Research Journal of Environmental Sciences

ISSN 1819-3412

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Environmental Sciences 5 (6): 573-586, 2011
ISSN 1819-3412 / DOI: 10.3923/rjes.2011.573.586

Biothanol Production from Enzymatically Saccharified Empty Fruit Bunches Hydrolysate using *Saccharomyces cerevisiae*

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ABSTRACT

Cellulosic ethanol production was carried out using Empty Fruit Bunches (EFB) via Separate Hydrolysis and Fermentation (SHF) approach. In this study, the EFB was initially pretreated with alkaline treatment using 1% (w/v) sodium hydroxide (NaOH) followed by mild acid hydrolysis using 0.7% (v/v) sulfuric acid (H_2SO_4) and enzymatic saccharification prior to fermentation with *Saccharomyces cerevisiae*. Pretreatment of EFB via alkaline and acid hydrolysis produced 13.38±0.18 g L^{-1} of xylose and 0.54±0.18 g L^{-1} of glucose. A total of 16.4±3.86 g L^{-1} and 3.85±2.2 g L^{-1} of glucose and xylose respectively was produced during the enzymatic saccharification. The EFB hydrolysate obtained was subjected to fermentation in batch mode and the influence of pH, temperature and rate of agitation on bioethanol production were evaluated. From optimization works of these various fermentation parameters, it was found that the highest ethanol yield ($Y_{p/s}$) was 0.51 g ethanol/g glucose achieved from 50 g L^{-1} of acid enzymatically saccharified EFB hydrolysate at pH 4, 30°C and 100 rpm was recorded at 72 h of incubation. This study showed that EFB generated from palm biomass can be used as a lignocellulosic ethanol production feedstock for the future.

Key words: Empty Fruit Bunches (EFB), pre-treatment, hydrolysis, fermentation, ethanol

INTRODUCTION

Malaysia is one of the largest producers of palm oil in the region and the product has contributed the biggest income to the country for many years. With the rapid growth of palm oil production in Malaysia, the amount of biomass residues generated also has shown a corresponding increase. As in 2010, the oil palm planted area in the country is 4.8 million hectares. The total average of 18.03 tones Fresh Fruit Bunches (FFB) per hectare palm plantation has been produced from the palm oil industry (Choo, 2011). Based on this figure, palm oil plantation areas has produced a total of more than 66.63 million tonnes of biomass residues such as Empty Fruit Bunches (EFB), mesocarp fiber, shell, palm kernel cakes, fronds, trunks and Palm Oil Mill Effluent (POME) in 2010 (Goh *et al*., 2009).

In a country that has a significant amount of agricultural activities, biomass can be a very promising alternative source of renewable energy. With increased awareness on reducing

greenhouse gas emissions, conversion of biomass residues into renewable energy such as ethanol, bio-oil, biogas, syngas and biohydrogen has attracted global attention. Biomass residues are valuable as an immediate and relatively cheap energy feedstock for development of a bio-energy industry (Ibeto *et al*., 2011). Besides, the utilization of biomass also offers an environmentally friendly way of disposing the unwanted by-products generated from palm oil mills.

EFB is one of the major biomass types generated in the oil palm industry. From 85.71 million tonnes of Fresh Fruit Bunches (FFB) produced in 2009, an estimated amount of 6.76 million tonnes of dried EFB was generated from palm oil mills. Generally, EFB is lignocelulosic materials consisting of a mixture of carbohydrate polymers, i.e., cellulose and hemicellulose (Taherzadeh and Karimi, 2007). It is estimated that EFB comprise of 44.2% cellulose, 33.5% hemicellulose and 20.4% lignin, respectively (Umikalsom *et al*., 1997; Aziz *et al*., 2002). Basically, cellulose is a polymer of α-D-1,4-linked anhydrous glucose unit. In contrast, hemicellulose is a random, amorphous copolymer comprising several sugar monomers such as glucose, fructose, xylose and mannose. Hence, EFB with high contents of cellulose and hemicellulose can be potentially converted into ethanol.

In most of the ethanol production using various different lignocellulosic biomass, two main processes i.e. pretreatment involving alkaline and acid hydrolysis and enzymatic saccharification to produce glucose and xylose followed by fermentation with yeast to produce ethanol (Demirbas, 2005). Ethanol production via fermentation is a complex biochemical process with yeast or bacteria utilizing fermentable sugar as substrate for their growth and converting them to ethanol, carbon dioxide and other metabolic end product. Several factors that can effect ethanol fermentation should be considered. During ethanol fermentation, most of the yeast cells used suffer from various stresses, including environmental stress such as glucose concentration, nutrient deficiency, temperature, rate of agitation and pH (Graves *et al*., 2006; Arisra *et al*., 2008; Yah *et al*., 2010).

Temperature in particular has a crucial effect, as it can affect the development of yeast used, yields of ethanol and the types of fermentation by-products (Sener *et al*., 2007). Temperature can also affect the sensitivity of the yeast in relation to alcohol concentration, growth rate, rate of fermentation, enzyme and membrane function (Torija *et al*., 2003). Moreover, pH and rate of agitation also have been reported to be important factors for ethanol production from lignocellulosic hydrolysate (Fleet and Heard, 1993; Liu and Shen, 2008). Thus, the purpose of this study was to explore the potential of EFB as an ethanol feedstock via Separate Hydrolysis and Fermentation (SHF) and to determine the optimum condition of physical parameters, i.e. pH, temperature and agitation rate that can affect the production of ethanol from EFB hydrolysate.

MATERIALS AND METHODS

EFB preparations: EFB was collected from a palm oil mill located in Padang Jawa, Klang. The bunch was dried at 100±5°C and cut into smaller pieces. It was then milled, sieved and separated in fractions using a test sieve shaker (Endecotts EFL, 2000). The particle size of EFB used for this study was 91-106 μm.

EFB characterization: Dried EFB was initially delignified according to ASTM 1104-56 to produce holocellulose, followed by removal of the hemicellulose fraction using ASTM D1103-60. For holocellulose, approximately 4.0 g of the ground EFB were mixed with distilled water and treated with 2.0 mL acetic acid and 5.0 g sodium chlorite at 70°C for 4 h. The mixture was then filtered

using filter paper and dried at 103°C for 24 h. Determination of holocellulose was carried out using dry weight method. A total of 2.0 g of dried holocellulose obtained were dissolved in 50 mL 17.5% sodium hydroxide solution and then a total of 70 mL were added in the mixture in purpose to separate hemicellulose from the holocellulose and leaving α-cellulose. The insoluble α-cellulose was filtered, then washed with 50 mL 8.3% sodium hydroxide and dried at 103°C for 24 h. Determination of α-cellulose was carried out using dry weight method.

Pretreatment: A total of 5 g of treated pulverized EFB was initially soaked with 1% (w/v) sodium hydroxide (NaOH) at 100°C for 2 h. The treated EFB was then washed with hot water prior to drying the sample at 103°C for 24 h. For the acid hydrolysis process, a total of 5 g of dried EFB was hydrolysed with 100 mL 0.7% (v/v) H_2SO_4 and autoclaved at 125°C for 120 min (Hirayama HVE-50). The treated EFB was then washed with hot water prior to drying at 103°C for 24 h.

For enzymatic saccharification EFB from the acid hydrolysis process was soaked in a 100 mL of acetate buffer solution (pH 4.8) and mixed with cellulase (70 FPU mL^{-1}) (Novozymes) at a temperature of 48°C and rate of agitation 150 rpm for 48 h. The EFB hydrolysate obtained was used for fermentation and determination of optimum fermentation process parameters.

Inoculums preparation: *Saccharomyces cerevisiae* was initially grown on Yeast-peptone-glucose (YPG) and was incubated at a temperature of 35°C and a rate of agitation 150 rpm for 18 to 24 h (Innova 40). In this study, YPG medium consisted of (g L^{-1}); 10 g yeast extract, 20 g peptone and 20 g glucose. After the incubation period, the cells were then harvested by centrifugation at 3000 rpm at 4°C for 15 min (Hettich, Universal 32 R). The pellet was then rinsed twice with sterilized saline solution before being re-suspended in sterilized saline solution to yield an Optical Density (OD) of 1.0 at 600 nm (Hirayama Model U-200). The standardized *S. cerevisiae* was used for subsequent study.

Determination of optimum parameters: Fermentation of EFB hydrolysate from the pretreatment process was carried out by using *S. cerevisea* ATCC 24860. A total of 150 mL of EFB hydrolysate from enzymatic saccharification was prepared in a 250 mL conical flask. A total of 10% (v/v) of standardized active *S. cerevisiae* was added to the hydrolysate prior to incubation into a shaker. To determine the effect of physical parameters on ethanol production, three series of experiments were carried out. For each series of experiments, EFB hydrolysate was harvested every 12 to 24 h interval. The harvested samples were filtered using a 0.45 μm membrane filter and then the filtered samples were put in 2.5 mL vials prior to being analyzed.

Effect of initial pH: The first series of experiments was to determine the effect of initial pH on ethanol production. The ranges of pH used were pH 4, 6, 7 and 8. Initially, the pH of EFB hydrolysate was adjusted using 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH) prior to inoculating with standardized *S. cerevisiae*. The samples were incubated at 30°C with an agitation rate of 100 rpm.

Effect of temperature: A second series of experiments was to determine the effect of temperature on ethanol production. Three different temperatures, i.e., 30, 35 and 40°C were used in this study and the EFB hydrolysate was incubated at the optimum pH obtained from the pH optimization study and a rate of agitation of 100 rpm.

Effect of rate of agitation: The third series of experiments was to determine the effect of rate of agitation on ethanol production. The EFB hydrolysate was incubated at different rates of agitation, i.e., 50, 100 and 150 rpm at the optimum pH and temperature obtained from the previous series of tests.

Products analysis: The fermentable sugar and ethanol concentrations in the EFB hydrolysate were determined using High Performance Liquid Chromatography (HPLC) (Waters, 2707); Sugar Pack ™1 column: 6.5×300 mm, detector temperature: 35°C, column temperature: 75°C, flow rate: 0.5 mL min^{-1} and injector volume of 1 μL. The ethanol yield ($Y_{p/s}$) was calculated as the actual ethanol produced and expressed as g ethanol per g total of sugar utilized and the ethanol fermentation efficiency (%) was calculated based on the ratio of ethanol yield obtained against theoretical maximum ethanol yield. All the kinetics on ethanol fermentation was calculated based on the following formulas (1 to 4):

$$\text{Fermentation efficiency (\%)} = \frac{(\text{Ethanol, g L}^{-1})}{(\text{Glucose, gL}^{-1})\times 0.51}\times 100 \quad (1)$$

$$\text{Ethanol yield (Y p/s)} = \frac{(\text{Ethano, gL}^{-1})}{(\text{Glucose, gL}^{-1})} \quad (2)$$

$$\text{Substrate consumption rate} = \frac{\Delta S}{\Delta t} \quad (3)$$

$$\text{Product formation rate} = \frac{\Delta P}{\Delta t} \quad (4)$$

where, S is the initial substrate (g L^{-1}), P is the actual ethanol produced (g L^{-1}) and t is the time at the optimum ethanol produced (h).

Statistical analysis: All the samples were prepared in triplicate. A t-test was carried out to determine the significant differences between the control and the experimental parameters. The statistical analysis was performed using Minitab 14.3 software.

RESULTS

Pretreatment of EFB: Fermentable sugar from pre-treated EFB: Characterization of pulverized EFB was carried out to determine major component present in the EFB. Table 1 shows the chemical composition of the untreated EFB. Based on the results obtained, it was found that holocellulose fraction of EFB was 83.27±6.11% of total biomass which consist of 54.17±6.55% alpha-cellulose and 29.10±4.49% hemicelluloses. High content of cellulose presence in EFB indicated its potential as a sugar source for ethanol production. Table 2 shows the fermentatble sugar extracted from the EFB. A total of 34.49 g L^{-1} of fermentable sugar was obtained from both acid hydrolysis and enzymatic saccharification (Table 2). The result also indicated that 73.58% of EFB was converted into fermentable sugar consisting of glucose, xylose and fructose with 46.75, 51.6 and 0.52%, respectively. During acid hydrolysis, the highest sugar concentration was xylose at

Table 1: Chemical composition of Empty Fruit Bunches (EFB)

Chemical compound	Treated EFB (%)
Lignin	15.13±6.10
Holocellulose	83.27±6.11
α-cellulose	54.17±6.55
Hemicellulose	29.10±4.49
Ash	2.86±1.20

Table 2: Concentration of fermentable sugars obtained from acid hydrolysis and enzymatic saccharification of EFB

	Pre-treatment (g L^{-1})		
Fermentable sugar	Total (g L^{-1})	Acid hydrolysis	Enzymatic
Glucose	0.54±0.2	16.4±3.86	16.58±1.90
Xylose	13.38±0.18	3.85±2.2	17.72±1.11
Fructose	0.19±0.17	*ND	0.19±1.70
			34.49±5.70

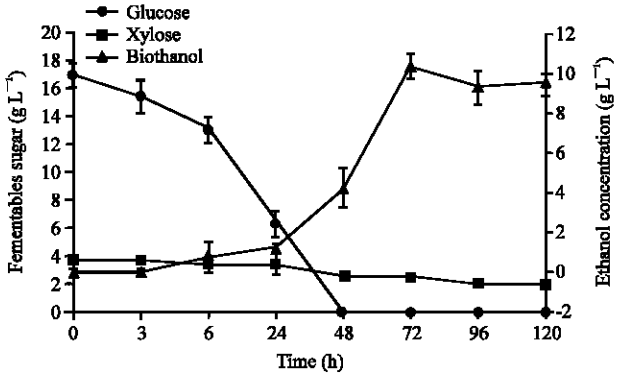

Fig. 1: Ethanol production from EFB hydrolysate incubated at pH 4, 30°C and rate of agitation 100 rpm

13.38±0.18 g L^{-1}, followed by glucose with a concentration of 0.54±0.2 g L^{-1}. In contrast, enzymatic saccharification produced the highest glucose concentration at 16.4 g L^{-1} followed by xylose, 4.1 g L^{-1} (Table 2).

Ethanol from EFB hydrolysate: Figure 1 shows the ethanol production profile from enzymatically saccharified EFB hydrolysate. Results showed that ethanol production was increased with increasing of time after 6 h and remained constant after 72 h of incubation. The profile also indicated that all glucose was completely consumed within 48 h of incubation (Fig. 1). The study also showed that the rate of glucose consumption rate was higher than xylose. The highest ethanol concentration was achieved after 72 h of incubation with 10.28 g L^{-1}, with an ethanol yield ($Y_{p/s}$) of 0.51 g ethanol/g glucose.

Determination of optimum parameter: Effect of initial pH: The effect of different pH on glucose consumption by *S. cerevisiae* and ethanol yield using EFB hydrolysate is shown in Fig. 2 and 3. The highest ethanol yield was obtained at pH 4 with a maximum ethanol concentration of 10.29 g L^{-1}

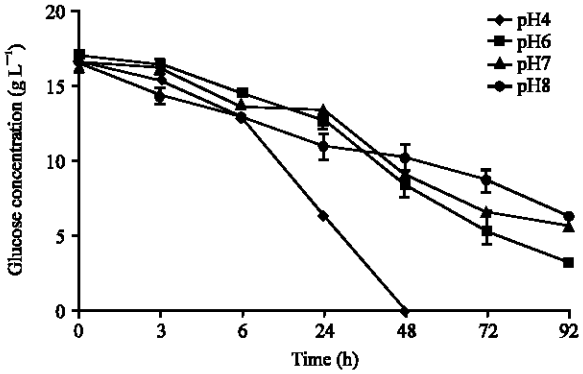

Fig. 2: Glucose consumption by *S. cerevisiae* at different initial pH incubated at 30°C and agitated at 100 rpm

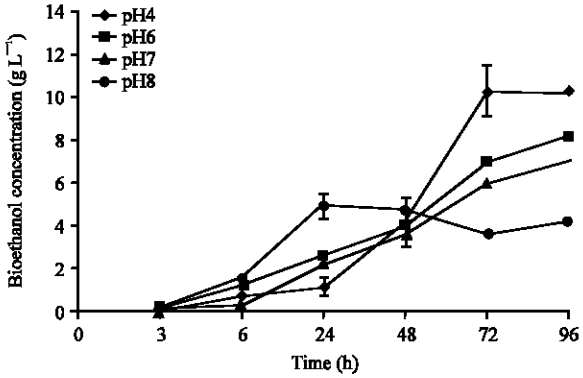

Fig. 3: Ethanol production from EFB hydrolysate at different pH incubated at 30°C and agitated at 100 rpm

Table 3: Effect of different pH on ethanol production from EFB hydrolysate at optimum condition

pH	Glucose consumption rate (g h^{-1})	Ethanol formation rate (g h^{-1})	Ethanol concentration (g L^{-1})	Y p/s (g g^{-1})	fermentation efficiency (%)
4	0.24	0.14	10.29	0.50	119.00
6	0.16	0.11	8.20	0.37	95.12
7	0.13	0.09	7.12	0.33	86.07
8	0.11	0.20	4.20	0.22	49.82

followed by 8.2 g L^{-1} ethanol at pH 6 at 72 h of incubation (Table 3). The respective ethanol yield ($Y_{p/s}$) for both these pH were 0.5 and 0.37 g ethanol/g glucose. Fermentation of EFB hydrolysate at initial pH 7 and 8 showed the lower ethanol concentration corresponding to ethanol yield ($Y_{p/s}$) of 0.33 and 0.22 g ethanol/g glucose respectively. Figure 2 also showed that glucose consumption rate was the highest at initial pH 4 in which it showed complete glucose utilization over 48 h of incubation. The fermentation efficiency was calculated based on the ratio of ethanol yield obtained against theoretical maximum ethanol yield. The highest ethanol fermentation efficiency was obtained at pH 4 with 119%. The high fermentation efficiency value may be due to the presence of other simple sugars such as mannose and arabinose in the hydrolysate, thus contribute to a

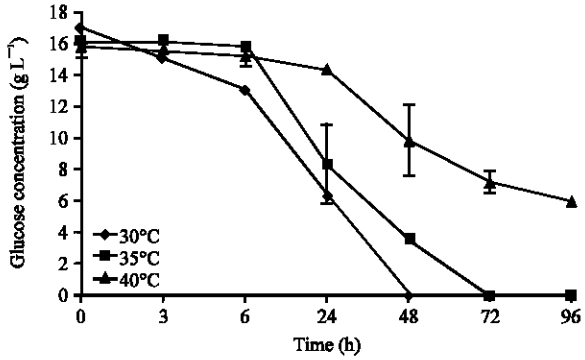

Fig. 4: Glucose consumption by *S. cerevisiae* at different temperatures incubated at pH 4 and rate of agitation 100 rpm

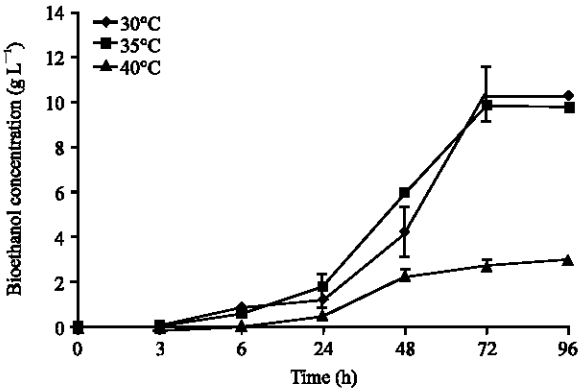

Fig. 5: Ethanol production from EFB hydrolysate at different temperatures incubated at pH 4 and rate of agitation 100 rpm

Table 4: Effect of different temperatures on ethanol production from EFB hydrolysate at optimum condition

Temperature	Glucose consumption rate (g h^{-1})	Ethanol formation rate (g h^{-1})	Ethanol concentration (g L^{-1})	Y p/s (g g^{-1})	Fermentation efficiency (%)
30	0.24	0.14	10.29	0.50	119.00
35	0.23	0.14	9.86	0.63	118.00
40	0.12	0.04	2.90	0.18	3.57

higher concentration of alcohol obtained in the process. As fermentation of EFB hydrolysate at pH 4 showed the maximum ethanol production, this pH value was used in all the following experiments.

Effect of temperature: The effect of different temperatures on glucose consumption by *S. cerevisiae* and ethanol production from EFB hydrolysate is shown in Fig. 4 and 5. The highest ethanol concentration was 10.29 g L^{-1} obtained when EFB hydrolysate was incubated at 30°C, followed by 9.86 g L^{-1} at 35°C which corresponded to an ethanol yield ($Y_{p/s}$) of 0.51 and 0.54 g ethanol/g glucose, respectively (Table 4). Based on the calculation, ethanol conversion at 30°C and 35°C was higher with an ethanol yield of 52.4 and 47.6%, respectively (Table 4). Even though

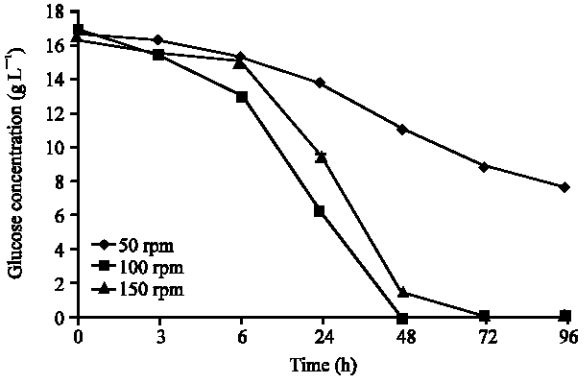

Fig. 6: Glucose consumption by *S. cerevisiae* at different rate of agitations incubated at pH 4 and 30°C for 96 h of incubation

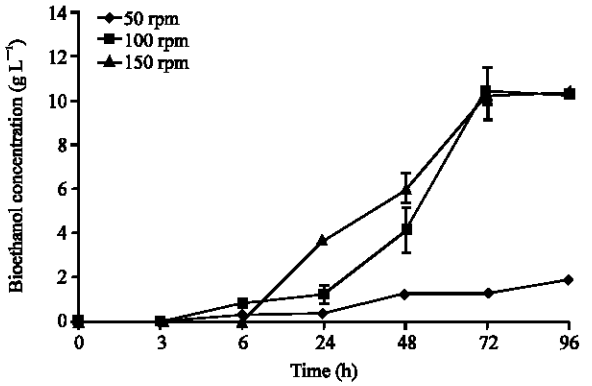

Fig. 7: Ethanol production from EFB hydrolysate at different rate of agitations incubated at pH 4 and 30°C for 96 h of incubation

ethanol concentration and yields obtained from both 30 and 35°C were comparable, however, studied indicated that there were not statistically significant ($p \leq 0.05$) between both temperatures. Fermentation at 40°C showed the lowest ethanol production rate i.e. ethanol production with an ethanol yield ($Y_{p/s}$) of 0.36 g ethanol/g glucose compared to the other two temperature at 30 and 35°C. This indicated that fermentation at higher temperature was able to inhibit ethanol production. As fermentation of EFB hydrolysate at 30°C showed the maximum ethanol production, thus temperature of 30°C was used in all following experiments.

Effect of rate of agitation: In assessing the effect of agitation rate in ethanol production, it was found that the maximum ethanol concentration 10.35 g L^{-1} was obtained at agitation rate of 150 rpm, followed by 10.29 g L^{-1} at agitation rate of 100 rpm which corresponded to an ethanol yield ($Y_{p/s}$) of 0.53 g and 0.5 g ethanol/g glucose respectively (Table 5). However, even though the maximum ethanol was obtained at 150 rpm, statistical analysis indicated that there was no significant difference on ethanol production between 100 and 150 rpm with p value: 0.787. Fermentation at lower rate of agitation produced lesser concentration of ethanol with 1.86 g L^{-1} ethanol at 72 h of incubation (Fig. 6, 7). The results suggested that higher ethanol production could be obtained by using higher agitation rate during the process.

Table 5: Effect of different rate of agitations (rpm) on ethanol production from EFB hydrolysate at optimum condition

Temperature	Glucose consumption rate (g h^{-1})	Ethanol formation rate (g h^{-1})	Ethanol concentration (g L^{-1})	Y p/s (g g^{-1})	Fermentation efficiency (%)
50	0.11	0.03	1.86	0.06	21.50
100	0.24	0.14	10.29	0.50	119.00
150	0.23	0.14	10.35	0.53	122.47

DISCUSSION

Pretreatment and ethanol production from EFB: Lignocellulosic ethanol is considered one of the most potential next generation automotive fuels for the future (Kumar *et al*., 2009). Lignocellulosic ethanol can be produced from various feedstocks such as lignocellulosic biomass, crop residue, starchy materials, sucrose-containing feedstock and microalgae (Nigam and Singh, 2011). In generally, the lignocellulosic biomass should undergoes several process consisting of pretreatment, hydrolysis, enzymatic saccharification and fermentation of sugar to ethanol (Sun and Cheng, 2002). The results obtained from the study indicated that combination of alkaline, dilute acid hydrolysis and enzymatic saccharification was found to be a promising approach to extract fermentable sugar from EFB as feedstock for ethanol production.

According to Sun and Cheng (2002), pretreatment of lignocelluloses material using alkaline treatment will increase internal surface area and porosity, decrease the degree of polymerization and crystallinity, separate structural linkages between lignin and carbohydrates and disrupt the lignin structure. This will enhance the EFB's structure and made it accessible to the attack during acid hydrolysis and improve enzymatic saccharification (Sudiyani *et al*., 2010). Dilute-acid hydrolysis is probably commonly method applied to produce sugar from biomass particularly from EFB (Millati *et al*., 2011). Generally, the proton in the mixture could catalyze and scissor the **β**-1,4, linkage of glucose and xylose monomer, acetyl group and other products in cellulose and hemicellulose in the biomass (Najafpour *et al*., 2007; Taherzadeh and Karimi, 2007). In Table 2, results show that the major sugar obtained from the acid hydrolysis was xylose. Similar observation has been made by Aziz *et al*. (2002), where they found that xylose is the major sugar produced during acid hydrolysis from EFB press fiber.

Table 2 also indicats that glucose was the dominant sugar produce during enzymatic saccharification. In general, during enzymatic hydrolysis, cellulose is degraded by cellulases to reducing sugars that can be fermented by yeast or bacteria to ethanol (Sun and Cheng, 2002). Enzymatic hydrolysis of cellulose entails three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugar and desorption of cellulase (Lynd *et al*., 2002). According to Coughlan and Ljungdahl (1998) at least three major groups of cellulase are involved in hydrolysis process i.e., a) endoglucanase which attacks regions of crystallinity in the cellulose, creating free chain-ends, b) cellobiohyrolase which degrades the molecule further by removing cellobiose units from free chain-ends and c) **β**-glucosidase which hydrolyzes cellobiose to glucose.

In order to produce ethanol from EFB, the fermentation of enzymatically saccharified EFB hydrolysate was fermented using *S. cerevisea*. Based on the Fig. 1, fermentation profile shows that all the glucose was completely consumed within 48 h. Based on the enzymatic saccharification study, it was found that glucose is the major monomer sugar obtained from the process. Thus, glucose is considered as a substrate in the fermentation process. This indicated that glucose extracted from EFB was able to be converted into ethanol by using *S. cerevisiae*. Current study

indicats that ethanol production yields from EFB hydrolysate is comparable with ethanol production yield from other lignocellulosic materials such as sweet sorghum, baggases, molasses and corncob. Based on the results obtained, it was found that ethanol production yield for EFB hydrolysate was 0.51 g ethanol/g glucose at 72 h of incubation. Similar results has been obtained by Millati *et al.* (2011), who reported that for ethanol production yield from second stage pretreated EFB in batch fermentation by *S. cerevisiae* was 0.46 g ethanol/g glucose at 48 h of incubation. In addition, for the ethanol production yield from sweet sorghum stem juice in batch fermentation by *S. cerevisiae*, the ethanol yield and ethanol efficiency were 0.5 g ethanol/g glucose and 91.71%, respectively (Khongsay *et al.*, 2010). Besides, study by Nadir *et al.* (2009) on ethanol production from enzymatic hydrolyzed sweet sorghum using *S. cerevisiae* showed that the highest ethanol yield of 0.64 g ethanol/g glucose was obtained after 64 h of incubation. Similar results has also been reported on ethanol production from molasses using *S. cerevisiae* GC-IIB31 which indicated that the ethanol yield of the fermentation was 0.63 g ethanol/g glucose (Mariam *et al.*, 2009). On the other hand, for the batch fermentation of corncob using *S. cerevisiae* 316, the ethanol yield of 0.47 g ethanol/g glucose was obtained within 18 h of fermentation (Chen *et al.*, 2007).

Determination of optimum parameter: The effect of initial pH has been reported to showed a significant influence on fermentation, mainly on yeast growth, fermentation rate and by-product formation (Chaudhary and Qazi, 2006; Sheela *et al.*, 2008; Manikandan *et al.*, 2008). The results obtained from this study shows that the most suitable initial pH value for ethanol production from EFB was pH 4. It was found that increment of pH value was able to reduce ethanol production and glucose consumption rate. This current study is in agreement with other studies reported that yeast grows and fermentation process performs best in natural or slightly acidic environment (Noor *et al.*, 2005; Manikandan *et al.*, 2008). According to Periyasamay *et al.* (2009), the suitable pH value for ethanol production from molasses using *S. cerevisiae* was pH 4 with maximum ethanol yield 0.53 g ethanol/g glucose. Similar result has also been reported by Sheela *et al.* (2008) on ethanol production from molasses by using indigenous yeast strain with ethanol yield 0.5 g ethanol/g glucose and ethanol efficiency of 99%. Moreover, ethanol production from water hyacinth and enzymatic saccharified sunflower stalk by *S. cerevisiae* also showed that the optimum ethanol production yield was obtained at pH 4 and 3.2 with ethanol yield of 0.29 and 0.28 g ethanol/g glucose, respectively (Satyanagalakshmi *et al.*, 2011; Vaithanomsat *et al.*, 2009).

This study indicates that ethanol production at higher pH value was lower. The lower ethanol productivity may be due to the lower metabolic rate of yeast cell used (Mariam *et al.*, 2009). Increment of pH value will increase the permeability of the cell membrane resulted reduction of the rate of sugar fermented enzyme production. The lower ethanol yield and sugar conversion obtained at higher pH value were also probably due to the formation of undesired product such as glycerol and organic acid during the fermentation process (Pramanik, 2003). According to Munene *et al.* (2002), increasing of pH value in the molasses hydrolysate will reduce alcohol hydrogenase activity in *S. cerevisiae* cell and lead to the formation of acetic acid instead of ethanol in the process.

According to Beltran *et al.* (2008), temperature used in fermentation has a significant influence on ethanol production performances, microbial growth and fermentation kinetics and lipid metabolisms in yeast. Based on the Table 4, this current study indicated that the most suitable temperature for ethanol production from enzymatically saccharified EFB was at 30°C. The data obtained is in agreement with reported by other researchers on effect of temperature towards

ethanol production from lignocellulosic biomass. In generally, the optimum temperature for ethanol production from cellulosic materials is between 30 to 40°C (Sheela *et al.*, 2008; Neelakandan and Usharani, 2009; Ratanapongleka *et al.*, 2010; Somda *et al.*, 2011). Study by Manikandan *et al.* (2008) on the effect of temperature towards ethanol production from banana peel by *S. cerevisiae* Strain 4 indicated that the maximum ethanol production of 0.09 g ethanol/g glucose was obtained at 33°C. Similar results have also been reported on ethanol production from water hyachinth which indicated that the maximum ethanol concentration was obtained at 30°C with ethanol efficiency of 59.3% (*Satyanagalakshmi et al.*, 2011). On the other hand, for ethanol production from molasses and sweet sorghum by *S. serevisiae*, maximum ethanol yield was obtained at 30°C with ethanol yield of 0.44 and 0.48 g ethanol/g glucose respectively (Mariam *et al.*, 2009; Khongsay *et al.*, 2010).

In this study, fermentation at high temperature (40°C) has shown a significant influence on ethanol production. Table 4 and Fig. 5 indicate that ethanol production yield from EFB hydrolysate is lower at temperature 40°C. The similar observation on effect of temperature towards ethanol production lignocellulosic materials has also been reported by previous studies. Fermentation of sugarcane molasses, baggases, corncob and sweet sorghum hydrolysate by *S. cerevisiea* indicated that further increase of temperature more than 40°C will reduce the ethanol production (Periyasamay *et al.*, 2009; Yah *et al.*, 2010; Umamaheswari *et al.*, 2010). A lower ethanol production by yeast at 40°C may be attributed to the loss of enzyme activity at higher temperature (Pramanik, 2003). An excessive high temperature may disrupt enzyme and alter the structure of the membrane and decrease its functionality resulted in low ethanol production (Lucero *et al.*, 2000; Sener *et al.*, 2007). On the other hand, increasing of fermentation temperature will increase production of acetic acid, glycerol, succinic acid and acetaldehyde which will cause toxicity to the yeast cell and reduced ethanol production (Torija *et al.*, 2003).

Mechanical agitation also plays an important role for ethanol production, yeast cell viability and uniform mixing of cells and nutrient within the medium components during fermentation in the fermentor (Boswell *et al.*, 2002; Noor *et al.*, 2003; Arisra *et al.*, 2008). This current study indicats that results obtained are in agreement with those reported by previous studies which explained that ethanol production yield using mechanical agitation was higher than without or lower agitation rate (Noor and Hameed, 1999). According to Silva *et al.* (2009), their study on fermentation of rice straw hemicelluloses hydrolysate by yeast *Pichia stipitis* showed the highest ethanol production of 0.42 g ethanol/g glucose was obtained at 100 rpm (Silva *et al.*, 2009). In addition, for the batch fermentation of wood hydrolysate by *P. stipitis* indicated that the highest ethanol production was obtained at 150 rpm with 0.37 g/g glucose after 72 h of incubation (Stoutenburg *et al.*, 2008).

CONCLUSIONS

A combination of integrated pretreatment processes such as alkaline treatment, acid hydrolysis and enzymatic saccharification was able to extract 73% of fermentable sugar from EFB. Suitable fermentation conditions are crucial in producing an optimum ethanol yield from EFB hydrolysate. In this study, the optimum ethanol yield from EFB hydrolysate which ranges from 9.55 to 10.32 g L^{-1} can be achieved at pH 4 and 30°C with an agitation rate of 100 rpm to 150 rpm for 72 h of incubation. Further study on other parameters such as nutrient and solid loading in Simultaneous Saccharification Fermentation (SSF) will be carried out to make ethanol production from palm biomass is produce in economical viable and in sustainable way.

ACKNOWLEDGMENT

The authors would like to thank Director-General of Malaysian Palm Oil Board (MPOB) for permission granted to publish this paper. Thanks are also due to all staff of Energy and Environment Unit, MPOB for their valuable assistance and technical supports.

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