Effect of Physical Parameters on Second Generation Bio-Ethanol Production from Oil Palm Frond by *Saccharomyces cerevisiae*

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The aim of this work was to develop a suitable bioprocess to maximize production of second generation bio-ethanol in submerged fermentation by using fermentable sugars derived from oil palm frond (OPF) through the solid state fermentation (SSF) system. The strain *Saccharomyces cerevisiae* was selected, and fermentation conditions were refined at the laboratory scale. Following optimization of inoculums size of *S. cerevisiae* and concentration of fermentable sugars (growth medium), yields of ethanol production as high as 23.10 g/ L were obtained, compared to 5.61 g/L before optimization. *S. cerevisiae* cells were able to assimilate the majority of the fermentable sugars from OPF. The results demonstrated that the culture conditions in fermentation process can significantly influence the ethanol yield in the flask system by using fermentable sugars obtained from the enzyme hydrolysis of OPF as fermentation medium.

Keywords: Bio-ethanol; Fermentation; Oil palm frond; Reducing sugars; Saccharomyces cerevisiae

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INTRODUCTION

Because the energy crisis is rising along with the global population, alternatives to fossil fuels must be developed. To overcome these problems, many scientists have tried to convert eco-friendly biomass into fuel ethanol as an alternative to fossil fuels (Lee *et al*. 2001). Development of bioethanol production from lignocelluloses is considered an important process that may increase energy efficiency and reduce the costs of the biomass option to mitigate greenhouse gas (GHG) emissions (Sheehan *et al*. 2003).

Ethanol production from lignocellulosic materials has been the subject of research and development in many countries in the last decades (Purwadi and Taherzadeh 2008). The main motivation for this subject has been to develop an alternative renewable transportation liquid fuel from lignocellulosic resources. Ethanol is an attractive alternative fuel because it is not only a renewable bio-based resource but also is oxygen-ated, thereby providing the potential to reduce particulate emissions in compression-ignition engines (Hansen *et al*. 2005). Ethanol can be produced from biomass by the hydrolysis and sugar fermentation processes. Biomass wastes contain a complex mixture of carbohydrate polymers from the plant cell walls, known as cellulose, hemicellulose, and lignin. The cellulose and the hemicellulose portions are broken down (hydrolysed) by enzymes or dilute acids into monomer sugar, which is then fermented into ethanol. Lignin, also present in the biomass, is normally used as a fuel for boilers in the ethanol

production plants. There are three principle methods of extracting sugars from biomass. These are concentrated acid hydrolysis, dilute acid hydrolysis, and enzymatic hydrolysis.

Malaysia is one of the main countries that export the palm oil in the world besides Indonesia and Ghana (Mohamad 2008). Oil palm plantation has generated huge quantities of lignocelluloses resources that are consistent in quality and quantities throughout the year. The oil palm frond (OPF) is produced at a rate of 26.2 million tonnes per annum from oil palm plantations. Therefore, OPF is a solid agro-waste that is abundantly available on oil palm plantations in Malaysia (WanRosli *et al*. 2007). Currently, the disposal of OPF is by direct decaying in the natural environment or by burning on site, with only a small amount being composted. These practices are creating environmental problems, and alternative ways to utilize and/or dispose of OPF are needed. Enzymatic hydrolysis of OPF could produce reducing sugars, such as glucose, fructose, xylose, mannose, and sucrose (Lange and Simatupang 1994). According to WanRosli *et al*. (2007), the main monomer of OPF is glucose. *Saccharomyces cerevisiae* is a type of yeast which can assimilate glucose as carbon sources to produce ethanol in anaerobic fermentation (Matsushika *et al*. 2009). Thus, the OPF has great potential to be used as substrate for the production of bioethanol in Malaysia. Bioethanol is carbon 'neutral' and free from sulfur and aromatics; therefore it can be considered as not harmful to living organisms. Thus, by using bioethanol as fuel, we can effectively protect our next generation against the upheaval resulting from global warming in the future (Goh *et al*. 2010).

The aim of the present work was to enhance the ethanol production by using the fermentable sugars obtained from OPF in submerged fermentation system. Important variables of the process were explored, including the concentration of reducing sugars and the size of inoculums used in the fermentation process.

EXPERIMENTAL

Materials and Methods

Substrate preparation

Processed OPF fiber strands were obtained from a local palm oil mill in Sungai Bakap, Penang, Malaysia. OPF obtained was dried in an oven at 50°C and milled with a grinder machine (Rong Tsong Precision Technology Corporation, Taiwan) to a particle size of 0.5 mm.

Microorganisms maintenance

Aspergillus niger USM AI1 and *Saccharomyces cerevisiae* were obtained from the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia (USM), Penang, Malaysia. The *A. niger* USM AI1 was grown on Potato Dextrose Agar (Himedia, India) slants and incubated at 30°C for 5 days until sporulate. The yeast used, *S. cerevisiae* was grown on Malt Extract Agar (Merck, Germany) slants and incubated at 30°C for 3 days. All the culture slants were kept at 4°C until further used.

Enzymatic hydrolysis of OPF

Enzymatic hydrolysis of OPF was carried out by using SSF. SSF was conducted by using an aluminum tray (30 cm x 20 cm x 6 cm). A total of 60.0 g of OPF with the

size of 0.5 mm was weighed and put into the tray. The tray containing approximately 0.8 cm high of the milled OPF layer was sterilized in an autoclave at 121°C for 20 minutes. Sterilized OPF was inoculated with 12 mL of *A. niger* USM AI1 inoculum (1 x 10^7 spores / mL), and the initial moisture content of the substrate was adjusted to the ratio of 1: 1.6 (w/v) by using sterile distilled water. The contents were mixed thoroughly by using a sterile spatula and incubated at room temperature (30 ± 2ºC). Sample as a whole tray in triplicates, were withdrawn after 4 d of cultivation.

Extraction of fermentable sugar

The crude fermentable sugars from the fermented materials were extracted by a simple contact method. The 60.0 g of fermented OPF was added with 1 L distilled water. Contents were mixed by spatula every 20 min for 2 h at room temperature (30 ± 2ºC). The suspension was filtered using filter paper (Whatman, No.1) under vacuum condition, and then it was further centrifuged for 30 min at 5000 rpm, 4°C (Sigma 4k15, Sartorius, Germany) to obtain the clear crude fermentable sugar. The crude fermentable sugar was concentrated by using rotary-evaporator (Eyela OSB 2100, China), and then used as the crude fermentable sugars for analysis. The concentrated fermentable sugar was kept in the freezer as a fermentation medium source for the production of bioethanol.

Ethanol fermentation in flask system

Ethanol fermentation was carried out using the modified method of Choo (2002).The 100 mL of fermentable sugars obtained were fermented in a 250 mL conical flask. Submerged fermentation in flask system was conducted with 200 rpm agitation at 30°C. The concentrated fermentable sugars were further diluted in the range of 2, 4, 6, and 8% (w/v) and the initial pH medium was adjusted to 5, inoculum size of *S. cerevisiae* in the range of 6, 8, 10, 12% (v/v) with concentration of inoculum of OD_{540}= 0.5 (approximately 0.65 g dry cell/ L) were used in this study. Samples were collected at regular intervals of 6 h for 72 h. The collected sample was then centrifuged at 5000 rpm for 20 min at 4°C (Sigma 4k15, Sartorius, Germany). The supernatant obtained will be used to determine the ethanol produced and fermentable sugar consumed in this system. The experiments were carried out in triplicates, and the results were expressed as mean of three experiments.

Analytical Methods

Analysis of fermentable sugar

Fermentable sugar obtained was measured by the Nelson and Somogyi (Breuil and Saddler 1984) procedure. The fermentable sugar obtained was further characterized by using high performance liquid chromatography (HPLC) (Waters Corporation, USA), pump equipped with an automatic injector, 10 µL injection capacity loop, 4.6 mm x 250 mm (C-18) High-Performance Carbohydrate Analysis Cartridge Column (Waters, Ireland), and chromatography computing integrator with ELSD detector (Polymer Laboratories, UK). The mobile phase used was acetonitrile and the flow rate was adjusted to 1.0 mL min^{-1} at room temperature (30 ± 2°C).

Ethanol analytical methods

Ethanol production was estimated by gas chromatography Clarus 500 (Perkin Elmer,USA) with J &W GC column, USA (30.0 m in length, Inner diameter of 0.32 mm, and film of 0.1 µm). The temperature of the column and the injector were set at 200°C.

The pressure of injector and column was adjusted to 5 kg/ cm^2. Nitrogen gas was used as gas carrier in this analysis.

Biomass of S. cerevisiae

Biomass was expressed as dry cell weight calibrated by optical density at 540 nm using a spectrophotometer (Genesys 10 uv, Spectronic Unicam, USA). The equation of the dependency as illustrated in the linear equation below, where *y* is a reading obtained from the spectrophotometer.

$$\text{Dry cell weight (g/L)} = \frac{y\ (\text{optical density}_{540}) + 0.0316}{0.8221} \quad (1)$$

Conversion rate of reducing sugars to ethanol

The conversion rate of reducing sugar to ethanol was calculated as the concentration of the ethanol production contained in the fermentation medium divided by the consumption of reducing sugar by *S. cerevisiae*, as illustrated in Eq. 2:

$$\text{Conversion rate of reducing sugars to ethanol (\%)} = \frac{\text{Ethanol production (g/L)}}{\text{Consumption of reducing sugar (g/L)}} \times 100\% \quad (2)$$

Statistical Analysis

The data were analyzed by student *t*-test by using Statistical Package for the Social Sciences (SPSS version 12.0) software (SPSS, Chicago, IL, USA). Statistical significance was assumed at the 0.05 levels ($p<0.05$).

RESULTS AND DISCUSSION

The dry milled OPF was used as solid substrate for this experiment to obtain the fermentable sugar for the second generation bio-ethanol production. OPF was chosen as a substrate for SSF mainly due to its content of the main components of the lignocellulosic complex (Goh *et al*. 2010). Rozman *et al*. (1998) and WanRosli *et al*. (2007) have reported that the lignin content in the OPF is 18.3% and 15.2%, respectively. The lignin content in the OPF is lower compared to other hardwood plants. For example, the lignin content in rye (a grain) is 25.3% (Garcia-Cubero *et al*. 2009), and eucalyptus oil (eucalyptus) is 22% (Alcaide *et al*. 1990). Ash content in dry OPF is about 2.25% (Lim *et al*. 2012). Ma *et al*. (2009) reported that ash content in dry paddy straw is as high as 16.2%. Therefore, low ash content in the OPF has made it suitable for use as substrates in the SSF processes. In our previous study (Lim *et al*., 2011), the percentage of holocellulose in OPF was found to be 60.71%. Holocellulose obtained from OPF was higher compared to the percentage of holocellulose derived from rye (54.3%) (Garcia-Cubero *et al*. 2009), dry rice straw (49.6%) (Ma *et al*. 2009), and dried orange peel (30.0%) (Mamma *et al*. 2008). Therefore, OPF shows potential as a substrate particularly in the SSF process for fermentable sugar production.

Table 1 shows the yield of monosaccharides and total fermentable sugar obtained from OPF after four days of fermentation by *A. niger* USM AI1. OPF released about 25.13% (w/w) of total reducing sugars, containing xylose (3.34%), fructose (4.40%), and

mainly glucose of 17.39%. The presence of glucose is an important key; it is a preferred carbon source for the bioethanol production process because glucose can be converted to ethanol with higher yield (Dien *et al*. 2006).

Table 1. Yield of Monosaccharides and Total Sugars of OPF after Enzymatic Hydrolysis by *A. niger* USM AI1 in SSF

Substrate	Monomeric sugars (%, w/w)			Total sugars (% g/ g OPF)
	Glucose	Fructose	Xylose	
Oil palm frond	17.39	4.40	3.34	25.13

The cultivations in the current work were carried out with *S. cerevisiae* [6% (v/v) of inoculums size] in fermentable sugar obtained from enzymatic hydrolysis from OPF without adding any nutrient in the flask system. The profile of ethanol production by *S. cerevisiae* in the flask system before improvement is shown in Fig. 1. It was observed that the concentration of ethanol increased with time, regardless of carbon source, until 54 h of cultivation time. After 54 h of cultivation time, the maximum yield of ethanol was 5.61 ± 0.36 g / L. The concentration of reducing sugars was reduced during ethanol fermentation, and it became constant after 48 h, at which point it was recorded as 3.61 ± 0.52 g / L. This indicated that a total of 81.95% of the reducing sugar can be fermented by *S. cerevisiae* yeast cell. The conversion rate of reducing sugar to ethanol production was found to be 34.23%. Thus, this observation was similar with the finding of Gupta *et al*. (2009), who have reported the rate of sugar consumption in the process of ethanol fermentation. The results also showed that *S. cerevisiae* was able to ferment sugars derived from OPF by *A. niger* USM AI1 in SSF to produce ethanol without adding any additional nutritional sources. The optimal growth of the yeast cell was 1.01 ± 0.05 g / L at 54 h of cultivation time.

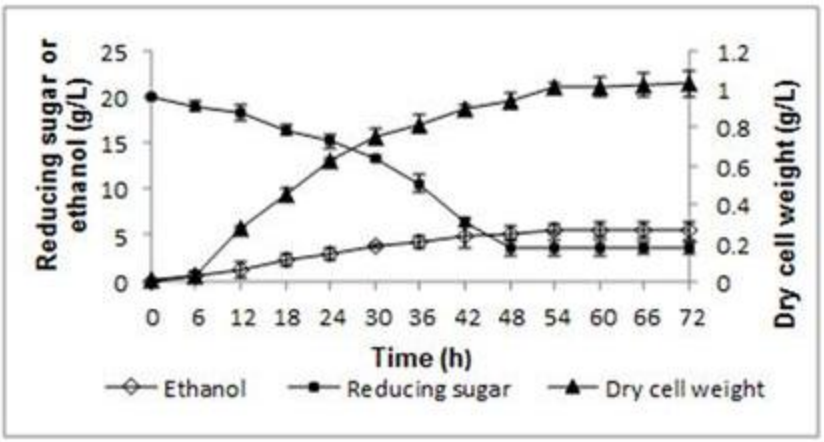

Fig. 1. Profile of ethanol production, reducing sugar consumption, and dry cell weight of *S. cerevisiae* in flask system before improvement of the physical parameter

Effect of time course

The time course of the ethanol fermentation of reducing sugar hydrolyzate from OPF was performed with various inoculums concentration of *S. cerevisiae*, and the results are presented in Fig. 2. A range of 6 to 12 % (v/v) of inoculums size was studied to enhance the utilization of fermentable sugar, and thereby improving ethanol production

(Fig. 2). Studies on the effect of inoculum's size on ethanol production showed that 10% (v/v) was the optimum level. With this ratio, the consumption of sugar by yeast cells was the highest compared to other percentage of inoculum size used. However, at the optimum inoculum level, the concentration of reducing sugar remained constant after 42 h of fermentation process, and reached a final concentration of 2.90 ± 0.09 g / L (Fig. 2a). It was found that 85.50% of reducing sugars were assimilated by the yeast in this study.

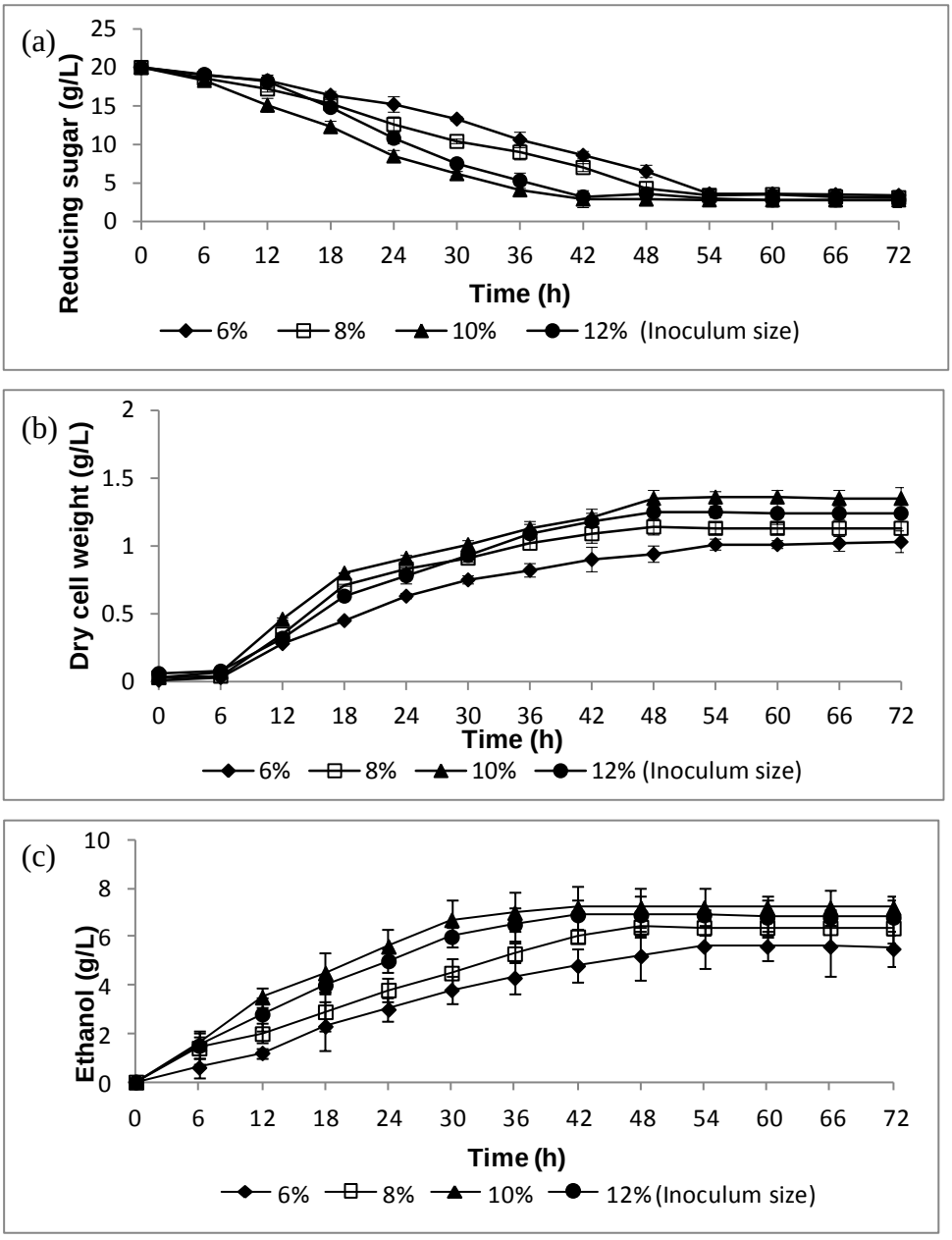

Fig. 2. Effect of inoculums size of *S. cerevisiae* on (a) reducing sugar uptake, (b) biomass production, (c) ethanol production

Effect of inoculum size

The effect of different inoculum size on the production of ethanol is shown in Figs. 2b and 2c, respectively. The rate of yeast cell growth depended on the size of the initial inoculum used. The results show the higher inoculum size was used, the more

yeast cell biomass was produced. This is likely due to the higher inoculum size (not exceeding the optimum level), which makes it possible for cells to multiply rapidly in the fermentation medium (Fig. 2b). Oliva *et al*. (2005) and Tomas-Pejo *et al.* (2009) have reported that the optimum level of inoculum size will increase the viability of the cells in the ethanol fermentation process. In this study, after 48 h of fermentation time, the optimum inoculum size of 10% (v/v) reached the maximum level biomass of yeast cell (1.35 ± 0.06 g / L) compared with other inoculum sizes. However, an increase in inoculum size to 12% (v/v) (beyond the optimal level) caused the biomass of the yeast cell to drop to 1.25 ± 0.04 g / L after 48 h of fermentation time.

Figure 2c shows that the different inoculum sizes were able to influence the production of ethanol. The rate of ethanol production at different inoculum size showed significant differences ($P < 0.05$). When 10% (v/v) of inoculum size was used, the production of ethanol was at a maximum of 7.15 ± 0.91 g / L after 42 h of fermentation time. The results showed the percentage of reducing sugar converted to ethanol was 41.80%. By contrast, when 12% (v/v) of inoculum size was used, the production of ethanol was not able to be increased, showing only 6.91 ± 0.53 g / L after 42 h of fermentation time. Mojovic *et al*. (2006) reported that increasing the inoculum size over the optimum level will not increase the final concentration of ethanol in ethanol fermentation process. Additionally, Nagodawithana and Steinkraus (1976) have reported that there is an optimal level of inoculum size in fermentation process of ethanol. When inoculum size increased, the rate of yeast growth and cell survival will decrease. According to Nagodawithna and Steinkraus (1976), at a higher inoculum size than the optimal level, the additional nutrient is needed to maintain the cell survival. Nevertheless, lower concentration of inoculums size may not be sufficient for initiating of the cell growth, and yet affect ethanol production.

Effect of concentration of reducing sugars

In order to enhance the production of bioethanol, we further performed the fermentation process at different concentrations of reducing sugars and constant inoculum size of 10% (v/v). For that purpose, 2 to 8 % (w/v) of reducing sugar was used in this study. It was noted that when compared statistically, the ethanol concentration obtained was significantly different ($P < 0.05$) at different concentrations of reducing sugars used in the fermentation process. When 2% (w/v) of reducing sugar was used as medium, about 85.51% of the sugars in the medium can be used by the yeast cell (Fig. 3a). The percentage of conversion of the reducing sugars to ethanol and ethanol production were 41.80% and 7.15 ± 0.91 g / L, respectively, after 42 h of fermentation time (Fig. 3c). This is expected because lower sugar concentrations will cause the cell respiration and fermentation process to occur at the same time, resulting in a less efficient fermentation process.

When 6% (w/v) concentration of reducing sugar was used, the ethanol fermentation process was found to be more efficient compared with the 2, 4, and 8% (w/v) concentrations of reducing sugar considered in this study. The percentage of sugar consumption was recorded as 84.72%. The conversion rate of reducing sugar to ethanol was 45.44%. Siqueira *et al*. (2008) have reported that theoretically 50% is the maximum percentage for converting reducing sugar to ethanol through fermentation. The results obtained showed OPF to be an effective substrate in providing glucose for second generation bioethanol fermentation.

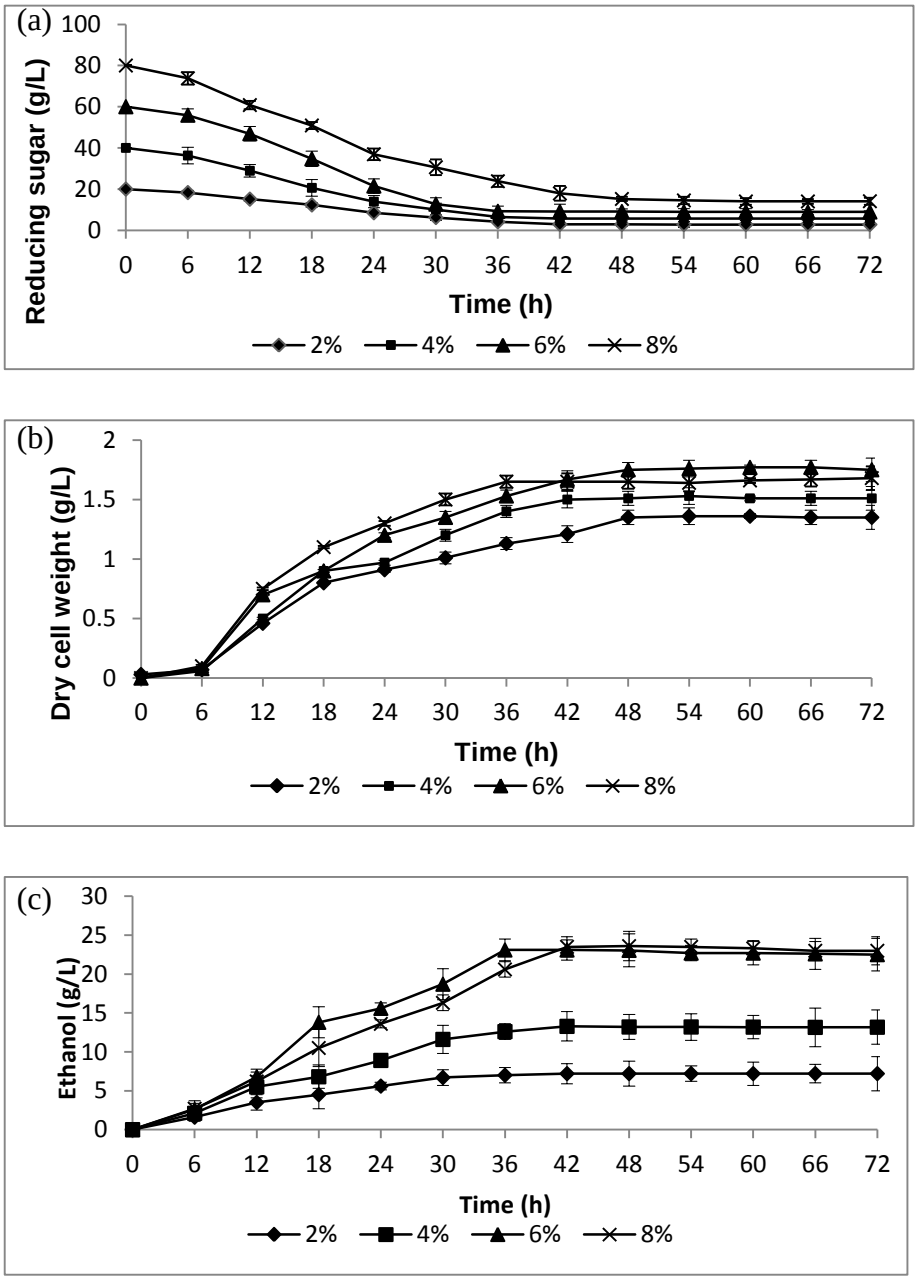

Fig. 3. Effect of initial concentration of reducing sugar for *S. cerevisiae* on (a) reducing sugar uptake, (b) biomass production, (c) ethanol production

On the other hand, the ethanol obtained was about 23.10 ± 1.27 g / L (Fig. 3C) after 36 h of fermentation. According to Siqueira *et al*. (2008), an optimal concentration of reducing sugars is able to increase the production of ethanol in ethanol fermentation process. In addition, the time required to reach maximum ethanol production has been shortened from 42 h to 36 h.

Figure 3b shows that the maximum yeast cell biomass at 6% (w/v) (optimum concentration) of reducing sugar was higher than with other percentages of reducing sugar used as fermentation medium. Optimal concentration of reducing sugars is an important key for converting the sugars to ethanol particularly in high concentrations

(Lim 2011). It was noted that at 8% (w/v) concentration of reducing sugar used as medium, the efficiency of utilization of reducing sugars and the percentage of sugars consumption were decreased compared to the optimal reducing sugar concentration (6%) both recorded as 35.80% and 82.41% respectively.

Generally, the lower percentage of conversion reducing sugar to ethanol indicates that the fermentation process may cause inhibition problems. According to Jones *et al*. (1981), reducing sugars that do not consist of pure glucose can also affect the production of ethanol. Reducing sugars obtained from this study is composed of 3.34% xylose. Therefore, higher concentrations of reducing sugar used as fermentation medium will also increase the concentration of xylose, and this is probably one of the reasons that can explain the observed decline of the conversion percentage of reducing sugars (hexose) to ethanol in this study. In addition, the higher osmotic pressure due to higher concentrations of fermentation medium also can inhibit ethanol diffuse out from the yeast cell. According to D'Amore and Stewart (1987), accumulation of intracellular ethanol is toxic to yeast cell. Therefore, increase of osmotic pressure in the yeast cell will reduce the yeast cell growth, and yet ethanol production will decline in the fermentation process (Sanchez and Cardona 2008).

Effect of time course after optimization

Figure 4 presents the time course of the ethanol fermentation after improvement of the physical parameters in this study. The maximum ethanol production was obtained (23.10 g/L) at 30°C, 10% (v/v) of inoculum size, 6% (w/v) concentration of reducing sugars, 200 rpm of agitation speed, and 36 h of cultivation time. Moreover, we found that the conversion of reducing sugars to ethanol was increased from 34.23% to 45.44% after improvement of the physical parameter conditions. The conversion percentage was increased by 11.21%. The results obtained are similar with results performed by Mojovic *et al*. (2006) who have reported growth parameter can influence the ethanol production yield in submerged fermentation. Hence, the inoculum size and concentration of fermentable sugars are significant parameters affecting ethanol fermentation process.

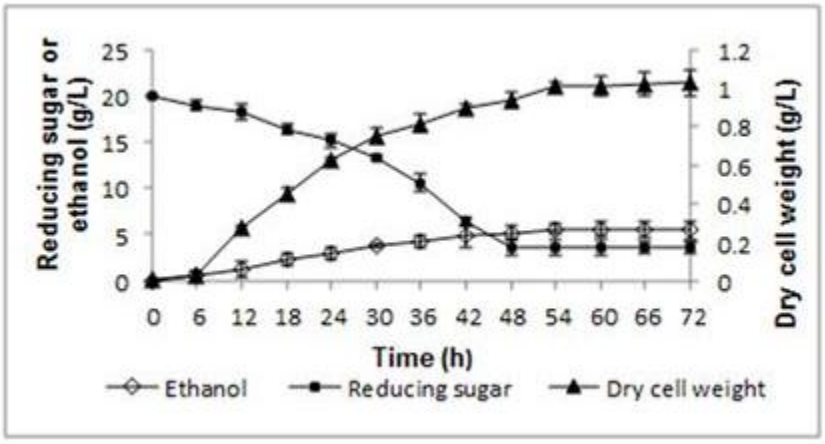

Fig. 4. Profile of ethanol production, reducing sugar consumption, and dry cell weight of *S. cerevisiae* in flask system after improvement of the physical parameter

CONCLUSIONS

1. *Saccharomyces cerevisiae* was able to assimilate the fermentable sugars obtained from OPF by *Aspergillus niger* USM AI1 under solid state fermentation into bio-ethanol.
2. Improvement of important cultivation variable resulted in higher yield of ethanol production.
3. The optimum conditions were determined to be 6% (w/v) of reducing sugars used as fermentation medium, 10% (v/v) of inoculums size, and 36 h of cultivation time.
4. These results also suggest that Malaysia generates a great deal of OPF waste in oil palm plantation, which has the potential to be converted into second-generation bio-ethanol as an alternative energy source.

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