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# Optimization of Bioethanol Production from Empty Fruit Bunches by Co-culture of *Saccharomyces cerevisae* and *Aspergillus niger* using Statistical Experimental Design

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**Production of bioethanol from oil palm empty fruit bunches (EFB) by *Saccharomyces cerevisae* and *Aspergillus niger* is among the ways of reducing environmental pollution and consumption of crude oil. This study used sequential optimization approach based on statistical experimental design including Plackett-Burman (PB) design, one-factor-at-a-time (OFAT) and face-centered central composite design (FCCCD). Among the parameters tested, pH, temperature, inoculum size, potassium dihydrogen phosphate ($KH_2PO_4$), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) and peptone showed positive effects while yeast extract, malt extract, potassium chloride, urea and agitation were influencing the production negatively. The three parameters chosen for determination of the optimum values by response surface methodology (RSM) based on the FCCCD were pH, $KH_2PO_4$ and agitation. Although agitation showed negative effects it was considered in FCCCD due to the mixing effect of fermnetation. The validity of the model was verified and the optimum value of pH 5.5, agitation of 150rpm and 0.3% of $KH_2PO_4$ led to a maximum bioethanol production of 7.4 g/l. The yield of bioethanol was determined based on the reducing sugar (16.85 g/l) obtained from the EFB.**

**Key words:** Bioethanol production, Optimization, Face-Centered Central Composite Design, Empty fruit bunches, *Saccharomyces cerevisae*, *Aspergillus niger*.

Alternative sources of energy such as bioethanol have attracted worldwide interest due to depletion of the world's energy supply[1]. Bioethanol has been used as a modern biofuel which is applied directly as a gasoline improver (gasoline substituent) in the form of ETBE (ethyl-tertiary butyl ether) for currently added synthetically-produced octane enhancers to reduce the emissions of exhaust gasses[2]. The reduction of carbon dioxide emission make bioethanol safe for the environment and its use as a fuel can reduce utilization of petroleum and greenhouse gas emission. Bioethanol is different from fossil fuel because it is a renewable fuel produced through fermentation of sugars[3]. Current production of bioethanol using food crops such as corn and sugarcane has resulted in competition with food supply since the biggest issue facing humankind today is a growing demand in food which directly correlated with the population increase[4]. This problem has resulted in a search to find a cheap and more abundant material to replace the use of food crops as starting materials for bioethanol production[5].

The lignocellulosic biomass includes wood chips, agricultural residues, paper wastes,

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other fibrous plant material etc., could serve as an ideal, inexpensive, abundant and non-food sources for an alternative to bioethanol production[6]. Lignocellulosic materials consist of lignin, cellulose and hemicelluloses. Cellulose and hemicelluloses are the main components of lignocellulosic which can easily be converted to sugar and further used for ethanol production[7]. As Malaysia is the main exporter of palm oil in the world, the utilization of lignocellulosic materials such as empty fruit bunches can be used as substrate for bioethanol production[8]. Large quantities of EFB are available during processing of fresh fruit bunches (FFB) since Malaysia has approximately 362 palm oil mills, processing about 82 million tonnes of FFB with annual estimated production of 33 million tonnes of crop residues in form of EFB, fibers and shells[3]. Fermentation of hydrolysis product which is reducing sugar to bioethanol involves microorganisms that use the sugars for food to produce ethanol and other by-products[1]. The most commonly used microbe has been *Saccharomyces cerevisae* (*S.cerevisae*)[9-11]. *S. cerevisae* has several advantages for ethanol production such as low pHand oxygen requirement, and high tolerance to ethanol and inhibitors[12]. This yeast can grow on simple sugars, such as glucose and also generally recognized as safe (GRAS) to be used as a food additive for human consumption[1]. Besides *S.cerevisae,* fungi like *Aspergillus* sp., *Nuerospora crassa, Trichoderma viride* and *Monilia*sp. have also been used for bioethanol production[1]. A study conducted showed that ethanol yields increase several fold in co-culture of *A. niger* and *S. cerevisae* due to the synergistic metabolic interactions between the species and these results indicate that fermentation to ethanol can be conducted efficiently by co-culture of *A.niger* and *S.cerevisae*[13].

Considering the fact of cost of fermentation medium and process conditions that play greater role in ensuring the suitable environmental for growth of microorganisms, the present investigation was aimed at evaluating the effects of medium components and process conditions on bioethanol production. In this study, factors that affect the bioethanol production were screened by Plackett-Burman design while one-factor-at-a-time approach used to obtain the possible optimum levels of the factors. The interactions between the factors and response were determined through optimization process by response surface methodology. As there are limited reports on the fermentation of EFB to bioethanol, this research is conducted to develop a sustainable technology by using oil palm empty fruit bunches (EFB) as renewable raw materials as well the utilization of EFB can help solve the disposal problem and minimize the environmental threat.

## MATERIALS AND METHODS

### Chemicals

All chemicals used were of analytical grade and commercially available in Malaysia.

### Microorganism and preparation of inoculums

*Saccharomyces cerevisae* and *Aspergillus niger* F44 was obtained from laboratory stock of Bioenvironmental Engineering Lab, IIUM. The *S.cerevisae* strains were maintained on 3.9% of potato dextrose agar (PDA) plates at 32°C for 2 days while *A.niger* strain for 5 days and subcultured every three weeks. It was then maintained and preserved at 4°C until further used.

Inoculum preparation for *S.cerevisae* was done in the laminar flow to ensure no contamination. Yeast malt (YM) media was used as nutrient broth for the microorganisms, which contained 3g of yeast extract, 3g of malt extract and 10g glucose followed by addition of 1 litre distilled water. The mixture was then heated on hot plate to mix thoroughly by using magnetic stirrer. The broth was then autoclaved at 121°C for 15 minutes and cooled at room temperature. A wire loop was sterilized under the flame of bunsen burner and cooled to room temperature where one loop full of cell from PDA plate was immersed in the broth in order to prepare yeasts inoculum. The mixture was incubated at 30°C and agitation of 150 rpm for one day. The inoculum was stored at 4°C chiller for only 14 days shelf life. The inoculum size was set to have an initial concentration of $3\times10^6$ cells per ml.

Preparation of inoculums for *A.niger* was by allowing the culture to be grown on PDA plate at temperature 32°C for 5 days. Spore suspension inoculum was prepared by washing the fungal culture grown on PDA plate. To prepare inoculum, all flasks, funnels, filter papers, distilled water was sterilized to avoid contamination. Each PDA plate

culture after full growth, was gently scrapped with sterilized distilled water using sterilized glass rod. The suspended fungal spores were then filtered using Whatman number 1 filter paper into an Erlenmeyer flask that contain yeast malt media as prepared for *S.cerevisae*. The inoculum size was set to have an initial concentration of $2.45x10^6$ spores/ml.

**Fermentation medium preparation and bioethanol production**

Fermentation medium was prepared using reducing sugar obtained from hydrolysis process of previous report which contained about 16.85g/l reducing sugar[14]. Others medium constituents and process conditions were based on statistical experimental design. Incubations were carried out in 150ml Erlenmeyer flasks according to the design matrix. The flasks were incubated for 3 days under orbital shaking.

**Analytical method**

Ethanol concentration was measured by Gas Chromatography Mass Spectrometer (GCMS) where 1µl of the derivatized sample was injected using a splitless mode by an Agilent 7890 A (Agilent Technology) coupled with MSD quadrupole detector 5975 C and the autosampler was equipped with a 30m x 0.25mm i.d. fused silica capillary column with a chemically bonded 0.50µm HP 5-MS Ultra Inert. The injector temperature and the purge flow-rate were 270°C and 20ml $min^{-1}$ respectively; turning on the purge after 60 s. The gas flow rate through the column was 1ml min{ [1], The gas flow rate through the column was 1 ml $min^{-1}$, the column temperature was held at 70 °C for 2 minutes, then increased by 40 °C $min^{-1}$ to 320 °C, and held there for 1 min. The column effluent was introduced into MSD quadrupole detector 5975 C mass spectrometer, where the transfer line and the ion source temperatures were 250°C and 200°C, respectively.

**Medium optimization of bioethanol production by statistical approach**

**Selection of important media components and process conditions by Plackett- Burman design**

Based on Plackett-Burman design, each variable was examined at two levels: low level (-1) and high level (+1). In this study, Design Expert 6.0 (Start Ease Inc., Minneapolis,MN) was used to generate a set of 12 experimental designs. Table 1 shows the medium components and process conditions as well as levels of each variable used in the experimental design, whereas Table 2 represents the design matrix. The Plackett-Burman design was based on linear equation model (1):

$$Y = B_0 + \Sigma B_1 X_1 \quad ...(1)$$

where Y is the response (bioethanol production), $\beta_0$ is the model intercept, $\beta_1$ is the linear coefficient, and $X_1$ is the level of the independent variable. However, this model does not describe the interaction among variables and only used to screen and evaluate the important variables that influence the response. The parameters for media components selected for the experiment were yeast extract, malt extract, peptone and urea as nitrogen source, $KH_2PO_4$ and KCl as K/P source, $MgSO_4.7H_2O$ as Mg source respectively. Other process parameters were incubation temperature, pH, agitation rate and inoculum size.

**Experimental design of "one-factor-at-a-time" method**

Following Plackett-Burman design, two variables namely agitation and pH were selected for one-factor-at-a-time (OFAT) approach to evaluate the possible optimum levels of the process conditions. In this study, the concentration of $MgSO_4.7H_2O$, peptone, temperature, inoculum size and $KH_2PO_4$ were fixed at 0.1%, 0.1%, 30° C, 4% and 0.2%, respectively. The variables investigated include agitation where it was varied from 25 to 100 rpm and the pH was varied from 4 to 10.

**Response surface methodology (RSM)**

RSM was utilized to optimize the fermentation process and face-centered central composite design (FCCCD) under the RSM was adopted in order to fit a second order model. RSM involves three important steps; performing the statistically designed experiments, estimating the coefficients in mathematical model, and predicting the response and checking the adequacy of the model. FCCCD was used to optimize three factors namely pH, agitation and $KH_2PO_4$, to find a set of 20 experimental runs with six replicated center points. The independent variables were studied at three different levels, low (-1), medium (0) and high (+1). The experimental design used for the study is shown in Table 2. The remaining factors

$MgSO_4.7H_2O$, peptone, temperature, time and inoculum size were fixed at 0.1%, 0.1%, 30°C, 72 hours and 4% respectively. Experiments were conducted in 150 ml Erlenmeyer flasks and the relationship between dependent and independent variable is explained by the following second order polynomial equation (2):

$$Y=B_0+B_1X_1+B_2X_2+B_3X_3+B_{12}X_1X_2+B_{13}X_1X_3+B_{23}X_2X_3+B_{11}X_1^2B_{22}X_2^2+B_{33}X_3^2 \quad ...(2)$$

where Y is the dependent variable (bioethanol production); $X_1$, $X_2$ and $X_3$ are independent variables (agitation, pH and $KH_2PO_4$); $\beta_0$ is an intercept term; $\beta_1$, $\beta_2$ and $\beta_3$ are linear coefficients; $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ are the interaction coefficients; and $\beta_{11}$, $\beta_{22}$ and $\beta_{33}$ are the quadratic coefficients.

The developed regression model was evaluated by analyzing the values of regression coefficients, ANOVA (analysis of variance), *p*-values and F-values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, $R^2$. The fitted polynomial equation was then expressed in the form of contour and surface plots in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

**Validation of the experimental model**

The statistical model was validated with respect to all the three variables. Three experiments were conducted to determine the bioethanol production and the results were compared with the predicted values.

## RESULTS AND DISCUSSION

**Evaluation of the Media Constituents and Process Conditions for Bioethanol Production Using the PlackettBurman Design**

Plackett-Burman (PB) design has been employed to evaluate the significant effect of the media constituents and process conditions on the production of bioethanol using EFB as a substrate as shown in Table 1. The main effect of each constituent on the cellulase production was calculated as the difference between the average measurement calculated at the higher (+) and lower (-) levels of the constituent.The results in Fig.1 showed that $KH_2PO_4$, $MgSO_4.7H_20$, peptone, temperature, inoculum size and pH have the positive effects on the bioethanol production. On the other hand yeast extract, malt extract, urea, agitation and KCl have the negative effects on the response.

The consistency of the influences by the parameters on each media constituent and process conditions would be helpful to decide which parameters should be evaluated in the next phase of study. Yeast extract, malt extract, urea, and KCl are excluded due to their negative effects on bioethanol production. However, agitation was considered for further studies because agitation could be beneficial to the growth and performance

**Table 1.** Plackett-Burman experimental design for evaluation of 11 components and the design response

| Run | A % | B % | C % | D % | E % | F % | G % | H. p C | J % | K | L rpm | BE (g/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 0.1 | 0 | 0.2 | 0.1 | 0 | 0.2 | 30 | 3 | 4 | 150 | 6.0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 3 | 4 | 50 | 10.0 |
| 3 | 1 | 0.1 | 0 | 0.2 | 0 | 0 | 0 | 37 | 5 | 6 | 50 | 12.0 |
| 4 | 1 | 0 | 0.6 | 0.2 | 0 | 0.1 | 0 | 30 | 3 | 6 | 150 | 4.0 |
| 5 | 1 | 0 | 0 | 0 | 0.1 | 0.1 | 0.2 | 30 | 5 | 6 | 50 | 12.0 |
| 6 | 1 | 0.1 | 0.6 | 0 | 0.1 | 0.1 | 0 | 37 | 3 | 4 | 50 | 3.0 |
| 7 | 0 | 0 | 0.6 | 0.2 | 0.1 | 0 | 0.2 | 37 | 3 | 6 | 50 | 9.0 |
| 8 | 0 | 0 | 0 | 0.2 | 0.1 | 0.1 | 0 | 37 | 5 | 4 | 150 | 13.0 |
| 9 | 0 | 0.1 | 0.6 | 0.2 | 0 | 0.1 | 0.2 | 30 | 5 | 4 | 50 | 3.0 |
| 10 | 1 | 0 | 0.6 | 0 | 0 | 0 | 0.2 | 37 | 5 | 4 | 150 | 2.0 |
| 11 | 0 | 0.1 | 0 | 0 | 0 | 0.1 | 0.2 | 37 | 3 | 6 | 150 | 9.0 |
| 12 | 0 | 0.1 | 0.6 | 0 | 0.1 | 0 | 0 | 30 | 5 | 6 | 150 | 3.0 |

[A, yeast extract; B, malt extract; C, urea; D, $KH_2PO_4$; E, $MgSO_4.7H_20$; F, Peptone; G, KCl; H, Temperature; J, Inoculum size; K, pH; L, agitation, BE; bioethanol]

of the microbial cells by improving the mass transfer characteristics with respect to substrates, products/by-products and oxygen.A small concentration of oxygen must be provided to the fermenting yeast, as it is a necessary component in the biosynthesis of several bioproducts[13].

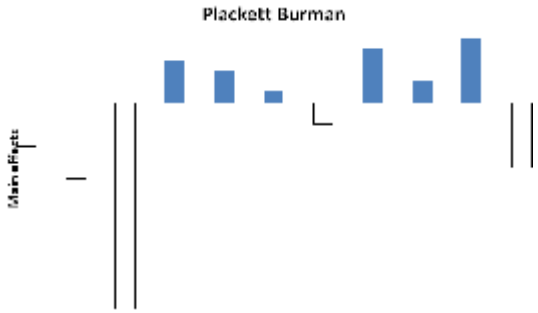



**Fig. 1.** Main Effects of the medium and process parameters on bioethanol production based on Plackett-Burman experimental results. [A, yeast extract; B, malt extract; C, urea; D, $KH_2PO_4$; E, $MgSO_4.7H_20$; F, Peptone; G, KCl; H, Temperature; J, Inoculum size; K, pH; L, agitation]

Urea a nitrogen source, might have negative effects on bioethanol production because it can react with ethanol yielding ethyl carbamate (urethane) as a product, resulting in lower ethanol concentration[15]. Furthermore, addition of yeast extract failed to enhanced the ethanol productivity from sugarcane juice[16]. This result is similar with the present study, where yeast extract and urea as nitrogen source have negative effect on bioethanol production. However, based on other literature, yeast extract has been considered an important nutritional source for ethanol production because it contains mixture of amino acid, vitamins and magnesium[17]. Yeast extract has protective effects on growth, viability and fermentation, which stimulates the fermentation rate and ethanol production. The only nitrogen source that shows positive effect in this study is peptone. This indicates peptone alone is sufficient enough to provide the nitrogen requirement and may aid in reducing the overall cost.

The other factor that shows positive main effect was temperature. In this study, two range of temperatures was employed which is 30°C and 37°C. However, further experiment for this study was conducted at temperature of 30°C due to several literature reports that 30°C is the most favourable temperature for bioethanol production[18-19]. Though, there are also several literatures where 35p C was used as the optimum temperature for bioethanol production[20,21]. Limtonget al[22] indicated that the concentrations of ethanol were almost the same at 30°C and 37°C and increase in the temperature from 40 to 45°C resulted in decreased ethanol concentration.

As for inoculum size, it was further decided to be 4% in between the range selected in PlackettBurman design. The selection of 4% is similar to what was used by Kabbashiet al[22]. on bioethanol production from oil palm empty fruit bunches by solid state bioconversion. Finally, two factors namely pH and agitation were selected for further optimization by OFAT to investigate the possible level of each factor for higher bioethanol production by statistical optimization. Besides that, temperature (30°C), inoculum size (4%), $MgSO_4.7H_20$ (0.1%), peptone (0.1%) and $KH_2PO_4$, which showed positive effects were also considered in this study.

**Determination of possible level for selected process conditions: one-factor-at-a-time (OFAT)**

Seven media constituents and four process conditions were screened by PB design to determine the effective range of parameters for bioethanol production, in which two factors, agitation and pH were further sorted by one-factor-at-a-time (OFAT). Bioethanol production was influenced by different pH level. Fig.2 shows high bioethanol production of 7.0 g/l at pH 4 to 6, however it decrease to 3.0 g/l when pH is 8 and 10. This result might be due to differences in pH optimum by each microorganism. Common yeast such as *Saccharomyces cerevisae* can survive at a pH range of 4.5-6, whereas fungi such as *Aspergillusniger* can survive at a pH range 5 to 7.

Neelakandan and Usharani (2009), studied the effect of pH on bioethanol production using immobilized yeast cell by *S.cerevisae*and found that ethanol yield increased significantly from 4 to 6 and the maximum ethanol yield of 6.91% was obtained at pH 6[24]. The inhibitory effect of pH (at the high level) on the ethanol yield could be due to the lower ATP production during the metabolic changes in *S.cerevisae*. During the investigation for ethanol production from various waste resources viz., bread residue, citrus peel kitchen

garbage, *S.cerevisae* was found to grow well within the range of pH 4[11].

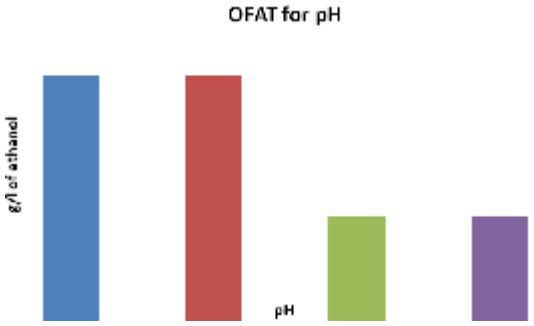



**Fig. 2.** Effect of different pH (4-10) 0n bioethanol production by *Saccharomyces cerevisae* and *Aspergillus niger*

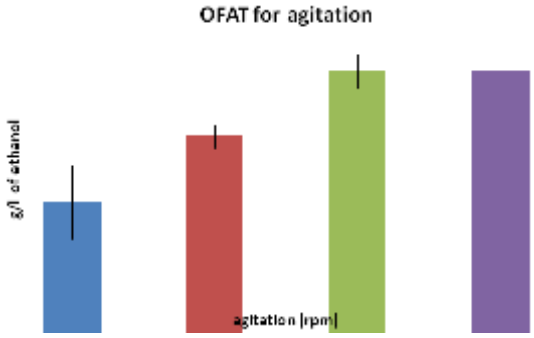



**Fig. 3.** Effect of different agitation (25-100 rpm) on bioethanol production by *Saccharomyces cerevisae* and *Aspergillus niger*

Fig.3 shows the effect of agitation on bioethanol production. High concentration of bioethanol of 4 g/l was obtained at 75 and 100 rpm. However agitation rates below 75 rpm showed decreasing trends in the amount of bioethanol. This result is in contrast with the result obtained by Rodmui *et al.*,[25] which obtained high ethanol productivity at 50 rpm. Mohd Asyraf *et al.*,[19] studied the effect of rate of agitation and found the maximum ethanol concentration 10.35 g/l was obtained at agitation rate of 150 rpm followed by 10.29 g/l at agitation rate of 100 rpm. Therefore, further optimization by FCCCD was needed to determine the optimum value of agitation for high bioethanol production.

**Optimization of Media Constituents and Process Conditions by Face Central Composite Design (FCCCD) under the Response Surface Methodology**

An experimental design, faced centered central composite design (FCCCD), was employed to optimize the three independent variables, agitation, pH and $KH_2PO_4$. The results of the experiment are shown in Table 2. The highest concentration of bioethanol obtained from FCCCD was 8.3 g/l which is observed in run 16 with agitation of 150 rpm, pH 6 and 0.35% of $KH_2PO_4$. A polynomial regression equation was developed under response surface methodology (RSM) to analyze the factor interaction by identifying the significant factors contributing to the regression model and to determine the optimal values of the most significant independent variables.

The effects of three independent variables on bioethanol production were predicted by the following polynomial regression equation:

$$Y\,(\text{bioethanol, g/l}) = +7.54 + 0.12\,A - 0.89\,B - 0.17\,C - 1.35\,A^2 - 2.00\,B^2 - 0.5\,C^2 + 0.012\,AB - 0.46\,AC + 0.11\,BC \quad ...(3)$$

where the bioethanol production (Y) is a function of agitation (A), pH (B) and $KH_2PO_4$ (C).

Analysis of variance (ANOVA) of the response surface, quadratic polynomial model is shown in Table 3. It is evident from the results that the model is significant ($p$<0.0001) and the 'Lack of Fit' of the model is not significant (0.6992). In this case B (pH), $A^2$, $B^2$, AC are found to be significant model terms, while agitation (A) and $KH_2PO_4$ (C) are not significant. The coefficient of determination ($R^2$) is 0.9508 which ensures a satisfactory data and indicated that approximately 95.08% of the variability in the dependent variable (bioethanol production) could be explained by the model. The "Predicted R-Squared" of 0.8252 is in reasonable agreement with the "Adjusted R-Squared" of 0.9065. These values indicated that the correlation between the experimental and the predicted values has high degree of correlation. 'Adequate Precision' measures the signal to noise ratio and it should be greater than 4. In this model the ratio of 12.70 indicates the adequacy of signal and that interpret the fitness of the model as well.

The three dimensional (3D) response surfaces are presented in Fig.4. These plots are the graphical representation of the regression equation used to determine the optimum values of the variables within the considered ranges[26]. An elliptical response surface in the entire region was found from the second order quadratic equation for bioethanol production with interaction of pH and $KH_2PO_4$ (Fig.4a), agitation and pH (Fig.4b) and

**Table 2.** Experimental and predicted values of bieothanol production by experimental design using FCCCD

| Run | Agitation (rpm) | pH | $KH_2PO_4$ (%) | Bioethanol (g/l) | |
|---|---|---|---|---|---|
| | | | | Experimental | Predicted |
| 1 | 100 | 4 | 0.2 | 4.1 | 4.3 |
| 2 | 200 | 4 | 0.2 | 5.4 | 5.44 |
| 3 | 100 | 8 | 0.2 | 2.4 | 2.27 |
| 4 | 200 | 8 | 0.2 | 3.7 | 3.46 |
| 5 | 100 | 4 | 0.5 | 4.5 | 4.66 |
| 6 | 200 | 4 | 0.5 | 3.9 | 3.95 |
| 7 | 100 | 8 | 0.5 | 3.2 | 3.08 |
| 8 | 200 | 8 | 0.5 | 2.7 | 2.42 |
| 9 | 100 | 6 | 0.35 | 6.2 | 6.07 |
| 10 | 200 | 6 | 0.35 | 5.9 | 6.31 |
| 11 | 150 | 4 | 0.35 | 6.9 | 6.43 |
| 12 | 150 | 8 | 0.35 | 3.9 | 4.65 |
| 13 | 150 | 6 | 0.2 | 7.1 | 7.21 |
| 14 | 150 | 6 | 0.5 | 6.7 | 6.87 |
| 15 | 150 | 6 | 0.35 | 8.1 | 7.54 |
| 16 | 150 | 6 | 0.35 | 8.3 | 7.54 |
| 17 | 150 | 6 | 0.35 | 7.7 | 7.54 |
| 18 | 150 | 6 | 0.35 | 7.9 | 7.54 |
| 19 | 150 | 6 | 0.35 | 7.3 | 7.54 |
| 20 | 150 | 6 | 0.35 | 6.5 | 7.54 |

**Table 3.** Analysis of variance (ANOVA) for the polynomial model

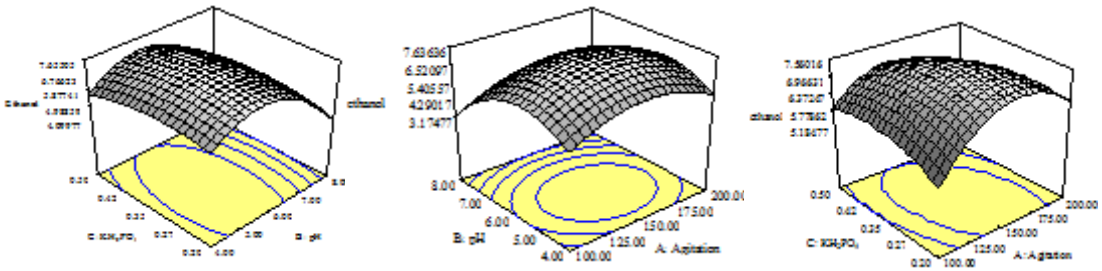
| Source | Sum ofSquares | DF | MeanSquare | FValue | Prob> F | |
|---|---|---|---|---|---|---|
| Model | 66.39593 | 9 | 7.377326 | 21.47025 | < 0.0001 | Significant |
| A | 0.144 | 1 | 0.144 | 0.419084 | 0.5320 | |
| B | 7.921 | 1 | 7.921 | 23.05251 | 0.0007* | |
| C | 0.289 | 1 | 0.289 | 0.841078 | 0.3807 | |
| A2 | 4.978182 | 1 | 4.978182 | 14.48802 | 0.0034* | |
| B2 | 10.95006 | 1 | 10.95006 | 31.86798 | 0.0002* | |
| C2 | 0.675057 | 1 | 0.675057 | 1.96462 | 0.1913 | |
| AB | 0.00125 | 1 | 0.00125 | 0.003638 | 0.9531 | |
| AC | 1.71125 | 1 | 1.71125 | 4.980256 | 0.0497** | |
| BC | 0.10125 | 1 | 0.10125 | 0.294668 | 0.5991 | |
| Residual | 3.436068 | 10 | 0.343607 | | | |
| Lack of Fit | 1.302735 | 5 | 0.260547 | 0.610657 | 0.6992 | not significant |

$R^2$=0.9508, adjusted $R^2$=0.9065

**Table 4.** Validation of the experimental model

| Run | Agitation (rpm) | pH | $KH_2PO_4$ (%) | Bioethanol production (g/l) | |
|---|---|---|---|---|---|
| | | | | Predicted | Experimental |
| 1 | 150 | 5.5 | 0.31 | 7.6 | 7.4 |
| 2 | 125 | 5 | 0.25 | 6.87 | 6.3 |
| 3 | 100 | 6 | 0.25 | 5.66 | 5.2 |

agitation and $KH_2PO_4$ (Fig.4c). Plots show that bioethanol production is considerably affected by varying the pH, agitation and $KH_2PO_4$. The maximum production was obtained at the point of intersection of major and minor axes of the ellipse. The production decreased at the maximum and minimum value of bioethanol of 7.44 g/l was predicted from the response surface at pH of about 5.5 and agitation of 150 rpm (Fig.4b). From the figure it can be concluded that further increase in the pH and agitation resulted in lower amount of bioethanol. Lower ethanol productivity at high pH may be due to the formation of undesired product such as organic acid during the fermentation process. As for agitation, Mohd Asyraf *et al.* (2011) stated that ethanol production yield was higher without or lower agitation rate.




(a) (b) (c)

**Fig. 4.** The 3D response surface curves; (a) pH and $KH_2PO_4$ at fixed amount of agitation (b) pH and agitation at fixed amount of $KH_2PO_4$and (c) agitation and $KH_2PO_4$ at fixed amount of pH

Fig.4c shows the predicted bioethanol production of 7.38 g/l based on the interaction of agitation of about 155 rpm and $KH_2PO_4$ of about 0.32 % at fixed pH of 6. Similarly, the interaction of pH (5.8) and $KH_2PO_4$ (0.26%) gave the maximum bioethanol production of 7.54 g/l (Fig.4a). In order to verify the optimizations results and to validate the developed second order quadratic model, a set of experiments were performed according to the process conditions presented in Table 4. The validation experiment shows a quite similarity between the predicted and the experimental results and the highest bioethanol production of 7.4 g/l was obtained at the optimum conditions (pH 5.5, agitation 150 rpm and 0.3% $KH_2PO_4$), which is slightly less than the predicted value.

The literature showed that highest production of bioethanol obtained was 120.68±0.54 g/l when sweet sorghum juice containing total sugar of 280 g/l, 3 g /l yeast extract and 5g/l peptone was used[15]. Moreover Gupta et al[27]studied fermentation of acid and enzymatic hydrolysate in the presence of 18.24 g/l and 37.47g/l sugars with *Pichiastipitis* and *Saccharomyces cerevisae* which produced 7.13 g/l and 18.52 g/l ethanol respectively. Bioconversion of EFB by solid state bioconversion gave 14.1% bioethanol with optimum condition of 60% moisture, pH of 7, inoculum size of 4% and co-substrate concentration of 2%[23]. Mohd Asyraf *et al.*,[19] studied the effect of pH, agitation and temperature on bioethanol production from EFB. The bioethanol obtained ranges from 9.55 to 10.32 g/L which can be achieved at pH 4, temperature of 30°C and agitation rate of 100 rpm to 150 rpm for 72 hrs of incubation. In another study, the maximum ethanol concentration of 24.17 g/l was obtained at the optimum conditions of temperature (38°C), pH 5.45 and reducing sugar concentration of 75 g/l[11]. The optimum conditions for maximum bioethanol production (7.62%) from cashew apple juice using immobilized yeast cells (*S.cerevisae*) were at temperature of 32.5°C, pH 6.0, substrate concentration 10% and inoculum level of 8%[24].

As far as we are concerned, there are no reports for enhancing ethanol productivity from EFB through analyzing the influence of the added mineral elements on the ethanol productivity. Yu et al[28]studied the effect of culture medium on ethanol productivity from stalk juice of sweet sorghum and found out based on the developed regression equation that the maximum ethanol productivity of 119.12 g/l h was achievable using the optimized medium consisting of 0.77 g/l phosphorus ($KH_2PO_4$), 2.15 g/l nitrogen, and pH of 6.39. A study on different nitrogen sources was

evaluated and the best ethanol concentration (15.1 g/l) was achieved when urea was used as a single nitrogen source[29]. Limtong et al[22] studied the effects of potassium source ($KH_2PO_4$) at 0-0.1% added to a sugar cane juice medium and found the highest ethanol concentration of 7.65% (w/v) which was attained by fermentation with 0.05% $KH_2PO_4$. In the present study, the concentration of bioethanol obtained was low compared to other studies due to the low amount of reducing sugar (16.85 g/l) present during the fermentation process. The optimization conditions of 150 rpm, pH of 5.5 and 0.3 % of $KH_2PO_4$ only produced 7.4 g/l of bioethanol with productivity of 0.103 g/lh after 72 hours. The ethanol yield for this experiment was 0.43 g ethanol $g^{-1}$ total sugar while the theoretical yield is 0.51 g/g. The amount of reducing sugar concentration present during fermentation is directly related to the product formation, and influences the yield and productivity values. This is because reducing sugar is part of carbon source used by the microorganisms for the cell growth and the remaining reducing sugar would then be used for product formation. Besides that, not all sugars in media were utilized by yeasts and some of the sugars might be used for maintenance and converted to other by-products. It has been demonstrated in other bioconversion studies that the yield and productivity of the process are improved when the initial sugar concentration is increased, of course, up to a certain limit.

## CONCLUSION

The developed optimization conditions used in the fermentation process were agitation of 150 rpm, $KH_2PO_4$ of 0.3 % (w/v) and pH of 5.5 which resulted in 7.4 g/l concentration of bioethanol with productivity of 0.103 g/L.h and ethanol yield of 0.43 g ethanol $g^{-1}$ total sugar. Finally, it can be concluded that empty fruit bunches from oil palm industry could be an alternative resource for production of bioethanol through hydrolysed sugar obtained by locally produced palm oil mill effluent (POME) based cellulase. This study might contribute to the economic development of Malaysia by producing bioethanol which is commercially valuable and at the same time by minimizing the cost of oil palm solid waste management.

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