PRODUCTION OF FERMENTABLE SUGARS FROM OIL PALM EMPTY FRUIT BUNCH USING CRUDE CELLULASE COCKTAILS WITH *TRICHODERMA ASPERELLUM* UPM1 AND *ASPERGILLUS FUMIGATUS* UPM2 FOR BIOETHANOL PRODUCTION

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Utilization of oil palm empty fruit bunch (OPEFB) for bioethanol production with crude cellulase cocktails from locally isolated fungi was studied. Enzymatic saccharification of alkaline pretreated OPEFB was done using different cellulase enzyme preparations. Crude cellulase cocktails from *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 produced 8.37 g/L reducing sugars with 0.17 g/g yield. Production of bioethanol from OPEFB hydrolysate using Baker's yeast produced approximately 0.59 g/L ethanol, corresponding to 13.8% of the theoretical yield. High reducing sugars concentration in the final fermentation samples resulted from accumulation of non-fermentable sugars such as xylose and cellobiose that were not consumed by the yeast. The results obtained support the possible utilization of OPEFB biomass for bioethanol production in the future.

Keywords: Oil palm empty fruit bunch (OPEFB); Crude cellulase enzymes; Lignocellulosic bioethanol; Locally isolated fungi

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INTRODUCTION

Bioethanol was first produced from edible feedstocks containing readily fermentable sugars, such as sugarcane, corn, and starch (Goh *et al.* 2010). However, production of bioethanol from food-based material has triggered conflicts between food production and fuel production, which has resulted in an expensive bioethanol selling price (Goh *et al.* 2010; Badger 2002). Driven by its favorable potential economics, exploitation of lignocellulosic biomass as feedstock to bioethanol production has prompted the initiation of many research projects. To date, bioethanol has successfully been produced in the United States and Brazil using wood, switchgrass, wheat straw, sugarcane bagasse, and municipal solid waste (MSW) (Badger 2002). In Malaysia, palm oil is a huge and profitable agricultural commodity. In 2005, approximately 55.73 million tones of oil palm biomass were produced (Shuit *et al.* 2009). The palm oil industry generates enormous quantities of biomass ranging from palm fiber, palm kernel cake (PKC), decanter cake, fronds, trunks, shells, and oil palm empty fruit bunch (OPEFB) (Shuit *et al.* 2009; Yusoff 2006; Joseph 2010). OPEFB is obtained from the process of stripping off fruit from fresh fruit bunches (Law *et al.* 2007; Umikalsom *et al.* 1997). OPEFB contains approximately 54.4% of cellulose, 28% hemicelluloses, and 17.6% lignin (Umikalsom *et al.* 1997).

It is current practice to burn OPEFB to produce piles of ash, which are redistributed to the plantation as fertilizer and soil conditioner (Yusoff 2006). Meanwhile, some of the disposed OPEFB has been directly used as a mulching agent for palm oil trees to promote vegetative growth and improve foliar nutrient levels. To more fully extend the utilization of OPEFB, fiberboard has been successfully produced from it. When considering the potential utilization of OPEFB in the future, some researchers have suggested production of value-added products such as bioethanol, fermentable sugars, and compost. In addition, utilization of OPEFB for environmentally friendly biofuel production, mainly as bioethanol, has attracted interest among researchers. Besides, it was estimated by Yano *et al.* (2009) that 1 to 2% of biofuel can be substituted by ethanol produced from OPEFB in Malaysia and Indonesia. Furthermore, Goh *et al.* (2010) showed that the country's energy demand can be fulfilled with bioethanol if available lignocellulosic biomass could be fully converted into bioethanol, thus reducing 19% of CO_2 emissions in Malaysia. Since OPEFB is rich in sugars, it is a suitable feedstock for second-generation bioethanol, *i.e.* ethanol from biomass.

Production of bioethanol from lignocellulosic biomass involves two stages: (1) hydrolysis of biomass and (2) ethanol fermentation. Hydrolysis of biomass can be done either using acid hydrolysis or enzymatic hydrolysis (Sukumaran *et al.* 2009). However, acid hydrolysis of biomass causes difficulties in the recovery of sugars and the need to employ an environmentally unfriendly process, since it produces hazardous acid waste (Sukumaran *et al.* 2009). Enzymatic hydrolysis is a more environmental friendly and efficient way to convert lignocellulosic biomass into bioethanol, even though it is expensive (Sukumaran *et al.* 2009; Badger 2002). Some studies have suggested the in-house production of cellulases in order to reduce the cost of production. Enzymatic hydrolysis requires relatively low energy and mild process conditions (Badger 2002). Cellulose degradation by cellulases requires synergistic action from all the cellulase components, exo-glucanase(FPase), endo-glucanase(CMCase), and β-glucosidase, starting with the action of endo-glucanase on cellulose amorphous region. Endo-glucanase will produce nick-openings in the cellulose structure and expose the reducing and non-reducing ends of cellulose chains. Upon the exposure, exo-glucanase will act on the exposed reducing and non-reducing ends to produce the cellobiose unit which will be cleaved by the action of β-glucosidase (Sun and Cheng 2002).

Thus, the aim of our study was to investigate the uses of cellulases from single and mixed culture in the enzymatic hydrolysis of OPEFB into fermentable sugars using locally isolated fungi namely *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2. Subsequent production of bioethanol from OPEFB hydrolysate using Baker's yeast was also evaluated.

MATERIALS AND METHODS

Microorganisms

Trichoderma asperellum UPM1 and *Aspergillus fumigatus* UPM2 were isolated from rotten oil palm fruit bunch. A suspension of spores of each fungus was obtained by growing them on potato dextrose agar (PDA) at 30°C for 7 days and harvesting the spores with sterile distilled water. Species identification was done through 18S rDNA methodology (Nurul Kartini 2010).

Substrate

Oil palm empty fruit bunches (OPEFB) were obtained from a palm oil factory in Dengkil, Selangor, Malaysia. They were first washed and dried before being ground to the particle size of 10 mm. Later, the substrate was subjected to 2% NaOH alkaline pretreatment according to Umikalsom *et al.* (1997) before being used as a carbon source for fungal cellulase production. For saccharification purposes, 2% NaOH alkaline pretreated OPEFB (10 mm) were further ground to a particle size of 0.5 mm. Pre-treatment using 2% of sodium hydroxide was able to sufficiently reduce the lignin content from approximately 17.6% of lignin to 12.1% of lignin.

Fungi Cultivation and Cellulase Enzymes Preparation

Fungi were grown in 250 mL shake flasks containing 100 mL Mandel basal medium (Mandel and Weber 1969). Samples of basal medium were inoculated with a specified amount of spores/ mL of each fungi spores suspension for pure culture and mixed culture system and were incubated at 30°C with an agitation speed of 150 rpm on a rotary orbital shaker. Culture filtrates were collected after 7 days of incubation in the cases of *Trichoderma asperellum* UPM1 and mixed culture, and for *Aspergillus fumigatus* UPM2, the filtrate was collected after 4 days of incubation. Solid materials and fungal biomass were separated from culture filtrate by centrifugation (3500 rpm, 20 min). Clear supernatants obtained were analyzed for total protein and activities of cellulase enzymes (FPase, CMCase, and β-glucosidase).

Saccharification of OPEFB

Enzymatic saccharification of alkaline pretreated OPEFB (0.5 mm) were carried out in a 100 mL shake flask incubated 50°C and agitated at 100 rpm. Saccharification was started adding 2 mL of respective cellulase enzymes prepared earlier into 18 mL of 0.05 M sodium acetate buffer (pH 5.0) containing 1.0 g of alkaline pretreated OPEFB (0.5 mm). For mixed cellulase enzyme, equal volumes of *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 cellulase enzymes were used for the saccharification experiment. Samples were withdrawn at regular time intervals for analysis of reducing sugars.

Ethanol Fermentation

Ethanol fermentation was done according to Cheng *et al.* (2009) with slight modification. Pre-culture medium contained (g/L): glucose (10.0), yeast extract (1.0), KH_2PO_4 (2.0), $(NH_4)_2SO_4$ (2.0), and $MgSO_4.7H_2O$ (0.4) with initial pH adjustment to pH 5. Development of inoculums was done by inoculating a loopful of Baker's yeast into pre-culture media and incubating for 6 h at 30°C, 200 rpm. Hydrolysate of OPEFB obtained after saccharification was supplemented with other nutrients prior to ethanol fermentation similar to pre-culture medium without the glucose. Ethanol produced was estimated by gas chromatography using a flame ionization detector and BP 20 column.

Analytical Methods

The gravimetric method described by Goering and Van Soest (1970) was used to determine cellulose, hemicellulose, lignin, and ash content in alkaline treated OPEFB (0.5 mm). Total soluble protein content was measured using the Lowry method, as described by Lowry *et al.* (1951). Activities of FPase, CMCase, and β-glucosidase were measured according to Wood and Bhat (1998) and Ariffin *et al.* (2006).

Withdrawn samples from saccharification experiments were centrifuged (10,000 rpm, 10 min) to obtain clear supernatant. Supernatants were subjected to reducing sugars analysis using dinitrosalicyclic acid (DNS) as described by Miller (1959) and Ariffin *et al.* (2006). Glucose, xylose, and cellobiose were detected using HPLC (JASCO, Japan) with an RI-detector (JASCO, Japan). The samples were filtered through a 0.20 μm filter prior to injection. Simple sugars such as glucose, xylose, and cellobiose were separated using a Rezex (00H-0130-K0) column (Phenomenex, USA) at 80°C with degassed deionized water as mobile phase at a flow rate 0.5 mL/min.

Ethanol concentration was detected using a gas chromatograph (Model GC-17A, Shimadzu, Japan), employing a BP 20 column, FID detector, and hydrogen gas as carrier. Ethanol was detected with the following conditions: injection temperature of 150°C, initial oven temperature of 40°C to a final temperature of 200°C, and flow rate of carrier gas of 5 mL min^{-1}. An internal standard of 1% of 1-propanol solution was used determined ethanol concentration in samples. All experiments were done in duplicates and repeated twice for confirmation.

RESULTS AND DISCUSSION

Saccharification of OPEFB Using Various Source of Cellulases

Figure 1 shows the production of reducing sugars during saccharification of alkaline-pretreated OPEFB (0.5 mm). Cellulase enzymes solutions were prepared from *Trichoderma asperellum* UPM1, *Aspergillus fumigatus* UPM2, mixed culture of both isolated fungi, and mixed cellulase enzymes solution from both fungi.

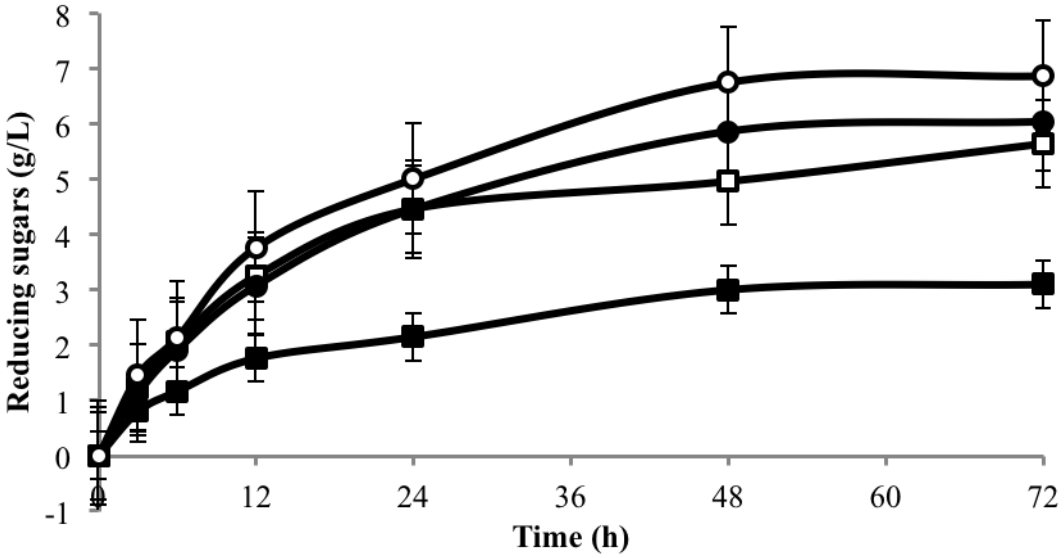

Fig. 1. Reducing sugar profiles from saccharification of OPEFB using cellulase enzymes from (■) *T. asperellum* UPM1, (□) *A. fumigatus* UPM2, (●) Mixed culture of *T. asperellum* UPM1 and *A. fumigatus* UPM2, and (○) mixed cellulase enzymes of *T. asperellum* UPM 1 and *A. fumigatus* UPM 2

Saccharification of alkaline-pretreated OPEFB was done to evaluate potential cellulases preparation that can give maximum reducing sugar from OPEFB hydrolysis. Based on the results obtained, the first 24 h of saccharification showed acceleration of

reducing sugars production in all cellulase enzymes solution preparation but production became constant after 48 h. The maximum reducing sugars production was observed for mixed cellulases preparation with 6.86 g/L at 72 h of incubation. On the contrary, cellulase enzymes preparation from *Trichoderma asperellum* UPM1 showed very low reducing sugars production, 3.09 g/L. On the other hand, cellulases preparation from *Aspergillus fumigatus* UPM2 and mixed culture generated approximately 5.63 g/L and 6.03 g/L, respectively.

Corresponding to the results obtained during saccharification in Fig. 1, Table 1 shows a summary of saccharification results for all cellulases preparations. Similarly, the mixed cellulases enzyme preparation gave maximum yield of reducing sugars per gram substrate and reducing sugars per gram of carbohydrate contained in the substrate with 0.14 g/g and 0.17 g/g, respectively. By contrast, *Trichoderma asperellum* UPM1 only generated 0.06 g/g of reducing sugars per gram substrate and 0.08 g/g reducing sugars per gram carbohydrate. Although FPase enzyme activity in *Aspergillus fumigatus* UPM2 cellulase preparation was high compared to the other cellulases preparation, it did not result in high yield and reducing sugars production. Likewise, for *Trichoderma asperellum* UPM1, the cellulases preparation that had high activity in β-glucosidase (0.68 U/mL) doubled the activity from mixed cellulases enzyme preparation. A high concentration of reducing sugar in the samples may be attributed not only to cellulase activity but also to the activities of other hydrolytic enzymes, such as hemicellulases that produced reducing sugars other than glucose, such as xylose.

Table 1. Summary of Production of Reducing Sugars during Saccharification of Alkaline-Pretreated OPEFB using Various Cellulases Preparations

Cellulase enzymes	FPase (U/mL)	CMCase (U/mL)	β-glucosidase (U/mL)	Reducing sugars (g/L)	Yield (g RS/g substrate)	Yield (g RS/g CHO)
T. asperellum UPM1	0.20±0.03	9.28±0.00	0.68±0.00	3.09±0.42	0.06±0.01	0.08±0.01
A. fumigatus UPM2	0.44±0.01	17.55±0.76	0.03±0.00	5.63±0.00	0.11±0.00	0.14±0.00
Mixed culture	0.39±0.02	18.04±0.14	0.23±0.00	6.03±1.46	0.12±0.03	0.15±0.04
Mixed cellulase	0.28±0.00	13.63±0.78	0.35±0.01	6.86±1.48	0.14±0.03	0.17±0.04

*Carbohydrate (CHO) in alkaline-pretreated OPEFB was approximately 0.75% based on determination of NDF and ADF in the fiber.
*Values are means of duplicate with standard deviation.

The results obtained were comparable to a previous study done by Baharuddin *et al.* (2009). The study obtained 4.37 g/L of reducing sugars and 0.09 g/g yield from saccharification of OPEFB using *A. niger* EB4 cellulase enzyme. Another study done by Umikalsom *et al.* (1997) obtained a maximum concentration of reducing sugars of 38.5 g/L at a cellulase enzymes loading of 10 U/mL FPase, 285 U/mL CMCase, and 60 U/mL β-glucosidase produced by *Chaetomium globosum* during saccharification of pretreated OPEFB fibers. The yield of the saccharification process was 0.76 g/g substrate.

According to Jabbar *et al.* (2004), the key to obtaining maximum saccharification is to have the right proportion of cellulases components that work synergistically. Many have contributed suggestions regarding the proportion of the cellulases enzyme component for saccharification of cellulosic material. Sukumaran *et al.* (2009) also stated that the use of a better "enzymes cocktail" may reduce the production cost of bioethanol

from lignocellulosic biomass. In addition, Meyer *et al.* (2009) suggested that identifying efficient minimal enzyme mixtures in lignocelluloses hydrolysis is crucial in order to obtain successful and feasible degradation of lignocelluloses and fibrous materials.

Sukumaran *et al.* (2009) had reported on the effect of enzymes loading ratio and incubation time on hydrolysis of alkali-pretreated sugarcane bagasse. It showed that above a given threshold concentration of cellulases with respect to type of biomass and degree of polymerization, enzyme concentration might have lesser effect on cellulose hydrolysis compared to the effect of reaction time. They also suggested that even at low enzyme loading, more efficient hydrolysis can be achieved with a prolonged incubation time. Increasing dosage of cellulases in an enzymatic process to a certain extent would significantly increase the cost of a process even though it enhances the yield and rate of hydrolysis (Sun and Cheng 2002). On the contrary, the hydrolysis rate decreases with time as the enzymatic reaction proceeds (Gupta and Lee 2009).

SEM of OPEFB Fibers

Hydrolysed samples from all cellulases preparation were dried for fiber study under a scanning electron microscope (SEM). Figure 2 shows images of raw, pretreated, and saccharified OPEFB fibers.

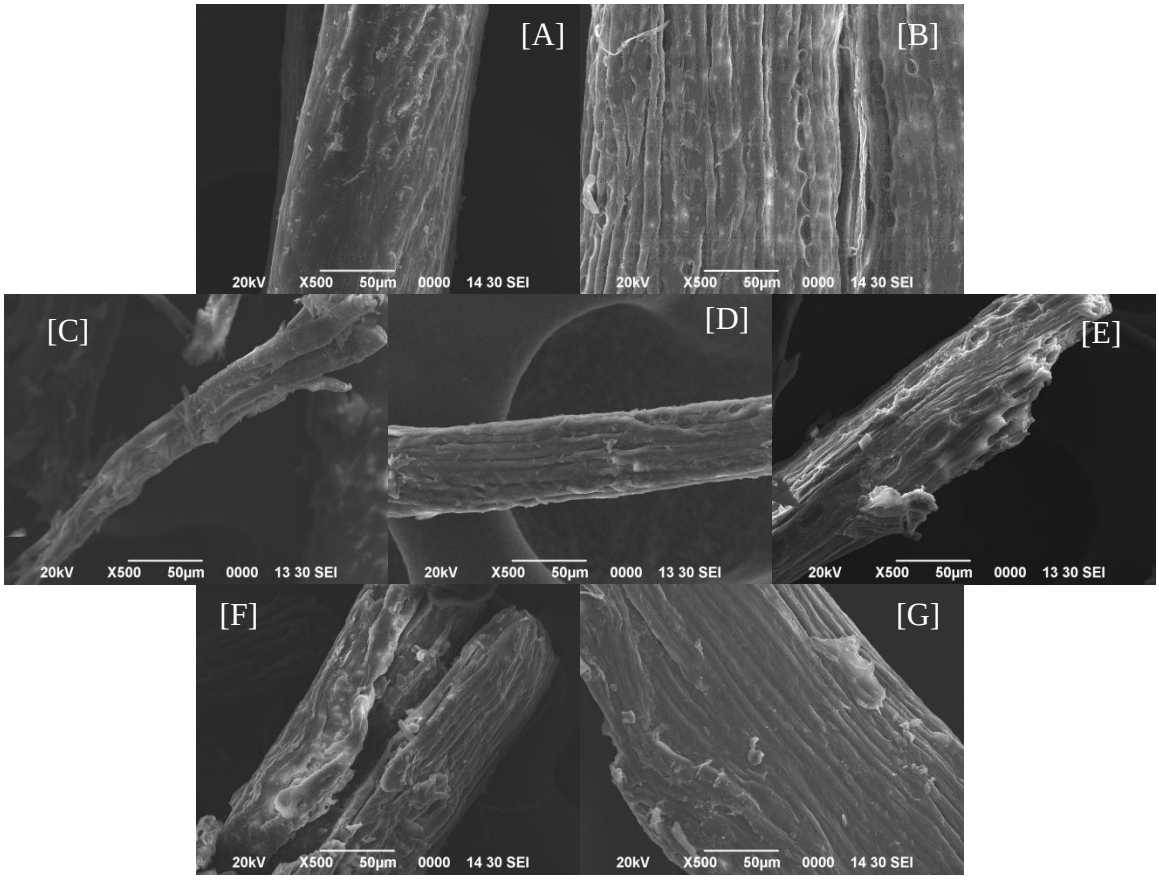

Fig. 2. Images of various OPEFB fibers under Scanning Electron Microscope (SEM), 1000x magnification. [A] Raw OPEFB (10 mm), [B] 2% NaOH pre-treated OPEFB (10 mm), [C] 2% NaOH pre-treated OPEFB (0.5 mm), [D] after saccharification with *Trichoderma asperellum* UPM 1 cellulases, [E] after saccharification with *Aspergillus fumigatus* UPM 2 cellulases, [F] after saccharification with mixed cultures cellulases, [G] after saccharification with mixture of cellulases from both locally isolated fungi

Raw shredded OPEFB (10 mm) showed a smooth surface without any fragmentation, as seen in comparison to 2% sodium hydroxide (NaOH) alkaline-treated OPEFB. Two percent sodium hydroxide alkaline-treated OPEFB (10 mm) on the other hand showed more defined and opened structures in which pores within the fiber structure can be observed. Pores observed on the fiber were previously associated with silica bodies imbedded in the lignocelluloses fiber. Likewise, pores also could be observed for 2% NaOH alkaline pretreated OPEFB (0.5 mm) with smaller size of fibers. Pretreatment also increased the surface area of fiber structure compared to untreated OPEFB (raw).

After saccharification with cellulases, remarkable changes were observed in every enzyme-treated preparation, especially for *Aspergillus fumigatus* UPM2 and mixed cultures cellulases. Both cellulases preparation showed disrupted fiber structure, resulting from enzymatic hydrolysis of the cellulose structure. Although qualitatively *Aspergillus fumigatus* UPM2 cellulases preparation gave the most disrupted structure of OPEFB, the reducing sugars produced from saccharification did not reveal the same. On the contrary, the mixed cellulases preparation that gave the highest yield and production of reducing sugars showed a more even surface under SEM. This observation was attributed to there having been strong synergistic action of FPase, CMCase, and β-glucosidase on the rough and loose parts of the fiber surfaces.

According to Gupta and Lee (2009) and Fan *et al.* (1987), a deformed structure of cellulose resulted from the pretreatment. In dilute alkaline media such as sodium hydroxide, lignocellulosic fiber structures swell, leading to an increase in internal surface area and hence to a decrease crystallinity and degree of polymerization. The treatment also separates structural linkages between lignin and carbohydrates, thus disrupting the lignin structure. Mechanical comminution such as grinding will result in a reduction of cellulose crystallinity and size of fiber, and an increase in surface area that is accessible for enzymatic action.

Remarkable changes on cellulose structure were observed on OPEFB fibers that had undergone pretreatment, such as alkaline pretreatment and mechanical grinding, as shown in Fig. 2 (B and C) compared to raw untreated OPEFB (A). As stated by Law *et al.* (2007), mechanical treatment and washing also affected silica content of OPEFB fiber structure. Silica bodies may perform multiple functions beyond the nutritional requirements of the plant. The removal revealed that the bottom of the silica-crater is perforated, which suggests that the silica bodies originate from the interior of the fibrous strand itself. Perforated fiber may enhance and facilitate access of cellulases to cellulose polymer for hydrolysis purposes.

Cellulase hydrolysed OPEFB fibers such as those shown in Fig. 2 (D, E. F and G), exhibited reasonably disrupted structures of fiber in comparison with initial unhydrolysed fiber by cellulase (Fig. 2 C). Unlike any other enzymes, cellulases need to penetrate into the cellulose polymer to access and hydrolyze it (Sukumaran *et al.* 2009). Cellulases binding domains must be attached to the polymer to allow the catalytic domain to degrade the polymer into sugar monomers. A synergistic effect of cellulase enzymes system consisting of endo-glucanse, exo-glucanase, and β-glucosidase determines the production of sugars from biomass. Effective synergistic action translates into a high production of sugars from lignocellulosic biomass. However, the availability of pores within the substrate must be large enough to accommodate large and small cellulase molecules to ensure a synergistic action of the enzymes system (Converse *et al.* 1988; Tanaka *et al.* 1988).

Disrupted OPEFB fiber structures were due to several reasons, such as pretreatment and enzyme hydrolysis, to which the fibers had been subjected during preliminary handling (pretreatment) and during hydrolysis process. Disrupted fibers structure facilitates the cellulase hydrolysis process, nevertheless its still does not definitely determine the yield and/or production of sugars monomers after hydrolysis.

Ethanol Production from OPEFB Hydrolysate

Figures 3 and 4 show ethanol production using glucose and OPEFB hydrolysate as a carbon source, respectively.

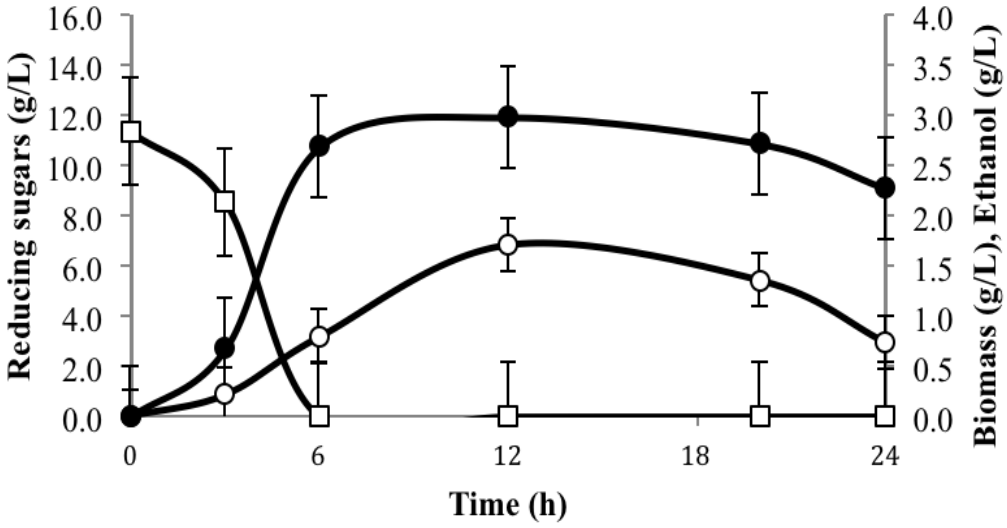

Fig. 3. Bioethanol fermentation profile using glucose as carbon source. (□, glucose;○, dry cell weight and●, ethanol)

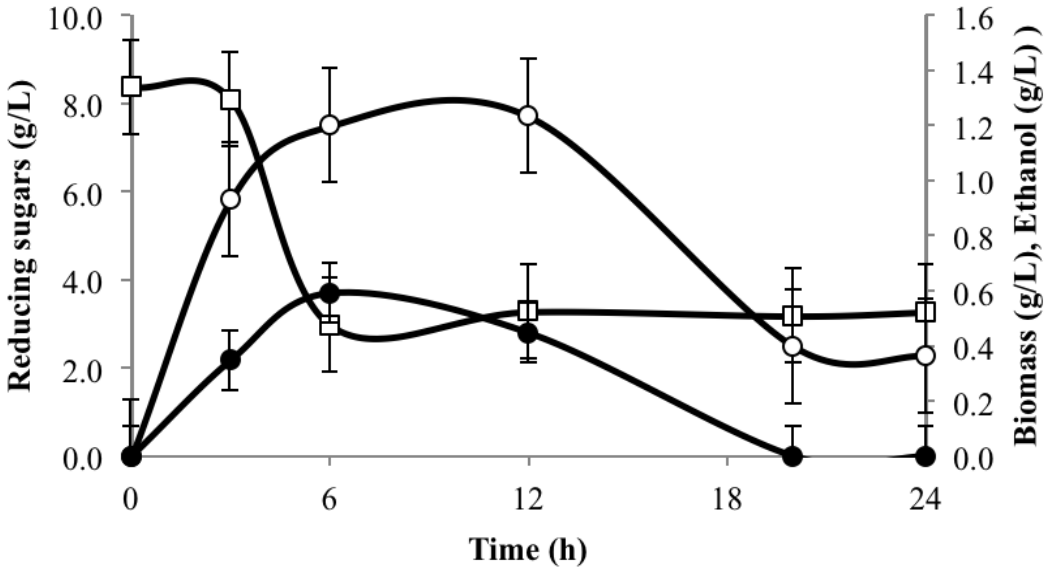

Fig. 4. Bioethanol fermentation using OPEFB hydrolysate as carbon source. (□, glucose; ○, dry cell weight and●, ethanol)

Ethanol was immediately produced after 3 h of incubation and became constant after 6 h for both carbon sources. However in OPEFB hydrolysate (Fig. 4), a decline of ethanol production was observed at 20 h of fermentation. In correlation to reducing sugars consumption, glucose was fully consumed after 6 h incubation. In contrast, in the case of OPEFB hydrolysate, reducing sugars accumulated and declined during the first 6 h, but not all were consumed by yeast. This indicates that only some sugars were successfully converted to ethanol in OPEFB hydrolysate by Baker's yeast. Yeast cells biomass in glucose slowly increased during the first 3 h and reached log phase at 12 h of incubation. A decline in yeast biomass was observed after 20 h, which resulted in reduced ethanol production. Differently in OPEFB hydrolysate, yeast biomass drastically increased after inoculation and reached log phase at 6 h. After 12 h fermentation, yeast biomass declined, accompanied by a reduction in ethanol production.

Table 2 shows production of ethanol from OPEFB hydrolysate and glucose by Baker's Yeast. The results were unsurprising from the standpoint of ethanol production. In ethanol fermentation by yeast, the most preferable carbon source for ethanol production by yeast is glucose. Based on the results, pure glucose produced a high concentration of bioethanol up to 2.98 g/L with 51.32% of the theoretical yield, compared to OPEFB hydrolysate with 0.59 g/L and 13.8% theoretical yield after 24 h bioethanol fermentation by yeast. Very low percentage of theoretical yield (based on glucose concentration) and bioethanol yield per gram reducing sugars was obtained.

Table 2. Ethanol Production from OPEFB Hydrolysate and Glucose by Baker's Yeast

Substrate	Reducing sugars (g/L)	Ethanol (g/L)	Ethanol Yield (g/g RS)	*Theoretical Yield (%)
Glucose	11.37±0.30	2.98±0.15	0.26±0.02	51.32±2.93
OPEFB hydrolysate	8.37±0.85	0.59±0.06	0.11±0.03	13.8±3.57

* Values are means of duplicate with ± standard deviation.

Apparently, OPEFB hydrolysate contained not only glucose but also other sugars derived from hemicelluloses, such as xylose. In relation, Table 3 shows the type of sugars present in the OPEFB hydrolysate. The reducing sugar content comes from cellobiose as the major sugar type, followed by glucose and xylose. Xyloses are 5-carbon sugars that cannot be used by yeast for ethanol fermentation. Preferentially, yeast requires readily metabolized 6-carbon sugars such as glucose or mannose. Therefore, the use of an engineered fermentable strain to utilize other types of sugar instead of glucose will definitely increase the conversion percentage (based on theoretical yield) from sugar hydrolysate, as discussed by other researchers (Martin *et al.* 2002; Yanase *et al.* 2005).

According to Cheng *et al.* (2009), at a concentration of 50 g/L of glucose, Baker's yeast is able to produce 18 g/L, with an ethanol yield of 0.81 g/g. Martin *et al.* (2002) studied ethanol production by recombinant xylose, utilizing *S. cerevisiae* with sugarcane bagasse hydrolysate as the carbon source. According to the study, at a concentration of 20.8 g/L of glucose and 26.6 g/L of total sugar, the yeast produced up to 6.0 g/L ethanol with an ethanol theoretical yield of 0.29 g/g. Other research done by Sukumaran *et al.* (2009), showed that ethanol fermentation by *S. cerevisiae* at reducing sugars concentration 60 g/L, yielded 12.34 g/L ethanol and doubled (at 120 g/L) the reducing sugars concentration.

An attempt of ethanol fermentation using OPEFB hydrolysate proved that even at very low concentration of glucose, yeast is able to produce ethanol at very low concentration. Many of the studies done for ethanol fermentation have used excessive concentrations of glucose, from 20 g/L to 200 g/L, to obtain high concentrations of ethanol. Excessive amounts of glucose made possible the conversion of glucose to ethanol by yeast with high theoretical yield due to surplus carbon source for survival. Glucose is easily and readily metabolized as a carbon source by many organisms. Yeast consumes glucose as carbon source for survival and production other metabolic products such as ethanol and carbon dioxide.

However, according to Cheng *et al.* (2009), excessive concentration of glucose (high feeding rate) in ethanol fermentation may cause catabolic repression and glucose overflow metabolism in yeast. A high substrate loading was also found to affect pH, viscosity, and activity of the medium. A high concentration of ethanol also inhibits yeast growth and proliferation. Most yeast can withstand up to 11% of ethanol concentration in the medium.

Sugars consumption by Baker's yeast during ethanol production of OPEFB hydrolysate was investigated to help account for the high levels of reducing sugars contained in fermentation broth after ethanol fermentation. Samples from the initial and final hour of ethanol fermentation were injected into an HPLC device to detect sugar composition in the fermentation broth. Types of sugar detected in the fermentation broth are shown in Table 3. During the initial hour of bioethanol production (0 h), substantial amounts of sugars were detected. Initially, fermentation broth contained 9.27 g/L of cellobiose, 3.21 g/L glucose, and 0.96 g/L of xylose. However after 24 h of bioethanol production, residual sugars of xylose and cellobiose were still detected at 0.97 g/L and 9.39 g/L, respectively. However after 24 h of ethanol fermentation, residual sugars of xylose and cellobiose were still detectable. This proved that only glucose sugar was consumed by the yeast for ethanol conversion, not xylose and cellobiose. Constant accumulation of xylose sugar can be observed throughout ethanol fermentation of OPEFB hydrolysate, as shown in Table 3. According to Prasad *et al.* (2007), *S. cerevisiae* is able to take up xylose sugars with the systems uses for glucose sugar. However, it suffered inefficient uptake and cannot be utilized due to the absence of an active isomerization system to convert xylose to xylulose (Prasad *et al.* 2007; Jeffries 1990). Xylose utilization to ethanol requires isomerization of xylose to xylulose and subsequent conversion of the xylulose produced to glyceraldehydes-3-phosphate and fructose-6-phosphate in the pentose phosphate pathway (Meinander *et al.* 1999).

Table 3. Types of Sugars Concentration during Initial and Final Hour of Ethanol Fermentation by Baker's Yeast

Sugars	Concentration g/L	
	Initial (0 h)	Final (24 h)
Cellobiose	9.27	9.39
Glucose	3.21	0.00
Xylose	0.96	0.97

Results shown also indicate the low cellulase enzyme loading during saccharification of OPEFB. Low enzyme loading specifically for β-glucosidase activity resulted in a high accumulation of cellobiose (Gupta and Lee 2009). Gupta and Lee (2009) had

found that high adsorption of β-glucosidase by filter paper reduced the enzyme availability in the liquid, thus causing high accumulation of cellobiose based on enzymatic hydrolysis of filter paper.

According to Taherzadeh and Karimi (2008), utilization of cellobiose is crucial in ethanol production from lignocellulosic materials. Utilization of cellobiose eliminates the need for a high activity of β-glucosidase enzymes. Thus, this will not only be beneficial for the saccharification process but also improve total effectiveness of ethanol conversion from lignocellulosic matter. Promising glucose and cellobiose fermenting yeast that can be utilized for ethanol conversion is *Brettanomyces custersii*. Other research done by Yanase *et al.* (2005), showed that the recombinant microbe of *Zymobacter palmae* bearing β-glucosidase gene is able to ferment both glucose and cellobiose into ethanol with growth of organism. However, glucose was preferentially utilized by the microbes compared to cellobiose with a faster rate of fermentation. Fermentation of cellobiose by *Z. palmae* achieved 95% theoretical yield with no glucose liberated and production of other metabolites.

CONCLUSIONS

1. Crude cellulases cocktail from local isolates of *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 was found to be successful to produce the highest level of reducing sugars from OPEFB (8.37 g/L).
2. Scanning electron microscopy (SEM) of raw, pre-treated, and saccharified OPEFB fibers showed equivalent results with sugars produced.
3. The detected level of ethanol was low during the fermentation of OPEFB hydrolysate as compared with similar tests in which glucose was used as a model reference to check the ability of Baker's yeast as the fermenting microbe. Hence, Baker's yeast was found to be unsuitable for the use as ethanol fermenting microbe for sugar hydrolysate comprising C5 sugar monomers. However, the conversion of OPEFB to bioethanol was proven to be possible, as certain improvement should be done, especially in the use of appropriate fermenting microbes to fully utilize the sugar produced.

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