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Production of Cellulase in Solid-State Fermentation with *Trichoderma reesei* MCG 80 on Wheat Straw

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ABSTRACT

It is an accepted fact that ethanol production from lignocellulosic materials is not economical as yet because of the high cost of cellulase production. To reduce the cost of cellulase production, lignocellulosic material (wheat straw [WS]), a comparatively much cheaper substrate, was used instead of costly substrates (pure cellulose or lactose). A pan bioreactor was developed for solid-state fermentation (SSF) that required a small capital investment. High yields of complete cellulase system were obtained compared to that in the liquid-state fermentation (LSF) from WS, when treated with 4.25% NaOH at 121°C for 1 h and mixed with Mandels' medium. A complete cellulase system is defined as one in which the ratio of β-glucosidase activity to filter paper activity in the enzyme solution is close to 1.0. The cellulase system derived from SSF using the pan bioreactor gave more than 85% hydrolysis of delignified WS. The prototype pan bioreactor requires further improvements so that optimum quantity of substrate can be fermented to obtain high yields of complete cellulase system per unit space. The SSF process provides a means for the production of complete cellulase system for the economical bioconversion of renewable biomass into ethanol.

Index Entries: Cellulase; hydrolytic potential; lignocelluloses; solid-state fermentation; *Trichoderma reesei* MCG 80.

INTRODUCTION

Economical production of ethanol from lignocellulosic materials is hindered because of the high cost of cellulase production *(1,2)*. One effective approach to reduce the cost of enzyme production is to replace pure cellulose or lactose with relatively cheeper substrates, such as lignocellulosic materials. There have been reports of successful attempts to produce cellulase on lignocellulosics *(1–4)*. The development of technology with minimum capital investment is another approach

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to reduce the cost of cellulase production. In our earlier work *(4)*, we were able to produce cellulase in a solid-state fermentation (SSF) process that required relatively inexpensive equipment compared to the conventional fermenter used for liquid-state fermentation (LSF) process. SSF is a process by which insoluble substrate is fermented with sufficient moisture, but without free water. In LSF, the insoluble substrate is fermented in a slurry of 1–5% suspension *(4)*.

For complete hydrolysis of cellulose to glucose, cellulase systems must contain the following enzymes:

1. Endo-1,4-β-glucanase (1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4);
2. Exo-1,4-β-glucanase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91); and
3. β-glucosidase (EC 3.2.1.21, β-D-glucoside glucohydrolase or cellobiase).

Since the hydrolysis of cellulose is owing to the synergistic action of endo- and exoglucanases, we refer to their synergistic activity as filter paper activity (FPA) in this article. The cellulase systems of hypercellulase mutants of *Trichoderma reesei* are generally deficient in β-glucosidase *(3,5–7)*. During the hydrolysis of cellulose with this type of enzyme system, cellobiose accumulates, which inhibits the action of cellulases *(8–10)*. From the hydrolysis results reported by various researchers, we can deduce that a cellulase system having a ratio of β-glucosidase activity (βGA) to FPA close to 1.0 is necessary to obtain the highest rate of hydrolysis and highest glucose content in the hydrolysate *(3,5–7)*.

In this article, we present our efforts to produce a cellulase system using *T. reesei* MCG 80 which has a βGA-to-FPA ratio close to 1.0 by varying the medium composition and pretreatment parameters of wheat straw (WS) for better exposure of cellulose to growing microorganism.

MATERIALS AND METHODS

Microorganism

T. reesei MCG 80 (NRRL#12368) was obtained from J. L. Swezey, ARS Patent Culture Collection, USDA (Peoria, IL). Its culture was maintained on delignified WS agar medium in Petri plates *(4)*.

Substrate and Nutrients

WS, ground to 20-mesh size, was pretreated with various concentrations of sodium hydroxide at 121°C for 1 h. Nutrients described by Mandels and Weber *(11)* were used in the culture medium, but proteose peptone was replaced by yeast extract, potassium phosphate concentration was variable and is described where necessary in the text, and the urea was omitted from the culture medium. The quantity of nutrients required was proportional to the total carbohydrate content (70%) of the WS. WS fermentation medium for SSF was prepared as explained by Chahal *(4)*. Four grams of WS pretreated and mixed with Mandels' medium was dispensed in each Erlenmeyer flask of 500-mL capacity. Twenty grams of WS were used in the pan bioreactor.

Enzyme Assay

The enzyme from SSF was extracted by mixing the whole content of the flask (4 g WS) in a total of 100 mL water and then shaking for 1 h at 200 rpm at room

temperature and then centrifuged at 10,000 rpm (11,000*g*) for 30 min. FPA was expressed in international units (IU = μmol of glucose released/min) from 50 mg filter paper after 60 min of incubation at 50°C according to the method described by Mandels et al. *(12)*, which was approved by the International Union of Pure and Applied Chemistry (IUPAC) *(13)*. The activity of β-glucosidase was measured in IU using 0.5% salicin solution for 30 min of incubation at 50°C. The ratio of enzyme activities was defined as β-glucosidase activity divided by FPA.

Hydrolysis and Analysis of Sugars

The hydrolysis of delignified WS was done in 5% (w/v) concentration with enzyme loading of 20 IU FPA/g delignified WS at 45°C in 0.05*M* citrate buffer at pH of 4.8. The quantities of glucose, cellobiose, xylose, and arabinose in the hydrolysate of delignified WS were determined by Beckman 344 HPLC equipped with Altex 156 Refractive Index detector and Spherogel 7.5% carbohydrate column with a flow rate of 0.5 mL/min in the mobile phase of deionized and degassed water at 90°C.

Delignification of WS

The WS was delignified by the method described by Toyama and Ogawa *(14)*.

Experimental Design

An orthogonal composite design was applied to explore the response surfaces of activities of filter paper and β-glucosidase *(15–18)*. This design requires that the distance from the center point to each of the star points be such that all effects and interactions estimated in the second-order model are orthogonal to one another. This distance, *d*, is 1.414 for two factors, and the model has the following form:

$$y = a_0 + a_1X_1 + a_2X_2 + a_{11}X_1^2 + a_{22}X_2^2 + a_{12}X_1X_2 \quad (1)$$

where y is the response variable, a_is are the coefficients of the equation, and X_i is the normalized value (dimensionless) of factor i, normalized according to the following equation:

$$X_i = \{V_i - [(H_i + L_i/2)]/[(H_i - L_i/2)]\} \quad (2)$$

where, H_i = upper limit of factor i, L_i = lower limit of factor i, and V_i = value of operating variable i.

Linear regression analysis was applied to fit the data to experimental values and the coefficients, a_is, were determined at 95% confidence level.

RESULTS AND DISCUSSION

Effect of NaOH Concentration for the Pretreatment of WS and KH_2PO_4 Concentration in the Medium for Growth

Sodium hydroxide was used to pretreat the WS to expose the cellulose for mycelial growth. When too little NaOH is used, not much cellulose is exposed. Whereas, when NaOH is used in a very high concentration, there is a possibility that certain chemicals are produced during the heating cycle of the pretreatment process that are toxic to mycelial growth or high concentrations of hemicelluloses are released that inhibit the cellulase production. Therefore, there was a need to

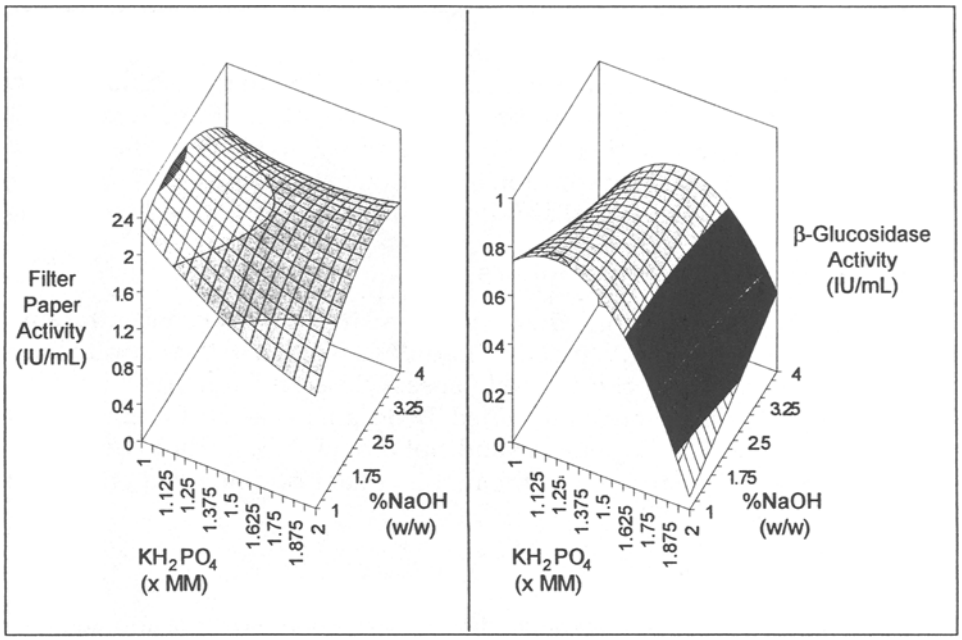

Fig. 1. FPA and βGA as a function of NaOH concentration required for the pretreatment of WS and the potassium phosphate concentration required in growth medium in LSF process.

find the best NaOH concentration required for the pretreatment of WS for the maximum production of cellulase. The potassium phosphate in the medium is a source of phosphorus for the growth of *T. reesei*, and it also acts as a buffer, preventing a fast drop in pH. The pH below 4.0 is not favorable for the biosynthesis of β-glucosidase. Therefore, there was a need for a medium that had a proper buffering capacity.

Experiments were designed with two factors having normalized $-d$ and $+d$ values representing 1.0 and 4.0% (w/w) for NaOH concentration for the pretreatment of WS, and 1.0 and 2.0 times the concentration of KH_2PO_4 required in Mandels' medium (×MM), respectively. MM contains 2 g KH_2PO_4 for 10 g carbohydrates.

Figure 1 shows the filter paper and βGAs activities in the LSF as functions of concentrations of NaOH (%w/w) and KH_2PO_4 (×MM). The increase in concentration of NaOH used for pretreatment of WS resulted in an increase in FPA. The highest FPA value, 2.4 IU/mL, was achieved when phosphate was in low concentration and the NaOH concentration used for the pretreatment of WS was about 2% (w/w). The increase in phosphate concentration decreased the FPA regardless of the concentration of the NaOH used for the pretreatment of WS. There was a sharp drop in βGA when phosphate concentration in the medium was high regardless of NaOH concentration required for the pretreatment of WS. The highest βGA, 0.8 IU/mL, was obtained at low concentration of NaOH (around 1%) used for the pretreatment with 1.25 x MM phosphate concentration in the growth medium. The best ratio of the activities obtained was 0.4.

When the fermentation was carried out in the SSF, the activities of both the enzymes increased considerably (Fig. 2). The FPA of 6 IU/mL was obtained for low-phosphate concentrations and high concentrations of NaOH for the pretreatment

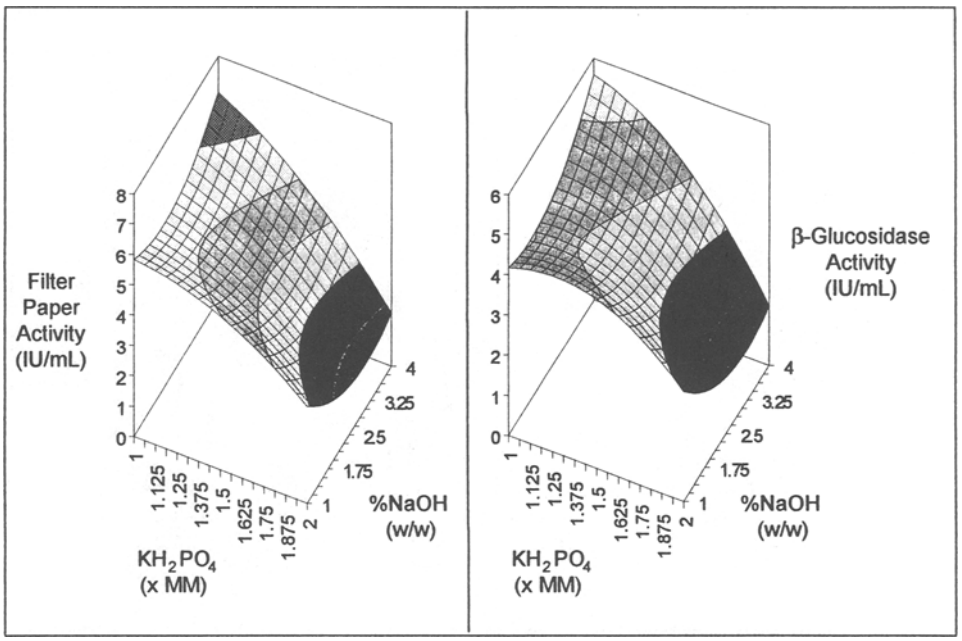

Fig. 2. FPA and βGA as a function of NaOH concentration required for the pretreatment of WS and the potassium phosphate concentration required in growth medium in SSF process.

of WS. The case was similar for βGA production. A value of about 5 IU/mL was achieved for low-phosphate concentration and high concentration of NaOH for pretreatment of WS. The ratio of activities in this case was about 0.8, significantly higher than that in LSF. The higher yields and ratio of activities obtained suggested further exploration of the SSF technology.

Effect of Water Content and NaOH Concentration in the Pretreatment of WS

It was necessary to determine the best water content to WS ratio in the pretreatment of the WS along with NaOH concentrations, because it was assumed that with higher water content in the pretreatment process, a better exposure to cellulose could be achieved with proper sodium hydroxide concentration. Therefore, it was decided to do another orthogonal composite design around the center point of 4.25% (w/w) of NaOH and 6-mL volume of NaOH/4 g WS with low and high value equivalents of 2.5 and 6.0 for % NaOH and low and high value equivalents of 4 and 8 for volume of NaOH solution (mL) required for the pretreatment. At the time of fermentation, the final water content in WS culture medium would be 76% after adding nutrient solution and inoculum. Figure 3 shows the FPA and βGA responses for % NaOH and volume of NaOH solution used for the pretreatment of 4 g WS. There was a maximum obtained in FPA (higher than 5 IU/mL) for % NaOH between 4.25 and 6.31, and water content between 4 and 6 mL/4 g WS. The βGA values were high for high NaOH concentrations regardless of the volume of NaOH solution used for the pretreatment. Although high βGA could

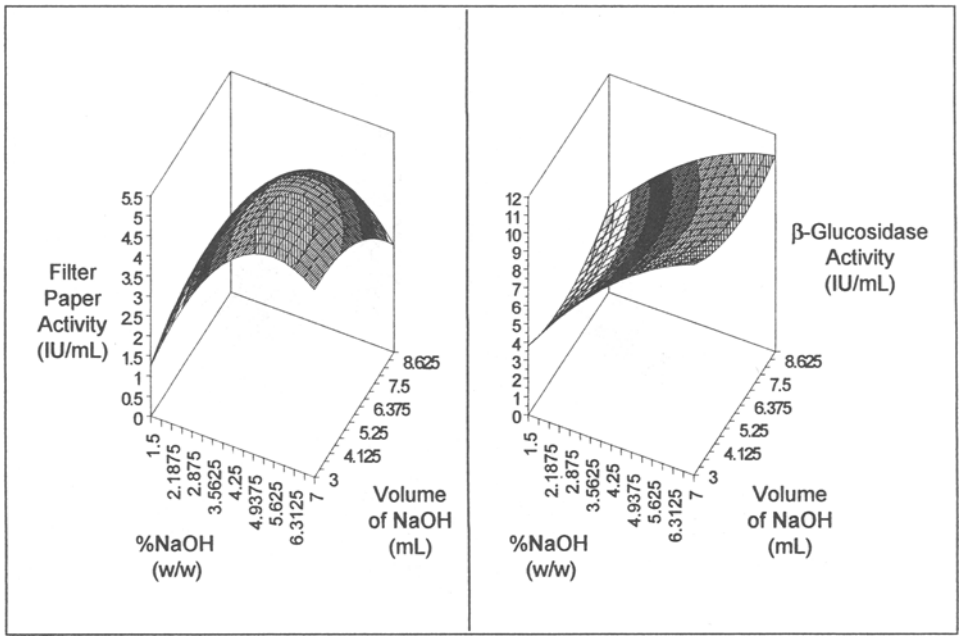

Fig. 3. FPA and βGA as a function of NaOH concentration and the volume of NaOH solution required for the pretreatment of WS in SSF process.

have been obtained by pretreating WS with higher NaOH concentration, it would not affect the overall hydrolysis of the cellulose, because the ratio of activities higher than 1.0 in the cellulase system does not increase the hydrolytic potential of the cellulase system *(3,5–7)*.

Development of Pan Bioreactor

Now there was a need to develop some means to grow *T. reesei* in such a way that a larger scale SSF could be tested. Therefore, a pan bioreactor was developed, the shape and the dimensions of which are shown in Fig. 4. In this bioreactor, a screen (60 mesh) was placed in the middle of the pan. A bed of pretreated WS mixed with medium was spread on the top of this screen. Forced aeration may be supplied from the bottom port leading to a cross with four slits on each side for uniform distribution of air under the screen (insert in Fig. 4). The upper port is for the exit of forced air or natural convectional air movement.

One set of experiments in flasks and two sets of experiments in pan bioreactors (one with forced aeration) were arranged by selecting the following best conditions (although some of the βGA was sacrificed by minimizing the NaOH concentration required for the pretreatment): the pretreatment used 4.25% NaOH (w/w) with 6 mL water/4 g WS, at 121°C for 1 h, the phosphate concentration was 1 × MM (= 2.0 g KH_2PO_4/10 g carbohydrates), the final moisture content of the WS medium was 76% at the time of fermentation, and the loading of WS in the Erlenmeyer flask and the pan bioreactor was 4 and 20 g, respectively. One set of the pan bioreactors was forced aerated. The results of LSF and SSF processes are summarized in Table 1. The best way to compare the activities of enzyme was to report the yields of activities, such as FPA and βGA/g WS supplied.

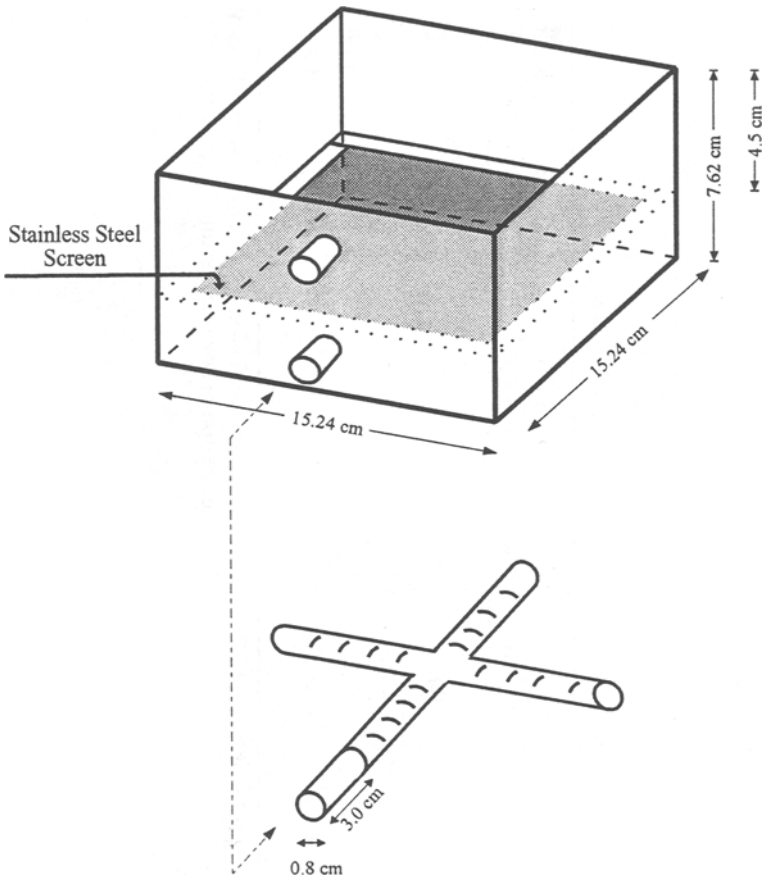

Fig. 4. Pan Bioreactor for the SSF of WS.

The FPA and βGA/g WS and their ratio in LSF in flasks were lower than that of SSF in flasks. In SSF the FPA/g WS in flask and pan bioreactor was the same after 21 d of fermentation. However, the βGA/g WS was slightly higher in the flask than in the pan bioreactor (Table 1). The values in the forced aerated pans were lower than the unforced aerated pan, probably because of the drying of the WS, which restricted the growth of the microorganism in certain regions of the pan bioreactor. It was decided to use humidified air in the future experiments. The ratios of the activities were close to 1.0 in all of the cases of SSF.

Hydrolytic Potential of the Cellulase System Produced in SSF

Figure 5 shows the hydrolysis of 5% delignified WS when loaded with the enzyme extracted from the SSF in the pan bioreactor at a concentration of 20 IU FPA/g delignified WS. A total of 4.33 g sugars/100 mL of hydrolysate were produced in 94 h of hydrolysis at 45°C with shaking at the rate of 200 rpm. The hydrolysate of 5 g delignified WS contained 4.33 g total sugars (3.2 g glucose, 0.8 g xylose, 0.08 g arabinose, and 0.25 g cellobiose) in 100 mL of solution. This represents the hydrolytic potential of over 85% with this cellulase system. Because of high βGA, very little accumulation of cellobiose (0.25 g/100 mL) in the hydrolysate was recorded with this cellulase system (Fig. 5). The accumulation of cellobiose in

Table 1

Cellulase Enzyme Yields in LSF and SSF in Erlenmeyer Flasks and Pan Bioreactors

Type of bioreactors	Amount of WS/ bioreactor, g	FPA[a] IU/mL	FPA[a] Total, IU	βGA[a] IU/mL	βGA[a] Total, IU	FPA, IU/g WS[b]	βGA, IU/g WS[b]	Ratio, β-GA/FPA
LSF								
Erlenmeyer flask	4	2.0	400	0.8	160	100	40	0.40
SSF								
Erlenmeyer flask	4	6.55	655	7.72	772	164	194	1.18
Pan bioreactor	20	6.53	3266	7.11	3555	163	178	1.09
Pan Bioreactor (Forced Aeration)	20	5.82	2912	5.61	2807	146	141	0.97

[a]Enzyme extraction: In Erlenmeyer flasks: 200 mL total water/4 g of WS in LSF process. In Erlenmeyer flasks: 100 mL total water/4 g of WS in SSF process. In Pan bioreactors: 500 mL total water/20 g of WS in SSF process. For high concentration of enzyme per unit volume, the volume of water required for extraction of enzyme should be reduced accordingly.

[b]WS contains 70% carbohydrate (30–35% cellulose, and 35–40% hemicelluloses).

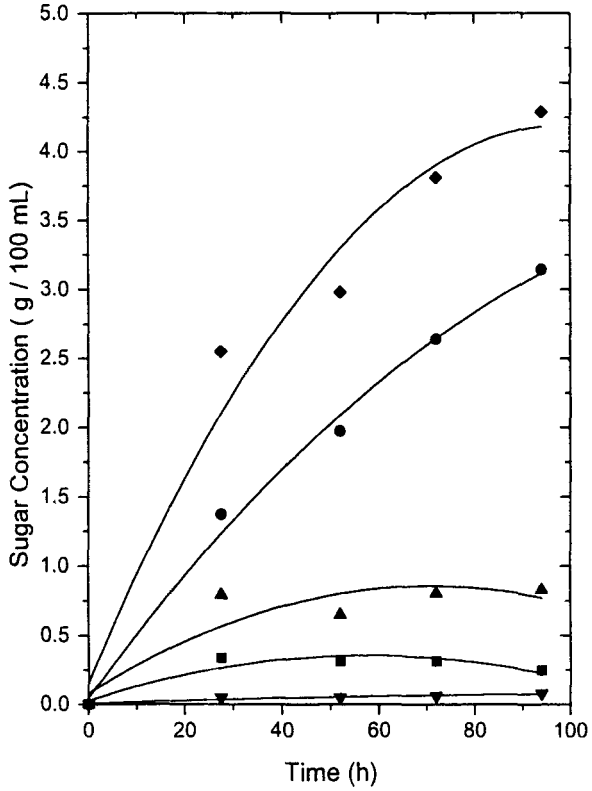

Fig. 5. Hydrolysis of delignified wheat straw by the enzyme system produced by 21 d of solid state fermentation in pan bioreactor. ■, Cellobiose; ●, glucose; ▲, xylose; ▼, arabinose; and ◆, total sugars.

the hydrolysate of cellulose is known to inhibit the cellulase action on cellulose *(8–10)*. The content of residual lignin in the delignified WS was not estimated; therefore, the hydrolytic potential of the cellulase system produced in SSF could be considered more than the reported 85% level.

Advantages of SSF Over LSF

The cellulase system produced in the conventional LSF is normally deficient in the βGA, and thus the ratio is usually <0.5. Therefore, it is necessary to add β-glucosidase from other sources to complement the cellulase system to obtain higher conversions of cellulose into sugars *(3,5–7)*. The LSF cellulase system is often very dilute, so there is a need for ultrafiltration to concentrate the enzyme so that reasonable loadings of the enzyme to substrate can be achieved, especially when the goal is to obtain reasonable glucose concentrations for subsequent ethanol fermentation. Furthermore, to achieve a large amount of enzyme, a large expensive fermenter is required to produce cellulase system in LSF.

In this study, SSF was achieved with minimum water (76% moisture content), thus requiring a very small fermenter (pan bioreactor). The cellulase system can be extracted by a plate and frame filtering unit by adding just enough water as required to achieve the desired enzyme concentration per unit volume. The cellulase system produced by SSF usually contains high βGA. Therefore, the

enzyme ratio close to 1.0 is easily obtained. The overall equipment cost for SSF is relatively less than that for LSF.

CONCLUSIONS

The following conclusions can be made as a result of this study:

1. A low-cost lignocellulosic substrate, WS, was successfully used instead of costly substrate (e.g., pure cellulose or lactose) to reduce the cost of cellulase production.
2. The SSF process appeared promising for obtaining high yields of a complete cellulase system and may be superior to the one obtained by LSF process.
3. To reduce the cost of enzyme production still further, a prototype biorector has been developed for SSF, which requires little capital investment. This prototype pan bioreactor requires some further modifications so that an optimum quantity of substrate, lignocellulosic materials, can be fermented to obtain high yields of complete cellulase system.
4. The SSF process provides a means for the production of complete cellulase system for the economical bioconversion of renewable biomass into ethanol.

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