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Kinetics of the Enzymatic Saccharification of Pretreated Tapioca Waste *(Manihot esculenta)* and Water Hyacinth *(Eichhornia crassipes)*

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ABSTRACT

Studies were carried out on saccharification of pretreated tapioca waste and water hyacinth under two different conditions: using microbial enzymes (cellulase from *Myrothecium verrucaria, Coprinus comatus, Pleurotus florida,* and *Cellulomonas* sp.) and solid-state fermentation. The rate of saccharification was determined at different temperatures, pH, substrate concentration, and incubation period. It was found that as the source of the enzyme varies, the optimal temperature and pH for the saccharification varies. Among the two different treatments, enzymatic saccharification was found to be the most efficient. Among the various cellulase sources tested, *M. verrucaria* cellulase was found to be the most efficient one followed by *C. comatus, P. florida,* and finally *Cellulomonas* sp.

Index Entries: Cellulose; cellulase; bioconversion; kinetics; biodegradation.

INTRODUCTION

During the last few years, a great interest has been brought to the enzymatic hydrolysis of cellulose. However, in spite of all the research work done on this subject, no detailed work for the industrial production of sugars from waste is available so far. The two major bottlenecks are related to enzyme production and substrate preparation. Problems linked to enzymes production are beginning to be solved. In most cases, problems

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related to substrate preparation still remain. The economy of many fermentation products depends mainly on the cost of the carbohydrate raw material *(1)*. The cost of the carbohydrate raw materials used will, therefore, play a major role in the future scope and importance of the fermentation industry. Lignocellulose conversion offers the potential for less expensive fermentable sugars. The most studied substrates are agricultural wastes such as wheat straw *(2)* or sugar cane bagasse *(3)*, which present several inconveniences, like seasonal availability. In this article, results are presented describing the saccharification of pretreated tapioca waste and water hyacinth, with the aim of producing sugars for subsequent use in ethanol production.

Lignocellulosic biomass cannot be saccharified by enzymes to high yields without any pretreatment *(4)*. The obstacles to enzymic saccharification are lignin and the crystalline cellulose in the cellulose waste *(5–7)*. Various pretreatment techniques have been used to enhance the susceptibility of the substrate to enzymes. These include mechanical, nonmechanical, and chemical methods *(8–13)*. In a recent review on lignocellulosic conversion and the future of fermentation biotechnology, emphasis was given to testing the fermentability of the sugars produced upon hydrolysis of lignocellulosic residues. The reducing sugar content of the saccharified lignocellulosic hydrolysate can be used as carbohydrate raw material for the fermentation production of alcohol and single-cell protein *(14)*. The present investigation focusses on the potential of reducing sugars in the cellulosic-waste hydrolysate as carbohydrate raw material for the production of alcohol. Various conditions were standardized to obtain the maximum saccharification.

MATERIALS AND METHODS

Cellulose Source

Twenty g each of 2% sodium hydroxide pretreated tapioca stem, 10% sodium hydroxide pretreated tapioca leaf, peracetic acid pretreated tapioca petiole and water hyacinth were used as cellulose source.

The bioconversion of these cellulose wastes was carried out by two different methods: solid state fermentation; and with cellulase enzyme.

Media for Enzyme Production

The media containing sodium nitrite 0.2%, potassium chloride 0.05%, magnesium sulphate 0.05%, dipotassium hydrogen phosphate 0.1%, ammonium molybdate 0.1%, cellulose (filter paper strips) 1.0% (pH of the medium was 6.0 for *M. verrucaria*, 8.0 for *C. comatus*, and 7.0 for *P. florida*) and a medium containing disodium hydrogen phosphate 1.88%, potassium dihydrogen phosphate 0.36%, magnesium sulphate 0.03%, sodium

chloride 0.04%, ammonium dihydrogen phosphate 0.1%, and cellulose (filter paper strips) 1% (pH 7.0) was used for enzyme production in fungi and bacteria, respectively.

Solid State Fermentation

Substrate

The previously mentioned substrates were used for the solid-state fermentation.

Media

The previously mentioned media were prepared using the substrate as the cellulose source.

Microorganism

The five different microorganisms used for the study are the following: *M. verrucaria; C. comatus; P. florida; Cellulomonas* sp.; *Phanerochaetous chrysosporium*. The inoculum for the fermentation studies were prepared *(15)*.

Fermentation

Twenty g each of the cellulose wastes were mixed separately with 80 mL of the proposed media in 250 mL conical flasks separately. All these samples were sterilized at 121°C for 20 min. After sterilization, the selected fungi and bacteria were inoculated in their respective media. All of them were incubated for 14 d. After the incubation period, all the samples were mixed with 220 mL of 0.2*M* solution of acetate buffer having pH 4.6. All the samples were centrifuged at 10,000 rpm for 30 min. The supernatant was collected in each case and the amount of total sugars, reducing sugars, and glucose were estimated. Estimation of total sugars was carried out by the procedure of Dubois *(16)*. Reducing sugars were estimated using the procedure of Miller *(17)*. The glucose level was estimated by the method of Malik and Singh *(18)*. The experiment was repeated by the addition of a 0.5-mm disk of *P. chrysosporium*.

Enzymatic Saccharification of Cellulose Waste

Production of Cellulase

The physicochemical conditions for the optimal production of cellulase from the previously mentioned microbes were carried out as mentioned *(15)*. The enzyme was partially purified using sephadex G-25 and DEAE-sepharose CL-6B *(19)*.

Saccharification

Five grams each of the powdered samples of cellulosic wastes were mixed separately with 60 mL of enzyme and 40 mL of 0.2*M* acetate buffer

having the appropriate pH in a set of 250 mL Erlenmeyer conical flasks and incubated at the optimum temperature for 24 h. Blanks were run using enzyme, buffer, and water and another one using samples, buffer, and water. After the incubation period, the reaction mixture was centrifuged at 10,000 rpm for 30 min and the supernatant was collected. The amount of total sugars *(16)*, reducing sugars *(17)* and glucose *(18)* were estimated. The percentage of saccharification (DS) was calculated using the formula

$$DS(\%) = C \times 0.89 \times 100 / m$$

Where C is the sugar concentration in the hydrolysate estimated as glucose in mg/mL; m is the amount of cellulose in mg/mL (the factor 0.89 is used to convert monosaccharides to polysaccharides because of water uptake during hydrolysis).

The effect of temperature, pH, substrate concentration, and incubation period on enzymatic saccharification was also examined by changing the temperature, pH, substrate concentration, and incubation period of the incubation mixture.

RESULTS AND DISCUSSION

Effect of pH

The results of the effect of pH on the activity of the enzyme using tapioca stem as the cellulose source are given in Fig. 1. The results showed that the pH of the saccharification medium depends upon the enzyme source *(20)*. The enzyme secreted by *M. verrucaria* gave the highest saccharification rate at pH 4.6 for all the cellulosic wastes studied. On the other hand, the enzyme from *C. comatus* gave the highest percentage of reducing sugars at pH 5.0 for all the cellulosic wastes, whereas the enzyme from *P. florida* and *Cellulomonas* sp. showed a pH optimum at 4.8. Even though the pH optimum for *M. verrucaria* was 4.6, it was active in the pH range 4.2–5.0. Similarly the enzyme from *C. comatus* could give saccharification in the pH range 4.6–5.2. *Pleurotus florida* showed a pH range of 4.4–5.0 for saccharification. The optimum pH for the growth of *C. comatus* was at an alkaline pH, but the optimal pH for saccharification was found to be at an acidic pH.

Effect of Temperature

The results of the effect of temperature on the activities of the enzyme using tapioca stem as the cellulose source are given in Fig. 2. The optimum temperature for bioconversion varied with the enzyme source *(21)*. The enzyme secreted by *M. verrucaria* and *P. florida* released the maximum amount of reducing sugars from the cellulosic samples at 40°C. On the other hand, the highest percentage of saccharification of the cellulosic

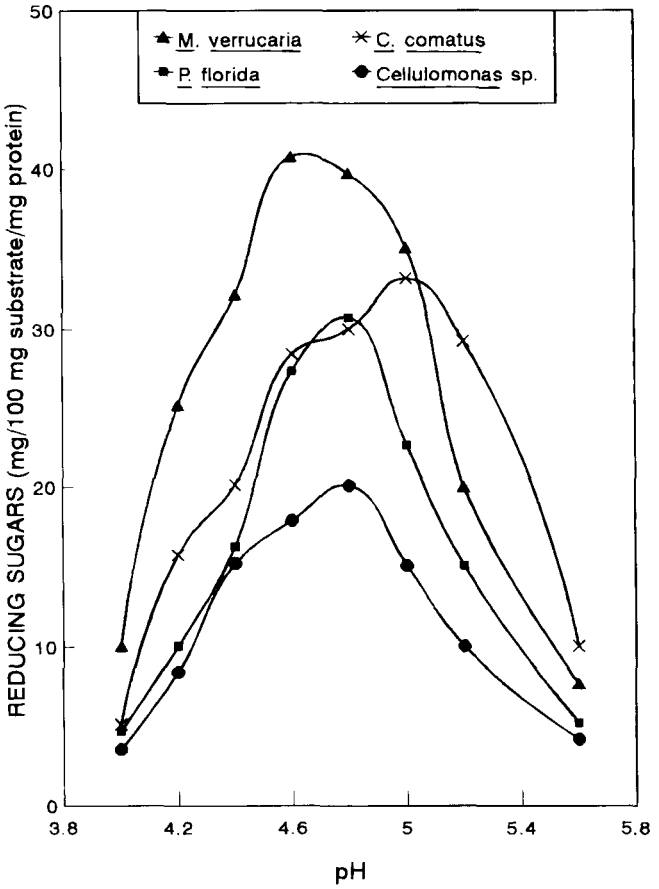

Fig. 1. Effect of pH on activity of the enzyme.

wastes was given by the enzyme from *C. comatus* at 50°C. Whereas cellulase from *Cellulomonas* sp. gave the maximum saccharification rate at 45°C. Above 50°C, the saccharification rate was found to be decreased. Cellulase from *C. comatus* preferred a higher temperature for bioconversion while *M. verrucaria* as well as *P. florida* preferred lower temperatures.

Effect of Substrate Concentration

The results of the effect of substrate concentration using tapioca stem as cellulose source on the activity of the enzyme are given in Fig. 3. As the concentration of the various cellulosic samples increased, the yield of reducing sugars was found to be increased, but the percentage of saccharification was decreased correspondingly. The same trend in reducing sugars as well as percentage of saccharification was observed for all the

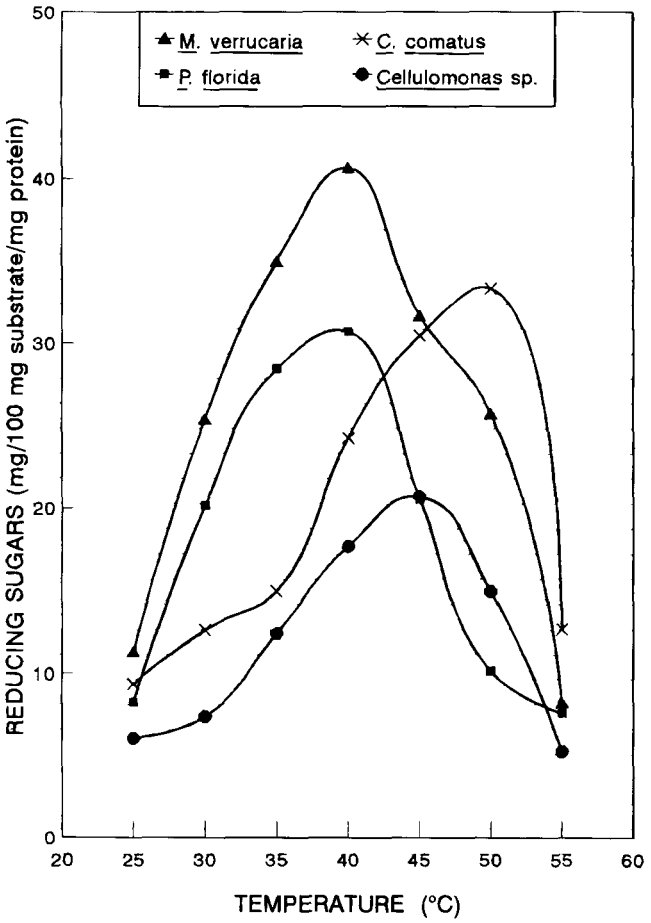

Fig. 2. Effect of temperature on activity of the enzyme.

enzymes from different sources. The enzyme from all the microbes gave the highest percentage of saccharification at 2% substrate concentration. Thereafter, the percentage of saccharification gradually decreased. The percentage of saccharification gradually increased from the substrate concentration 1.8 to 2.0%. The highest percentage of saccharification and hence the maximum amount of reducing sugars were given by the enzyme from *M. verrucaria.*

Effect of Incubation Period

The results of the effect of incubation period on the activity of the enzyme using tapioca stem as cellulose source are given in Fig. 4. When the incubation period was increased, the yield of reducing sugars was also increased. The rate of saccharification of all the cellulosic wastes increased almost in a uniform manner up to 12 h of incubation using the enzyme from various microbes. After 12 h of incubation, the saccharification per-

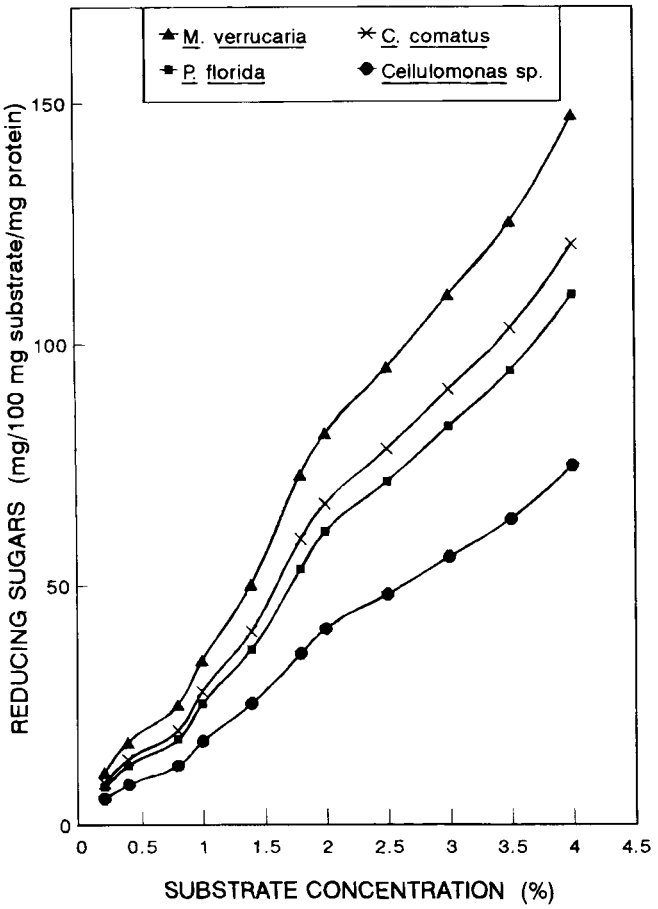

Fig. 3. Effect of substrate concentration on activity of the enzyme.

unit time was decreased. Tapioca stem gave the highest yield of reducing sugars in 12 h of incubation followed by tapioca petiole, water hyacinth, and tapioca leaf. The enzyme secreated by *M. verrucaria* gave the maximum rate of saccharification during 12 h of incubation. This was followed by *C. comatus, P. florida,* and *Cellulomonas* sp. The yield of reducing sugars was maximum for tapioca stem, when the saccharification was carried out by the enzyme from *M. verrucaria* and minimum for tapioca leaf, when the saccharification was carried out by the enzyme from *Cellulomonas.*

Bioconversion of Tapioca Waste and Water Hyacinth

Solid State Fermentation by the Action of Microorganisms

The results of the effect of solid state fermentation of tapioca waste and water hyacinth are given in Table 1. *M. verrucaria* was found to give maximum fermentation. The efficiency of the microbes for bioconversion

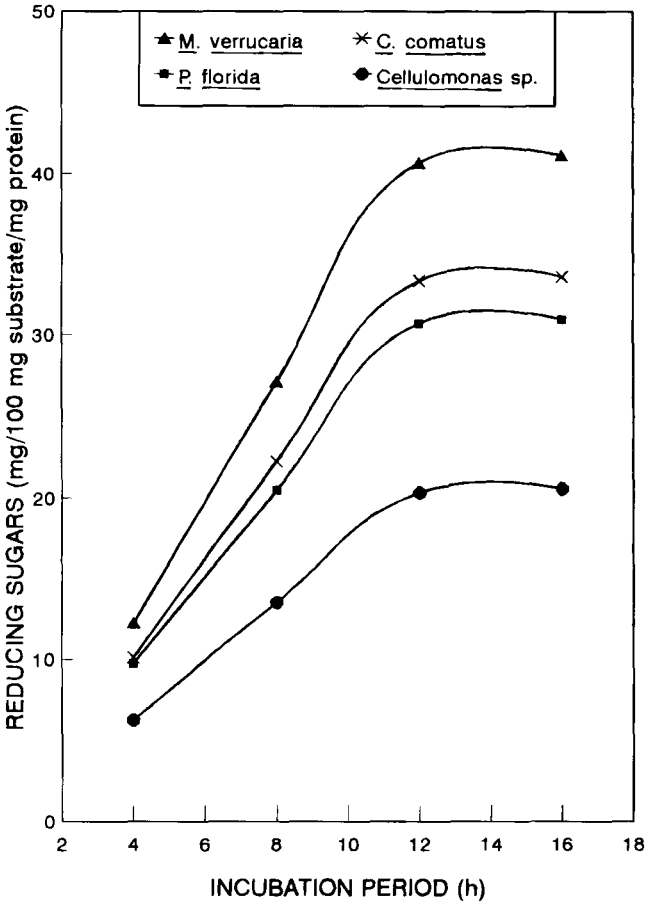

Fig. 4. Effect of incubation period on activity of the enzyme.

decreased in the order of *M. verrucaria, C. comatus, P. florida,* and *Cellulomonas* sp. Similarly, among the various cellulosic wastes, tapioca stem gave the highest percentage of fermentation. The ability of the cellulosic wastes to undergo fermentation increased in the order of tapioca leaf < water hyacinth < tapioca petiole < tapioca stem. The highest percentage of fermentation was achieved by *M. verrucaria* on tapioca stem and the lowest by *Cellulomonas* sp. on tapioca leaf. The yield of total sugars was higher than reducing sugars and glucose in all cases. However, in the case of fermentation carried out by fungi, the amount of reducing sugars produced was almost equal to the amount of glucose. But in the case of bacterial fermentation, the reducing sugars released from the cellulosic wastes were higher than that of glucose.

Table 1
Solid-State Fermentation of Cellulose Waste[a,b]

Cellulose Waste	Organism				Percentage of Saccharification			
	M. v.	C. c.	P. f.	Cell.	M. v.	C. c.	P. f.	Cell.
Tapioca stem	38.50 ± 0.34	31.90 ± 0.19	28.25 ± 0.14	17.40 ± 0.10	34.64	28.70	25.41	15.65
Tapioca petiole	33.05 ± 0.19	29.50 ± 0.30	26.96 ± 0.16	16.25 ± 0.07	29.73	26.54	24.25	14.62
Tapioca leaf	31.50 ± 0.25	27.72 ± 0.16	24.35 ± 0.14	13.94 ± 0.20	28.34	24.94	21.90	12.54
Water hyacinth	32.90 ± 0.39	29.45 ± 0.23	26.55 ± 0.21	16.15 ± 0.09	29.60	26.49	23.88	14.53

[a] Average of five values in each case ± SEM.

[b] Values are expressed as mg of total sugars per 100 mg substrate per mg protein.

Abbreviations: M. v.—*M. verrucaria*; C. c.—*C. comatus*; P. f.—*P. florida*; and Cell.—*Cellulomonas* sp.

Table 2
Solid-State Fermentation by Cellulolytic and Ligninolytic Microbes[a,b]

	Organism				Percentage of Saccharification			
Cellulose Waste	A	B	C	D	A	B	C	D
Tapioca stem	40.18 ± 0.52	33.10 ± 0.26	30.30 ± 0.45	19.40 ± 0.23	36.15	29.78	27.26	17.45
Tapioca petiole	35.06 ± 0.45	30.98 ± 0.27	28.05 ± 0.11	18.35 ± 0.22	31.54	27.87	25.23	16.51
Tapioca leaf	33.95 ± 0.50	29.50 ± 0.20	26.50 ± 0.47	15.17 ± 0.21	30.54	26.54	23.84	13.64
Water hyacinth	35.00 ± 0.49	30.65 ± 0.21	28.00 ± 0.44	18.00 ± 0.27	31.49	27.57	25.19	16.19

[a] Average of 5 values in each case ± SEM.

[b] Values are expressed as mg of total sugars per 100 mg substrate per mg protein.

Abbreviations: A—M. v. + P. c.; B—C. c. + P. c.; C—P. f. + P. c.; D—Cell. + P. c.; M. v.—*M. verrucaria*; C. c.—*C. comatus*; P. f.—*P. florida*; Cell.—*Cellulomonas* sp.; and P. c.—*P. chrysosporium*.

Table 3
Bioconversion of Cellulose Waste by Enzyme[a,b]

Cellulose Waste	Organism				Percentage of Saccharification			
	M. v.	C. c.	P. f.	Cell.	M. v.	C. c.	P. f.	Cell.
Tapioca stem	42.80 ± 0.38	35.90 ± 0.14	32.30 ± 0.51	21.50 ± 0.19	38.51	32.30	29.06	19.34
Tapioca petiole	37.95 ± 0.34	33.70 ± 0.50	30.10 ± 0.54	20.90 ± 0.31	34.14	30.32	27.08	18.80
Tapioca leaf	36.30 ± 0.43	31.85 ± 0.44	28.65 ± 0.25	17.50 ± 0.14	32.66	28.65	25.77	15.74
Water hyacinth	37.50 ± 0.33	32.65 ± 0.58	30.01 ± 0.12	19.85 ± 0.13	33.74	29.37	27.00	17.86

[a] Average of 5 values in each case ± SEM.

[b] Values are expressed as mg of total sugars per 100 mg substrate per mg protein.

Abbreviations: M. v.—*M. verrucaria;* C. c.—*C. comatus;* P. f.—*P. florida;* and Cell.—*Cellulomonas* sp.

Action of Cellulolytic Microbes and Ligninolytic Fungi

The results of the solid-state fermentation of cellulosic wastes, carried out by the combined action of ligninolytic and cellulolytic microbes, are given in Table 2. The combined action of cellulolytic and ligninolytic organisms on tapioca wastes and water hyacinth showed that the presence of ligninolytic organisms in the fermentation medium favored the rate of fermentation. The presence of ligninolytic organisms in the fermentation medium increased the release of total sugars, reducing sugars and glucose in all cases. The highest percentage of bioconversion was given by *M. verrucaria*. In this case also, tapioca stem gave the highest amount of reducing sugar.

Action of Cellulase Enzyme on Cellulose Waste

The results of the enzymatic saccharification of the cellulosic wastes are given in Table 3. Bioconversion of wastes by the enzyme showed that the yield of total sugars, reducing sugars, and glucose were higher in the enzymatic conversion than in solid-state fermentation. On the other hand, the yield of glucose produced by the enzymatic saccharification of cellulosic wastes by *Cellulomonas* sp. was significantly low. The yield of total sugars was much higher than reducing sugars and glucose in all cases. But the difference between reducing sugars and glucose was not significant for the enzymatic saccharification of cellulose waste by fungi. The highest saccharification was given by the enzyme from *M. verrucaria*. Tapioca stem gave the maximum yield of total sugars, reducing sugars and glucose.

CONCLUSION

Solid-state fermentation was one of the efficient methods of bioconversion of lignocellulosic wastes under optimum environmental conditions. Microorganisms secrete a number of hydrolytic enzymes and attack a number of complex compounds to simple compounds. Cellulase, being the major hydrolytic enzyme, contributed considerably to the solid-state fermentation. The end product of this process consisted of a mixture of simple sugars like glucose. In the present study, the amounts of reducing sugars and glucose are the same, indicating that glucose is the only reducing sugar released during solid-state fermentation. Solid-state fermentation in the presence of the fungi *P. chrysosporium* was found to be good. Because lignin in the reaction mixture was hydrolyzed, the cellulolytic activity of the microbes was also enhanced. The differential effect of microbial attack on different substrates was mainly due to the biochemical composition and structure of the substrates *(22)*. Tapioca-stem waste contained maximum cellulose and hence showed higher level of fermentation.

In the present study, it was found that fermentation rate was higher when pure enzyme was used. Enzymatic hydrolysis of cellulose for sugar

production offers advantages like higher conversion, minimal by-product formation, low-energy requirement and mild operating conditions over other chemical conversions *(23)*.

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