# Final Announcement

MPOB International Palm Oil Congress and Exhibition

INTERNATIONAL PALM OIL CONGRESS AND EXHIBITION

# PIPOC 2015

# Oil Palm: Powering the World, Sustaining the Future

**6-8 OCTOBER 2015**
Kuala Lumpur Convention Centre
Kuala Lumpur, Malaysia

Organised by
Malaysian Palm Oil Board (MPOB)
Ministry of Plantation Industries
and Commodities, Malaysia

# CONGRESS PROGRAMME AT A GLANCE

## Sunday • 4 October 2015

Golf
Congress Pre-registration
Technical Tours Registration

## Monday • 5 October 2015

Pre-registration
Poster Set Up
Exhibition Set Up
Technical Tours
Congress Mixer

## Tuesday • 6 October 2015

Registration
Congress Official Opening
Keynote Address
Plenary Lectures
Evening Forum

## Wednesday • 7 October 2015

**Concurrent Conferences:**

- Agriculture, Biotechnology & Sustainability Conference
- Chemistry, Processing Technology & Bio-energy Conference
- Food, Health & Nutrition Conference
- Oleo & Specialty Chemicals Conference
- Global Economics & Marketing Conference

**Congress Dinner**

## Thursday • 8 October 2015

**Concurrent Conferences:**

- Agriculture, Biotechnology & Sustainability Conference
- Chemistry, Processing Technology & Bio-energy Conference
- Food, Health & Nutrition Conference
- Oleo & Specialty Chemicals Conference
- Global Economics & Marketing Conference

# PRETREATMENT OF CELLULOSIC MATERIAL FROM OIL PALM TRUNKS AND OPTIMIZATION OF FERMENTATION VARIABLES FOR BIOETHANOL PRODUCTION

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## ABSTRACT

Indonesia is the largest producer of palm oil in the world. In the plantation of oil palm, the trees require *replanting* every *20-25 years*, consequently an enormous quantity of oil palm trunks are produced creating disposal problem. Since Oil Palm Trunks (OPT) are rich in cellulosic material, an alternative usage for bioethanol production is investigated in this study. Pretreatment is an essential step in the conversion of ligno-cellulosic biomass to fermentable sugars. In this study, pretreatment processes of ligno-cellulosic biomass from Oil Palm Trunk (OPT) vascular bundle were compared. Alkali pretreatment, microwave-alkali, and alkaline peroxide combined with autoclave were used to decrease the lignin content. Morphology and structural changes of biomass were also analyzed by Scanning Electron Microscope (SEM). The result showed that a significant lignin removal up to 83.26% and also cellulose released up to 80.74% were obtained in free starch vascular bundle treated with alkaline peroxide combined with autoclave step. The treated OPT vascular bundle were then subjected to enzymatic hydrolysis to produce reducing sugar. Optimization condition of Simultaneous Saccharification and Co-Fermentation (SSCF) process was further performed using Response Surface Methodology (RSM) to obtain the highest yield of ethanol production. Effect of three independent variables in a defined range of temperature (25, 30, 35$^{O}$C), cell ratio of *S. cerevisiae* and *P. stipitis* (0,25:0,75; 0.50:0.50; 0.75:0.25) and fermentation time (3, 5, 7 days) on ethanol production was investigated. The results of this study confirm that oil palm trunk (OPT) can be a potential feedstocks for bioethanol production.

Keywords: oil palm trunks; pretreatmenat; optimization; response surface methodology

# I. INTRODUCTION

Bioethanol is one of renewable biomass energy produced via sugar fermentation and can be a potential source of sustainable fuel. It has been proposed that an alternative feedstock for biofuel is wasted crops, replacing the traditional starch crop and can avoid conflicts with human food uses. Carbohydrates in the wasted crops, cellulose and hemicelluloses are the main potential feedstock for producing bioethanol.

Indonesia is one of the largest producer of palm oil in the world. In 2010, oil palm plantations produced 22 million tonnes of Crude Palm Oil (CPO), while in 2011 was 23.5 million tonnes. By 2020, Indonesia plans to double the current production of CPO to 40 million tonnes annually and expand its oil palm plantation portfolio by additional 4 million hectares. Along with the growing of palm oil industry, it creates the availability of palm oil residue including oil palm trunk (OPT) waste which is considered as a great potential source of renewable energy. Oil palm trunk contains approximately 70% sugar and 30% (w/w) cellulosic residue. This indicates that OPT is a very promising material to be used as feedstock for second-generation ethanol production.

In general, the production of ethanol from lignocellulose requires several stages, including delignification, saccharification to liberate fermentable hexoses and pentoses of polysaccharides, released sugar fermentation and distillation stage to separate the ethanol. Delignification is the essential step to effectively prepare cellulose to be used by fermenting microorganism for ethanol production. In this study, some pretreatment methods, i.e. alkali, alkali-microwave, and alkaline peroxide combined with autoclav were investigated. In addition, Simultaneous Saccharification and Co-Fermentation (SSCF) system was chosen to maximize the utilization of hexose and pentose sugars by *Saccharomyces cerevisiae* NCYC 479 and *Pichia stipitis* NCYC 1541. The SSCF system

has some additional advantages such as (1) the existence of yeast and enzyme complexes together reduce the accumulation of glucose and short cellulose oligomers which can inhibit the enzyme, thus will be able to increase the yield of ethanol and saccharification rate, (2) use of one bioreactor can reduce investment costs, (3) the presence of ethanol in the bioreactor and the rapid consumption of sugar by yeast reduce the risk of contamination (Ferreira et al., 2010). Simultaneous saccharification and fermentation of lignocellulosic ethanol production is influenced by many factors, such as, cell ratio, temperature and fermentation time. In this study, Response Surface Methodology (RSM) with a full factorial central composite design (CCD) was applied to optimize fermentation to maximize ethanol production and fermentation efficiency. This method intend to find an appropriate function in order to predict the response (ethanol content and fermentation efficiency) and determine the value of the independent variables (cell ratio, temperature and fermentation time) that provide optimal response.

## II. MATERIALS AND METHODS

### *A. Materials*

Oil palm trunk (OPT) estimated to be 25 years old were collected from plantation of *PT. Sampoerna Agro Tbk.* Indonesia. Ten cm in thickness of disks trunk, was taken from middle part of each trunk, which ranged from 10- 12 m in length. Laboratory-scale crusher was used to make disks trunk into small particle. The sap was squeezed from the disks using a laboratory-scale press at 250 Bar. The free sugars remaining in disks were removed by twice washing with distilled water. The moisture content of crushed disks was reduced to <5% using oven ($60^{o}$C for 48 h). The dried disks was pounded manually into small pieces until the mixed fiber was ready to separate into parenchyma (PA) and vascular bundle (VB) by using 30 mesh (0.6 mm) and 80 mesh screen (0.2 mm). The

retained particles on 80 mesh screen (VB) were used as the raw material for ethanol production.

### *B. Alkali pretreatment*

Ten percent of raw materials (dried VB) were pretreated with dilute sodium hydroxide (NaOH) at concentration of 5% (w/v) NaOH. Then, the mixtures were heated at 150$^{o}$C for 3 h. The mixture was filtered to separate solid residues and thoroughly washed with distilled water to neutral pH. Finally, it was dried in the oven at 105°C for 48 h.

### *C. Alkali-peroxide Pretreatment*

Raw materials were pretreated with 250 ml of $H_2O_2$ 5% (v/v) solution in autoclavable bottle. The mixture was adjusted to pH 11.5 using sodium hydroxide (NaOH) and incubated at room temperature for 3 days. To remove the moisture, it was heated at 121$^{o}$C for 15 minutes, 1 atm, using autoclave. After heating, the raw material slurry was filtered to recover the insoluble solids. The solids were washed with distilled water until the pH of the solid became neutral. The washed treated raw materials were dried in a drying oven at 105$^{o}$C for 48 h. After drying, the moisture content of treated raw materials were measured.

### *D. Alkali-Microwave Pretreatment*

Raw materials (dried VB) were pretreated with dilute sodium hydroxide (NaOH) at concentration of 5% (w/v) NaOH and at solid loading of 10%. Then, the mixtures were placed in a open 250 ml glass beaker and exposed to microwave radiation at 400 watt for 30 minutes and 800 watt for 80 minutes. The mixture was filtered to separate solid residues out. The solid residues were thoroughly washed with distilled water to neutral pH and dried in the oven at 105°C for 48 h.

### *E. Chemical Analysis methods*

The moisture content of raw material was determined by drying at 105$^{o}$C for 48 h. The chemical composition of oven-dried untreated and pretreated raw material was analyzed following the National Renewable Energy Laboratory (NREL) Chemical Analysis and Testing Standard Procedure. The cellulose and hemicellulose content were determined by the methods of Van Soest (1991). The lignin and starch content were analyzed according to Sluiter *et al*., (2011) and Sluiter (2008) in NREL Chemical Analysis and Testing Standard Procedure. The ethanol content was determined using ethanol assay kit under standard condition according to the manufacturer's instruction (Megazyme, K-ETOH 01/I4, Ireland). Monosaccharide components were quantified by high performance liquid chromatography (HPLC; Shimadzu corp. Kyoto, Japan), with a refractive index detector (Shimadzu RID-10A) on a CLC-$NH_2$(M) 25 cm operated at room temperature. These analytical value are shown as the means of duplicate experiment. Scanning electron microscopy (SEM) (FEI inspect-500) was employed to investigate the morphological properties untreated and treated raw material. The specimen for SEM was prepared by Au-Pd coating.

### *F. Enzymatic hydrolysis*

The treated raw material was hydrolyzed using a commercial complex cellulase (Cellic Ctec2, Novozyme, Denmark) and supplemented with complex hemicellulase Cellic Htec2 (Novozyme, Denmark). Cellic Ctec2 activity was estimated as 134 FPU/g enzyme according to the filter paper assay (Adney, 2008) using National Renewable Energy Laboratory (NREL) Chemical Analysis and Testing Standard Procedure. The xylanase activity of Cellic Htec2 was estimated 110 units/ml according to the assay method of Bailey (1992). Enzymatic digestibility of treated raw material was performed at 50$^{o}$C with shaking 120 rpm in 250 ml flask containing 5% (w/v) treated raw material, in the presence

of 50 mM sodium acetate buffer (pH 5) with working volume of 10 ml. The reaction was initiated by mixing of 25 FPU of Cellic Ctec2 and 1.6 units/ml of Cellic Htec2 per gram substrate. The released reducing sugar concentration was analyzed based on the amount of liberated reducing sugars using 3,5-dinitrosalisylic acid (DNS) method (Miller, 1959).

***G. Microorganism and Inoculum preparation***

Hexose-utilizing yeast, *Saccharomyces cerevisiae* NCYC 479 and pentose-utilizing yeast, *Pichia stipitis* NCYC 1541 was obtained from National Collection of Yeast Culture (NCYC), Norwich, UK. The culture was maintained at $4^{o}$C on a yeast peptone dextrose agar consisiting of yeast extract, 10 g $l^{-1}$; peptone 20 g $l^{-1}$; glucose 20 g $l^{-1}$and agar, 15 g $l^{-1}$ at pH 5.0. Cells were grown in 150 ml Erlenmeyer flask containing 50 ml of YPD medium (1% yeast extract, 2% peptone and 2% glucose) in shaker incubator at $30^{o}$C with 100 rpm. Following 20 h growth, broth was centrifuged and inoculum was prepared corresponding to 1.0 g $l^{-1}$cells.

***H. Optimization Simultaneous saccharification and co-fermentation (SSCF) by RSM***

Fermentation were carried out in 10% (w/v) treated raw material with 10 ml working volume containing yeast extract 0.5% (w/v) and $(NH_4)_2SO_4$ 1.0% (w/v) dissolved in sodium acetate 50 mM pH 5.0. The pre-hydrolysis reactions were incubated at $50^{o}$C, 100 rpm for 8 h with Cellic Ctec2 25FPU$g^{-1}$ substrate and 1.6 nkat/g substrate of xylanase, before inoculation of 20 h grown yeast cultures.

The experimental design and statistical analysis of fermentation were performed according to the RSM using Design-Expert software (Trial Version 7.1.5, Stat-Ease, Minneapolis, 2008). Central Composite Design (CCD) (Box and Wilson, 1951) was employed to study the combined effect of three independent variables namely cell ratio of *S. cerevisiae* and *P. stipitis* (0.25:0.75; 0.50:0.50; 0.75:0.25) $X_1$, temperature (25, 30 and $35^{o}$C) $X_2$ and fermentation time (3, 5, 7 days) $X_3$. The dependent variable selected for this

study was ethanol concentration, Y (%, v/v). In the CCD, the total number of experimental combinations was $2^K$ +2K + $n_0$, where K is the number of independent variables and $n_0$ is the number of repetitions of the experiments at the center point, which indicated that 20 experiments were required for this procedure. The CCD contains a total of 20 experiments with five level full factorial design and replications of the central points and axial points.

## III. RESULTS AND DISCUSSION

### *A. Separation of vascular bundle and parenchyma*

Separation of vascular bundle (VB) and parenchyma (PA) of oil palm trunks was performed by cutting, size reduction, pressing, drying and sieving. According to Dervish et al., (2013) vascular bundle has higher density in the central part than the bottom of oil palm trunks. The chemical composition of palm trunks after 30 mesh (595 µm) sieving is presented in Table 1.

Further sieving by using 80 mesh (177 µm) was performed to separate starch from VB. Noor et al., (1999) stated that the starch palm trunks size approximately 14.5 µm. By further sieving (80 mesh), it is expected that starch will be eliminated. The chemical composition after 80 mesh (177 µm) sieving is presented in Table 2.

It was found that the level of lignin and cellulose was higher in ≥ 177 µm particles than in particle which has size less than 177 µm. Thus, it is assumed that particle that did not pass in 80 mesh sieving is VB with high cellulose content. However, it still contain starch about 20.19%, which is may decrease the effectiveness of delignification. Delignification in this study was done chemically and physically (heating). The effectiveness of delignification will decrease due to starch gelatinization which occurs during heat treatment. Gelatinization temperature of palm starch is 52.4-72.1$^{o}$C (Noor et

al., 1999). To solve this problem, enzymatic hydrolysis using α-amylase is selected to be applied to decrease starch content in the preparation of raw material prior to delignification. Table 3 shows the chemical composition of raw material after enzymatic hydrolysis. Hydrolysis treatment led to a reduction of lignin and starch, while an increase in cellulose and hemicellulose. Starch has been reduced to 29.47%, while cellulose and hemicelluloses has been increased until 8.51% and 5.28%, respectively.

### *B. Delignification*

There are three methods of delignification which are applied to the lignocellulosic palm trunks, which are alkali and heat treatment (NaOH 5% w/v, oven 150$^{o}$C for 3 hours); alkali-microwave (NaOH 5% w/v, 400 watt for 30 min); alkaline peroxide and heat treatment (NaOH 5% w/v, 121$^{o}$C for 15 minutes). Alkaline conditions are chosen because of its advantages which include: (1) impact on the degradation of sugars is smaller when compared to the delignification in acidic conditions, (2) can dissolve the lignin greater than in acidic conditions, and (3) a small effect on the crystallinity of cellulose (Harmsen et al., 2010 and Singh et al., 2014). The comparison of lignin content before and after delignification is presented in Table 4. The result shows that combination of alkali and heat treatment does not cause a significant lignin reduction, as well as in the alkali treatment with microwave. The significance lignin reduction (80.88%) was found in alkaline peroxide and heat treatment. Figure 1 shows the physical change of raw materials before and after delignification. Figure 1 provides an insight into the variation structure change affected by some methods of pretreatment. Alkali treatment with a hot oven cause physical changes in the surface (Figure 1A and B). Lignocellulose surface of the bulge-shaped section contain an accumulation of lignin (Gauld et al., 1995). Fig. 1B shows that lignin has been degraded in the lignocellulosic surface. Gauld et al., (1995) also explained that

the lignin is a compound that glue the vessels and restrict file microfibril cellulose in plant cell walls. A thin layer between cellulose microfibrils also be a complex of lignin and hemicellulose. Figure 1C shows that microwaves-alkali treatment led to the degradation heat penetration due to the interaction of microwaves with water molecules is higher than the heat penetration of hot oven. Physical changes of raw materials after delignification with alkaline peroxide and heat treatment (1D) shows to be differ significantly from the condition of raw materials before treatment (Figure 1A). Figure 1D shows that alkaline peroxide and heat treatment causes the degradation of lignin in the surface as well as between the cellulose microfibrils more leverage than the treatment of alkali with hot oven or by microwaves. It is proven that cellulose microfibrils have successfully broken down. Singh et al., (2014) explained that the removal of lignin causes cell separation vessel beam, thus forming the cellular structure are linked to each other long in the longitudinal direction. Lignin decomposition may occur due to rupture α-aryl ether bond of monomer polyphenols constituent lignin. Therefore, treatment with hot alkaline peroxide delignification was chosen as the appropriate method to be applied to lignocellulose palm trunks. The advantages of alkaline peroxide delignification method with heat treatment is not require high pressure and expensive equipment, and can significantly degrade lignin (Carvalheiro et al., 2008). Changes in the chemical composition of raw materials, after delignification with alkaline peroxide and heat treatment ($121^{o}$C, 1 atm, 15 minutes) are presented in Table 5.

Table 5 explains that alkaline peroxide decreased lignin content to 3.39%, and reduced hemicellulose content to 10.41%. In addition, alkaline peroxide increased cellulose content to 72.42%, suggesting that cellulose is readily hydrolyzed into sugars. Singh et al., (2014) stated that the acetyl group is a side chain of the main structure of xylan. The removal of acetyl groups from the raw materials can improve the accessibility

of the enzyme to the cellulose and xylan. Table 6 shows the effect of alkaline peroxide and heat treatment on digestibility of substrate. As seen in Table 6 the content of glucose in raw materials increased after delignification. It was confirmed that the lignin reduction will increase the accessibility of cellulase to substrate.

### *C. Optimization of ethanol production efficiency using RSM*

Optimization of fermentation process parameters for *S. cerevisiae* and *P. stipitis* co-culture was performed by using RSM. Parameters examined in this study were: cell ratio between *S. cerevisiae* and *P. stipitis*, culture temperature and fermentation time. Cell ratio between *S. cerevisiae* and *P. stipitis* related to assimilation rate of glucose and xylose by the yeasts. Thus, this parameter is considered a major factor on ethanol production efficiency. Temperature and fermentation time is also key factor on yeast growth. Therefore, these parameters were also optimized for ethanol fermentation. Saccharification and co-fermentation simultaneously must be preceded by pre-hydrolysis. Pre-hydrolysis intended for fermentation can be initiated on the condition of the substrate and the yeast mix well (Prawitwong et al., 2012). Pre-hydrolysis in this study was conducted at 50°C for 8 hours. Pre-hydrolysis processes are evaluated based on the changes of physical substrate and sugar content. Inoculation of yeast into the substrate is performed after the substrate achieving a sugar content that can be used to initiate the fermentation. Cell ratio of *S. cerevisiae/P. stipitis*, culture temperature, and fermentation time were analyzed to determine its relationship to the response prior to the optimization stage. Optimization stage is done after the independent and dependent variables proved to have a quadratic relationship (Montgomery, 2005). Optimization of saccharification and co-fermentation is done based on the central composite design response surface method. Data experimental

results were analyzed using Design Expert program, to predict regression (statistical model) response data. The independent variables i.e. cell ratio, temperature, and fermentation time were optimized, whereas the observed response is ethanol and fermentation efficiency. The efficiency of fermentation is an additional response, which is the development of content data of ethanol. Fermentation efficiency value was calculated based on the concentration of ethanol divided by glucose levels change until the end of fermentation (Δ substrate), and multiplied by 100%. Optimization experiment design and ethanol production performance of *S. cerevisiae* and *P. stipitis* co-culture are presented in Table 7. Statistical model in Software Design Expert consists of a quadratic model, linear, 2FI (interaction of two factors) and cubic (Gasperz, 1992) . Selection of the most appropriate statistical model to determine the optimum response is based on the evaluation order of the sum of squares (sequential model of sum squares), inaccuracies testing model (lack of fit test) and summary statistics (model statistical summary) (Montgomery, 2005). Table 8 provides the profile of xylose and glucose of *S. cerevisiae/P. stipitis* co-culture fermentation.

### *D. Effect of Temperature and Fermentation Time on Ethanol Production*

Figure 2 shows the quadratic relationship between temperature and ethanol content which shows the levels of ethanol is about 1.20% (v/v) under 25$^{O}$C, 3 days fermentation with *S. cerevisiae* ratio of 0.50. The ethanol concentration increased towards optimum temperature around 32,5$^{O}$C (1.83% v/v) and decreased at 35°C (1.36% v/v). This result is in accordance with Lin et al. (2012) that found the optimum temperature of *S. cerevisiae* was 30-40$^{O}$C for ethanol production. High temperature approaching 40°C can interferes transport activity in cells resulting in the production of ethanol decrease. Whereas, *S. cerevisiae* growth rate will decrease under 25°C due to low cell tolerance to ethanol (Lin et al., 2012). Figure 2 also describes a quadratic relationship between the fermentation time

towards ethanol production. The optimum ethanol production (1.83% (v/v) could be achieved under 25$^{O}$C for 5 days fermentation and decreased to1.52% (v / v) after 7 days fermentation.

### *E. Effect of Temperature and Cell Ratio on Ethanol Production*

A quadratic relationship was found between the cell ratio and ethanol production of *S. cerevisiae/P. stipitis* in simultaneous saccharification and co-fermentation (SSCF) process. The ethanol production about 1.16% (v/v) at cell ratio *S. cerevisiae/P. stipitis* of 0.25, 30 °C, 3 days fermentation, then increased when cells reach a ratio of 0.50 (1.66% v/v) and decreased to 1.49% (v/v) at cell ratio of 0.75 (Figure 3). The cell ratio at a certain temperature significantly affected the amount of carbon source consumed by *S. cerevisiae and P. stipitis*, so it can affect the amount of ethanol content. When cell ratio is 0.50:0.50 at 30°C for 5 days fermentation, *S. cerevisiae* and *Pichia stipitis* together actively utilize xylose and glucose as shown in Table 8. Xylose content decreased from 0.626 % (v/v) to 0% and glucose content decreased from 6.163 % to 0.0027%. When cell ratio of *S. cerevisiae* is 0.75 at 35$^{O}$C, and 7 days fermentation, the concentration of ethanol reached 1.34 % (v/v). In this condition, xylose content decreased from 1.062 % (v/v) to 0.694 % (v/v), suggesting that higher consumption rate of glucose was performed by *S. cerevisiae* whereas *P. stipitis* could not consume the xylose maximally.

### *F. Effect of Fermentation Time and Cell ratio on Ethanol Production*

A quadratic relationship between fermentation time and cell ratio was observed on ethanol production. Figure 4 shows that the ethanol increased by the increasing of the cell ratio, then decreased after reaching maximum ethanol content. The same phenomenon was also observed for fermentation time toward ethanol production.

### *G. Effect of Temperature and Fermentation Time on Fermentation Efficiency*

Fermentation efficiency can reach about 60% at cell ratio of 0.5, 35°C, and 3 days f. This result is in accordance with the data presence in Table 7 that shows the fermentation efficiency about 62.63% under the cell ratio of *S. cerevisiae/P. stipitis* at 0.75:0.25, 35$^{O}$C, and 3 days fermentation. *S. cerevisiae* more active in using glucose than *P. stipitis* at 35$^{O}$C since consumption of glucose occurred during co-fermentation process but not for xylose (Table 8).

However, under the same cell ratio and temperature, the fermentation efficiency decreased to 45% when longer incubation (7 days) was performed (Figure 5). This is due to the longer of fermentation time, the lower of ethanol can be produced because ethanol will be consumed by *S. cerevisiae* as a carbon source to generate energy. This relationship can be explained by analysis of variance (data not shown) which shows that there is a correlation between temperature and fermentation time on the efficiency of fermentation. The value of the correlation coefficient is -0.97. The negative values reflecting inverse relationship, meaning that the higher of temperature and the shorter of fermentation time, the fermentation efficiency will increase. The result can be explained by the data shown in Table 8. When the temperature is 35$^{O}$C, 3 days fermentation and cell ratio of 0.75: 0.25, the fermentation efficiency reached 62.63%, but when the temperature is 25°C, 7 days fermentation, with the same cell ratios, the fermentation efficiency decreased to 40.16%.

### *H. The Effect of Temperature and Cell Ratio on Fermentation Efficiency*

Figure 6 shows the optimum fermentation efficiency in the range of 30°C, the cell ratio of 0.50: 0.50 with 5 days fermentation. *S. cerevisiae* and *P. stipitis* can ferment together at a temperature and duration of fermentation. Results of analysis of variance

shows that there is no correlation between the ratio of cells with long fermentation on the efficiency of fermentation

### *I. Effect of Long Fermentation Time and Cell Ratio on Fermentation Efficiency*

Figure 7 shows that the cell ratio of 0.25:0.75, 3 days fermentation and under $30^{O}$C achieved fermentation efficiency about 41.5%. When the cell ratio at 0.50:0.50, fermentation efficiency increase until 54% and decreased to 50% when the cell ratio of 0.75:0.25. These data illustrate the quadratic relationship between the cell ratio and efficiency of fermentation. Quadratic relationship is also found between fermentation time and fermentation efficiency. Fermentation efficiency reached 39%, 3 days fermentation, $30^{O}$C, cell ratio of 0.25:0.75, and increased to 47% for 5 days fermentation, and decreased to approximately 25% for 7 days fermentation.

### *J. Model validation for optimal simultaneous saccharification and co-fermentation of ethanol*

Optimization of saccharification and co-fermentation simultaneously (SSCF) temperature treatment, fermentation time and cell ratio were statistically analyzed using Design-Expert software version 7. The model predicted the optimal conditions as follows: cell ratio of *S. cerevisiae/P. stipitis* at 0.54; temperature at $33.45^{O}$C; and fermentation time at 4.22 days. The maximum yield prediction on ethanol and fermentation efficiency under optimal conditions is shown in Table 9. The optimal condition at temperature $33.45^{O}$C, fermentation time 4.22 days and cell ratio of *S. cerevisiae* at 0.54 chosen by the program based on the levels of ethanol fermentation and highest efficiency. Validation is done by comparing the response of the actual experimental results with the predicted value of the program. Suitability of variables at optimal point is tested and repeated three times based on temperature variables $33.45^{O}$C, 4.22 days and cell ratio of *S. cerevisiae* at 0.54.

Validation shows that the difference of actual experimental results and the predicted value of the program Design Expert of ethanol content is 1.22% and 0.72% for fermentation efficiency (Table 10). The differences between experimental value and predicted value was found less than 5%, indicating the value of the independent variables optimal point is quite suitable to produce an optimal response. Sun et al., (2011) reported the results of the experimental validation and predictive value of the program has an error rate of less than 5%, proving that the value of the optimum point variables have a high suitability.

## Conclusion

The SSCF process by *S. cerevisiae* and *P. stipitis* was systematically optimized using design experiment. Alkaline peroxide combined with heat treatment, successfully removed lignin until 93.22% of the treated raw material. Optimization of SSCF condition using DoE model show that the co-culture can work together to produce maximum ethanol and fermentation efficiency at 33,45$^{O}$C, 4.22 days and cell ratio 0.54: 0.46 (*S. cerevisiae* NCYC 479 and *P. stipitis* NCYC 1541). This study could provide a strategy for improvement of efficient ethanol production in SSCF process of oil palm trunk.

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## TABLES

**TABLE 1**. Chemical composition analysis of raw material after 30 mesh sieving

| Compound | weight (g) | lignin (% b/b) | cellulose (% b/b) | hemicellulose (% b/b) | starch (% b/b) |
|---|---|---|---|---|---|
| ≥ 595 µm | 36 | 19.40 | 33.90 | 14.30 | 25.18 |
| ≤ 595 µm | 64 | 18.58 | 30.21 | 14.52 | 35.00 |

**TABLE 2**. Chemical composition analysis of raw material after 80 mesh sieving

| Compound | weight (g) | lignin (% b/b) | cellulose (% b/b) | hemicellulose (% b/b) | starch (% b/b) |
|---|---|---|---|---|---|
| ≥ 177µm | 26 | 22.49 | 41.13 | 14.20 | 20.19 |
| ≤ 177um | 74 | 19.06 | 28.19 | 14.70 | 36.84 |

**TABLE 3**. Chemical composition analysis of raw material after enzymatic hydrolysis

| Treatment | weight (g) | lignin (% b/b) | cellulose (% b/b) | hemicellulose (% b/b) | starch (% b/b) |
|---|---|---|---|---|---|
| Before hydrolysis | 100 | 22.49 | 41.13 | 14.20 | 20.19 |
| After hydrolysis | 72,27 | 17.73 | 51.45 | 16.63 | 12.24 |

**TABLE 4**. The lignin content before and after delignification

| Treatment | Lignin (% w/w) |
|---|---|
| 1. Control | 17.73 |
| 2. Alkali and heat treatment | 18.76 |
| 3. Microwave-alkali treatment | 18.23 |
| 4. Alkaline peroxide and heat treatment | 3.39 |

**TABLE 5**. Chemical composition of raw materials after alkaline peroxide and heat treatment

| Treatment | weight (g) | Lignin (% b/b) | cellulose (% b/b) | hemicellulose (% b/b) | Starch (% b/b) |
|---|---|---|---|---|---|
| Before | 100 | 17.73 | 51.45 | 16.63 | 12.24 |
| After | 40 | 3.39 | 72.42 | 10.41 | 12.21 |

**TABLE 6**. Effect of alkaline peroxide and heat treatment on glucose

| Treatment | lignin (% w/w) | glucose (% w/v) | Increasing of glucose (%) |
|---|---|---|---|
| Before delignification | 17.73 | 0.36 | 93.22 |
| After delignification | 3.39 | 5.31 | |

*hydrolysis of 5% (b/v ) substrate, with 10 ml working volume using cellulose (Cellic Ctec2) 25 FPU/g substrate, pH buffer 5, at $50^{o}$C for 72 h.

**TABLE 7.** Optimization experiment design and ethanol production performance of *S. cerevisiae* and *P. stipitis* co-culture by simultaneous saccharification and co-fermentation (SSCF)

| No. | Temperature ($^{o}$C) | Fermentation time (days) | Cell ratio | Variable | | | Ethanol (% v/v) ($Y_1$) | Fermentation Efficiency (%) ($Y_2$) |
|---|---|---|---|---|---|---|---|---|
| | | | | $X_1$ | $X_2$ | $X_3$ | | |
| 1 | 35 | 7 | 0,75 | +1 | +1 | +1 | 1,34 | 30,03 |
| 2 | 35 | 7 | 0,25 | +1 | +1 | -1 | 0,87 | 18,61 |
| 3 | 35 | 3 | 0,75 | +1 | -1 | +1 | 1,50 | 62,63 |
| 4 | 35 | 3 | 0,25 | +1 | -1 | -1 | 1,45 | 49,21 |
| 5 | 25 | 7 | 0,75 | -1 | +1 | +1 | 1,34 | 40,16 |
| 6 | 25 | 7 | 0,25 | -1 | +1 | -1 | 0,64 | 19,18 |
| 7 | 25 | 3 | 0,75 | -1 | -1 | +1 | 0,67 | 24,17 |
| 8 | 25 | 3 | 0,25 | -1 | -1 | -1 | 0,58 | 20,99 |
| 9 | 38 | 5 | 0,50 | +1,682 | 0 | 0 | 0,88 | 69,46 |
| 10 | 22 | 5 | 0,50 | -1.682 | 0 | 0 | 1,34 | 46,50 |
| 11 | 30 | 8 days, 10 h | 0,50 | 0 | +1,682 | 0 | 0,78 | 22,41 |
| 12 | 30 | 1 days, 15 h | 0,50 | 0 | -1.682 | 0 | 0,82 | 53,43 |
| 13 | 30 | 5 | 0,92 | 0 | 0 | +1,682 | 1,25 | 36,42 |
| 14 | 30 | 5 | 0,08 | 0 | 0 | -1.682 | 0,78 | 22,53 |
| 15 | 30 | 5 | 0,50 | 0 | 0 | 0 | 2,18 | 63,20 |
| 16 | 30 | 5 | 0,50 | 0 | 0 | 0 | 1,77 | 51,32 |
| 17 | 30 | 5 | 0,50 | 0 | 0 | 0 | 1,84 | 53,36 |
| 18 | 30 | 5 | 0,50 | 0 | 0 | 0 | 2,25 | 65,24 |
| 19 | 30 | 5 | 0,50 | 0 | 0 | 0 | 1,89 | 55,20 |
| 20 | 30 | 5 | 0,50 | 0 | 0 | 0 | 2,00 | 57,99 |

**TABEL 8**. Xylose and glucose content of *S. cerevisiae / P. stipitis* simultaneous saccharification and co-fermentation (SSCF) process

| No. | Temp ($^{o}$C) | Fermentation (day) | Cell ratio | Simultaneous saccharification and co-fermentation | | | | | | substrat | ethanol (%) | Fermentation efficiency (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Before | | | After | | | | | |
| | | | | xylose | glucose | Total | xylose | glucose | Total | | | |
| 1 | 35 | 7 | 0,75:0,25 | 1,062 | 8,435 | 9,497 | 0,694 | 0,054 | 0,748 | 8,749 | 1,34 | 30,03 |
| 2 | 35 | 7 | 0,25:0,75 | 1,062 | 8,435 | 9,497 | 0,295 | 0,037 | 0,332 | 9,165 | 0,87 | 18,61 |
| 3 | 35 | 3 | 0,75:0,25 | 0,759 | 7,002 | 7,761 | 0,970 | 2,095 | 3,065 | 4,696 | 1,50 | 62,63 |
| 4 | 35 | 3 | 0,25:0,75 | 0,759 | 7,002 | 7,761 | 0,915 | 1,068 | 1,983 | 5,778 | 1,45 | 49,21 |
| 5 | 25 | 7 | 0,75:0,25 | 0,841 | 5,702 | 6,543 | 0,000 | 0,000 | 0,000 | 6,543 | 1,34 | 40,16 |
| 6 | 25 | 7 | 0,25:0,75 | 0,841 | 5,702 | 6,543 | 0,000 | 0,000 | 0,000 | 6,543 | 0,64 | 19.18 |
| 7 | 25 | 3 | 0,75:0,25 | 0,419 | 5,042 | 5,461 | 0,000 | 0,026 | 0,026 | 5,435 | 0,67 | 24,17 |
| 8 | 25 | 3 | 0,25:0,75 | 0,419 | 5,042 | 5,461 | 0,000 | 0,042 | 0,042 | 5,419 | 0,58 | 20,99 |
| 9 | 38 | 5 | 0,50:0,50 | 1,124 | 10,319 | 11,443 | 1,201 | 7,758 | 8,959 | 2,484 | 0,88 | 69,46 |
| 10 | 22 | 5 | 0,50:0,50 | 0,780 | 4,920 | 5,700 | 0,000 | 0,050 | 0,050 | 5,650 | 1,34 | 46,50 |
| 11 | 30 | 8 days, 10 h | 0,50:0,50 | 0,691 | 6,160 | 6,851 | 0,000 | 0,0027 | 0,027 | 6,824 | 0,78 | 22,41 |
| 12 | 30 | 1days, 15 h | 0,50:0,50 | 0,340 | 4,358 | 4,698 | 0,660 | 1,029 | 1,689 | 3,009 | 0,82 | 53,43 |
| 13 | 30 | 5 | 0,92:0,08 | 0,626 | 6,163 | 6,789 | 0,000 | 0,060 | 0,060 | 6,729 | 1,25 | 36,42 |
| 14 | 30 | 5 | 0,08:0,92 | 0,626 | 6,163 | 6,789 | 0,000 | 0,000 | 0,000 | 6,789 | 0,78 | 22,53 |
| 15 | 30 | 5 | 0,50:0,50 | 0,626 | 6,163 | 6,789 | 0,000 | 0,026 | 0,026 | 6,763 | 2,18 | 63,20 |
| 16 | 30 | 5 | 0,50:0,50 | 0,626 | 6,163 | 6,789 | 0,000 | 0,027 | 0,027 | 6,762 | 1,77 | 51,32 |
| 17 | 30 | 5 | 0,50:0,50 | 0,626 | 6,163 | 6,789 | 0,000 | 0,028 | 0,028 | 6,761 | 1,84 | 53,36 |
| 18 | 30 | 5 | 0,50:0,50 | 0,626 | 6,163 | 6,789 | 0,000 | 0,027 | 0,027 | 6,762 | 2,25 | 65,24 |
| 19 | 30 | 5 | 0,50:0,50 | 0,626 | 6,163 | 6,789 | 0,000 | 0,075 | 0,075 | 6,714 | 1,89 | 55,20 |
| 20 | 30 | 5 | 0,50:0,50 | 0,626 | 6,163 | 6,789 | 0,000 | 0,026 | 0,026 | 6,763 | 2,00 | 57,99 |

**TABLE 9**. Optimization predicted by *Design Expert* program

| Response | Optimum point of SSCF under optimal conditions (33,45$^{o}$C, 4,22 days and cell ratio of *S. cerevisiae* 0,54) |
|---|---|
| Etanol (%, v/v) | 1,905 |
| Fermentation efficiency | 66,628 |

**TABLE 10.** Experimental validation

| Variables of SSCF | | | Ethanol (%) | | | Fermentation efficiency (%) | | |
|---|---|---|---|---|---|---|---|---|
| Temperature ($^{o}$C) | Fermentation time (days) | Cel ratio | Experiment | Predicted | % difference | Experiment | Predicted | % difference |
| 33,45 | 4,22 | 0,54 | 1,87 | 1,91 | 2,09 | 65,91 | 66,62 | 1,07 |
| 33,45 | 4,22 | 0,54 | 1,90 | 1,91 | 0,52 | 66,14 | 66,62 | 0,72 |
| 33,45 | 4,22 | 0,54 | 1,89 | 1,91 | 1,05 | 66,37 | 66,62 | 0,38 |
| | average | | 1,89 | 1,91 | 1,22 | 66,14 | 66,62 | 0,72 |

*Figure 1*. SEM images of untreated raw materials (A); pretreated raw materials with NaOH 5%, 150$^{o}$C for 3 hour (B); pretreated raw materials with microwave-alkali (NaOH 5% w/v, 121$^{o}$C for 15 minutes) (C); pretreated raw materials with $H_2O_2$ 5%; pH 11.5; 121$^{o}$C for 15 min (D).

*Figure 2*. Response surface of ethanol production showing the influence of temperature and fermentation time for *S. cerevisiae/P. stipitis* in simultaneous saccharification and co-fermentation (SSCF) process

*Figure 3*. Response surface of ethanol production showing the influence of cell ratio and temperature for *S. cerevisiae/P. stipitis* in simultaneous saccharification and co-fermentation (SSCF) process

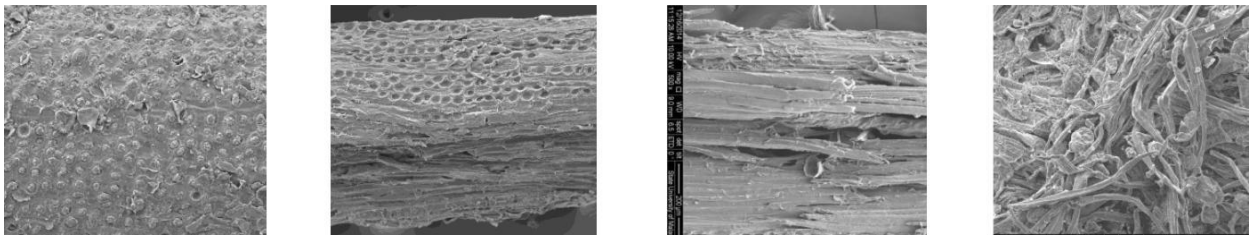
*Figure 4*. Response surface of ethanol production showing the influence of cell ratio and fermentation time for *S. cerevisiae/P. stipitis* in simultaneous saccharification and co-fermentation (SSCF) process

*Figure 5*. Response surface of fermentation efficiency showing the influence of temperature and fermentation time for *S. cerevisiae/P. stipitis* in simultaneous saccharification and co-fermentation (SSCF) process

*Figure 6*. Response surface of fermentation efficiency showing the influence of temperature and cell ratio for *S. cerevisiae/P. stipitis* in simultaneous saccharification and co-fermentation (SSCF) process

*Figure 7*. Response surface of fermentation efficiency showing the influence of fermentation time and cell ratio for *S. cerevisiae/P. stipitis* in simultaneous saccharification and co-fermentation (SSCF) process

Figure 1



A B C D

Figure 2

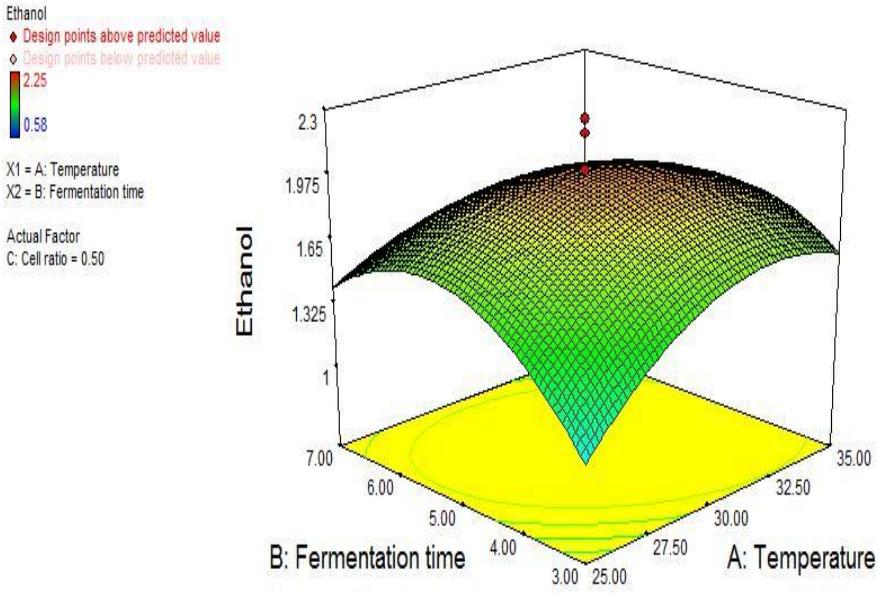



Figure 3

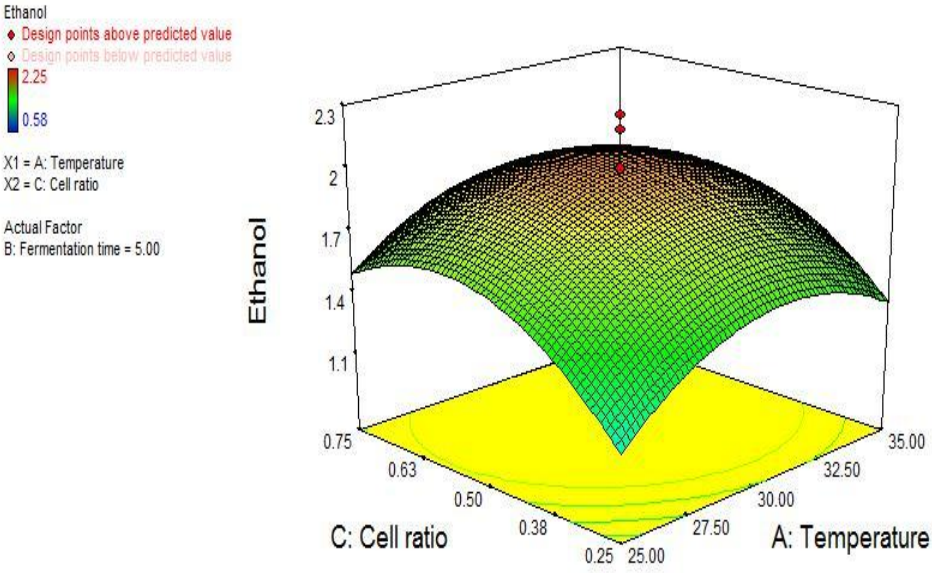


Figure 4

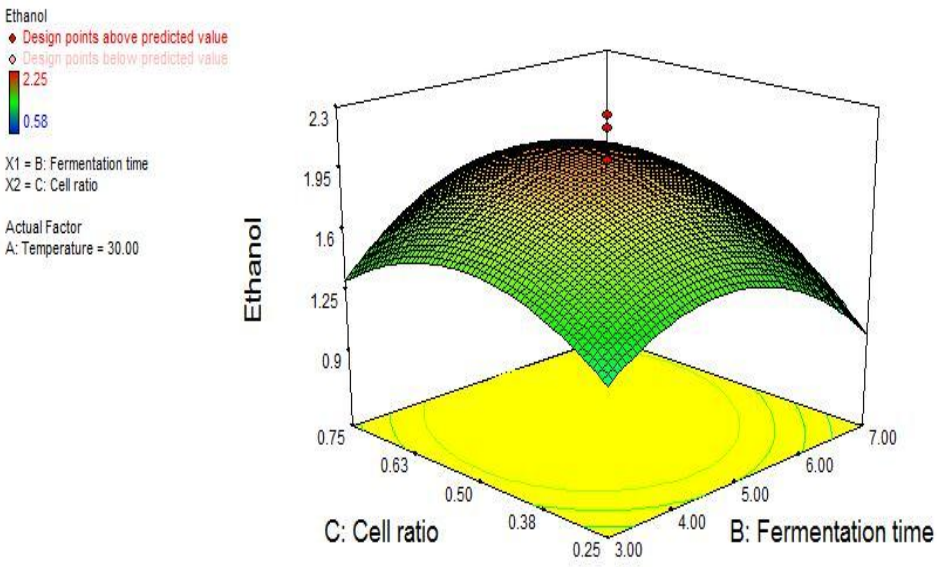



Figure 5

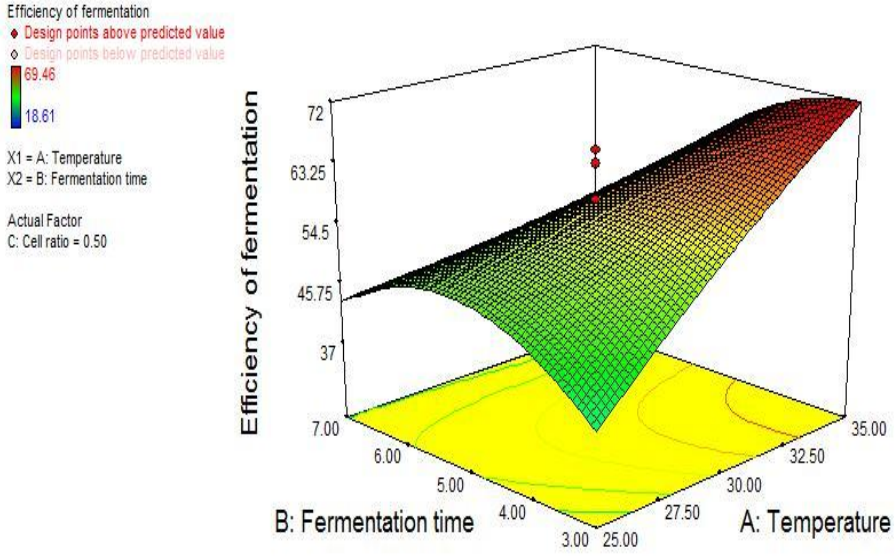


Figure 6

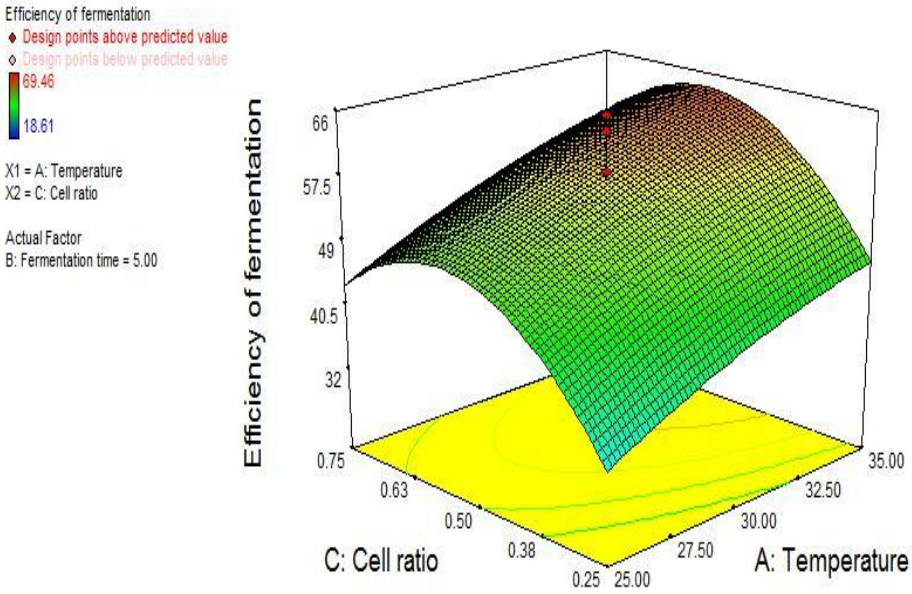



Figure 7