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Enzymatic Hydrolysis of Palm Oil Mill Effluent Solid Using Mixed Cellulases from Locally Isolated Fungi

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Abstract: In order to optimize the enzymatic hydrolysis of POME solid, the effects of substrate pretreatment using varying concentrations of sodium hydroxide and sulfuric acid, crude enzyme from both strains in different ratio and pH reaction were studied. The best experimental conditions found to degrade POME solids were 12 h incubation time, 0.5% (v/v) sulfuric acid pretreatment, crude enzymes mixture from *Aspergillus niger* EB5 and *Trichoderma* sp. EB6 (1.75 mL Asp+0.25 mL *Tri* with the total cellulase activity equal to 14.76 IU) and incubation pH at 5.0. Under these conditions, the reducing sugar concentration reached 23 g L^{-1} with the hydrolysis yield and productivity at 32% and 1.90 g L^{-1} h^{-1}, respectively. The bioconversion of POME solid to reducing sugar by the mixture of crude enzyme from the strains was relatively higher by almost 2 folds as compared to commercial cellulase. The results suggested that the crude cellulases mixture from locally isolated fungi has potential for hydrolyzing the abundant agriculture residues from the palm oil industry.

Key words: Enzymatic hydrolysis, Palm Oil Mill Effluent (POME), *Aspergillus niger* EB5, *Trichoderma* sp. EB6, cellulases

INTRODUCTION

Lignocellulose biomass is the most abundance organic raw material in the world. Cellulose present in renewable lignocellulosic material is considered to be the most abundant organic substrate for the production of glucose, fuel and as chemical feed stock (Krishna, 1999). However, the physical structure of cellulose in native lignocellulose is intrinsicly resistant to enzymatic attack and cellulose is further protected by the surrounding matrix of lignin, hemicellulose and pectin. Various pretreatment processes, including acid or alkali, steam explosion and extraction with aqueous organic solvents are under techno-economic evaluation (Berlin *et al*., 2005).

Oil palm is the leading agricultural crop in Malaysia with its planting areas keep increasing from about 3,499,012 ha in 2001 to 3,670,243 ha in 2002 (Malaysian Palm Oil Board, 2002). Besides palm oil and palm kernel, palm oil mills also generate large quantities of liquid waste that is commonly referred to as Palm Oil Mill Effluent (POME). POME disposal is a major problem in the palm oil industry. In the year 2004, more than 40 million tonnes of POME was generated from 372 mills in Malaysia. If the effluent is discharged untreated, it can certainly cause considerable environmental problems due to its high biochemical oxygen demand (25,000 mg L^{-1}), chemical oxygen demand (53,630 mg L^{-1}), oil and grease (8370 mg L^{-1}), total solids (43,635 mg L^{-1}) as well as suspended solids (19,020 mg L^{-1}) (Wu *et al*., 2007). The POME solid consist mainly debris from palm fruit mesocarp which is lignocellulosic material.

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Cellulase is a system containing endoglucanase, cellobiohydrolase and β-glucosidase. Cellulose is hydrolyzed to glucose recourse to the co-operation of three cellulases. In nature, lignocellulolytic microbes interact in mixed culture to degrade lignocellulose (Yang *et al*., 2004). The microorganisms which appear to be most promising at present are *Trichoderma reesei* mutant. However, it is of interest to examine a new microorganism to improve the cellulase production. *Aspergillus* species is known to be a good producer of cellulases (Jecu, 2000).

Many researches have been conducted on enzymatic hydrolysis of lignocellulosic substrate utilized cellulase from single strain or mixed cultures of cellulase producer but little information concerning on enzymatic hydrolysis by a mixture of crude cellulase from indigenous strains. Therefore, in the current study, we investigated the enzymatic hydrolysis of POME solid utilizing a mixture of crude cellulases from locally isolated fungi, *Aspergillus niger* EB5 and *Trichoderma* sp. EB6 for efficient cellulase system in degrading POME solid. The present research also describes the effect of substrate pretreatment, different ratio of cellulase mixture and incubation pH on the enzymatic hydrolysis of POME solids. In order to test the bioconversion effectiveness of POME solids by the mixed crude cellulase, enzymatic hydrolysis of POME using the mixed crude cellulases and commercial cellulase were performed simultaneously under the optimized conditions.

MATERIALS AND METHODS

Microorganism

The locally isolated fungus from laboratory collection, *Aspergillus niger* EB5 and *Trichoderma* sp. EB6 were used in this study. The culture was maintained at 4°C on Potato Dextrose Agar (PDA) plate.

Preparation of Palm Oil Mill Effluent (POME) as Substrate

Palm oil mill effluent was obtained from Seri Ulu Langat Palm Oil Mill at Dengkil, Selangor, Malaysia in year 2005. The POME solids were obtained by using freezing and thawing techniques. The POME was frozen at 0°C in a freezer for overnight followed by thawing on a sieve for another 24 h at room temperature to remove the liquid. The remaining solid present on the mesh was collected and oven dried at 50°C for 24 h.

Growth Medium

The basal medium contained per liter: 1.4 g $(NH_4)SO_4$, 2.0 g KH_2PO_4, 0.3 g Urea, 0.3 g $CaCl_2$, 0.3 g $MgSO_4.7H_2O$, 5.0×10^{-3} g $FeSO_4.7H_2O$, 1.6×10^{-3} g $MnSO_4.H_2O$, 1.4×10^{-3} g $ZnSO_4.7H_2O$, 2.0×10^{-3} g $CoCl_2$, 0.75 g peptone and 2.0 mL Tween 80.

Production of Crude Enzyme Extract

Spores from *Aspergillus niger* EB5 and *Trichoderma* sp. EB6 were produced on PDA agar plate incubated for 7 days at 30°C. In order to produce the enzyme, an appropriate number of spores were inoculated into 500 mL Erlenmeyer flask containing 100 mL of basal medium at pH 5.5. The medium was supplemented with 1% sugarcane bagasse as carbon source. The flasks were placed on a rotary orbital shaker and agitated at 200 rpm for 6 days at 37°C.

The fermentation broth was centrifuged and the crude enzyme (supernatant) was then precipitated using ammonium sulphate precipitation technique at 90%. Finally the solution was centrifuged at 5000 rpm for 20 min and the pellet obtained was washed with 10 mL of 0.1 M sodium acetate buffer. This solution was kept at 4°C prior to use for the hydrolysis of POME solid process.

Enzymatic Hydrolysis of POME Solid

The enzymatic hydrolysis of POME solid (at a concentration of 10% dry basis in a total volume of 100 mL) was carried out in 500 mL Erlenmeyer flask. Two milliliter of cellulase mixture from *Aspergilus niger* EB5 and *Trichoderma* sp. EB6 was added for enzymatic hydrolysis. The hydrolysis reaction was carried out in a 0.1 M sodium-acetate buffer medium (pH 5.0) at 50°C for 12 h on an orbital shaker at 150 rpm. Samples were taken periodically with a pipette. The samples were then centrifuged at 3500 rpm for 30 min in order to remove the residual solids remaining after cellulase hydrolysis. The content of reducing sugar and glucose were determined after appropriate dilution. Control experiment in which no enzymes added was performed simultaneously.

Sample Assays

The FPase activity was determined by measuring the reducing sugars produced from 1×6 cm^2 strip of Whatman filter paper No. 1 as substrate (Wood and Bhat, 1988). The endoglucanase (CMCase) was determined according to Mandels *et al*. (1974). The amount of reducing sugar released was determined using dinitrosalicyclic reagent (Miller, 1959). The activity of CMCase and FPase was expressed in International Units (IU), defined as the amount of enzyme required to produce 1 μmol glucose/min. The determination of β-glucosidase was done using the method described by Wood and Bhat (1988). One unit of β-glucosidase activity was defined as 1 μmol p-nitrophenol liberated per minute of reaction. The concentration of glucose in the broth was determined using Glucose Trinder Method (Sigma). The determination of cellulose, hemicellulose and lignin content was done according to the method of Gorring and Van Soest (1970).

RESULTS AND DISCUSSION

Preliminary study was conducted to determine the optimum temperature for cellulase enzyme reaction from the both cultures. It was shown that the optimum temperature of the three cellulases components of *A. niger* EB5 and *Trichoderma* sp. EB6 for the reaction was between 50-60°C (unpublished results). Thus, in this study temperature at 50°C was selected for the enzymatic hydrolysis process.

In this study, POME solid was hydrolysed to produce reducing sugar and glucose by the mixed crude cellulase of *A. niger* EB5 and *Trichoderma* sp. EB6. The enzyme profiles for both strains show these strains produce different ratios of enzyme activities (Table 1). In particular, unlike *Trichoderma* sp. EB6, *A. niger* EB5 exhibited high β-glucosidase activity. One of the problems related to the economic viability of the enzymatic hydrolysis of cellulose is due to low β-glucosidase levels in the culture filtrates containing cellulase enzyme by many fungi (Umikalsom *et al*., 1997). Presumably, β-glucosidase improves cellulose hydrolysis by reducing end-product inhibition caused by accumulation of cellobiose (Berlin *et al*., 2005). Therefore, it was assume by supplementing *Trichoderma* sp. cellulase with *Aspergillus* sp. enzymes might result in more effective enzyme mixture which the two enzymes preparations would complement each other as what have been done by Wen *et al*. (2005), which developed the co-culture of *T. reseei* and *A. phoenicis* to overcome the deficiency of β-glucosidase.

Table 1: Cellulases component in *Aspergillus niger* EB5 and *Trichoderma* sp. EB6

Strain	FPase	CMCase	β-glucosidase
	----------(U mL^{-1})----------		
Aspergillus niger EB5	1.38	3.76	6.00
Trichoderma sp. EB6	1.53	5.13	0.18

Table 2: Effect of varying concentration of chemical pretreatments on reducing sugar and glucose produced in enzymatic hydrolysis of POME solid

Chemicals	Reducing sugars (g L^{-1})	Glucose (g L^{-1})
NaOH, 0.5% (w/v)	8.9	4.5
NaOH, 1.0% (w/v)	5.3	5.2
NaOH, 2.0% (w/v)	4.9	0.2
NaOH, 4.0% (w/v)	3.9	0.3
H_2SO_4, 0.5% (v/v)	7.0	6.3
H_2SO_4, 1.0% (v/v)	8.5	4.4
H_2SO_4, 2.0% (v/v)	1.8	4.3
H_2SO_4, 4.0% (v/v)	1.8	4.0

Table 3: Effect of chemical pretreatments on cellulose, hemicellulose and lignin content (expressed as percent of dry matter) of POME solid

Treatments	Cellulose	Hemicellulose	Lignin
	----------(%)----------		
Untreated (raw POME solid)	39.56	23.33	25.02
0.5% H_2SO_4	45.92	25.92	16.14
1% NaOH	42.15	23.46	16.37

Substrate Pretreatment

It was observed that the lignin-to-cellulose ratio for the POME solid are relatively high in comparison with other agricultural fibrous residues (Table 3). Therefore, pretreatment is necessary for accelerating the hydrolysis process. Table 2 shown the production of glucose and reducing sugar from POME solid treated with different concentrations of sodium hydroxide and sulphuric acid solution. About 8.5 and 7.00 g L^{-1} of reducing sugars were detected at the end of the process when applying 1% (w/v) NaOH and 0.5% (w/v) NaOH, respectively. Different situations were observed when POME solid treated at 2 and 4% (w/v) NaOH where the amount of reducing sugar produced was less than 2.00 g L^{-1}. The highest glucose production for 1, 2 and 4% (v/v) H_2SO_4 pretreatment was 4.0 g L^{-1} and reducing sugar produced, 1.8 g L^{-1}. Pretreatment with H_2SO_4 at 1% (v/v) was comparable to that pretreatment using 0.5% NaOH in terms of reducing sugar and glucose production.

Low sugars were detected when POME solid pre-treated with 2 and 4% (w/v) of NaOH. This probably was due to the high concentration of alkaline treatment. In the production of cellulase, intense alkaline treatment may reduce enzyme production. Generally, alkaline pretreatment is more effective on agricultural residues and herbaceous crops than on wood materials. Peroxide pretreatment enhances enzymatic conversion through oxidative delignification and reduction of cellulose crystallinity. Increased lignin solubilization and cellulose availability were observed during the peroxide pretreatment of wheat straw (Silverstein *et al*., 2006).

The composition of the untreated POME or raw POME solid is shown in Table 3. The content of cellulose, hemicellulose and lignin in the POME solid were 39.56, 23.33 and 25.02%, respectively. Lignin is not attacked by the enzymes and shields the cellulose during hydrolysis (Silverstein *et al*., 2006). Therefore, substrate pretreatment is needed to accelerate enzymatic hydrolysis process. Table 3 also shows the effect of chemical pretreatment on cellulose, lignocellulose and lignin content. During pretreatment it is desirable that the cellulose portions of the biomass remains virtually unaffected and reduce the lignin content significantly. It is interesting to note that in this study the content of cellulose was increased for the pretreatment using 0.5% H_2SO_4 and 1% NaOH by 18 and 7.5%, respectively. The reduction of lignin, based on a comparison between the weight of lignin in the initial dry-weight sample before pretreatment and the weight of lignin in the solids remaining after the both pretreatment processes was as much as 36%. Increased lignin solubilization and cellulose availability were also observed during the peroxide pretreatment of wheat straw (Silverstein *et al*., 2006). Compared to untreated materials, effectively pretreated lignocellulosic materials are generally characterized by increased surface area accessible to cellulase enzyme (porosity) and solubilization

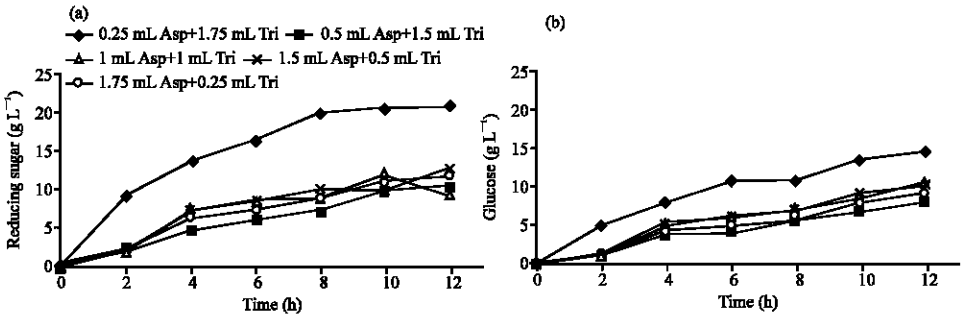

Fig. 1: Effect of different ratio of crude cellulase from *Aspergillus niger* EB5 and *Trichoderma* sp. EB6 during enzymatic pretreatment of POME solid. (a) Reducing sugar production and (b) Glucose production

and/or redistribution of lignin. Lignin distribution is thought to explain why dilute acid and steam explosion are effective pretreatment processes although lignin is not removed (Lynd *et al*., 2002). It is thought that lignin melts during pretreatment and coalesces upon cooling such that its properties are altered substantially.

Process Parameters Optimisation

Figure 1 shows glucose and reducing sugar production from pretreated POME solid at various ratio of cellulase enzyme. Ratio of 0.25 mL from *A. niger* EB5 with 1.75 mL of *Trichoderma* sp. EB6 with a total enzyme activity of 14.76 U gave the highest reducing sugar produced, 21.5 g L^{-1} and glucose (14.7 g L^{-1}) at 12 h incubation of saccharification process. Similar glucose production profile was observed with the range between 0.1 to 10.6 g L^{-1} when utilizing the cellulase enzyme ratio of 0.5 mL *Asp*: 1.5 mL *Tri* (total activity 15.83 U), 1 mL *Asp*: 1 mL *Tri* (total activity 17.98 U), 1.5 mL *Asp*: 0.5 mL *Tri* (total activity 20.13U) and 1.75 mL *Asp*: 0.5 mL *Tri* (total activity 21.21 U). The other combination ratios of enzyme show less than 15 g L^{-1} of reducing sugar released. The enzyme ratio of 0.25 mL from *A. niger* EB5 and 1.75 mL of *Trichoderma* sp. EB6 with a total enzyme activity of 14.76 U was found to be the satisfactory ratio in producing the maximum concentration of sugar. This may due to higher FPase and CMCase from *Trichoderma* sp. EB6 which is needed to assist the breakdown of cellulose and with a presence of β-glucosidase from *A. niger* EB5 may helps in converting cellobiose to glucose effectively. According to Imai *et al*. (2004), a mixed-enzyme system of cellulases from *T. viride* and *A. niger* improved hydrolytic activity of CMC over single-enzyme system.

The reducing sugar and glucose produced were not increased by prolonging the incubation period after 12 h (Fig. 1). This might be due to the excessive glucose production which produced an inhibitory effect that limited the hydrolysis rate. The faster conversion rate of POME solid to reducing sugar/glucose during the early stage of enzymatic hydrolysis might be due to smaller particle of cellulose favoring the access of enzyme to potential cleavage sites (Ortega *et al*., 2001). It can be noticed that reducing sugar production was almost constant after 8 h hydrolysis implying that the glucose produced suppressed cellulase activity. Hanif *et al*. (2004) reported that glucose enhanced the cell mass productivity but suppressed cellobiohydrolase (CBH) productivity and substrate consumption rate.

Figure 2 shows the production of glucose and reducing sugar from pretreated POME solid at variable p H. The maximum glucose concentration detected at pH 3.5, 4 and 5 were 13.8, 14.7 and 14.8 g L^{-1}, respectively. In contrast, only 11.6 g L^{-1} glucose was observed when the reaction was carried out at pH 6. The amount of reducing sugar produced was also lower at pH 6 with only 18.4 g L^{-1} compared to pH 3.5, 4 and 5 which released glucose at 21.3 21.4 and 22.8 g L^{-1}, respectively.

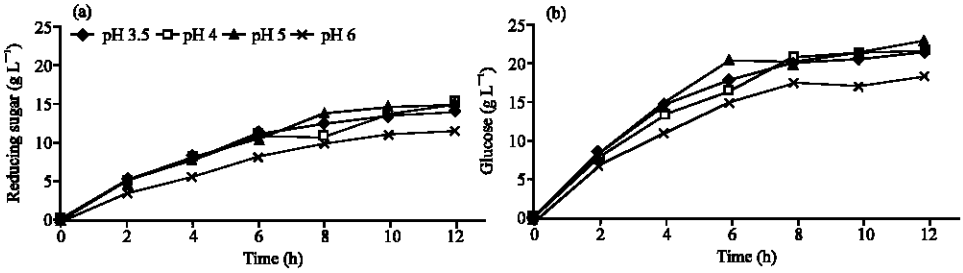

Fig. 2: Effect of different pH during enzymatic hydrolysis of POME solid at 13 h incubation. (a) Reducing sugar production and (b) Glucose production

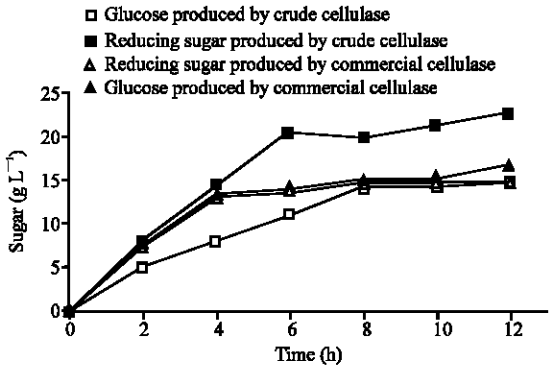

Fig. 3: Comparison for the enzymatic hydrolysis of POME solid using mixture of crude cellulase from isolated strains and commercial cellulase

The pH for cellulase reaction in the present study shows optimum in acidic condition. Thus, the sugar content reduced when the reaction carried out at pH 6. The results obtained are in agreement with report by Ortega *et al.* (2001) where the largest saccharification yield of carboxymethylcellulose was occurred at pH 4.0 followed by pH 4.5 and 5, while pH 6.0 gave the lowest yield. It was reported that maximum cellulase activity was obtained when the initial pH was adjusted to 4.5-5.5 in solid substrate fermentation of wheat straw and wheat bran by *Aspergilus niger* 38 (Jecu, 2000).

Comparative Study for Hydrolysis of POME Solids by Cellulases from the Isolated Strains and Commercial Cellulase

The experiment was performed in order to compare the saccharification efficiency of POME solid using crude cellulase enzyme and with the diluted commercial cellulase enzyme under optimized conditions. A commercial cellulase mixture, supplied by Novozymes A/S (Bagsværd, Denmark), consisting of Celluclast 1.5 L (65 FPU/g and 17 β-glucosidase IU/g) and supplemented with the β-glucosidase preparation (Novozym188, 376 β-glucosidase IU/g) was employed. These commercial enzymes were diluted to approximately the cellulase activity as in the mixture of cellulase produced by *A. niger* EB5 and *Trichoderma* sp. EB6. The profiles of reducing sugar and glucose production by the mixed crude cellulase from isolated strains and commercial cellulase for the hydrolysis of POME solid under the optimized conditions is shown in Fig. 3. Reducing sugar and glucose increased gradually within 6 h hydrolysis and became constant until the hydrolysis stopped at 12 h.

When the crude cellulase enzyme was employed, reducing sugar produced from POME solid at 12 h was higher compared to commercial cellulase with an amount of 22.8 and 16.8 g L^{-1}, respectively.

While the concentration of glucose produced was comparable for the both processes. The possible reason for this situation is the crude enzymes might contain other enzymes impurities or accessory hydrolytic enzymes beside cellulase resulting in a mixture of reducing sugar produced. The yield of saccharification obtained at the end of hydrolysis process using the crude enzyme was 32% reducing sugars and 23.6% glucose. Shen and Xia (2004) reported that the concentration of reducing sugar from enzymatic hydrolysis of treated corncob was 48.50 g L^{-1} and the hydrolysis yield was 69.5% by immobilized spores of *Aspergillus niger* ZU-07. However, the amount of sugar produced in enzymatic hydrolysis depends on the nature of lignocellulose substrate used such as cellulose and lignin content, substrate pretreatment, etc. The monosaccharide converted from POME solid could be used further in the fermentation of ethanol, organic acid and SCP, etc.

CONCLUSION

The mixture of crude cellulase from locally isolated fungi *Aspergillus niger* EB5 and *Trichoderma* sp. EB6 significantly hydrolysed POME solid into fermentable sugars. Besides substrate pretreatment, ratio of mixed crude cellulases from the locally isolated strains and pH incubation (pH 3.5-5.0) are important factors affecting enzymatic hydrolysis of POME solid. The hydrolysis efficiency of POME solid (in terms of reducing sugar and glucose produced) were 32 and 23.6%, respectively. The mixture of crude cellulase shows better performance in hydrolyzing POME solid to reducing sugars as compared to commercial enzyme.

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