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Solid Substrate and Submerged Culture Fermentation of Sugar Cane Bagasse for the Production of Cellulase and Reducing Sugars by a Local Isolate, *Aspergillus terreus* SUK-1

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Abstract Several process parameters were studied to ascertain the effect on degradation of sugar cane bagasse in relation to the production of cellulase enzyme and reducing sugars by Solid Substrate Fermentation (SSF) and Submerged Culture Fermentation (SCF) of *Aspergillus terreus* SUK-1. The effect of air-flow rate (0– 1.3 v/v/m), of different ratios of substrate weight to liquid volume (1:6, 1:10, 1:20, and 1:30 w/v, g/ml), scale-up effect (10, 20, and 100 times of 1:10 ratio, w/v) and the effect of temperature (30, 40, 50, and 60°C) in SSF were studied. Air-flow rate of 1.0 v/v/m gave the highest enzyme activity (FPase 0.25 IU/ml, CMCase 1.24 IU/ml) and reducing sugars concentration (0.72 mg/ml). Experiment using 1:10 ratio (w/v) was found to support maximum cellulase activity (FPase 0.58 IU/ml, CMCase 1.97 IU/ml) and reducing sugar concentration (1.23 mg/ml). Scaling-up the ratio of 1:10 (w/v) by a factor of 20 gave the highest cellulase activity (FPase 0.71 IU/ml, CMCase 2.25 IU/ml) and reducing sugar concentration (3.67 mg/ml). The optimum temperature for cellulase activity and reducing sugar production was 50°C (FPase 0.792 IU/ml, CMCase 2.25 IU/ml and 3.85 mg/ml for reducing sugar concentration). For SCF, the activity of cellulase enzyme and reducing sugar concentration was found to be lower than that obtained for SSF. The highest cellulase activity obtained in SCF was 50% lower than the highest cellulase activity in SSF, while for reducing sugar concentration, the highest concentration obtained in SCF was 90% lower than that obtained in SSF.

Key words: Local isolate, sugar cane bagasse, solid substrate fermentation, submerged culture fermentation

Cellulolytic microorganisms have been widely employed for the degradation of cellulosic material and agricultural wastes, the end products of the whole process being the substrate residue, cellulase enzyme, and fermentable sugars. The most efficient bioconversion of cellulose to produce cellulase enzyme and reducing sugars reported by Gadgill *et al.* [2] is via submerged culture fermentation either by a direct one-step process or by a multistage process. The well established SCF enzymatic process poses a major hindrance to its industrial application, because of its high cost of enzyme production [18]. An efficient production method employing a cheap and easily available substrate and a good cellulolytic microorganism will reduce the cost of enzyme production and will improve the economy of the overall cellulose bioconversion [1]. Based on the above report, Solid Substrate Fermentation (SSF) as a technique appears to be an efficient production method. Employment of production of concentrated enzyme solution in simple cultivation equipment using SSF has been reported [1, 8]. In the present study, we report a local strain isolated from palm oil waste, *Aspergillus terreus* SUK-1 [4], which is capable of degrading cellulosic materials. The general properties of crude enzyme preparation from *A. terreus* SUK-1 has been reported by Omar *et al.* [10]. The objective of the present paper is also to compare SCF and SSF in producing cellulase enzyme and reducing sugars, specifically pertaining to the effect of air-flow rate, the effect of substrate weight and liquid volume ratio, scale-up effect, and the effect of temperature in SSF, using sugar cane bagasse as the cheap and easily available substrate.

MATERIALS AND METHODS

Microorganism and Estimation of Growth

The local isolate *A. terreus* SUK-1 was obtained from the stock culture of the Microbiology Department (Faculty of Life science, UKM). The culture was maintained on Potato

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Dextrose Agar slants which was allowed to grow at 30°C for 3 days and then for 4 more days at room temperature (27°C) before storing at 4°C. Spore suspension of 10^7 spores/ml was used as inoculum. Estimation of growth was carried out by inspection during all the experiments.

Substrate

The substrate sugar cane bagasse was used as the sole carbon source. It was washed, dried, and milled (1–2 mm). No chemical pretreatment was carried out.

Medium

The medium containing $(NH_4)_2SO_4$ and urea was used as the nitrogen source (as developed by Mandels and Reese [7]). The medium is composed of $(NH_4)_2SO_4$ 1.4 g; KH_2PO_4 2.0 g; $CaCl_2$ 0.3 g; $MgSO_4$ 0.3 g; $FeSO_4 \cdot 7H_2O$ 0.005 g; $MnSO_4 \cdot 7H_2O$ 0.0016 g; $ZnSO_4 \cdot 7H_2O$ 0.0014 g; $CoCl_2$ 0.002 g; protease peptone 0.75 g; and Tween 80 1.0 g; per liter. The pH of the medium was adjusted to 5.5 at room temperature.

Cultivation under SCF

One-and-a-half grams of the substrate were dispensed in 200 ml of the medium (0.75% w/v) in 500-ml flasks. Air-flow rate was set in the range of 0.5–1.0 v/v/m and the flasks were agitated at 100 rpm using an orbital shaker (Certomat. R, Germany).

Cultivation and Optimization of Parameter for SSF

Effect of air-flow rate in SSF. Five grams of milled sugar cane bagasse was dispensed in 100 ml of Mandels medium (to obtain the ratio 1:20 w/v) in 500-ml flasks (8 flasks). Air at different flow rates (0–1.3 v/v/m) were sparged through the medium throughout the cultivation.

Effect of moisture content in SSF. Four ratios of substrate weight to medium volume (g:ml) were investigated (1:6, 1:10, 1:20, and 1:30). Air-flow rate was set on 1.0 v/v/m.

Effect of scale-up SSF. Three scale-up factors were used (10, 20, and 100) based on a 1:10 ratio (w/v, 6/60 g/ml) as scale-up factor 1. Five-hundred-ml flasks containing sterile water was used in all experiments to moisturize air inlet (1.0 v/v/m), and a sparger system for distributing air inside the cultures.

Effect of temperature in SSF. The solid media of 1:10 ratio (w/v) was subjected to different temperatures (40, 50, and 60°C) in a heated water baths (Certomat. W.R, Germany). Air at room temperature (27°C) was sparged through the culture at the rate of 1.0 v/v/m. Air was supplied into the cultures using sterile silicon tubes connected to an air pump (Precision, SR-7500). The air was passed through calibrated flow-meters (Gantt flow-meter) and there was a sterilized membrane filter (0.22 μm pore size) to ensure a sterile air supply reached the cultures. The fermentations were carried out for an 8-day duration for all experiments. For each experiment, 8 flasks were employed, and a whole flask per day was taken for analyzing cellulase activity, reducing sugars, and percentage of degradation.

Sampling

In SCF. The contents of each flask were filtered through Whatman no. 1 filter paper and then dried at 60°C for 24 h to obtain the percentage of degradation of bagasse substrate. The liquid portion was centrifuged at 10,000 rpm for 15 min and the supernatant was used for assaying cellulase activity and reducing sugars [3].

In SSF. For each gram of initial substrate weight, 10 ml of distilled water was used to dispense the fermented moldy pith [8]. The dispensed pith was shaken at 130 rpm at room temperature (27°C) for 1 h. The mixture was filtered through nylon cloth of 200 mesh and dried at 60°C for 24 h to measure the percentage of degradation of bagasse substrate. The liquid portion was centrifuged at 10,000 rpm for 15 min and the supernatant was used for assaying cellulase activity, β-glucosidase activity, and reducing sugars.

The percentage degradation was calculated as follows:

$$\% \text{ degradation} = \frac{\text{I.wt} - \text{F.wt}}{\text{I.wt}} 100$$

where, I.wt is the initial weight of the substrate and F.wt is the final weight after fermentation (dry basis), assuming weight contribution by biomass to be equal in all samples.

Analysis

The endoglucanase activity was measured as carboxymethyl cellulase (CMCase) using 3% carboxymethyl cellulose in 1.5 M acetate buffer, pH 5.0, while exoglucanase was measured as filter paper activity (FPase) using Whatman no. 1 filter paper (according to Mandels *et al.* [6]). Absorbances were read using a spectrophotometer (Hitachi 2000, Japan). β-Glucosidase activity was assayed according to the method of Wood and Bhat [19] using ρ-nitrophenyl-β-D-glucopyranoside as substrate. Incubation of 1 ml of the enzyme with 1 ml of the substrate in 0.2 M phosphate buffer at pH 6.0 for 20 min at 37°C was carried out. The activities are expressed as international units (IU), defined as the amount of enzyme required to produce one mmole of glucose or ρ-nitrophenol (in the case of β-glucosidase) per minute. The amount of reducing sugars released by the hydrolysis of bagasse was measured using the Nelson-Somogyi method [9, 15].

RESULTS AND DISCUSSION

Effect of Air-Flow Rate

SSF, operated at an optimized air-flow rate of 1.0 v/v/m, gave significantly higher values for cellulase activity, β-

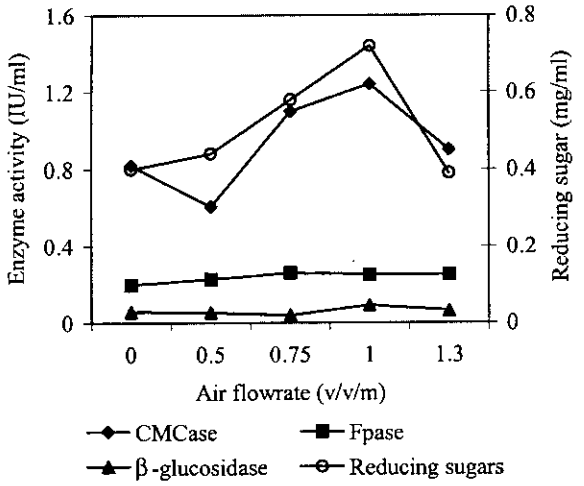

Fig. 1. Effect of aeration on maximum enzyme activities and reducing sugar concentration in SSF.
0.0 v/v/m: at 6th day of fermentation. 0.5 v/v/m: at 4th day of fermentation. 0.75 v/v/m: at 6th day of fermentation. 1.0 v/v/m: at 4th day of fermentation. 1.3 v/v/m: at 7th day of fermentation.

glucosidase activity, as well as reducing sugar production compared to SCF (at optimized air-flow rate). The highest activities of FPase, CMCase, and β-glucosidase were 0.25, 1.24, and 0.094 IU/ml, respectively (Fig. 1). Reducing sugar concentration was 0.72 mg/ml. These values of cellulase activity and reducing sugar concentration were obtained after 4 days of fermentation. For SSF, enzyme activities and reducing sugar concentrations were found to decrease at 1.3 v/v/m. Sargantanis *et al.* [13] claimed that the increase in air-flow rate produced a smoother effect on biomass production. The SSF performance improves at high air-flow rates, but the biomass content does not reach

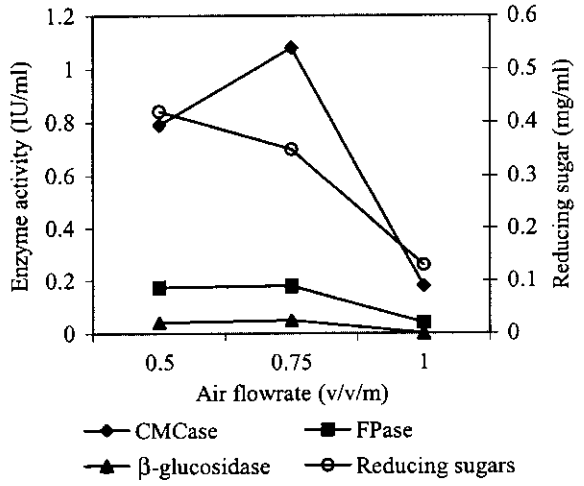

Fig. 2. Effect of aeration on maximum enzyme activities and reducing sugars concentration in SCF.
0.5 v/v/m: at 6th day of fermentation. 0.75 v/v/m: at 7th day of fermentation. 1.0 v/v/m: at 2nd day of fermentation. *Symbols are the same as in Fig. 1.

Table 1. Comparison of SSF and SCF for the production of cellulase enzyme, β-glucosidase (β-Glu), and reducing sugars at optimized air-flow rates (0.75 v/v/m in SCF and 1.0 v/v/m in SSF).

	Enzyme activity (IU/ml)			Reducing sugars concentration (mg/ml)
	FPase	CMCase	β-Glu	
SSF	0.25	1.24	0.09	0.72
SCF	0.18	1.08	0.05	0.42

the best levels due to reduction in moisture content caused by the high air-flow rate.

In the case of SCF, the data shows that an air-flow rate of 0.75 v/v/m gave the highest activity of cellulase enzyme (Fig. 2), whereas 0.5 v/v/m was better for reducing sugar production (0.42 mg/ml). The highest activities of FPase, CMCase, and β-glucosidase were 0.183, 1.075, and 0.048 IU/ml, respectively. These amounts were produced after 7 days of fermentation. When the air-flow rate was increased to 1.0 v/v/m, high foam formation was observed and the experiment was stopped due to contamination and reduction in enzymatic activity.

Comparison between SSF and SCF based on cellulase enzyme activities, β-glucosidase activity, and reducing sugar concentration at two air-flow rates was carried out (Table 1). All the enzyme activities obtained in SSF were found to be more active than those obtained in SCF. This may be due to the greater amount of solids in SSF than in SCF (10% dry matter versus 0.075%). However, it must be mentioned that the total units obtained in SCF was several times more than that obtained in SSF (it was also recorded by Rao *et al.* [12]). Hence, although the enzyme activity in the solution obtained by SSF was almost twice the activity of the culture filtrate obtained by the SCF, the total activity was much less. It is also important to mention that the productivity of cellulase in SSF is much better than that in SCF. These results support the effectiveness of aeration in SSF.

Aeration fulfills four major functions in SSF, namely (a) to maintain aerobic conditions, (b) to desorb carbon dioxide, (c) to regulate substrate temperature, and (d) to regulate the moisture level. The gaseous environment may significantly affect the relative levels of biomass and enzyme production [17]. In aerobic SCF, oxygen supply is often the limiting growth factor due to the low solubility of oxygen in water. In contrast, a solid state process allows free access of atmospheric oxygen to the substrate. Therefore, aeration may be easier than in SCF because of the rapid rate of oxygen diffusion into the water film surrounding the insoluble substrate particles, and also because of the very high surface of contact between gas phase, substrate, and aerial mycelium [17].

Effect of Moisture Content in SSF

The maximum activities of cellulase were obtained when the moisture content was 10 times the weight of the substrate by volume (Fig. 3). When ratios increased up to

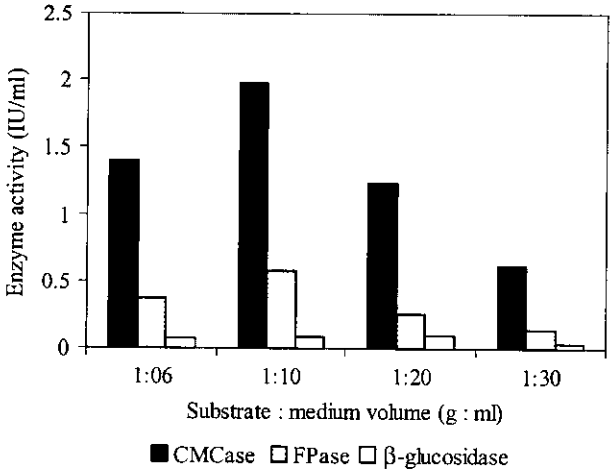

Fig. 3. Effect of substrate:medium ratio (w/v) on maximum enzyme activities in SSF.
1:6 at 7th day of fermentation. 1:10 at 4th day of fermentation. 1:20 at 4th day of fermentation. 1:30 at 7th day of fermentation.

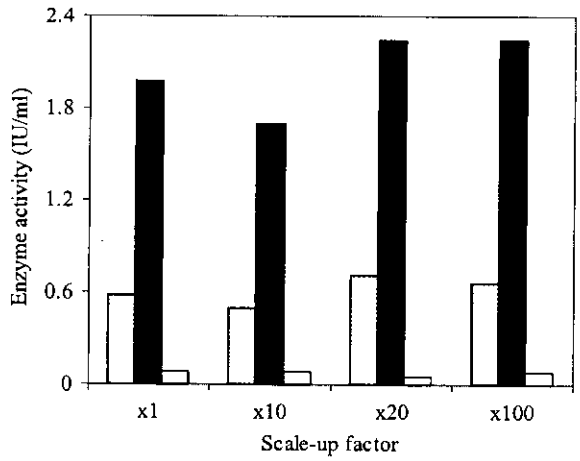

Fig. 5. Effect of scale-up of SSF process on maximum enzyme activities.
x1: 7th day of fermentation. x10: 4th day of fermentation. x20: 4th day of fermentation. x100: 7th day of fermentation. *Symbols are the same as in Fig. 3.

1:6 (w/v), the cultures appeared dry and the growth was low (no appreciable colonization of substrate), probably due to insufficient nutrients and water. A lower moisture level decreases porosity, enhances the formation of liquid mycelium, and creates an additional barrier to the diffusion of microorganism into the substrate [8]. Too much water decreases porosity and oxygen diffusion into the substrate mass, and can also favor contamination [11]. Experiments using 1:10 (w/v) ratio gave the highest FPase and CMCase activities of 0.58 and 1.975 IU/ml after 7 days of fermentation, respectively, and the maximum β-glucosidase activity (0.08 IU/ml) was obtained in 3 days. For reducing sugars, the maximum production was also obtained in 1:10 (w/v) ratio (Fig. 4), and the maximum concentration was 1.23 mg/ml after 4 days of fermentation.

Effect of Scale-up of SSF

From the obtained results, a scale-up factor of 20 gave the maximum activities of cellulase (0.71 IU/ml for FPase, 2.25 IU/ml for CMCase, and 0.036 IU/ml for β-glucosidase) after 4 days of fermentation (Fig. 5). In the case of 100 times, scale-up cellulase activities were found to be almost the same as that produced at the 20 times scale-up factor, but these activities were produced after 7 days of fermentation. This shows that 20 times scale-up is better for cellulase production compared to 100 times scale-up in terms of short fermentation time. The highest reducing sugar production was also obtained at 20 times scale-up. The maximum reducing sugar production was 3.67 mg/ml after 4 days of fermentation (Fig. 6).

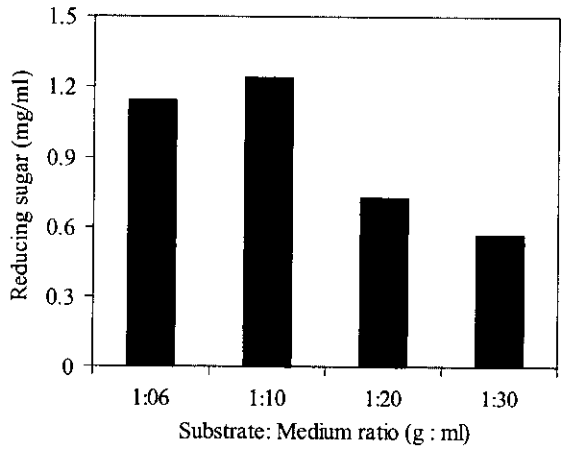

Fig. 4. Effect of substrate:medium ratio (w/v) on maximum reducing sugar concentration in SSF.
1:6 at 7th day of fermentation. 1:10 at 4th day of fermentation. 1:20 at 4th day of fermentation. 1:30 at 7th day of fermentation.

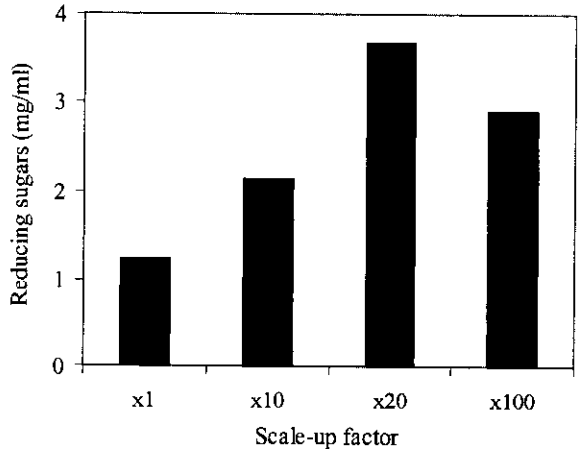

Fig. 6. Effect of scale-up of SSF on maximum reducing sugar concentration.
x1: 4th day of fermentation. x10: 7th day of fermentation. x20: 4th day of fermentation. x100: 7th day of fermentation.

Some factors such as heat build up, poor aeration, and few active contact between microorganism/enzymes and substrate work disadvantageously for large-scale of SSF [1]. Laukevics and Viesturs [5] claimed that moisture can be used for evaporative heat removal which is considered to be the most crucial factor in large-scale SSF processes. Moist air inlet and sparger system were used in all experiments for evaporative heat removal and regulation of moisture content in the cultures.

Effect of Initial Temperature on SSF

The highest cellulase activities (Fig. 7) were obtained at the temperature of 50°C (0.792 IU/ml for FPase, 2.25 IU/ml for CMCase in 3 days of fermentation, and 0.027 IU/ml for β-glucosidase in 7 days). The maximum reducing sugar production was 3.85 mg/ml in the fifth day of fermentation at a temperature of 50°C (Fig. 8). The best fungus growth was noticed at 30°C, and there was a high β-glucosidase activity after 3 days of fermentation and the maximum amount of reducing sugar obtained was 1.23 mg/ml at the fourth day of fermentation. Increasing the temperature up to 50°C, the enzyme was more active, since cellulase activity was the highest at 50°C (Fig. 7) and the reducing sugars concentration was also the highest at 50°C (Fig. 8). A high temperature inhibits germination of spores, but did not significantly affect mycelium growth [11]. Smith *et al.* [14] claimed that enzymes have an optimum temperature for maximum activity and, if the temperature rises above the optimum, activity decreased due to thermal denaturation of the enzyme. Yazdi *et al.* [20] found that the optimum temperature for cellulase activity from *Neurospora crassa* ranged between 45 and 65°C, and Steiner *et al.* [16] claimed that enzymes from *Penicillium purpurogenum* were stable up to 50°C. Also, Omar *et al.* [10] reported that

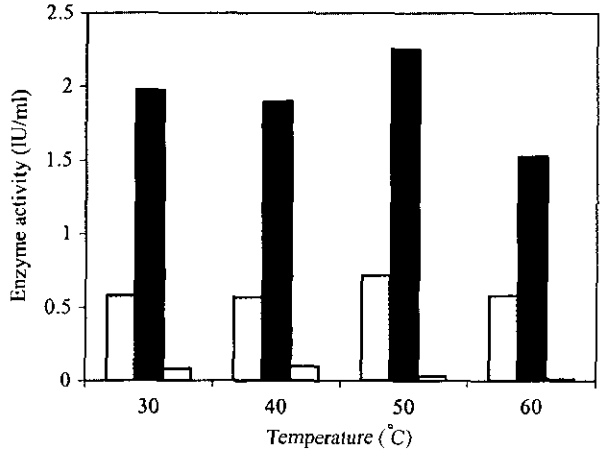

Fig. 7. Effect of temperature on maximum enzyme activities in SSF.
30: 7th day of fermentation. 40: 7th day of fermentation. 50: 3rd day of fermentation. 60: 4th day of fermentation. *Symbols are the same as in Fig. 3.

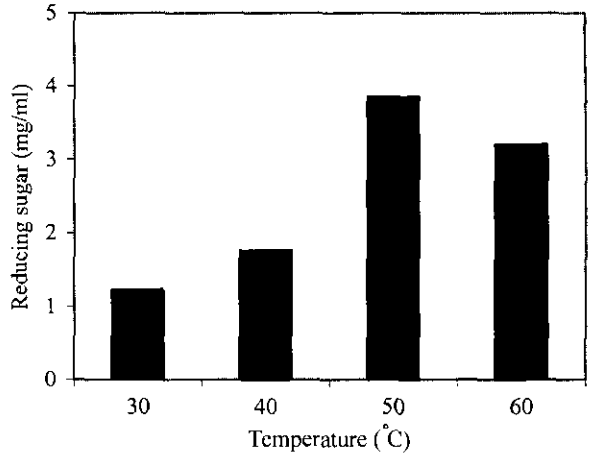

Fig. 8. Effect of temperature on maximum reducing sugar concentration in SSF.
30: 4th day of fermentation. 40: 7th day of fermentation. 50: 5th day of fermentation. 60: 5th day of fermentation.

cellulase from different *Aspergillus* sp. including *A. terreus* SUK-1 was stable up to 50°C. There was no appreciable temperature changes throughout the experiments due to continuous air supply through the cultures.

Comparison of SSF with SCF

From our investigations of the cultural conditions (i.e. airflow rate, substrate weight medium volume, and temperature) in SSF, it was found that using the local SSF is a process superior to SCF for cellulase enzyme and reducing sugar production of the *Aspergillus* strain. The data in Table 2 indicates that the activity of cellulase and reducing sugar concentration obtained in SSF cultures is higher than that obtained in SCF. The highest cellulase and β-glucosidase activities obtained in SCF (0.26 IU/ml FPase, 1.075 IU/ml CMCase, and 0.048 IU/ml β-glucosidase) was 50% lower than the highest activities obtained in SSF (0.792 IU/ml FPase, 2.25 IU/ml, and 0.097 IU/ml β-glucosidase). The highest reducing sugar concentration obtained in SCF (0.42 mg/ml) was around 90% lower than the highest concentration obtained in SSF (3.85 mg/ml). The productivity data of

Table 2. Results of cellulase and β-glucosidase activities, productivity, and reducing sugars concentration in SSF and SCF after optimizing SSF.

		SSF	SCF
Enzyme activity IU/ml	FPase	0.79	0.18
	CMCase	2.25	1.08
	β-Glu.	0.10	0.05
Enzyme productivity IU/l · h	FPase	11.00	1.09
	CMCase	31.25	6.40
	β-Glu.	1.35	0.28
Reducing sugar conc. (mg/ml)		3.85	0.42
% of degradation		29.00	33.00

cellulase enzyme in Table 2 were taken on the third day of fermentation in SSF and on the 7th day of fermentation in SCF. The limitation in SSF arises from enhanced cellulose degradation, which was only around 29% in our experiments, while degradation was more than 33% in SCF. This result was in support with the reports of others [5]. The limitation may be imposed by glucose repression of cellulase production and by a lower surface area for enzymatic reaction in SSF compared to SCF [11]. Solid substrate fermentations have an advantage of low capital cost and energy expenditure, as well as absence of waste water output and of foaming, which causes problems in SCF.

In conclusion, the best air-flow rate (for more activities of cellulase enzyme and reducing sugars production) for SSF was found to be 1.0 v/v/m, whilst for SCF 0.75 v/v/m was the best for cellulase production and 0.5 v/v/m in the case of reducing sugars production. In SSF, the best ratio of substrate weight to medium volume was 1:10 (w/v) with a scale-up factor of 20 times for the highest cellulase activity and reducing sugar production. The optimum temperature for growth of the microorganism in SSF was 30°C, while 50°C was better for reducing sugar production.

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