid ratio was 1:10) [15, 16]. Both treated (NaOH, H_2SO_4, and boiling) and non-treated chopped bagasse were further passed with steam treatment (121°C for 15 minutes in an autoclave). These substrates were used for enzyme production.

2.3. *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 ml of the suspension was pipetted into 50 ml of inoculum growth medium [17] and incubated in an orbital shaking incubator at 30°C and 200 rpm. After 36 hours growth, the medium was used as the inoculum for enzyme production.

2.4. *Culture conditions and enzyme production*

The standard mineral basal medium was used for growth and enzyme production as described by Copa-Patiño . The initial pH was adjusted to 5.0. Cultures were conducted in 250 ml Erlenmeyer flasks containing 100 ml mineral medium and two percent (w/v) sugarcane bagasse (pretreated) as a carbon source. The growth medium was sterilized at 121°C for 15 minutes, then inoculated with five ml spore suspension (10^6-10^7 spores) and incubated at 30°C on an orbital shaking incubator at 200 rpm for 72 hours. After cultivation the culture filtrate was centrifuged at 10,000 rpm for 15 minutes using lab centrifuge (Hettich, Model- D78532, Germany). The clear supernatant fluid was used directly for the determination of the enzyme activities**.**

2.5. *Chemical analysis*

Fermentable sugar i.e. reducing sugar was determined by dinitrosalicylic acid (DNS) method [18] 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.* [19].

2.6. *Enzyme assay*

Carboxymethylcellulase (CMCase) activity was estimated essentially according to IUPAC instructions [20] using one percent solution of carboxymethyl cellulose-sodium salt (Serva, Germany.) in 0.05*M* citrate buffer, pH 5.0 as substrate. One CMCase unit is the amount of enzyme necessary to produce one µmol reducing sugar as glucose equivalents per minute under the standard assay conditions. The assay was also carried out for different time interval to find out the optimum time for enzyme activity.

Filter paper activity (FPase) was determined essentially according to the IUPAC instructions and the liberated reducing sugars were estimated by the DNS method [18]. FPase activity corresponds to one µmol of reducing sugars as glucose equivalents librated per minute under the assay conditions.

Xylanase activity was determined according to Biley *et al.* [21] using one percent birch wood xylan (Roch, Germany) powder (in 0.05 *M* citrate buffer, pH 5.0) as substrate. One unit of xylanase activity was defined as the amount of enzyme which released one µmol reducing sugar as xylose equivalents per minute under the assay conditions. A time dependent assay was also conducted to find out the optimum time for enzyme activity.

2.7. *Saccharification of sugarcane bagasse*

Saccharification experiments were performed in 100 ml Erlenmayer flasks containing 10 ml of reaction mixture, which contained 200 mg (two percent dry wt) substrate and 10 ml enzyme solution in citrate buffer (0.05 *M*, pH 5.0). The flask were sealed with aluminum foil and incubated at 50^{o}C under continuous agitation at 150 rpm for 48 hours. Hydrolysates were transferred in screw-capped tubes, heated in a boiling water bath for 15 minutes and centrifuged to remove solid particles. The supernatant were used for analysis of released sugars. Degree of saccharification (D_oS) was calculated essentially described by Vallander and Eriksson [22] using the following equation:

$$D_oS = \frac{c\, v\, f_1}{m\, f_2} \times 100 \qquad (1)$$

where c is the sugar concentration in the hydrolysate estimated as total reducing sugars, in mg/ml; v is the liquid volume of the hydrolysates, in ml; 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 dry weight, in mg; and f_2 is the factor for the carbohydrate content of the substrate (total carbohydrate, mg/total substrate, mg).

2.8. *Effect of temperature on saccharification of bagasse using the crude enzyme of trichoderma sp.*

Saccharification study was carried out at various temperatures (20, 30, 40, 50 and 60ºC) to find out the best hydrolysis condition. Hence NaOH-treated chopped bagasse (200 mg) was added to the reaction mixture containing 10 ml enzyme solution in citrate buffer (0.05 *M*. pH 5.0). Saccharification was carried out for two hours and the sugars released were estimated by DNS method.

2.9. *Effect of pH on saccharification of bagasse using the crude enzyme of Trichoderma* **sp.**

The optimum pH for saccharification was also determined. Here NaOH-treated chopped bagasse (200 mg) was suspended in 10 ml enzyme solution in citrate buffer (pH 3.0-7.0). The saccharification was carried out for two hour at 50^{o}C in an orbital shaker at 150 rpm. The sugars released were estimated by DNS method.

2.10. *Statistical optimization of saccharification conditions by graeco-latin square design*

The optimum temperature, pH of citrate buffer, enzyme amount and percentages of substrate for saccharification of sugarcane bagasse were finally determined using Graeco-Latin square design. Assuming that the reaction mixture under study should contain four ingredients such as substrates (bagasse), enzyme, reaction pH and reaction temperature. These four ingredients were used at different level to create different saccharification condition such as substrates (bagasse) of different amount (1.5, 2.0 and 2.5% designated as A1, A2 and A3 respectively), enzyme of three variable amount (4.5, 5.0 and 5.5 ml designated as B1, B2 and B3 respectively), buffer of three types of pH (pH 4.5, 5.0 and 5.5 designated as C1, C2, C3 respectively) and three different temperature treatment (45, 50 and 55ºC designated as D1, D2 and D3 respectively). The optima were determined by averaging the resultant degree of saccharification (D_oS) obtained with the three respective hydrolysis reactions for a single variable of a particular factor.

2.11. *Saccharification kinetic studies*

The saccharification experiments were carried out for six hours and reducing sugar released was determined by DNS method at various time intervals. Initially the reaction mixture contained two percent 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.

3. Results and Discussion

3.1. *Enzyme activity of culture filtrate from four fungal isolates*

Among a large number of lignocellulosic wastes decomposing fungal isolates, four fungal isolates identified as *Trichoderma* sp., *Aspergillus niger*, *Cladosporium* sp. and *Curvularia* sp., were found to be potent in the case of CMCase, FPase and xylanase activity using reference medium supplied with two percent bagasse. The highest CMCase, FPase and xylanase activity were obtained by *Trichoderma* sp. which were 0.977, 0.110 and 9.280 U/ml as shown in Fig. 1. The other isolates were not potent hydrolase enzyme producer.

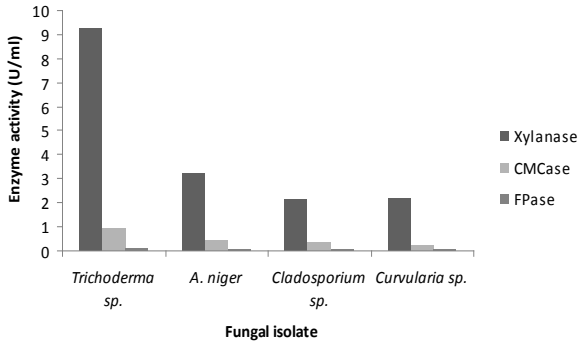

Fig. 1. CMCaes, FPase and xylanase activities of culture filtrate from four fungal isolates grown on reference medium supplied with 2% bagasse.

3.2. *Enzyme production on variously pretreated bagasse by Trichoderma* sp.

The organism *Trichoderma* sp. was grown in both treated and untreated bagasse used as carbon source in enzyme production media. Table 1 shows the list of various pretreatments that were applied to the substrate in this experiment.

Table 1. List of different pretreatment applied to sugarcane bagasse.

Pretreatment No.	Pretreatment type
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	Boiled short pieces bagasse
8	Boiled + steam treated short pieces bagasse
9	Short pieces bagasse
10	Steam treated short pieces bagasse

The effect of differentially treated bagasse on the production of enzyme is shown in Fig. 2 and Fig. 3. Enzyme production was enhanced by chemical pretreatments using alkali and acid. Further increase in enzyme production was achieved when the chemically pretreated substrate were subjected to additional stream treatment. The highest CMCase activity (0.977 U/ml) was obtained from steamed NaOH treated (short piece) bagasse, followed by steamed H_2SO_4 substrate (0.843 U/ml). The least enzyme activity was obtained from heat treated (steaming and boiling). The highest CMCase activity (0.977 U/ml) was obtained from steamed NaOH treated (short piece) bagasse, followed by steamed H_2SO_4 substrate (0.843 U/ml). The least enzyme activity was obtained from heat treated (steaming and boiling). Similarly, the highest FPase activity was observed when chemically pretreated substrates were subjected to steam treatment shown in Fig. 3.

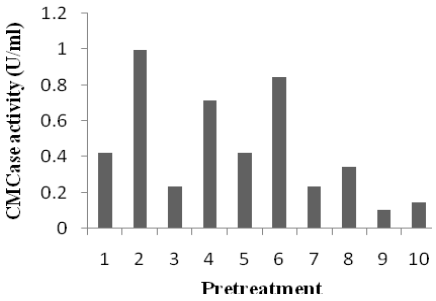

Fig. 2. Effect of different pretreatments of sugarcane bagasse on extracellular CMCase production by *Trichoderma* sp. in shake-flask culture

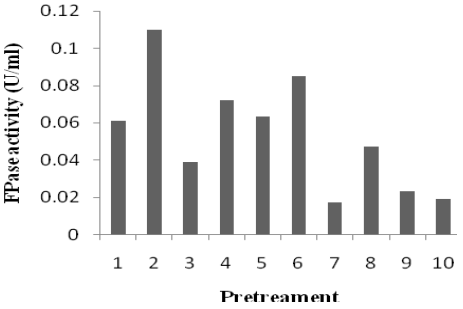

Fig. 3. Effect of different pretreatments of sugarcane bagasse on extracellular FPase production by *Trichoderma* sp. in shake-flask culture.

3.3. *Time course of enzyme production of Trichoderma* sp.

Time course of enzyme cellulase production by *Trichoderma* sp. was studied using steamed alkali-treated sugarcane bagasse at 30°C for six days the medium was (100 ml) inoculated with 5 ml of the spore suspension (10^6–10^7 spores/ml) and incubated in an orbital shaker incubator with an agitation rate of 200 rpm. Samples withdrawn at various time intervals were assayed for CMCase, FPase and xylanase activities. Fig. 4 shows the time-dependent enzyme production. CMCase biosynthesis was not detected up to 24 hours of incubation and then the enzyme activity increased sharply up to 72 hours and highest CMCase activity was found 1.31 U/ml at 144 hours. FPase production started after a large lag period (about 72 hours) and thereafter the enzyme synthesis increase sharply. The final FPase activity was 0.110 U/ml at 144 hours. Initially, xylanase production was low and at 96 hours a sharp increase in enzyme production was observed and after that the enzyme activity remained constant. The highest xylanase activity was found after 144 hours of incubation which was 13.250 U/ml.

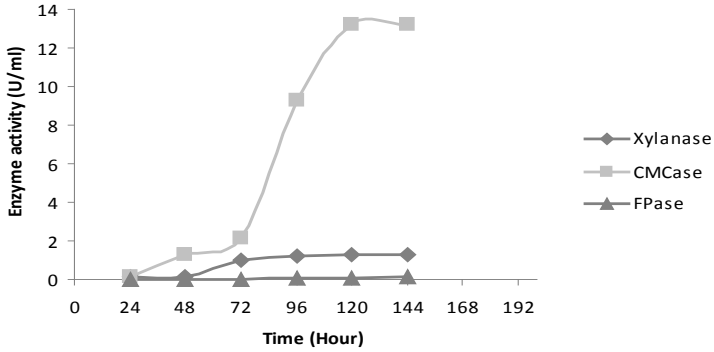

Fig. 4. Time course of CMCase, FPase and xylanase production in steamed NaOH-treated sugarcane bagasse by *Trichoderma* sp.

3.3. *Saccharification of sugarcane sagasse by four fungal isolates*

The degree of saccharification (D_oS) of sugarcane bagasse by crude enzyme of four fungal isolates were performed in 100 ml Erlenmayer flasks containing 10 ml of reaction mixture which contained 200 mg NaOH treated chopped bagasse in citrate buffer (0.05 M. pH 5.0). Each reaction mixture contained 5 ml enzyme of each type and 5 ml of citrate buffer and saccharification was carried out for 4 hours. The highest degree of saccharification (D_oS) of bagasse was achieved with crude enzymes of *Trichoderma* sp., which was 45.71%. The degree of saccharification (D_oS) of bagasse by *Aspergillus niger*, *Cladosporium* sp. and *Curvularia* sp. were 19.98%, 12.86% and 13.89%, respectively (Fig. 5).

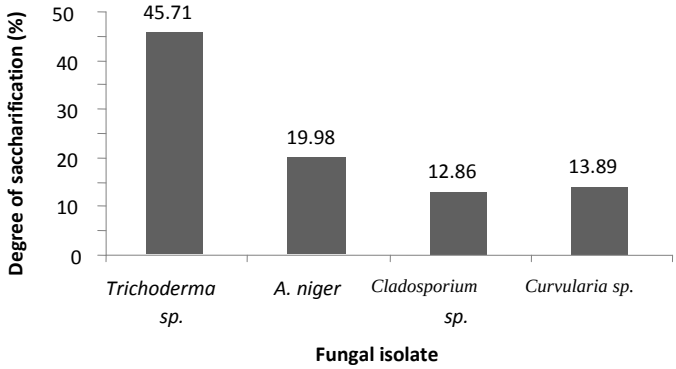

Fig. 5. The degree of saccharification (D_oS) of sugarcane bagasse by crude enzyme preparation of four fungal isolates.

3.5. *Effect of temperature and pH on enzymatic saccharification of bagasse using crude enzyme of Trichoderma* sp.

In this study the optimal hydrolysis temperature and optimum pH for saccharification of sugar cane bagasse to produce fermentable sugar were found intriguing for commercialization of fermentable sugar production process. Fig. 6 shows the effect of temperature on saccharification. Maximum hydrolysis of alkali-treated substrates occurred at 50^{o}C that correspond to degree of saccharification of 37.29% shown in Fig. 6. At 20, 30, 40, 50 and 60^{o}C the degree of hydrolysis of alkali-treated bagasse were 5.51%, 12.94%, 29.68% and 33.11% respectively, which were considerably lower than that what occurred at 60^{o}C . Fig. 7 shows the effect of pH on hydrolysis of alkali-treated sugar cane bagasse. The extent of hydrolysis in relation to reaction pH varied, and excellent hydrolysis was achieved at pH range between 4.0 and 6.0. However, the maximum degree of saccharification (D_oS 37.29%) of alkali-treated sugar cane bagasse occurred at pH 5.0.

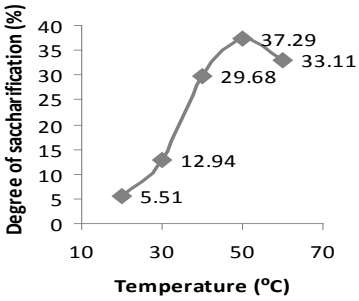

Fig. 6. Effect of temperature on enzymatic saccharification of sugar cane bagasse using crude enzyme of *Trichoderma* sp.

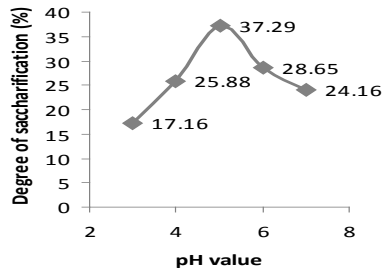

Fig. 7. Effect of pH on enzymatic saccharification of sugar cane bagasse using crude enzyme of *Trichoderma* sp.

3.6. *Statistical optimization of saccharification conditions using graeco-latin square technique*

For optimization of saccharification condition, Graeco-Latin Square technique was performed using four important parameters: substrates (bagasse) concentration, enzyme, reaction pH and reaction temperature. The design of experiment with the corresponding degree of saccharification (D_oS) was summarized in Table 2. The experiment arranged in Graeco-Latin Square Design consisted of nine different reaction conditions and each reaction condition of any particular factor occurred in three reaction setup.

According to Graeco-Latin Square Design the optimum conditions for saccharification of bagasse were 2.5% substrates (sugarcane bagasse), (2) 10 ml enzyme

solution (5.5 ml enzyme + 4.5 ml citrate buffer), reaction pH of 4.5, and reaction temperature of 45ºC.

Table 2. Graeco-Latin square design for optimization of saccharification condition.

Parameter		Medium serial number								
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
Substrates (mg)	A1	1.5			1.5			1.5		
	A2		2			2			2	
	A3			2.5			2.5			2.5
Enzyme (ml)	B1	4.5	4.5	4.5						
	B2				5	5	5			
	B3							5.5	5.5	5.5
pH	C1	4.5					4.5		4.5	
	C2		5		5					5
	C3			5.5		5.5		5.5		
Temperature (ºC)	D1	45				45				45
	D2			50	50				50	
	D3		55				55	55		
Degree of saccharification (%)		10.92	12.36	13.89	13.05	16.51	17.81	12.97	18.34	20.55

3.7. *Time course of enzymatic saccharification of alkali-pretreated bagasse by crude enzyme preparation of Trichoderma* sp.

The optimum reaction time for saccharification was determined by carrying out hydrolysis experiments under shaking condition (150 rpm) at 50^{o}C for various time intervals (from one to six hours) using 200 mg substrates and 10 ml enzyme solutions in citrate buffer (pH 5). Fig. 8 shows the effect of reaction time on hydrolysis of alkali-treated bagasse. The rate of hydrolysis in relation to reaction time varied. For first one hour the rate of saccharification was high. After two hours a sharp decrease in rate of saccharification was occurred and at hour two, the degree of saccharification (D_oS) was 37.29% and after that the rate of saccharification slowed down.

The rate and extent of saccharification depend on the nature and pretreatment of the substrate, enzyme and substrate concentration, product inhibition and enzyme stability [5, 23]. All of these interact to cause the rate of hydrolysis to fall of rapidly with time. As the more susceptible portions of the cellulose are hydrolyzed, the residue is increasingly crystalline and resistant, products accumulated and they competitively inhibit the enzymes. Since cellulose simultaneously decreases, the inhibitor/substrate ratio rises [24].

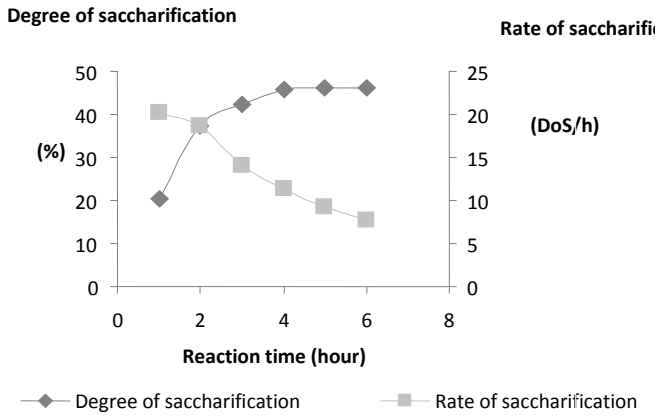

Fig. 8. Time course of enzymatic saccharification of alkali-pretreated bagasse by crude enzyme preparation of *Trichoderma* sp.

Pretreatment of the lignocellulosic substrate is essential for efficient enzymatic hydrolysis because of the various physical and chemical barriers that greatly inhibit the accessibility of the polysaccharide to hydrolytic enzymes [6, 23]. The ability to decrease the biomass crystallinity is responsible for the improved bagasse enzymatic saccharification rates and yields obtained [25].In this study, alkali-treated bagasse subsequently steam-treated was used as the substrate for saccharification experiments. The extent of hydrolysis depended on the reaction temperature and maximum hydrolysis was achieved at 50^{o}C above and below of this temperature the D_oS was reduced considerably.

4. Conclusion

The enzymatic saccharification of sugar cane bagasse depends on various factors like substrates concentration (normally two percent bagasse), amount of enzyme (normally 5 ml) used in reaction mixture, pH of buffer (normally 5) and treatment temperature (50ºC). By using Graeco-Latin square technique [13], it was found that increasing bagasse concentration to 2.50%, lowering buffer pH to 4.5, increasing crude enzyme amount to 5.5 ml and lowering treatment temperature to 45ºC optimum saccharification of sugarcane bagasse was found. At these optimized conditions the degree of saccharification (D_oS) of steamed alkali-treated chopped bagasse by the crude enzyme preparation from *Trichoderma* sp. was found 45.71%, while the other lignocellulosic waste decomposing fungal isolates were poor in enzymatic saccharification of sugar cane bagasse.

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