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Comparison of SHF and SSF processes using enzyme and dry yeast for optimization of bioethanol production from empty fruit bunch

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Abstract

Empty fruit bunch was considered as substrate for second generation of bioethanol because it consists of lignin, cellulose, and hemicelluloses. For lignocelluloses materials, it usually needs pretreatment and hydrolysis to convert cellulose into glucose. Two methods of enzymatic hydrolysis, Separated Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) were carried out in this study. The performance of both SHF and SSF was concerned to evaluate the effect of hydrolysis methods and enzyme concentration for producing ethanol. Pretreatment was conducted in a reactor using 10% sodium hydroxide at temperature 150°C during 30 minutes. Two kinds of enzyme, Cellic® CTec2 and Cellic® HTec2 from novozyme were added in 15% (gr/ml) of pretreated EFB at pH 4.8. Four concentration of enzyme Cellic® CTec2, 10, 20, 30, 40 FPU per gram biomass were performed in SHF and SSF processes respectively, while Cellic® HTec2 was added 20% from Cellic® CTec2. Contents of glucose, xylose, and ethanol were recorded every 24 hours. Using 40 FPU of concentration enzyme, it could be produce 4.74% of ethanol in 72 hours fermentation by SHF process and 6.05% of ethanol in 24 hours by SSF process. From this study, the SSF method was considered as a better process than SHF due to rapidly ethanol production and the highest concentration of produced ethanol.

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Keywords: bioethanol; empty fruit bunch; SHF; SSF

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1. Introduction

The increasing of world's population leads to the economic growth and industrial area development in various countries. Consequently, the supply of reserves energy is decreased, while energy production takes a long time because most of the energy comes from fossil sources. Currently, renewable energy starts to become a focus because it can be an alternative energy instead of fossil fuel.

One of alternative fuel is bioethanol that could be a promising alternative to petroleum-based transport fuel [1]. First generation of bioethanol is produced from starchy materials such as corn, cassava, wheat, soybean, etc and some others from sugar cane. Only sugar cane and corn starch has been produced for industrial scale [2]. The big problem of the first generation is the compatibility with food supply. Therefore, second generation bioethanol, which comes from lignocelluloses was developed for being alternative energy.

Lignocellulose is one of the renewable resources and abundant biopolymers in the earth [3]. In Indonesia, oil palm empty fruit bunch was considered as a potential feedstock that could be used for bioethanol production. Data in 2012, Indonesia produced 23,633,412 ton Crude Palm Oil (CPO) or approximately 25–35 million EFB produced, with estimated the palm oil industry produces 1.1 to 1.5 tones EFB for every tone CPO produced[4]. Usually, the same as other agricultural wastes, most of which only dispose as a natural fertilizer and the small amount use as a fuel of boiler.

As like as common lignocellulosic materials, EFB also contains lignin, cellulose, and hemicellulose. Lignin is a complex hydrophobic, cross linked aromatic polymer composed of three types of substituted phenols (phenylpropanoids). Hemicellulose is polysaccharide typically made up of chains of xylose. The side chains contain arabinose, galactose, mannose, glucose, acetyl and other sugar groups. While cellulose is important thing, it is protected by hemicelluloses and lignin. It is also a polysaccharide that consist a linear chain of several hundred to more than ten thousand d-glucose units linked by β-1,4 bonds[5,6].

Some pretreatment method was needed to damage the lignin and open the structure for efficiently bioconversion in lignocellulosicsubstrate [7]. Pretreatment also was conducted to increase surface area, decrease crystallinity, and increase porosity of materials [8]. In this paper, alkaline pretreatment with high pressure and temperature was chosen. The characteristic of the process is almost same as Ammonia Fiber Explosion (AFEX), alkaline thermal pretreatment which lignocellulosic materials are dissolved in ammonia solution at high temperature and pressure for a period [9]. Using alkaline pretreatment would increase cellulose digestibility and effective for lignin solubility on agricultural waste than wood materials [10, 11]. Sodium hydroxide was reported to reduce lignin from 24–55% to 20%[11].

Hydrolysis and fermentation is the next step conducted after pretreatment process for producing bioethanol. Separated Hydrolysis and Fermentation (SHF) was the conventional method that hydrolysis was carried out in the period and fermentation process after then. This process allowed hydrolysis process worked first to produce monosaccharide sugar, so sugar is ready when the fermentation begins. Through this method, each process would get optimum condition, *Saccharomyces cereviceae* at 32°C, and enzyme at 50°C[12,13]. Another method of hydrolysis and fermentation was Simultaneous Saccharification and Fermentation (SSF). In SSF, hydrolysis and fermentation located in a single reactor, enzyme and yeast put together, so glucose is rapidly converted into ethanol [7]. Wyman et al. reported that SSF process gives higher yield of ethanol than SHF because low residual sugar relieves inhibition on the cellulase enzyme [14].

In this study, empty fruit bunch (EFB) would be used as a raw material for producing bioethanol using two methods of hydrolysis, SHF and SSF, with four variation of enzyme concentration. Fermentation was carried out by dry yeast, *Saccharomyces cereviceae*. The performance of both SHF and SSF was concerned to evaluate the effect of hydrolysis methods and enzyme concentration in ethanol production.

2. Materials and methods

2.1. Materials

Empty Fruit Bunch used in this study was obtained from palm oil plantation in Palembang, South Sumatera Indonesia. After air-dried, physical pretreatment i.e. chipping and milling until 2 mm was conducted to maximize

contact area of the substrate. The moisture content was measured by Moisture Analyzer OHAUS MB 45 and stored in a dry place. The composition of materials was analyzed based on National Renewable Energy Laboratory (NREL) standard procedures [15]. It was shown in the table 1. Enzymes, Cellic® CTec2 and Cellic® HTec2 from Novozymes, Denmark were used in the saccharification process. The activity of Cellic® CTec2 was 144 FPU/g cellulose according to National Renewable Energy Laboratory (NREL)[16]. Dry yeast (*Saccharomyces cereviceae*) was employed in the fermentation process. All reagents are used in this paper were analytical grade, except the reagent of the pretreatment process, i.e. sodium hydroxide is industrial grade.

2.2. Pretreatment

Pretreatment was conducted in the pilot plant reactor CHEMEX in Research Center for Chemistry, Indonesian Institute of sciences. This reactor was equipped by cyclone, beltpress, washing tank, and buffer tank. Empty fruit bunch in small size was heated using NaOH solution 10% (kg/L) at 150°C for 30 minutes. A solid liquid ratio was 1:5. The pressure was controlled four bars at early heating. EFB was washed then until wash water turned to pH 7 and dried in the oven at 50–60°C overnight.

2.3. Separate Hydrolysis and Fermentation (SHF)

This method was performed on 15% (g/ml) of pretreated EFB in 0.05 M buffer citrate in the erlenmeyer flask. Both of the processes, enzymatic hydrolysis (saccharification) and fermentation, was carried out for 72 hours. Thus the total time processes were 144 hours. Duplicate process was arranged to get the best approach. The samples, containing pretreated EFB in the buffer citrate with pH 4.8, were closed and autoclaved at 121°C for 20 minutes. After it was cooling down, 10, 20, 30, 40 FPU of Cellic® CTec2 per gram dry biomass and 20%Cellic® HTec2 was added for each. All of the samples were placed in the shaking incubator. For hydrolysis process, temperature and velocity agitation was set 50°C and 150 rpm respectively. Sampling every 24 hour was employed to monitor producing sugar. Glucose and xylose were measured as a product in this hydrolysis.

Data of glucose form HPLC was required to get the percentage of yield (Yg). It was calculated by comparing the weight of measured glucose (Wgm) with the theoretical weight of glucose (Wgt). The theoretical weight of glucose is weight of glucose that should be obtained from pretreated substrate. Wgt and Yg is performed using following equations:

$$\%Yg = Wgm/Wgt\ x100\% \tag{1}$$

$$Wgt = Wc\ x\ Anhydro\ Correction \tag{2}$$

where *Wc* is weight of cellulose in pretreated substrate, *Anhydro correction* = 1.11 (for cellulose to glucose)[15,17].

The next process, fermentation, was conducted 72 hours after hydrolysis process. The temperature of shaking incubator was changed into 32°C. In the constant temperature, one percent (g/ml) of dry yeast, *Saccharomyces cereviceae*, was put in the each flask. Alcohol content, glucose, and xylose were monitored every 24 hour. The calculation percentage of yield in fermentation is as same as those in saccharification process i.e by comparing measured ethanol weight with the theoretical weight of ethanol. The anhydro correction is 0.51 (for glucose to ethanol)[18].

2.4. Simultaneous Saccharification and Fermentation (SSF)

Fifteen percent (g/ml) of pretreated EFB in 0.05 M buffer citrate in erlenmeyer flask was sterilized by autoclave at 121°C for 20 minutes. The each enzyme concentration as described in SHF was added together with 1% (g/ml) dry yeast, *Saccharomyces cereviceae*. The process was conducted in the shaking incubator under temperature condition 32°C with velocity agitation 150 rpm during 72 hours. Sugar and ethanol were monitored every 24 hour. Flow diagram of SHF and SSF process is shown in Fig. 1.

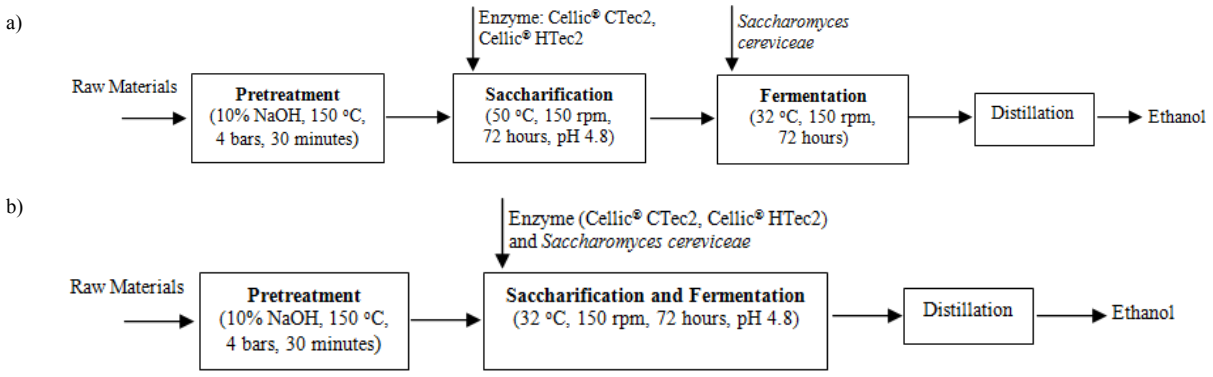

Fig. 1. Experimental set up of separate hydrolysis and fermentation; (a) simultaneous saccharification and fermentation; (b) processes.

2.5. Yeast Count

Yeast count was conducted to observe the yeast growth in the fermentation broth. Haemocytometer, a microscope with 40x objective, some pipettes, and glassware for dilutions, were need in this observation. Fermentation broth was shaken until the suspension was homogeneous for counting the yeast. Samples was taken a little using a sterilized pipette and diluted in distilled water until yeast suspension become translucent. Microscope was conditioned until each section in haemocytometer has below 5–6 cells.

2.6. HPLC Analysis

Glucose, xylose and ethanol were measured by High Performance Liquid Chromatography (HPLC) waters, USA. The mobile phase of this equipment is 5 mM H_2SO_4 at 0.6 ml/min and was equipped with AMINEX HPX 87H column, a guard column, an automated sampler, a gradient pump. A Relative Index (RI) was used as the detector. The oven temperature was maintained at 65°C.

3. Result and Discussion

3.1. Alkaline Pretreatment

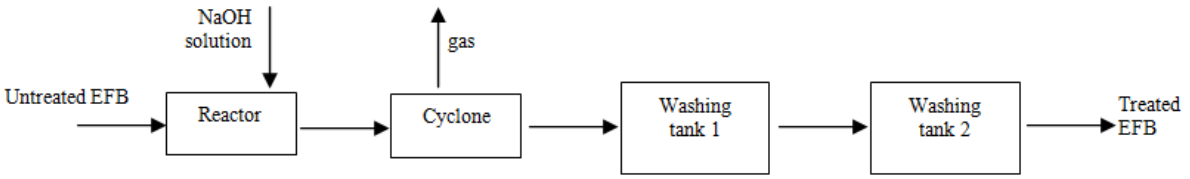

Fig. 2. Flow diagram process of EFB pretreatment

Fig. 2 shows the pretreatment process was carried out in this study. Small size of untreated EFB was mixed with 10% NaOH solution in batch reactor at 150°C during thirty minutes. The pressure was set 4 bars in the early heating and reached up to 8 bars at the end of process. Because the process was high temperature and pressure, it produced gas and need cyclone to separate solid include liquid and gas. The explosion occurred when all of the materials flow into cyclone. Two unit washing tank was prepared to wash and neutralize the solid materials. Treated EFB with high cellulose was defined by this process. Jeon et al. were able to reduce lignin content until 47% using this equipment[19].

This process of pretreatment has the same characteristic with Ammonia Fiber Explosion (AFEX). In AFEX, 5–15% aqueous ammonia was put in the reactor packed with biomass at temperature (160–180°C) during 14 minutes [20]. However, high cost and recovery of ammonia still became problems pretreatment in this area [21]. The odor of this substance was also considered. Therefore, the use of sodium hydroxide seems to be more favorable. This substance looks quite stable, safe, and odorless. For higher lignin such as EFB, alkaline was considered as an effective agent [22].

Table 1. Composition of EFB before and after pretreatment

Substrate	Lignin (%)	Cellulose (%)	Hemicellulose (%)
Untreated EFB	25.52	35.84	15.48
Treated EFB	15.70	73.24	7.81

The percentage of lignin decreased due to an effect of alkaline pretreatment. Over than 38% lignin removed and as a consequent the cellulose content increased. Lignin was considered as an inhibitor because they could restrict the hydrolysis enzyme to attack cellulose [22]. Furthermore, using sodium hydroxide would induce swelling leading on the increasing of surface area [23]. As an effect, degree of polymerization decreased and crystallinity occurred with a separation of structural linkages between lignin and carbohydrates. The lignin disruption then saponification of intermolecular ester bonds was occurred after that [10].

The use of alkaline pretreatment would also take effect in hemicelluloses content. Hemicelluloses decreased almost 50% after 30 minutes reaction on the pretreatment reactor. Mussatto et al. reported that lower hemicellulose and lignin content in the substrate would make higher efficiency of cellulose hydrolysis [17]. In this study, the process producing ethanol was coming from cellulose only due to the capability of *Saccharomyces cereviceae*. This microbe only changes glucose to ethanol. Because it was not converted to another substance, hemicellulose was detected as xylose at the end of process.

3.2. Separate Hydrolysis and Fermentation (SHF)

As described above, the SHF process was carried out in each step, which hydrolysis was followed by fermentation process. Enzymatic hydrolysis, often called saccharification, was performed in order to convert cellulose into glucose. High-cellulose pretreated EFB was attacked by combination of enzyme, Cellic® CTec2 and Cellic® HTec2. In this process, the existing cellulose expected was converted entirely within 72 hours.

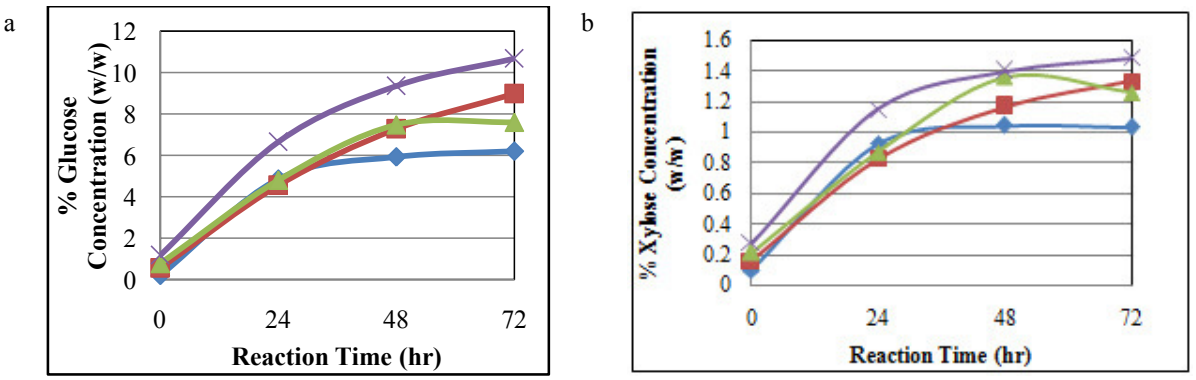

Fig. 3.Glucose Concentration; (a) and Xylose Concentration; (b) in SHF Process for Ethanol Production. This method was employed with (♦) 10 FPU (■) 20 FPU (▲) 30 FPU (ж) 40 FPU

The result of sugar from saccharification process could be seen in Fig. 3. This figure shows glucose and xylose obtained from HPLC analysis. Only glucose and xylose are reported since the others could be negligible. This analysis predicted the sugar released by enzyme performance. Cellulose and hemicellulose were converted into glucose and xylose respectively using combined enzyme. As shown in Fig.3, glucose and xylose concentration increased significantly with increasing of reaction time. Fig. 3(a) shows the glucose concentration based on the differences in enzyme concentration of substrate. Increasing the enzyme concentration would increase the concentration of glucose. The higher concentration of enzyme, the more cellulose could be converted. Therefore, it would increase a formed glucose. Concentration of 10 FPU enzymes showed a lowest value, i.e., 6.2% for glucose concentration in 72 hours. It is most likely due to the low enzymes concentration that was added, so that the enzymes were only able to convert some of the cellulose. In the concentration of 20 and 30 FPU, at the beginning of saccharification, the produced sugar value was almost equal to the concentration of 10 FPU, but at the end of the process provided a significant improvement. The concentration of 40 FPU produced the highest glucose in the 72 hours, about 10.67%. The resulted indicated that enzymatic hydrolysis was affected by enzymes concentration.

The percentage yield of glucose could be calculated using equation (1) and (2). By multiple mass of sample and percentage of cellulose in table 1, it could be found weight of cellulose (*Wc*). From eq.(2), it can be calculated that theoretical weight of glucose (*Wgt*) is 12.21 g. Based on the experiment shown in fig.2, producing glucose (*Wgm*) is about 10.67 g by 40 FPU of enzyme concentration. Therefore it was good enough for enzymatic hydrolysis process because yield of glucose (*Yg*) is about 87%. It can be said that 87% more of cellulose changes into glucose

Fig. 3(b) shows xylose concentration during 72 hour's process. As like as glucose concentration, xylose also increased during saccharification. The combination of two enzymes not only converted cellulose to glucose, but it also changed hemicellulose to xylose. However, the xylose concentration was lower compared with glucose concentration. The xylose concentration obtained in the range 1–1.5 % at the end of saccharification. As same as glucose concentration, the highest xylose was resulted from EFB by using 40 FPU of enzymes.

In SHF process, after 72 hours of enzymatic hydrolysis, process was continued by fermentation. Yeast (*Saccharomyces cereviceae)* plays a role to convert glucose into ethanol. Fermentation was also monitored 72-hour. Sampling was taken every 24 hours and analyzed the component i.e. produced ethanol, glucose, and xylose using HPLC equipment.

Fig. 4 shows the existing compound during the fermentation process. The three components were monitored i.e., xylose, glucose, and ethanol. The xylose appeared constant in the beginning until the last. As described above, xylose was not used by *Saccharomyces cereviceae* to produce ethanol in this fermentation process. *Saccharomyces cereviceae* only convert glucose, while xylose needs other microbes such as *Clostridium thermohydrosulfuricum*, *Zymomonasmobilis,* etc, to convert themselves to ethanol[25]. In this study, xylose was obtained as co-product at the end of fermentation.

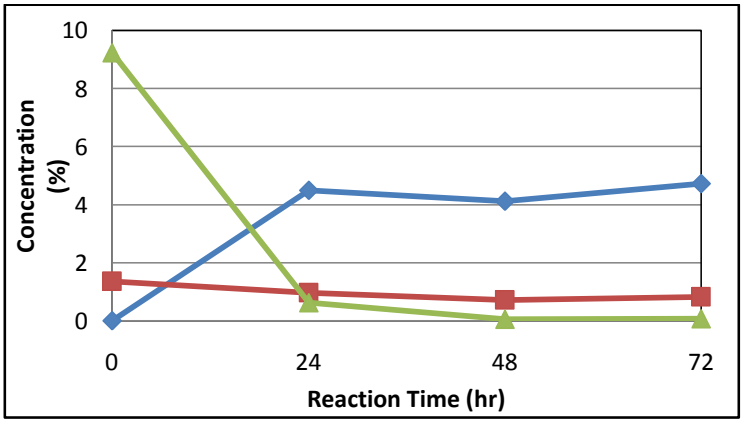

Fig. 4.Concentration of (♦) ethanol (■) xylose (▲) glucose in the fermentation of EFB using SHF method at concentration enzyme of 40 FPU.

The ethanol increased significantly during reaction time. In contrast, glucose decline sharply in the early fermentation (as long as 24 hours) and down constantly until 72 hours. It could be seen that there is no glucose in 72 hours. This means the glucose was already converted into ethanol. It is known that the maximum of theoretical ethanol yield is 0.51 g ethanol/g glucose. When saccharification was carried out using 40 FPU of enzyme, the glucose concentration was found 10.67 g. The theoretical of ethanol was 6.22% calculated by multiply theoretical weight of glucose from saccharification process with conversion 0.51. From Fig.4, the ethanol result from the process is 4.74%. So, by calculation, the yield of ethanol is 76%.

3.3. Simultaneous Saccharification and Fermentation (SSF)

Simultaneous Saccharification and fermentation (SSF) process was considered as preferably process because of reduced operation all costs, lower enzyme requirement and increased productivity [26]. SSF process can use single reactor and the same temperature for saccharification and fermentation process, so the reduction of operation cost can beachieved. Glucose produced from hydrolysis is simultaneously metabolized by microorganism, thereby alleviating problems caused by product inhibition in SSF process [27]. In this study, the variation of enzyme loading was conducted in SSF process, i.e; 10, 20, 30 and 40 FPU of Cellic® CTec2 per gram dry biomass. Glucose and xylose concentration during SSF process for each variation of enzyme loading can be seen in Table 2.

Table 2.Glucose and Xylose Concentration in SSF Process.

Time Reaction (hr)	10 FPU		20 FPU		30FPU		40 FPU	
	Glucose (%)	Xylose (%)	Glucose (%)	Xylose (%)	Glucose (%)	Xylose (%)	Glucose (%)	Xylose (%)
0	0.20	0.10	0.55	0.16	0.77	0.21	1.17	0.27
24	0.02	0.57	0	0.64	0	0.80	0	0.85
48	0	0.38	0	0.53	0	0.29	0	0.59
72	0.09	0.30	0	0.40	0	0.54	0	0.54

The differences were shown when the result was compared with the result of SHF. In the SSF process, glucose almost always depleted in every sampling time (Table 2). In zero hours of reaction time, there are present amount of glucose and xylose coming from the enzyme. The amount of sugar in enzyme can be regarded very small, and the value increased with increasing of enzyme concentration. SSF process allowed enzymes and yeast put together while working process was conducted at lower temperature. In this study, the used temperature was same with fermentation i.e., 32°C. More saving energy is one of the advantages of the SSF process. Time producing ethanol becomes shorter because the hydrolysis and fermentation were carried out at the same time.

When enzyme produced glucose, the yeast, *Saccharomyces cereviceae* changed to ethanol directly. It is why the concentration of glucose always zero in the 24, 48, 72 hour fermentation, except using 10 FPU of enzyme concentration. Probably 10 FPU of enzyme concentration was too little, so its performance was slow.

Table 2 also performs xylose concentration that increased in 24th hour. Cellic® CTec2 is complex enzymes that consist of cellulase, β-glucosidase, and hemicellulase whereas Cellic® HTec2 consists of endoxylanase with high specificity toward soluble hemicellulose and cellulase. Hemicellulose could be converted to xylose because Cellic® CTec2 has hemicellulase and Cellic® HTec2 has endoxylanase using combined enzymes [28]. After 24 hour, the xylose decreased. Probably the performance of enzyme to hydrolyse hemicellulose had become low, so the concentration was lower.

Fig. 5 shows the produced ethanol by *Saccharomyces cereviceae* during a period. The figures performed that increased concentrations of enzyme giving an effect to ethanol produced. Highest producing ethanol reached 6.05% using 40 FPU of enzymes. From fig.5, alcohol content was tending to decrease after 48 hours except using enzyme concentration 30 FPU, although the increasing was not really significant. It could be said that, optimum time reaction in SSF process was 48 hours. SSF process is more efficient than previous processes (SHF) because only with 24 hours, ethanol has been formed.

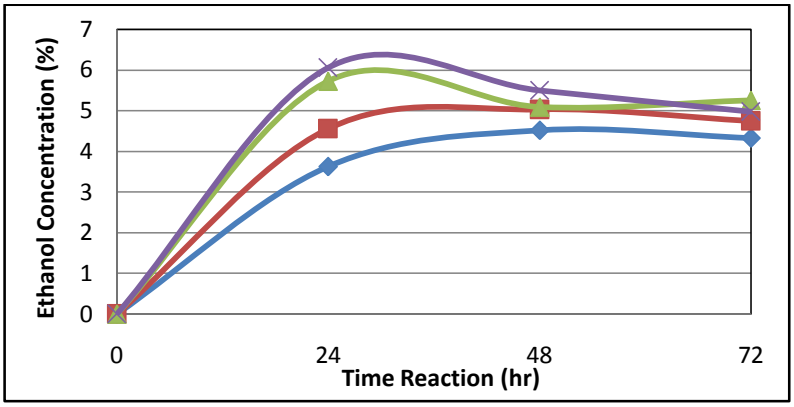

Fig. 5.Producing Ethanol from EFB by SSF Process. Legend: (♦) 10 FPU (■) 20 FPU (▲) 30 FPU (ж) 40 FPU

As described above, the theoretical of ethanol is 6.22%. In this SSF process, the highest concentration of ethanol could be found 6.05% by 40 FPU of enzyme. So, it can be calculated that the yield of ethanol is 97%. The resulted indicated that the process has high effectiveness.

During SSF process, growth of yeast was monitored every sampling hour using microscope. Dry yeast (*saccharomyces cereviceae*) was added in each flask together with Cellic® CTec2 and Cellic® HTec2. This process was necessary to minimize the contamination during SSF process. The yeast monitoring was also used for initial approach whether the saccharification and fermentation were working good or not. The growth of yeast during SSF process can be seen in Table 3.

Table 3. Yeast Count in SSF Process

Time Reaction	Yeast Count ($x10^{10}$)			
(hr)	10 FPU	20 FPU	30 FPU	40 FPU
0	1.52	1.52	1.52	1.52
24	0.68	0.82	0.84	1.06
48	0.30	0.34	0.24	0.32
72	0.14	0.16	0.18	0.18

Table 3 showed that in each SSF process with variation of enzyme loading (10, 20, 30, 40FPU) occurred decreasing amount of *Saccharomyces cereviceae*. The amount of yeast decreased because of the availability of glucose when SSF process. As perform in table 2, glucose depleted at every sampling time. In SSF process, the number of yeast was competing for food each other while conversion of cellulose to glucose is still ongoing. The available glucose as a food is still limited. Probably, some yeast was losing and eventually died. The decreasing amount of yeast is most likely due to alcohol content produced during SSF process also. Brown et. al shown the effect of ethanol on growth rate of yeast. They performed that the addition of ethanol concentration would cause an immediate reduction in growth rate[29]. Some people in their study were investigating potential microbial strain of *Saccharomyces cereviceae* which is having high ethanol tolerance [30].

From the result, if it was compared, SSF was preferred than SHF in the hydrolysis and fermentation of lignocellulose process because rapidly ethanol production and the highest concentration of produced ethanol. Table 4 shows detail comparison of SSF and SHF process. The yield of ethanol from SSF process was higher than SHF. Wyman et. al showed that using SSF process could increase yields and concentration of ethanol with less capital investment[14]. SSF process was more energy saving due to the process was carried out in the temperature 32°C.

Some reported that SSF could also reduce end-product inhibition of the hydrolysis by glucose and cellobiose [14,31].

Table 4. Comparison Yield of SHF and SSF Processes

	SHF	SSF
Weight of Substrate	15 g	15 g
Cellulose in Substrate	10.986 g	10.986 g
Measured Glucose	10.67 g	-
Theoretical Glucose	12.21 g	12.21 g
% Yield of Glucose	87 %	-
Measured Ethanol	4.74%	6.05 %
Theoretical Ethanol	6.22%	6.22 %
% Yield of Ethanol	76%(in 72 hour fermentation)	97%(in 24 hour of process)

4. Conclusion

Optimization process of bioethanol using Empty Fruit Bunch (EFB) was conducted in the area of hydrolysis and fermentation. Enzymatic hydrolysis and fermentation were studied in the two variation methods, Simultaneous Saccharification and Fermentation (SSF) and Separate Hydrolysis and Fermentation (SHF). Using 40 FPU of concentration enzyme, it could be produce 4.74% of ethanol in 72 hour fermentation by SHF process and 6.05% of ethanol in 24 hour by SSF process. From this study, the SSF method was considered as a better process than SHF due to rapidly ethanol production and the highest concentration of produced ethanol.

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