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The optimization of ethanol production from oil palm empty fruit bunches hydrolysate by using statistical experimental design

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Abstract: Ethanol is a gasoline substituting fuel which its market has continued to grow rapidly in recent years. Generally, ethanol is produced from a variety of raw materials and biomass is one modern source of renewable energy that obtains as a byproduct from the agriculture and forestry industries. The oil palm empty fruit bunch (OPEFB) is one of the potential lignocellulosic biomasses for ethanol production. In this study, ethanol production from OPEFB was investigated. Firstly, OPEFB was alkali-pretreatment with 15% (w/v) NaOH at 121˚C for 45 min and it provided of 67.2% cellulose, 22.8% hemicellulose and 7.2% lignin. The cellulose content in the alkali-pretreated OPEFB had increased 39.4% from untreated OPEFB, while the hemicellulose and lignin had decreased to 33.1% and 36.7% respectively. Secondly, alkali-pretreated OPEFB was hydrolyzed with 7% (v/v) H_2SO_4 at 140°C for 90 min. The highest glucose yield of 92.38% was obtained representing 55.54 g/L. Lastly, the glucose resulting was fermented to ethanol by *Saccharomyces cerevisiae*. Lastly, a response surface methodology (RSM) with a central composite design (CCD) was applied in the experimental design to optimize the ethanol production condition. A verification experiment indicated the optimal fermentation condition was as follows: 55 g/L initial glucose, pH 6.25, 2% inoculum size at 35±2ºC for 48 h. The highest ethanol yield of 40.76% (22.4 g/L, 0.41 g/g glucose) was over 80% of the theoretical ethanol yield produced from glucose fermentation, which was 28.05 g/L (0.51 g/g glucose). The ethanol yield achieved appears quite attractive and demonstrates that OPEFB have excellent potential resource of renewable energy. While the experimental design is useful for optimization designs with several variables and describing more complex behavior by including higher orders model components.

Keywords: Ethanol, oil palm empty fruit bunch, alkali pretreatment, response surface methodology

1. Introduction

Renewable and sustainable energy resources will play a crucial role in the future of human life. Bioethanol can be blended with gasoline to make ethanol fuel mixtures with "E numbers" using in gasoline engines which has become an alternative sustainable energy source. Recently, most of bioethanol production throughout the world is from food crops such as cassava and sugarcane [1-2], raising questions concerning the competition with food supply and arable land [3]. This has resulted in the promotion of lignocellulosic biomass as the raw materials for production of liquid biofuels.

Lignocellulose biomass (LB) is an alternative renewable resource which is abundant worldwide [4-5]. LB is mainly composed of 30-60% cellulose, a glucose polymer; 25-35% hemicellulose, a sugar heteropolymer and 15-20% lignin, an aromatic polymer [6]. The conversion of sugar from these materials to ethanol offers the opportunity to replace fossil fuels without competing with food [7]. In Thailand, oil palm empty fruit bunch (OPEFB) offers a potential feedstock for bioethanol production because there are many palm oil plantations producing OPEFB. The palm oil industry generates waste by-products for about 70–75% of the raw material including 50-59% oil palm empty fruit

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bunches (OPEFB), 12-14% palm pressed fiber (PPF), 1.0-5.0% palm kernels (PKS) and 8.0-11% palm nut shell those related to amount of the dry mass fraction in each palm oil production process. In addition, the solid wastes can be estimated to three million tons of OPEFB, over one million tons of PPF and around one million tons of PKS per year [8].

Ethanol production process from LB consists of three steps. First, pretreatment; pretreatment is needed to decrease the crystallinity of cellulose, increase reaction area, and to increase the porosity of the materials for efficient conversion to ethanol [5, 9]. An alkali pretreatment could be increased cellulose digestibility and effectively promote the lignin solubility of agricultural waste and wood materials [2]. Sodium hydroxide has been reported to reduce half of lignin [5]. Duangwang et al. [10] reported that the amount of cellulose of NaOH treated- OPEFB was highest (68.8 % of cellulose) and a minimal amount of lignin as well while pretreated with H_2SO_4 and water, the amount cellulose decreased because H_2SO_4 and water can degrade hemicellulose and also hydrolysis of cellulose. Second, hydrolysis; there are two types of hydrolysis, enzymatic hydrolysis and acid hydrolysis. Although enzymatic hydrolysis is environmentally friendly, releasing no toxins during the process and produces a high yield of ethanol. However, it is limited by price of hydrolytic enzymes. Acid hydrolysis is the conventional process used in ethanol production plants. Choojit et al. [11] studied of acid hydrolysis pineapple leaf residues by sulfuric and the result presented highly reproducible of 20.89 g/L glucose content. The last step in the process is fermentation with fermenting organisms that is used for the conversion of sugar to ethanol. The selection of strain for bioethanol production is made by considering their productivity, tolerance to ethanol, fermentation inhibitors and severe pH and temperature conditions. In most of the fermentation processes *Saccharomyces cerevisiae* is used. *S. cerevisiae* is an efficient bio-ethanol producer due to its high tolerance to ethanol, low optimum pH range and anaerobic conditions requirement [12]. Response surface methodology (RSM) is a collection of mathematical and statistical techniques for building an experiment. Based on the fit of an equation to the experimental data and can be described the trend of result for making statistical predictions. In addition, it can be applied successfully even response is affected by several variables. Moreover, this method can be evaluated interactive effects between the parameters and accordingly, investigate the effects of the parameters to the responses [13].

2. Materials and methods

The overall procedure for ethanol production from alkali-pretreated OPEFB is presented in Fig. 1.

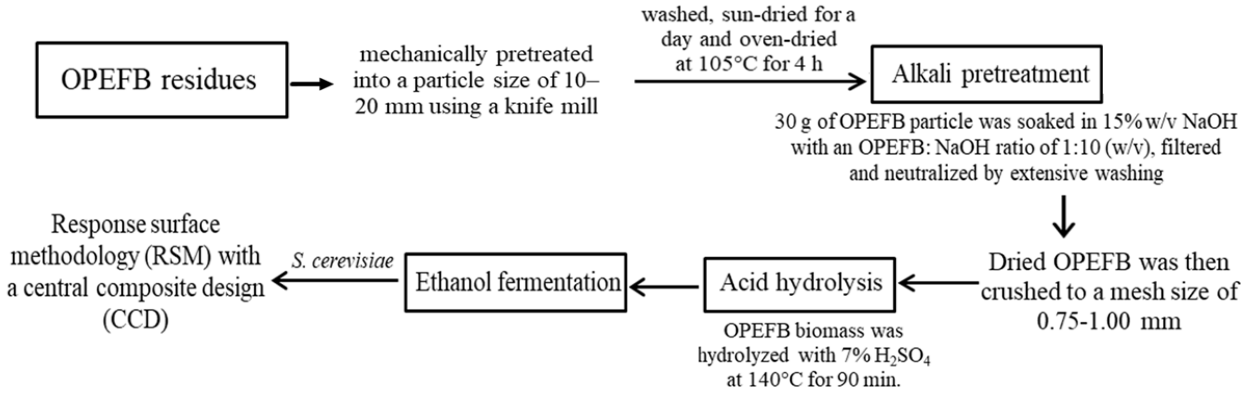

Fig. 1 – Flow diagram of the overall procedure for ethanol production from alkali-pretreated OPEFB by *Saccharomyces cerevisiae*

2.1 Raw material, reagents and microorganism

The OPEFB residues were obtained from Trang Palm Oil Industry Co., Ltd., Trang, Thailand. OPEFB was washed with tap water, sunlight drying and oven-dried at 105°C for 8 h until the moisture content was approximately 5% (w/w). The dried OPEFB was then mechanically pretreated by shredding into a particle size of 5–10 mm using a hammer mill and stored in a vacuum jar [14]. All chemicals and reagents were analytical grades which were purchased from Sigma–Aldrich, Co. LLC. *S. cerevisiae* TISTR 5596 was provided from the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. The culture condition for the inoculum preparation was prepared by transferring *S. cerevisiae* powder into YM broth (containing 40 g/L glucose, 3 g/L malt extract, 3 g/l yeast extract, 3 g/L peptone) and incubated in a rotary shaker with 160 rpm shaking speed at 30 °C for 24 h. This inoculum was obtained *S. cerevisiae* concentration of 10^8 CFU/mL.

2.2 Alkali pretreatment

Thirty grams of OPEFB were soaked in 15% w/v NaOH with an OPEFB: NaOH ratio of 1:10 (w/v) at 130°C for 40 min [10]. Then the solid-OPEFB was obtained by a filter and washed with distilled water until pH 7. The alkali pretreated OPEFB was dried in an oven at 80°C for 24 h. Then the dried alkali pretreated OPEFB was crushed to a mesh size of 0.5-1 mm using a hammer mill. Cellulose hemicellulose and lignin contents were determined by the AOAC methods [15].

2.3 Acid hydrolysis

The alkali pretreated OPEFB was carried out in 125 ml Erlenmeyer flasks with 7% (v/v) H_2SO_4 concentration at 140°C and for 90 min [16]. After hydrolysis, the acid hydrolysis solution was obtained by filtration. Then the acid hydrolysis solution was separated into two parts; the first part was analyzed for sugar content while second part was detoxified by over-liming with 1.2 g/L sodium hydroxide solution to pH 11. After that, the detoxified solution was precipitated and then centrifuged for 5 min. The clear fraction was then detoxified with activated charcoal (15% w/v) and incubation in a shaker with shaking for 200 rpm at 30°C for 30 min and then filtered. The detoxified acid hydrolysis solution was adjusted to several pH by adding sodium hydroxide or sulfuric acid to the desired level.

2.4 Ethanol fermentation

The Design expert software (Trial version 7.0, Stat-Ease, Inc., Minneapolis, USA) with a CCD design matrix was used for the design of ethanol fermentation experiments. The ethanol fermentation was carried in a 150 mL Duran bottle with a working volume of 50 mL which was purged of oxygen with nitrogen and immediately capped with an airlock. Four independent variables, namely of pH, temperature, initial glucose content, and inoculum size were 4-7, 20-40 °C, 40-100 g/L and 1-5%, respectively were used at five coded levels (-α, -1, 0, +1, +α), as shown in Table 1.

Table 1 - Variable codes and actual factor levels for the central composite design (CCD)

Independent variable	Unit	Code	Actual factor level				
			- α	**-1**	**0**	**+1**	**+α**
Initial glucose	g/L	*A*	40	55	70	85	100
pH	-	*B*	4	4.75	5.5	6.25	7
Temperature	°C	*C*	20	25	30	35	40
Inoculum size	%	*D*	1	2	3	4	5

The 2^3 factorial central composite experimental designs with six start points and three replicates at the central point had 28 experimental runs in the design (Table 2). Samples were incubated in incubation shaker at 150 rpm and the fermentation sample was conducted for five days and interval sampling at 6, 12, 24, 48, 72 and 96 h. The significance of each variable and their interactions, and fitting a predictive model to the experimental responses was based on the following second-order polynomial:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \sum_{j}^{k} \beta_{ij} x_i x_j \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Here Y is the observed response (ethanol yield, %; β_0 is the constant term; i, j and k are integers (in this case i is from 1 to 3, j is from 2 to 3, and k is the total number of factors, 3); β_i, β_{ii}, β_{ij} are, respectively, the coefficients for the linear, quadratic and interactive effects; and x_i and x_j are independent variables or factors, representing the acid concentration, hydrolysis temperature and hydrolysis time. The statistical software package Design Expert (Trial version 7.0) is used to analyze the results. The fit of the models is assessed from the coefficient of determination R^2 and the adjusted R^2. Experimental validation of the model-based optimum set-point for ethanol production was performed. The different initial glucose concentrations were prepared by using dilutions from stock glucose solution. First, preparation of 100 g/L initial glucose stock solution for 500 mL; pour 250 ml of deionized water into a 500 mL beaker and measure out 100 g of powdered glucose and add it to the beaker, then insert a stir bar and sit the beaker on a hot plate, stir the solution over heat for a few minutes until glucose dissolves in water. Finally, add more deionized water to bring the total volume up to 500 mL. The other initial glucose concentrations were prepared by calculating the volume of 100 g/L initial glucose stock solution that was required.

2.5 Analytical method

Glucose concentration in the sample was determined by high-performance liquid chromatography (HPLC, 1100, Hewlett Packard, Germany) using an APS-2 HYPERSIL column and RI detector. 75% of acetonitrile was used as the mobile phase (flow rate of 0.5 mL/min and temperature was maintained at 35°C). The ethanol concentration was

analyzed by gas chromatography (GC) according to the conditions of Duangwang et al. [13] using an HP-FFAP polyethylene glycol column (30 m × 0.25 mm) at 120°C and flame ionization detectors (FID) at 250°C with injector set at 150°C. Helium was used as carrier gas (flow rate of 2 mL/min). Furfural was analyzed by using an Aminex HP-87H column performing at 65°C with 0.005 N H_2SO_4 as the mobile phase with a flow rate of 0.6 mL/min and a UV detector (280 nm) Miliauskas et al. [17].

3. Results and discussion

3.1 Alkali pretreatment

The composition of OPEFB was determined The AOAC official methods. The raw OPEFB was investigated to be 48.2% cellulose, 34.1% hemicellulose, 19.6% lignin and 0.8% ash. Since, lignin is potential against to efficient enzymatic hydrolysis of complex sugars, because it could be intercepted their availability for enzymatic access [18], sodium hydroxide solution was broken the lignin cover and infest the cellulose crystalline structure [19]. Although, different methods of pretreatment techniques have been generated with the common objective of making biomass more susceptible to ethanol production, the pretreatment with alkaline solution has one of the most efficient process, due to its strong pretreatment effect and relatively simple process scheme.

The alkali pretreatment at 121˚C for 45 min with 15% (w/v) sodium hydroxide, the OPEFB composed of 67.2% cellulose, 22.8% hemicellulose, 7.2% lignin, and 0.3% ash. After the alkali pretreated, the cellulose content in OPEFB had increased from 39.4% in the untreated OPEFB, whereas the hemicellulose and lignin decreased to 33.1% and 36.7% respectively from the untreated OPEFB. This was related to previous work using alkali pretreatment of OPEFB at the optimum reaction NaOH concentration, temperature and reaction time of and 15% (w/v) and 130 ˚c, 40 min, respectively by presenting the maximum cellulose value of 68.8% [10] while with 4.7% sodium hydroxide solution and found that the cellulose content was increased twice times while lignin content decreased a half time [20]. Therefore, alkali pretreatment clearly affects the structure of OPEFB. In this work, the cellulose and hemicellulose contents were still high which was lower lignin content so this would be improved the efficiency of sugar production in hydrolysis step.

3.2 Acid hydrolysis

Although both enzymatic and acid hydrolysis is possible for biomass hydrolysis, enzymatic hydrolysis represents a more environmentally friendly process. However, cellulase enzymes (glucanase and xylanase) are known the high cost of cellulose enzymes represents a drawback for high capacity ethanol production. Therefore, acid hydrolysis has traditionally been employed to hydrolyze cellulose into fermentable sugars for industrial ethanol production. However, acid could be to be generated by fermentation inhibitors, such as furfural, and hydroxymethyl furfural (5-HMF) and phenolic compounds [21].

In this study, acid hydrolysis was used to convert alkali pretreated OPEFB cellulose into fermentable sugar using a 7% H_2SO_4 concentration as a catalyst at a temperature of 140°C and a reaction time of 90 minutes. A high glucose yield of 92.38% was obtained representing 55.54 g/L with a low by-product content of 2.28 g/L furfural and 1.987 g/L phenolic compounds. In previous work, Oliva et al. [22] demonstrated that furfural was a strong inhibitor of the growth of *Kluyveromyces marxianus* and ethanol fermentation. Furfural could be inhibited cell growth and affected growth rate of fermenting yeast depending on its concentration in the fermentation medium [23]. While the furfural concentration lower than 0.5 g/L showed a positive effect on cell growth and its concentrations above 2 g/L could be inhibited cell growth almost completely [24]. In addition, lignin degradation products presented more toxic to fermenting microorganisms than furfural and hydroxymethylfurfural [23].

As inhibitors problem, the fermentation of non-detoxified hydrolyzates lignocellulose was conducted to improve the ethanol fermentation process by Mussatto and Roberto, [23]; over-liming and the application of 15% activated charcoal is the combination of methods which has been developed to produce a high yield of ethanol. The results of the combination methods in this study showed that furfural and phenolic compounds were largely removed with the furfural being reduced to 0.51 g/L and phenolic compounds to 0.26 g/L with a sugar reduction of 15% or 47.20 g/L of sugars resulting. According to the study by Silva et al. [25], the pretreated sugarcane bagasse solution was applied with activated charcoal and observed that 30% of charcoal reduced the sugars by 31.3%. Jung et al. [26] reported that sulfuric acid-pretreated OPEFB solution was treated with activated carbon and the concentrations of acetic acid and furfural content was decreased by 65% and 11%, respectively.

3.3 Response surface of ethanol production

3.3.1 Optimization of ethanol production

Ethanol production is affected by various parameters. Screening of significant parameters is necessary to select the major parameters which affect the efficiency of the process. The biomass residue for ethanol production in this research was OPEFB hydrolysate which similarly to the biomass residue in Singh and Bishnoi, [26] which was alkali pretreated. To economize on the chemicals, reagents and microorganism used in the screening experiment this

investigation concentrated on optimizing four parameters, initial glucose (g/L), pH, inoculum size (%) and temperature (°C). Based on a CCD for ethanol production using *S. cerevisiae* TISTR 5596; A range of pH, temperature, initial sugar, and inoculum size were evaluated. The optimal pH in previous research for ethanol production by *S. cerevisiae* were: 4-7 [27-28], 3.9 4.1 [29], 5.5 [30], 3.6-6.8 [19] and 5.0 [31]. The range of temperature (25-35°C) needs to be set with consideration to the growth of *S. cerevisiae* from each culture collection while the range of initial sugar contents and inoculum levels must take into consideration the achievement of a high yield of ethanol at various sampling times as well as aiming for the most economical process. A CCD was developed to study and evaluate the main variable effects and the interactive effects of each parameter. CCD was also used to optimize the conditions for ethanol fermentation. Four variables were investigated: initial glucose content, pH, inoculum level and temperature. The observed ethanol in each experiment is shown in Table 2.

Table 2 - The CCD of the experiment and the observed response of the ethanol yield

	(A)	(B)	(C)	(D)	(Y)
Run	**Initial glucose (g/L)**	**pH**	**Temperature (ºC)**	**Inoculum level (%)**	**Ethanol yield (%)**
1	55	4.75	25	2	9.23
2	85	4.75	25	2	25.92
3	55	6.25	25	2	18.65
4	85	6.25	25	2	20.69
5	55	4.75	35	2	17.45
6	85	4.75	35	2	23.59
7	55	6.25	35	2	40.76
8	85	6.25	35	2	28.35
9	55	4.75	25	4	29.19
10	85	4.75	25	4	35.00
11	55	6.25	25	4	16.65
12	85	6.25	25	4	18.11
13	55	4.75	35	4	25.8
14	85	4.75	35	4	19.66
15	55	6.25	35	4	30.67
16	85	6.25	35	4	19.75
17	40	5.5	30	3	17.89
18	100	5.5	30	3	17.8
19	70	4.0	30	3	14.52
20	70	7.0	30	3	15.04
21	70	5.5	20	3	19.78
22	70	5.5	40	3	29.67
23	70	5.5	30	1	15.21
24	70	5.5	30	5	17.77
25	70	5.5	30	3	29.55
26	70	5.5	30	3	33.00
27	70	5.5	30	3	32.1
28	70	5.5	30	3	32.45

The conditions for ethanol production from alkali pretreated OPEFB in this work were optimized by an expert-designed program through numerical optimization. At optimal conditions, the value of the independent variables was initial glucose content, 55 g/L, pH 6.25 at 35°C, and inoculum size of 1.68%. The ethanol yield (Yp/s), obtained at the optimum was 0.41 g/g. The observed and model-predicted values of ethanol fermentation are shown in Table 4 and the predicted values for ethanol fermentation (Fig. 2)

The regression equation and regression coefficients were generated from the experimental responses using an expert-designed program employing analysis of variance (ANOVA). The quadratic model used to represent the ethanol response is given in Eq. (2).

$$Y = 31.78+0.10A+0.37B+2.18C+0.64D-2.65AB- 3.08AC+3.64BC-3.55BD-2.42CD-2.61A^2-3.37B^2 \quad \ldots\ldots \quad (2)$$

Where Y is the ethanol yield (%) while A, B, C, and D are the initial glucose content (g/L), pH, temperature (°C), and inoculum size (%), respectively. The significance and effects of each variable on ethanol yield are presented in Table 3.

Table - 3 ANOVA of the quadratic unreduced model for ethanol yield

Source	Sum of squares	DF	Mean square	F value	P Value
Model	1373.76	14	98.13	6.34	0.0010*
A-Initial glucose	0.26	1	0.26	0.017	0.8992
B-pH	3.25	1	3.25	0.21	0.6545
C-Temperature	114.28	1	114.28	7.38	0.0176*
D-Inoculum size	9.77	1	9.77	0.63	0.4414
AB	111.99	1	111.99	7.23	0.0186*
AC	152.09	1	152.09	9.82	0.0079*
AD	30.94	1	30.94	2.00	0.1810
BC	212.21	1	212.21	13.70	0.0027*
BD	201.14	1	201.14	12.99	0.0032*
CD	93.75	1	93.75	6.05	0.0287*
A^2	163.03	1	163.03	10.53	0.0064*
B^2	272.99	1	272.99	17.63	0.0010*
C^2	18.85	1	18.85	1.22	0.2899
D^2	208.17	1	208.17	13.44	0.0028
Residual	201.35	13	15.49		
Lack of Fit	194.33	10	19.43	8.31	0.0539
Pure Error	7.01	3	2.34	6.34	0.0010
Cor. Total	1575.11	27	98.13	0.017	0.8992

Table 4 - Observed and predicted values of ethanol yield.

Run no.	Actual Value	Predicted Value	Residual	Run no.	Actual Value	Predicted Value	Residual
1	9.23	9.23	8.00E-04	15	30.67	29.84	0.83
2	25.92	23.68	2.24	16	19.75	15.81	3.94
3	18.65	15.06	3.59	17	17.89	21.14	-3.25
4	20.69	18.93	1.76	18	17.8	21.56	-3.76
5	17.45	17.32	0.13	19	14.52	17.55	-3.03
6	23.59	19.43	4.16	20	15.04	19.02	-3.98
7	40.76	37.72	3.04	21	19.78	23.87	-4.09
8	28.35	29.25	-0.90	22	29.67	32.59	-2.92
9	29.19	25.22	3.97	23	15.21	18.72	-3.51
10	35.00	34.10	0.90	24	17.77	21.27	-3.5
11	16.65	16.87	-0.22	25	29.55	31.78	-2.23
12	18.11	15.17	2.94	26	33	31.78	1.22
13	25.8	23.62	2.18	27	32.1	31.78	0.32
14	19.66	20.18	-0.52	28	32.45	31.78	0.67

The significance of the model is shown by an R^2 value of 0.8722 and an adjusted R^2 value of 0.7345. The *F* value was high at 6.34 corresponding to a *P*-value of 0.0010 which was mentioned that the model was highly significant which confirmed that the model was a good fit to the data.

Moreover, it showed that some parameters had a smaller *P*-value less than 0.5 that mean that parameter had the significance affected on ethanol fermentation. The results showed that the interaction terms of AB, AC, BC, BD, and CD and the quadratic terms of A^2, B^2, and D^2 were significant for the ethanol production based on *P*-values that lower than 0.5. *P*-value was also used to determine the significance of the regression coefficients, with smaller *P*-values indicating higher significance of the corresponding coefficient. The results showed that the interaction terms of AB, AC, BC, BD, and CD and the quadratic terms of A^2, B^2, and D^2 were significant for the ethanol production based on P values that lower than 0.5. *P*-value was also used to determine the significance of the regression coefficients, with smaller P values indicating higher significance of the corresponding coefficient [16].

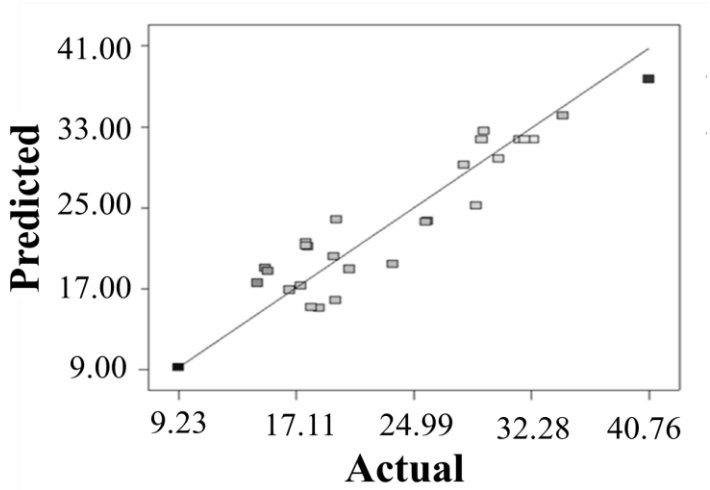

Fig. 2 - Experimental values and predicted values of ethanol yield for model equation (2)

The main parameter affecting the ethanol fermentation process in this work was the temperature which was agreed with the previous work [28] that temperature was the most important parameter with the largest *F* value of 161.275 which was higher than any for the other parameters. Likewise, Zhang et al. [27] found that temperature affected the fermentation process more than other parameters ($P < 0.0001$ with the highest F value of 137.72).

Many researchers have studied the ethanol yield from lignocellulosic materials. Singh and Bishnoi, [28] produced ethanol from microwave alkali pretreated rice straw using a statistical experimental design using *S. cerevisiae* and found that under optimum conditions of inoculum size of 3%, pH 5.75 at 30°C and urea concentration, 0.50 g/L the ethanol yield to consumed sugar was 0.50 g/g. The same researchers [27] also investigated the production of ethanol from pretreated wheat straw hydrolyzate using *S. cerevisiae*. Under optimum conditions (pH 5.5, the temperature of 30°C, inoculum level, 3.3% and TRS concentration, 6.5%) the ethanol production studied at bioreactor level produced an ethanol concentration of 16.4 g/L with an ethanol yield of 0.48 g/g obtained.

The study's output suggests that optimization by RSM can reduce the time needed to find the optimum conditions for the fermentation of ethanol because RSM is able to reduce the number of factorial experiments from 81 to 28. The application of RSM to investigate ethanol production does not just save on the cost of chemicals, raw materials and yeast, but can also make savings on energy consumption.

3.3.2 Response surface plot for ethanol yield

Graphical representations of the regression equations from Eq. (2) are shown in Fig. 3 and 4 (a)-(e). Three-dimensional surface plots and contour plots were drawn using RSM to predict the result. When two independent variables were fixed, the interaction effect of the other two independent variables on the dependent variable can be seen.

Fig. 3(a) and 4(a) showed the plot obtained by varying the initial glucose content and pH at the centre point of the temperature and inoculum level. The ethanol yield increases with increasing pH up to 5.5 but the ethanol yield decreases with further increases in pH from 5.5 to 7.0, which agrees with the finding of Lee et al. [32] that ethanol production reduces gradually when the pH value falls below 4.0 or increases above 5.0. A high pH level may cause a lower ethanol yield because of lower ATP production during the metabolic changes in *S. cerevisiae* [27, 33]. The cell proliferation and viability of *S. cerevisiae* were inhibited at low pH levels due to the increased proton gradient across the plasma membrane, resulting in an increase in the passive proton uptake rate. Further, a neutral intracellular pH was crucial for cell viability. When the pH decreased, the cell replication activity also decreased. The optimal external pH range for the growth of *S. cerevisiae* was 5.0–5.5 [34]. Fig. 3(b) and 4(b) presented the interaction between initial glucose content and temperature that pH and inoculum size maintained at the centre point. The interaction between initial glucose content and temperature was significant with a *P*-value of 0.0079. The temperature was a crucial factor for cell viability and the optimum temperature for the growth of *S. cerevisiae* has been found to be 30-35°C [20] or 28–35°C [14, 27]. This study found that increasing the temperature from 20 to 40°C at a lower initial glucose content caused the ethanol yield to increase. However, at higher initial glucose content, the ethanol yield gradually decreased when the temperature increased above 35°C. This finding was consistent with that of Singh and Bishnoi, [27] who found that temperatures above 35°C inhibited the growth of yeast. Fig. 3(c) and 4(c) presented the effect of temperature and pH on the ethanol yield. The ethanol yield increased with increasing pH and temperature across a certain range. The finding was similar to the result achieved by Zhang et al. [19]. Fig. 3(d) and 4(d) depicted the interaction effect of pH and the inoculum level on the ethanol yield. At an initial glucose content of 70 g/L and a temperature of 30°C (center point), when the pH was 4, 5.5, and 7, the ethanol yield increased with an increasing inoculum size up to 3% and then gradually decreased with further increases in the inoculum level to 5%. Fig. 3(e) and 4(e) displayed the

interaction effect of the inoculum size and the temperature on ethanol yield. The ethanol yield gradually increased with increasing temperature when the inoculum level was held at a low level of 1%. However, the ethanol yield was constant then gradually decreased with increasing temperature when the inoculum level was held at a high level of 5%. There was only a small difference in the ethanol yield for any changes in temperature and inoculum size which was consistent with the findings of Singh and Bishnoi, [27]. Moreover, Zhang et al. [19] reported, using low inoculum size and with high glucose content would have an inhibitory effect on yeast activity, which would consequently reduce the ethanol yield.

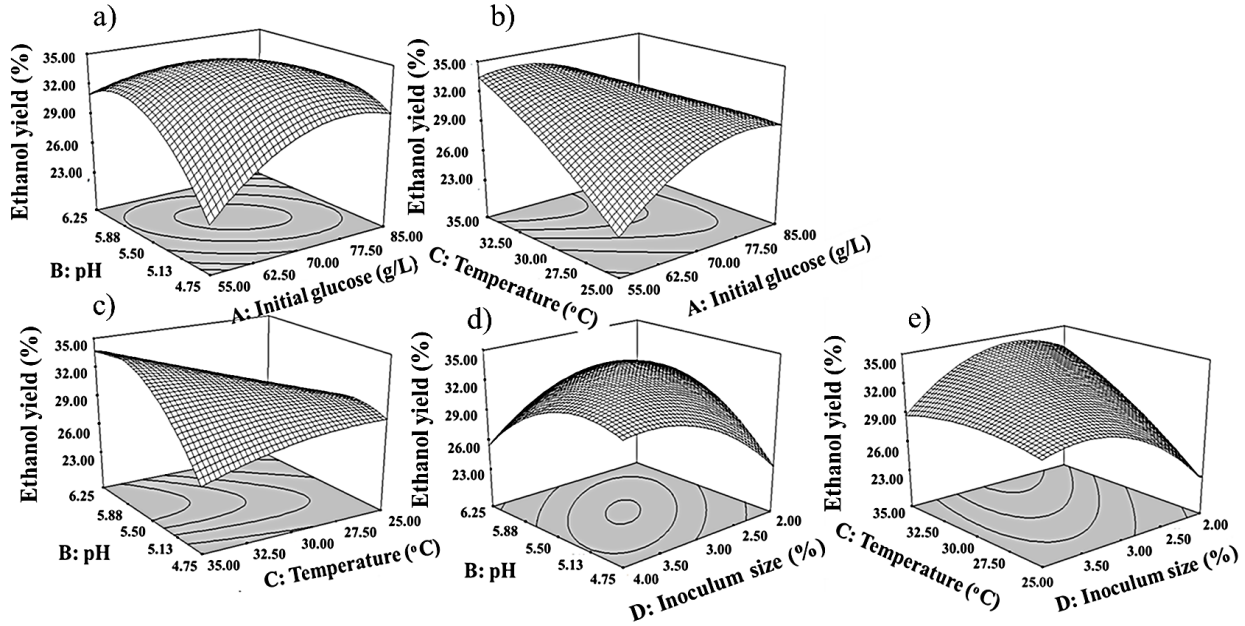

Fig. 3 - 3D response surface plots for ethanol production showing the interaction between (a) initial glucose and pH; (b) initial glucose and temperature; (c) pH and temperature; (d) pH and inoculum size; (e) temperature and inoculum size

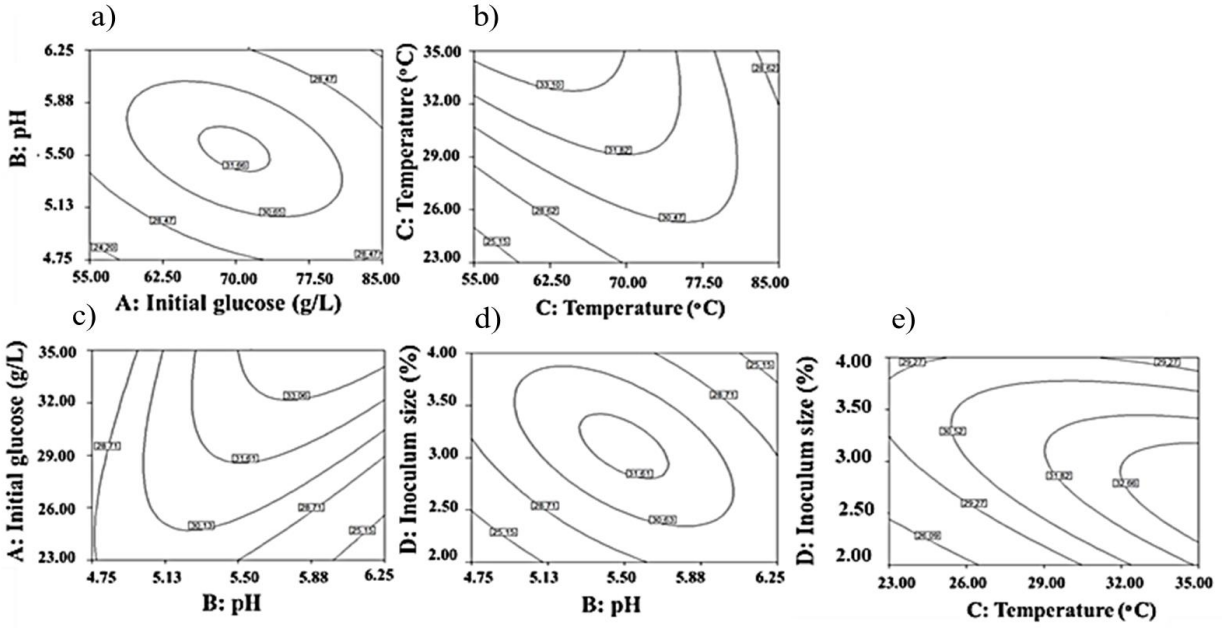

Fig. 4 - Contour plots for ethanol production showing the interaction between (a) initial glucose and pH; (b) initial glucose and temperature; (c) pH and temperature; (d) pH and inoculum size; (e) temperature and inoculum size

4. Summary

This study used RSM in a CCD type experimental design, using expert-designed software employing numerical optimization; to investigate the optimal conditions for ethanol production from resulting glucose of acid hydrolysis

alkali pretreated OPEFB. The OPEFB was shredded into the particle size of 10-0 mm then pretreatment with sodium hydroxide. OPEFB was alkali-pretreatment with 15% (w/v) NaOH at 121˚C for 45 min and provided of 67.2% cellulose, 22.8% hemicellulose and 7.2% lignin. The cellulose content in the alkali-pretreated OPEFB had increased 39.4% from untreated OPEFB, while the hemicellulose and lignin had decreased to 33.1% and 36.7% respectively. Glucose, the raw material for ethanol fermentation, was obtained by acid hydrolysis of the alkali pretreated OPEFB; alkali-pretreated OPEFB was hydrolyzed with 7% (v/v) H_2SO_4 at 140°C for 90 min. The highest glucose yield of 92.38% was obtained representing 55.54 g/L. Lastly, ethanol by *S. cerevisiae*. The optimum values of the four variables selected for testing were initial glucose content, 55 g/L, pH, 6.25, fermentation temperature, 35°C, and inoculum size 2%. The optimum ethanol yield was 0.41 g/g which was over 80% of the theoretical ethanol yield produced from glucose fermentation, which was 28.05 g/L (0.51 g/g glucose). Moreover, RSM-CCD presents an alternative experimental design in design of experiment and help to reduce number of experiment as compared to a full factorial design.

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