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Biocoversion of Oil Palm Empty Fruit Bunch by *Aspergillus niger* EB4 under Solid-state Fermentation

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ABSTRACT

Oil palm empty fruit bunch (OPEFB) is an abundant lignocellulosic waste material generated from the palm oil industry. In this study, a locally isolated strain *Aspergillus niger* EB4 was cultivated on a pre-treated OPEFB as substrate to produce cellulase in the solid-state fermentation (SSF) process. The cellulase recovered was then subjected to a saccharification process. The strain was grown on the pre-treated OPEFB in a 250 ml Erlenmeyer flask and a 192 L tray cabinet bioreactor, at 80% moisture content and incubated for nine days under a static condition. The activities of the crude cellulase extract in the tray bioreactor were 19.02 ± 0.85, 6.36 ± 0.38 and 4.56 ± 0.26 U/g for β-glucosidase, CMCase and FPase, respectively on day 6 of fermentation. These results were similar to the results obtained from the flask experiment. The results demonstrated the feasibility of solid substrate fermentation of the OPEFB in both flask and tray cabinet bioreactor for the cellulase production. The enzymatic hydrolysis of OPEFB at 5% (w/v) was performed by utilizing the partially purified and crude cellulase incubated at 40°C for seven days. The partially purified cellullases hydrolyzed the pre-treated OPEFB and released 7.7 g/l of reducing sugar which corresponded to a 15% conversion.

Keywords: Cellulase, oil palm empty fruit bunch, *Aspergillus niger*, solid-state fermentation

INTRODUCTION

Cellulase is responsible for the hydrolysis of cellulose and its production is the most important step in the economical production of bio-ethanol, single cell protein and other chemicals. It is a complex mixture of enzyme with different specificities to hydrolyze glycosidic bonds. At present, cellulase is widely used in food, animal feed, beverage, textile and laundry, pulp and paper, as well as agriculture and for research purposes.

Malaysia produces an abundant supply of lignocellulosic OPEFB waste which has not been satisfactorily utilized. Approximately

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2.4 millions tonnes of OPEFB are generated annually. OPEFB consists of mainly cellulose (50.4%), hemicellulose (21.9%), lignin (10%), and ash (17.7%) (Umi Kalsom *et al*., 1997).

The development of a technology with a minimum capital investment is another approach to reduce the cost involved in producing cellulase. This can be accomplished by producing cellulase in solid-state fermentation (SSF), which requires relatively inexpensive equipment as compared to the conventional fermentor in liquid-state fermentation (LSF). The SSF technique has also been shown to reduce energy requirement, improve product recovery, and decrease wastewater output (Hamidi-Esfahani *et al*., 2004). Moreover, these conditions favour the growth of filamentous fungi, which typically grow in nature on solid substrates, such as organic natural materials.

In this study, the production of cellulolytic enzyme by locally isolated *Aspergillus niger* EB4, cultivated on the pre-treated OPEFB in the SSF was investigated. The performance of the locally designed tray cabinet bioreactor for the production of cellulase and the feasibility of saccharification, using crude and partially purified cellulases on the OPEFB were also studied.

MATERIALS AND METHODS

Strain and Inoculum Preparation

The locally isolated fungus *Aspergillus niger* EB4 was obtained from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. The culture was maintained on potato dextrose agar (PDA) and incubated at room temperature (21˚C – 26˚C) for seven days. The inoculum was prepared aseptically by adding 10 ml of sterile distilled water, containing 0.1% Tween 80 on the mycelial agar. A spore suspension was measured using a haemocytometer. The inoculum was prepared freshly prior to the experiments. Pre-germination was carried out by inoculating 10% (v/v) of spore suspension to give an initial concentration of 1×10^6 spores per ml of basal medium at pH 5.5. Carboxymethylcellulose (CMC) at 1% (w/v) was added into the basal medium as a substrate. The cultures were pre-germinated on a rotary shaker at 180 rpm at 27 ± 2˚C for 24 h.

Substrate Treatment

The shredded oil palm empty fruit bunch (OPEFB) was obtained from a local palm oil mill in Selangor, Malaysia. The OPEFB was thoroughly washed to remove dust and then sun dried. The shredded OPEFB was reduced to 2 mm length, using a hammer mill. Then, the OPEFB fibres were further treated with 2% (w/v) of sodium hydroxide (NaOH). In this procedure, 50 g of OPEFB was soaked in 500 mL of 2% (w/v) NaOH at 30˚C for 4 h, followed by filtering and rinsing it with distilled water until it was completely free of alkali and then dried at 90˚C for 48 h. The cellulose, hemicellulose and lignin contents in the OPEFB were determined using the gravimetric method as described by Gorring and Van Soest (1970).

Solid-state Fermentation

The solid-state fermentation (SSF) was conducted in a 250 mL Erlenmeyer flask containing 5 g of pre-treated substrate. The flasks were autoclaved at 121˚C, 15 psi for 15 min. Each flask was added with 3 mL of nutrient supplement as follows: 0.5% (v/v) $(NH_4)_2SO_4$, 0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$, 1% (w/v) $CaCO_3$. An inoculum of blended mycelial suspension (10 mL) of *A. niger* EB4 was aseptically inoculated into the flask. At the initial stage of fermentation, the moisture content and pH was set at 80% and 7, respectively. All the flasks were incubated at 30˚C, under a static condition for nine days of fermentation. The control experiment was carried out by excluding the mycelial suspension in the mixture of substrate and fermentation medium. The solid-state fermentation was also performed in a stainless steel tray cabinet bioreactor (width 0.40 m; length 0.60 m; height 0.8 m) with the capacity of 192 L, as shown in *Fig. 1*. The bioreactor consisted of three

perforated trays. Meanwhile, the total weight of the OPEFB for the three perforated trays was 1.05 kg. The OPEFB was placed in a 3 L beaker, sterilized at 121˚C, 15 psi, for 15 min. After cooling, 0.49 L of sterile distilled water, 0.21 L nutrient supplement and 0.7 L pre-germinated fungus were added to the substrate to achieve 80% of the initial moisture content. The well-mixed substrate and fermentation medium were divided into three portions with the same weight and then poured onto each sterile tray to form a thin layer (less than 3 cm) in a sterile condition. Then, the trays were immediately stacked in the cabinet. Meanwhile, the solid state fermentation was carried out at the room temperature (21 – 26˚C) without aeration and static condition. The sampling was carried out aseptically using a sterile sampler. The samples, which were taken from each tray at approximately the same amount, were combined and kept at 4˚C prior to the analysis. The control experiment was conducted by excluding the mycelial suspension on the mixed substrate and fermentation medium.

Extraction Procedures and Analysis

The fermented substrate (5 g) was extracted using 50 mL of 0.01 M phosphate buffer (pH 7) at 4˚C. The extraction was done using a homogenizer at 9700 rpm for 4 min. The culture slurry was then centrifuged at 5000 rpm, at 4˚C for 20 minutes, and the supernatant was stored at -20˚C before the analysis (Shahrim *et al*., 2008; Latifian *et al*., 2007). The CMCase activity was measured according to Dong *et al*. (1992), while the FPase was determined based on Elshafaei *et al*. (1990). After 30 min of incubation at 50˚C, the reducing sugar, liberated in the reaction mixture was measured by the dinitrosalicyclic (DNS) method (Miller, 1959). β-glucosidase was determined based on the procedure suggested by Dong *et al*. (1992). The substrate used for the determination of β-glucosidase activity was p-nitrophenol-β-Glucopyranoside. One unit (U) of the enzyme activities was defined as the amount of enzyme required to liberate 1 µmol of product per min. The enzyme yield was expressed as U/g of dry substrate. The reducing sugars concentration

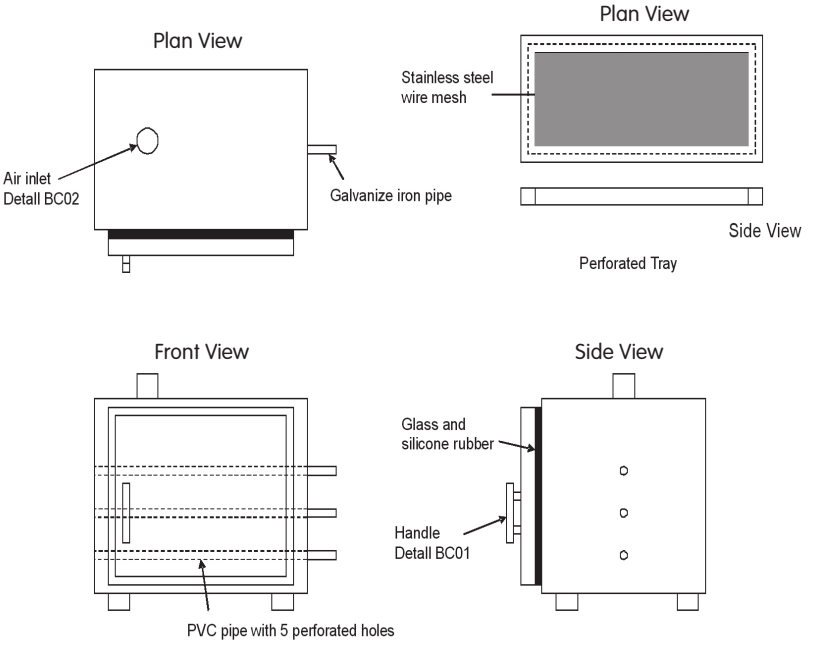

Fig. 1: Schematic diagram of tray bioreactor for SSF

was analyzed according to the DNS method (Miller, 1959). The soluble protein content was determined based on the method proposed by Lowry *et al*. (1951).

Cellulase Recovery and Saccharification of OPEFB

The crude cellulase of *A. niger* EB4 was obtained from the fermentation process in the tray bioreactor. The crude enzyme was assayed before subjecting to the saccharification process and precipitated using 80% ammonium sulphate saturation. The ammonium sulphate precipitation was done according to the method described by Harris (1989). The enzymatic saccharification of OPEFB was carried out in a water bath shaker (Protech Ltd.) at 40˚C and agitated at 100 rpm. For the saccharification experiment, 5 g of the treated OPEFB was placed into a 250 ml Erlenmayer flask, after which 30 ml of crude or partially purified enzyme in 70 ml 0.01 M phosphate buffer (pH 7) was added to obtain 5% (w/v) substrate suspension. Sodium azide (0.02%, w/v) was added to the reaction mixture to prevent bacterial or fungal contamination. Samples (3 ml) were collected every 24 h for the seven days of incubation. The collected sample was centrifuged for 10 min at 5000 rpm. The supernatant was used to determine the reducing sugars. The control experiment was done by excluding the enzyme in the flask and replacing with the buffer solution. The data were the average of three replicates.

RESULTS AND DISCUSSION

Soluble Protein, Reducing Sugars and pH Profile in Flask

The soluble protein of the culture extract during the fermentation is shown in *Fig. 2a*. The soluble protein was highest on the seventh day of fermentation. It should be noted that the increase of the protein content in the culture extract might probably be due to the growth of fungus and the secretion of enzymes such as cellulase. Other extra-cellular enzymes, such as hemicellulases involved in the degradation of the OPEFB, might have also contributed to the soluble protein content. The reducing sugar gradually increased and reached the maximum value on day 6 of fermentation (16.2 mg/g), indicating substrate conversion (*Fig. 2b*). The reducing sugar was found to decrease after day 6 and this correlates with the decrease of the enzyme activities (*Fig. 3*). However, the reducing sugar yield was not a linear function of the concentration of enzyme in the mixture. The pH of the fermented substrate was found to decrease towards the end of the fermentation period. After six days of fermentation, the final

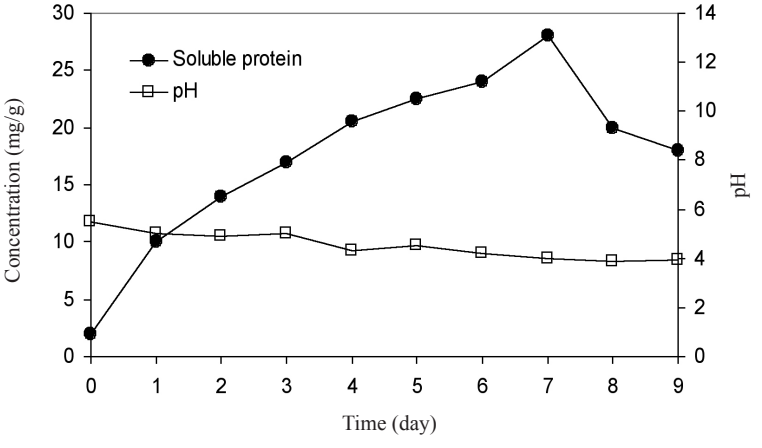

Fig. 2a: Profile of soluble protein, pH during SSF of A. niger *EB4 grown on OPEFB*

Fig. 2b: Profile of reducing sugar during SSF of A. niger *EB4 grown on OPEFB*

pH detected was in the range between 3.5 and 4.0 (*Fig. 2a*). Most of the filamentous fungi, especially *Aspergillus* and *Trichoderma* strains, were able to grow and metabolize in such a pH range (Jecu, 2000).

Cellulase Production in Solid-State Bioconversion

The lignocellulosic composition of the treated OPEFB used in this study consisted of cellulose (50.7%), followed by hemicellulose (20.4%) and lignin (10%). Based on the composition, the OPEFB could serve as a good substrate for the production of cellulase. Alkali treatment with 2% NaOH was applied on the substrate for delignification. It was reported that 2% NaOH could demolish the structure of cellulose (Yang *et al*., 2004). Filamentous fungi have the ability to penetrate effectively into the intracellular and intercellular spaces of the solid substrate. In the SSF, the pre-treatment of the solid substrate, by either mechanical or chemical means, was employed to improve its amenability to microbial modification by forming smaller permeable molecules, providing sites for easy microbial penetration (Lonsane *et al*., 1992).

The time course study of the cellulase activities of *A.niger* EB4 grown on pre-treated OPEFB in flask is shown in *Fig. 3*. In this study,

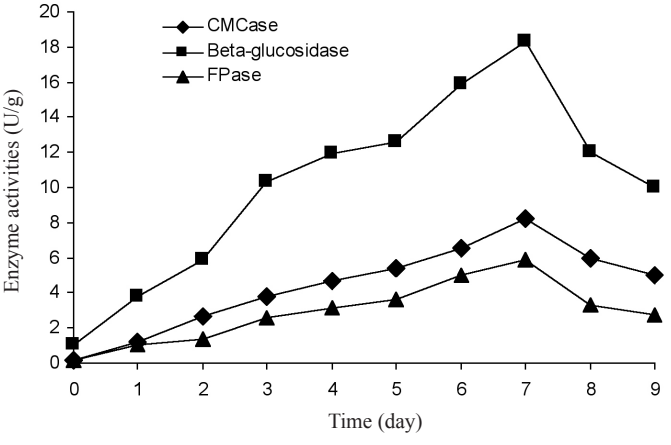

Fig. 3: Time course of the cellulase activities of Aspergillus niger *EB4 grown on OPEFB in SSF at 30˚C*

the cellulase activity was gradually increased and it achieved the maximum value on day six of cultivation and it then gradually declined. The time required to reach the maximum levels of the activity may be affected by several factors, including the presence of the different ratios of amorphous to crystalline cellulose (Gao *et al*., 2008). The highest activity for CMCase, FPase and BGDase were 8.24 U/g, 4.32 U/g and 19.07 U/g, respectively. The decrease in the activity after day 7 might be due to feedback repression by cellobiose. The cellulase activities produced by *A. niger* EB4 and grown on OPEFB in the SSF were comparable with other the cellulase producing fungi (Table 1). The SSF of rice chaff by a microbial consortium showed the highest FPase activity at 5.64 U/g (Yang *et al*., 2004). Alam *et al*. (2005) reported the highest cellulase activity of 0.0433 unit by *Trichoderma harzianum* grown on OPEFB in flask at day 9 in the SSF. The efficiency of enzymatic degradation is also dependent on the chemical composition of the substrate, and the composition of the individual enzymes of the total cellulase (Krishna, 1999; Gao *et al*., 2008).

The experiment of the SSF, using a tray bioreactor, was also conducted in this study. Although the tray bioreactor was conducted at the room temperature, the cellulase activities obtained were comparable to the activities in the flasks. On day 7, the highest FPase, CMCase and β-glucosidase obtained were 4.56, 6.36 and 19.02 U/g, respectively. It should be noted that the cellulase activities for the tray bioreactor were not much affected by uncontrolled temperature (room temperature). The results obtained in this study indicated the effectiveness of the tray bioreactor for the production of cellulase using a simple and economical SSF system.

Enzyme Recovery and Hydrolysis of OPEFB

The ammonium sulphate precipitation, at different saturations for cellulase from *A.niger* EB4, is shown in *Fig. 4*. The results showed that 80% ammonium sulphate saturation gave the highest concentration of the soluble protein and the cellulase activity. The enzyme activities of the crude and partially purified cellulase are shown in Table 2. For enzyme recovery, using 80% ammonium sulphate precipitation, it was shown that 8.1% (2.1 fold purification) was achieved for β-glucosidase, while CMCase showed 7.7% (2 fold purification). As for FPase, only 5.8% recovery with 1.5 fold purification was obtained. The sacharification of the OPEFB

TABLE 1
Comparison of cellulase production from different fungi in SSF

Substrate	Fungal strain	Enyme	Enzyme Yield (U/g substrate)	Reference
Rice bran	*Trichoderma reseei* MGG77	FPase	2.314	Laitifian *et al*. (2007)
Wheat ban	*A. ustus*	FPase CMCase β-glucosidase	5.6 14.0 12.9	Sharmala and Sreekantiah (1987)
Wheat straw	*Thermoascus aurantiacus*	FPase CMCase β-glucosidase	5.5 1709.0 79.0	Kalogeris *et al*. (2003)
Oil empty palm fruit bunch	*A. niger EB4*	FPase CMCase β-glucosidase	4.56 6.36 19.02	This study

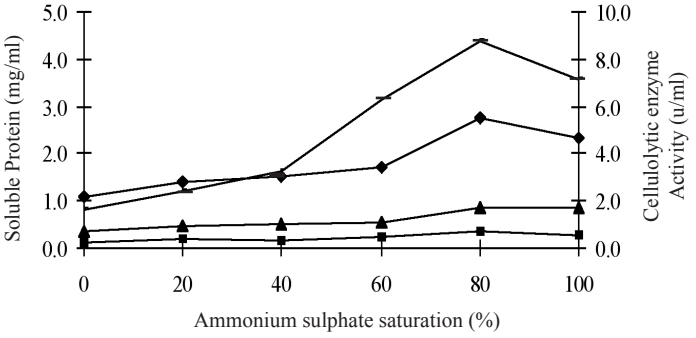

Fig. 4: Ammonium sulphate precipitation for cellulase at various saturations

TABLE 2
Partial purification of cellulase from *A.niger* EB4, using 80% ammonium sulphate saturation

Enzyme	Sample	Activity (U/ml)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
β-glucosidase	Crude filtrate	3.17	0.63	100	1.0
	Partially Purified	5.12	1.35	8.1	2.1
CMCase	Crude filtrate	1.06	0.21	100	1.0
	Partially Purified	1.64	0.43	7.7	2.0
FPase	Crude filtrate	0.53	0.11	100	1.0
	Partially Purified	0.62	0.16	5.8	1.5

was performed using crude and precipitated cellulase. The enzymatic hydrolysis process was monitored by the amount of reducing sugar released.

The profile for the saccharification of the pre-treated OPEFB, using crude and partially purified cellulase from *A. niger* EB4 is shown in *Fig. 5*. The enzymatic hydrolysis of the OPEFB showed a typical upward trend in the rapid release of soluble sugars for four days of fermentation and it then reached a plateau at day 5 onwards. The concentration of reducing sugars was constant after five days of incubation for both the crude and partially purified cellulases. The maximum reducing sugars produced from the OPEFB saccharification, using crude and partially purified cellulase from *A. niger* EB4 are listed in Table 3. The maximum production of the reducing sugars was 7.7 g/l, which corresponded to a yield of 0.15 g/g. The highest glucose concentration produced from the saccharification of the pre-treated OPEFB, obtained for both the crude and partially purified cellulose were 2.62 g/l and 4.87 g/l, respectively; these corresponded with the yield of 0.06 and 0.10 g/g, respectively. The partially purified enzyme gave a better yield of reducing sugars as compared to the crude enzyme in the saccharification experiment. The bioconversion of the pre-treated OPEFB into sugars by the partially purified cellulase was 15%. Baig *et al*. (2004) reported that enzymatic saccharification by precipitated cellulose, containing 0.2 U/ml FPase, 0.41 U/ml CMCase, 0.24 U/ml β-glucosidase from *T. lignorum,* gave banana waste conversion of 15%. The results obtained in this study proved that lignocellulose OPEFB could be used as a potential substrate for enzymatic saccharification.

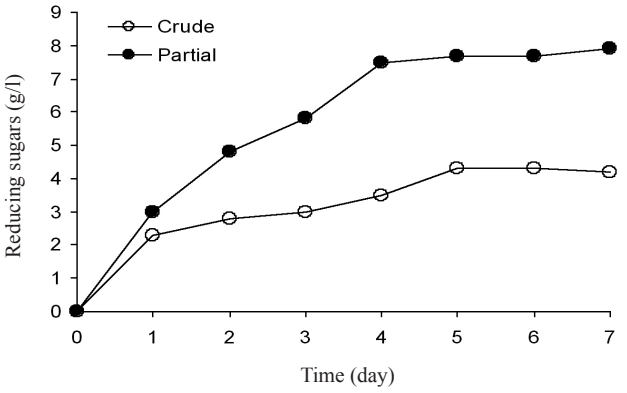

Fig. 5: Time course of the saccharification of the OPEFB, using crude and partially purified cellulase from A. niger *EB4*

TABLE 3
Reducing sugars production in enzymatic saccharification of OPEFB by the crude and partially purified cellulase from *A.niger EB4*

Enzyme	Total reducing sugars		
	Production (g/l)	Yield (g/g substrate)	Bioconversion (%)
Crude	4.37	0.09	9
Partially Purified	7.70	0.15	15

CONCLUSIONS

A. niger EB4 was successfully grown on the pre-treated OPEFB and produced cellulase in both the flask and tray cabinet bioreactors. The activities of the crude cellulase extract in the tray bioreactor were 19.02 ± 0.85, 6.36 ± 0.38 and 4.56 ± 0.26 U/g for β-glucosidase, CMCase and FPase, respectively, and these were almost similar to the results obtained in the flask. The saccharification of the OPEFB, by the partially purified enzyme, gave a higher yield of reducing sugars than the crude enzyme. The maximum production of reducing sugars was 7.7 g/l, which corresponded to 0.15 g/g of the reducing sugars yielded and 15% conversion. The present study indicated that the OPEFB was suitable to be converted into sugars by the cellulase produced from locally isolated fungus.

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