

Statistical Optimization of the Enzymatic Saccharification of the Oil Palm Empty Fruit Bunches

Rashid S. S. and Alam M. Z.

Abstract—A statistical optimization of the saccharification process of EFB was studied. The statistical analysis was done by applying faced centered central composite design (FCCCD) under response surface methodology (RSM). In this investigation, EFB dose, enzyme dose and saccharification period was examined, and the maximum 53.45% (w/w) yield of reducing sugar was found with 4% (w/v) of EFB, 10% (v/v) of enzyme after 120 hours of incubation. It can be calculated that the conversion rate of cellulose content of the substrate is more than 75% (w/w) which can be considered as a remarkable achievement. All the variables, linear, quadratic and interaction coefficient, were found to be highly significant, other than two coefficients, one quadratic and another interaction coefficient. The coefficient of determination (R^2) is 0.9898 that confirms a satisfactory data and indicated that approximately 98.98% of the variability in the dependent variable, saccharification of EFB, could be explained by this model.

Keywords—Face centered central composite design (FCCCD), Liquid state bioconversion (LSB), Palm oil mill effluent, *Trichoderma reesei* RUT C-30.

I. INTRODUCTION

WORLD is looking for the alternatives of the existing energy, fossil fuel, due to its drastic depletion and the reverse ecological impacts. Utilization of fossil fuel is thought to be the main precursor of the current global environmental down-gradation [1]-[3]. Therefore, feasibility of production of a wide range of alternative energy sources such as methanol [4], ethanol [5], butanol [6], biodiesel [7], bio-hydrogen [8] and wind [9] etc., is being sought by the scientific and business communities in the world, recent times. To produce these bio-fuels, lignocellulosic wastes are found to be one of the main candidates of being raw materials due to its degradability to the simplest bio-molecules, sugar monomer, dimer or oligomer, with which most of the bio-fuels such as ethanol [5], butanol [6], bio-hydrogen [8], can be produced through biodegradation/fermentation.

Biological wastes can be saccharified to produce easily digestible sugars for biofuel production through microbial

degradation/fermentation. Most of the biological solid wastes contain more than 50% (w/w) convertible carbohydrate. Alone cellulose content is about 50%, 62%, and 34.4% in sugarcane baggase, wood chips, rice husks respectively [10]-[12]. These cellulose contents can be used for alternative energy production, but the main challenge in this process is to get the easily digestible sugars through enzymatic saccharification. Hence, pretreatment has been an inevitable redundant steps of the saccharification process to make the cellulosic materials of the solid wastes accessible for microbial biodegradation, fermentation or enzymatic catalysis.

Pretreatment is an important tool for the saccharification process. Target of doing pretreatment of the lignocellulosic biomass is to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars. Many different pretreatment methods have been applied to facilitate the better hydrolysis of the lignocellulosic wastes. Pretreatment methods can be either physical, chemical or sometimes incorporated both [13]. Steam and water are not considered as chemical agents for pretreatment since extraneous chemicals are not added to the biomass. Physical pretreatment methods include comminution (mechanical reduction in biomass particulate size), steam explosion, and hydrothermolysis. Comminution, including dry, wet, and vibratory ball milling [14]-[16] and compression milling [17] is sometimes needed to make material handling easier through subsequent processing steps.

A 44 million tones of Empty fruit bunches (EFB) is generated in Malaysia during the production process of plam oil. EFB contains about 44% (w/w) cellulosic material. So, it would be an ideal material for the production of ethanol or other value added bioproducts such as cellulase enzyme, biohydrogen etc. But utilization of the cellulosic content effectively remains a very big challenge. Thus, an efficient pretreatment method together with the optimized saccharification process of the EFB is necessary for better utilization to produce different bio-products. Rashid et al. [18] already developed the pretreatment process to maximize the hydrolysis of the EFB, but the yield was not sufficient enough to proceed for the pilot scale processing. Therefore, in this study a statistical response surface methodology was adopted to enhance the saccharification of EFB through interaction study of the different contributing factors on the enzymatic hydrolysis process.

Rashid S. S. is with the Industrial Biotechnology, Faculty of Industrial Sciences and Technology (FIST), Universiti Malaysia Pahang (UMP), 26300 Gambang, Pahang, Malaysia (Corresponding author; e-mail: samiur@ump.edu.my).

Alam M. Z. is with the Bioenvironmental Engineering Research Unit (BERU), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia (IIUM), Gombak, 50728 Kuala Lumpur, Malaysia.

II. MATERIALS AND METHODS

A. Enzyme Preparation

Sample of crude cellulase enzyme was collected from the Environmental Engineering Laboratory Stock of International Islamic University Malaysia (IIUM). Crude cellulase enzyme is the broth of the bioconversion product of POME by *Trichoderma reesei* RUT C-30 using optimized media and process conditions in a 30 L bioreactor, according to Rashid et al. [19]. The fermentation broth of cellulase enzyme was first filtered using the bag filter with porosity of 250 μ m and stored at -20°C and thawed at 4°C overnight before micro- and ultra-filtration. After thawing, the enzyme solution was centrifuged at 4°C with 10,000g.

B. Collection, Preparation and Pretreatment of EFB

Sample of EFB was collected from East Oil Mill, Sime-Darby, Banting, Kuala Lumpur, Malaysia. Collected sample was preserved in the cold room at 4°C to avoid the unwanted bio-degradation by any microorganisms. Collected EFB was prepared and pretreated according to the study of Rashid et al. [18]. Collected EFB sample was washed with distilled water vigorously to remove all mud, dust and other unwanted substances. Washed sample was dried in oven at 105°C for 24h to get constant dry weight. Dried EFB fiber was ground with milling machine to obtain 1.0mm particle size. The EFB was pretreated with 3% NaOH at 100°C in the water bath for 2h having 5% of EFB.

C. Optimization of the Parameters for Enzymatic Saccharification of EFB

The process parameters for the enzymatic saccharification of EFB were performed using face centred central composite design (FCCCD) under the response surface methodology (RSM) in order to describe the nature of the response surface in the experimental design and elucidate the optimal conditions of the most significant independent variables.

Tween 80, particle size of the substrate, substrate dose, enzyme dose, agitation, saccharification duration were found to be the most influential parameters for the enzymatic saccharification of EFB using OFAT methodology from the previous study [18]. Tween 80, particle size and agitation were found to be effective at 0.1% (v/v), 1mm, and 150rpm respectively and the other three parameters, namely substrate dose, enzyme dose, saccharification duration were considered for further interactive investigation in the RSM experimental design. The factors were examined at three different levels (low, medium, high) with the codes (-1, 0, +1) as shown in Table I. According to the FCCCD for the three variables, 20 experimental runs including 6 centre points were executed and their observations were fitted to the following second order polynomial model:

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{23}BC + \beta_{13}AC$$

where, Y is the dependent variable (total reducing sugar); A, B and C are the independent variable (enzyme dose, EFB dose and saccharification duration); β_0 is the regression coefficient at the center point; β_1 , β_2 , and β_3 are the linear coefficients; β_{11} , β_{22} and β_{33} are the quadratic coefficients and β_{12} , β_{23} and β_{13} are the second order interaction coefficient.

The developed regression model was evaluated by analyzing the regression coefficient values, analysis of variance (ANOVA), p and F values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R². The statistical software package Design-Expert 6.0.8 (Stat Ease Inc., Minneapolis, USA) was used to identify the experimental design as well as to generate a regression model to predict the optimum combinations considering the effects of linear, quadratic and interaction on cellulase enzyme production. A final experiment was conducted to validate the FCCCD developed model.

TABLE I
THE FACE CENTERED CENTRAL COMPOSITE DESIGN (FCCCD) FOR THE OPTIMIZATION OF THE ENZYMATICAL SACCHARIFICATION OF EFB

| Run | A:EFB Dose | B:Enzyme Dose | C:Saccharification Time | Reducing Sugar (mg/g of EFB) | |
|-----|------------|---------------|-------------------------|------------------------------|--------------|
| | (%, w/v) | (%, v/v) | (day) | Predicted | Experimental |
| 1 | 12 | 10 | 1 | 289.04 | 289.72 |
| 2 | 8 | 7.5 | 3 | 280.84 | 269.11 |
| 3 | 8 | 7.5 | 3 | 280.84 | 277.35 |
| 4 | 8 | 7.5 | 3 | 280.84 | 283.61 |
| 5 | 8 | 7.5 | 1 | 264.86 | 264.27 |
| 6 | 8 | 7.5 | 3 | 280.84 | 274.37 |
| 7 | 4 | 10 | 5 | 528.60 | 534.52 |
| 8 | 8 | 7.5 | 3 | 280.84 | 282.47 |
| 9 | 8 | 5 | 3 | 248.09 | 251.76 |
| 10 | 4 | 10 | 1 | 377.67 | 377.57 |
| 11 | 12 | 5 | 5 | 271.27 | 269.82 |
| 12 | 4 | 5 | 5 | 380.37 | 378.14 |
| 13 | 4 | 7.5 | 3 | 331.78 | 320.71 |
| 14 | 8 | 7.5 | 5 | 360.11 | 366.91 |
| 15 | 12 | 7.5 | 3 | 232.91 | 250.20 |
| 16 | 8 | 7.5 | 3 | 280.84 | 285.74 |
| 17 | 12 | 5 | 1 | 231.70 | 224.23 |
| 18 | 12 | 10 | 5 | 340.74 | 331.70 |
| 19 | 8 | 10 | 3 | 350.87 | 353.41 |
| 20 | 4 | 5 | 1 | 241.58 | 249.06 |

D. Analytical Analysis

Reducing sugar of the assayed samples was estimated by dinitrosalicylic acid (DNS) method [20]. Residual cellulase activity of the reaction broth was determined by CMC (carboxymethyl cellulase) assay (CMCase) where CMC was used as a substrate. The method of determination of degradation of lignin, cellulose, and hemicellulose in EFB during saccharification was described.

The sequential fractionation of lignocellulosics was carried out according to Datta [21] with slight modifications. One gram of sample was suspended in 100ml distilled water, kept at 100°C for 2h in a water bath and filtered on a tare crucible, and residue was dried at 90°C till constant weight. Loss was considered as water soluble part. Dried residue was suspended in 100ml of 0.5M H₂SO₄ and after keeping for 2h at 100°C in a water bath, the contents were filtered, dried and weighed as described in the first step and loss in weight was represented as hemicellulose content. For cellulose and lignin estimations, 10ml of 72% (v/v) H₂SO₄ was added to the earlier mentioned dried residue and kept at 30°C for 1h on a rotary shaker at 200rpm. After incubation, the mixture was diluted up to 4% (v/v) of H₂SO₄ and autoclaved at 1.06kg/cm² for 40min. The contents were filtered, dried and weighed. The loss in weight was treated as cellulose, and the left over residue was considered as lignin.

For estimating the residual ash content, 1g of sample was kept at 550°C for 5h in a tare crucible and reweighed to calculate the residual ash content.

Degradations of lignin, cellulose and hemicellulose contents of EFB after saccharification by cellulase enzyme were determined by subtracting the remaining quantity from the initial quantity and expressed in percentage. Initial fractions of lignin, cellulose and hemicellulose were determined for the EFB before saccharification and the remaining contents were determined in EFB sample after saccharification.

III. RESULTS AND DISCUSSION

A. Statistical Optimization of the Saccharification Process by FCCCD Under RSM

Face centered central composite design (FCCCD), an experimental design, was applied to optimize the three independent variables, EFB dose, enzyme dose and incubation period, for the enzymatic saccharification of EFB. The ranges of these parameters were fixed through the study by OFAT method.

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 determine the optimal values of the most significant independent variables. The effects of the three independent variables, namely EFB dose, enzyme dose and incubation period, on the enzymatic saccharification of the EFB were predicted by the following polynomial regression equation:

$$Y (\text{Reducing Sugar, mg/gm of EFB}) = 220.11 + 10.21 * A - 10.23 * B - 3.39242 * C + 0.093965 * A^2 + 2.98 * B^2 + 7.91 * C^2 - 1.96890 * A * B - 3.10083 * A * C + 0.60654 * B * C$$

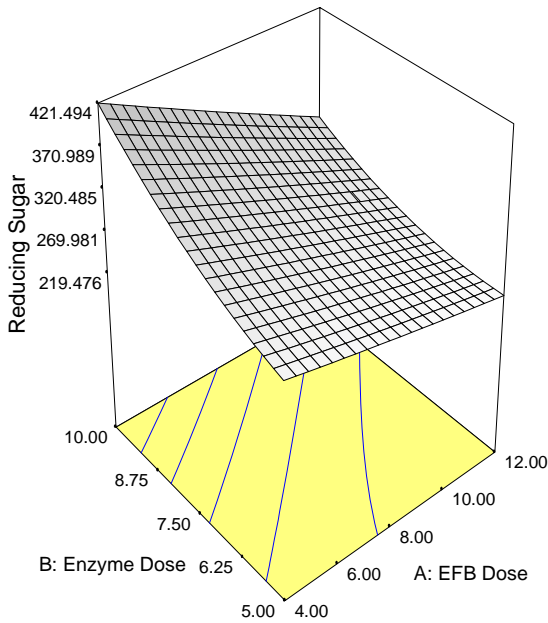
where, saccharification of EFB in term of reducing sugar content (Y) is a function of EFB dose (A), enzyme dose (B) and saccharification period (C).

The coefficient of determination (R^2) is 0.9898 which ensures a satisfactory data and indicated that approximately 98.98% of the variability in the dependent variable, saccharification of EFB, could be explained by this model. The adjusted R^2 is 0.9807, which is more suitable for comparing models with different numbers of independent variables. 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 43.074 indicates the adequacy of signal and that interpret the fitness of the model as well.

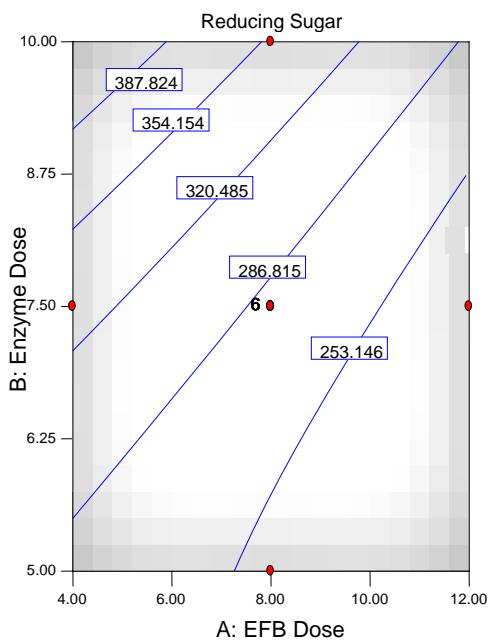
Analysis of variance (ANOVA) of the response surface, quadratic polynomial model is shown in Table II. It is evident from the results that the model is significant ($p < 0.0001$) and the 'Lack of Fit' of the model is non-significant (0.087). All the linear, quadratic and interaction coefficient of variables in this study were found to be significant, other than two coefficients. The quadratic and interaction coefficient of A² and BC, respectively, were non-significant in this model. All the linear and the AC interaction coefficient were significant at $p < 0.0001$ and the other quadratic coefficient of B² and C² and the interaction coefficient of AB were found to be significant at $p < 0.05$ level. So it can be concluded that the parameters of the saccharification condition under investigation can act as a limiting factor on the response, yield of reducing sugar during the enzymatic saccharification of EFB [22].

TABLE II
ANALYSIS OF VARIANCE (ANOVA) FOR THE POLYNOMIAL MODEL

| Source | SS | DF | Mean | F-value | p-value>F |
|----------------|----------|----|----------|----------|-----------|
| Model | 92367.64 | 9 | 10263.07 | 108.0141 | < 0.0001 |
| A | 24436.47 | 1 | 24436.47 | 257.1825 | < 0.0001 |
| B | 26409.64 | 1 | 26409.64 | 277.9492 | < 0.0001 |
| C | 22679.61 | 1 | 22679.61 | 238.6923 | < 0.0001 |
| A ² | 6.215865 | 1 | 6.215865 | 0.065419 | 0.8033 |
| B ² | 954.824 | 1 | 954.824 | 10.04908 | 0.0100 |
| C ² | 2753.178 | 1 | 2753.178 | 28.97592 | 0.0003 |
| AB | 3101.24 | 1 | 3101.24 | 32.63911 | 0.0002 |
| AC | 4922.965 | 1 | 4922.965 | 51.81192 | < 0.0001 |
| BC | 73.5789 | 1 | 73.5789 | 0.774384 | 0.3995 |
| Lack of Fit | 749.7273 | 5 | 149.9455 | 3.740532 | 0.0870 |



(a)

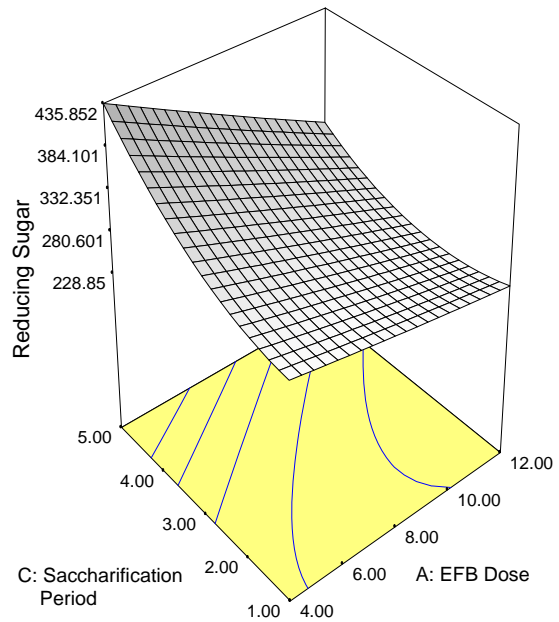


(b)

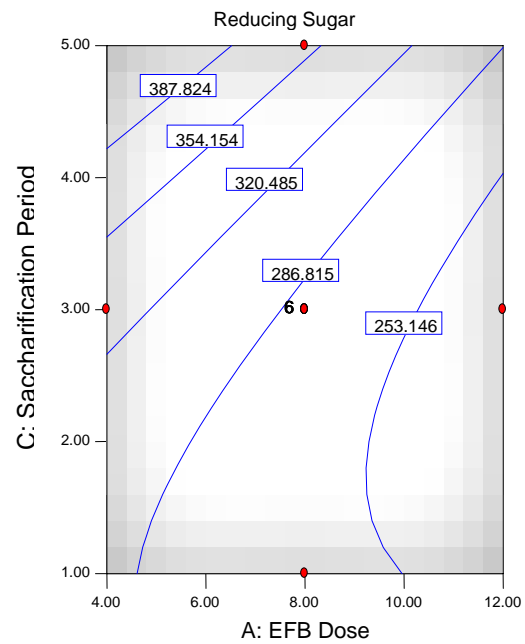
Fig. 1 Interaction of enzyme and EFB dose on saccharification (mg of reducing sugar/gm of EFB) (a) 3D response surface; (b) 2D contour plots

The three dimensional (3D) response surface and two dimensional (2D) contour plot of the interaction between enzyme dose and EFB dose is presented in Fig. 1. It was revealed that the reducing sugar yield was increased with the increment of enzyme dose and EFB dose, but there is a suppression of the yield even though the EFB dose is increased might be due to the saturation effect [23]. The

maximum yield of reducing sugar, 534.52mg/gm of EFB (Table I), was obtained with the EFB and enzyme dose of 4% (w/v) and 10% (v/v), respectively.



(a)



(b)

Fig. 2 Interaction of saccharification period and EFB dose on saccharification (mg of reducing sugar/gm of EFB): (a) 3D response surface; (b) 2D contour plots

The interaction of saccharification period and EFB dose was represented by the 3D response surface and 2D contour plot in Fig. 2. The graph shows that the yield of reducing

sugar increased with the increment of the saccharification period at the lower level of EFB, but at the higher level of EFB the yield reduced with the progression of the saccharification. This might indicate to use the lower dose of EFB in the saccharification process.

In the Fig. 3 the interaction of saccharification period and the enzyme dose was shown by the 3D response surface and 2D contour plot. The results indicated that the yield of reducing sugar increased with the increment of the enzyme dose and the saccharification period. The results in the Table I showed that with the highest enzyme dose (10%, v/v) the yield of reducing sugar was 377.57mg and 534.52mg after the saccharification period of 24hrs and 120hrs, respectively, where the EFB dose was 4% (w/v) for each of the instances.

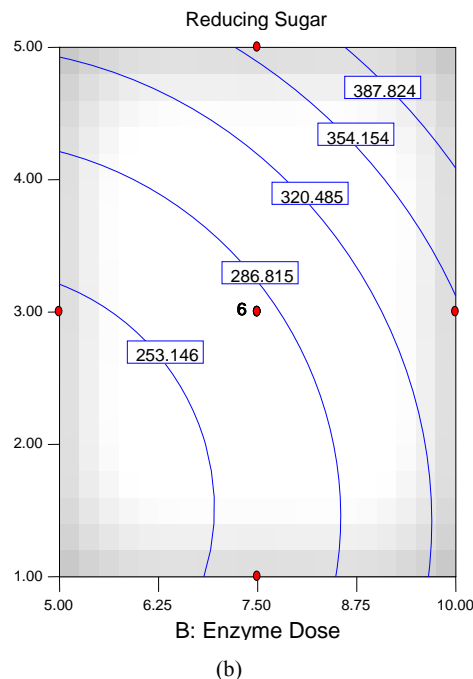
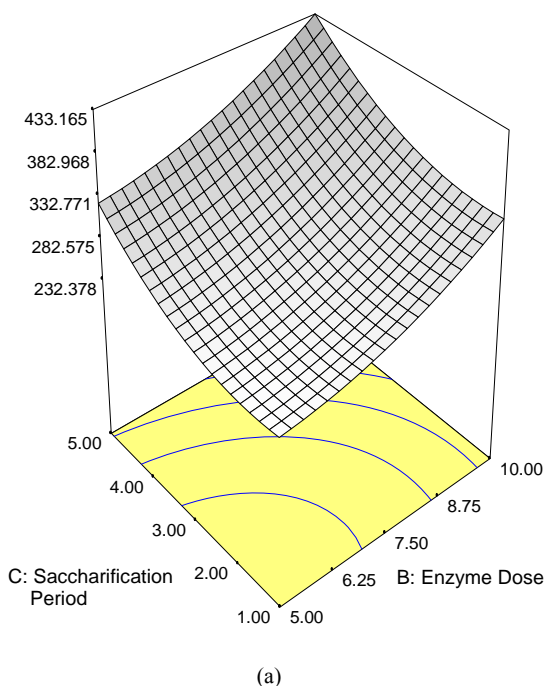


Fig. 3 Interaction of saccharification period and enzyme dose on saccharification (mg of reducing sugar/gm of EFB): (a) 3D response surface; (b) 2D contour plots

B. Validation of the Model Developed: Saccharification of EFB

A set of experiment was performed to verify the optimization results in order to validate the developed model. Predicted values of the different combinations of the parameters were calculated from the developed model. The process condition and combination for the saccharification of EFB composed of parameters of independent variables are shown in the Table III. The predicted and experimental process condition for the saccharification of EFB was found to be within the error percentage of ± 10 . So it can be concluded that the developed model is capable to predict the yield of saccharification of the EFB.

TABLE III
VALIDATION OF THE DEVELOPED MODEL ENZYMATIC EFB SACCHARIFICATION

| EFB Dose* | Enz Dose* | Time* | Predicted | Experimental | Error % |
|-----------|-----------|-------|-----------|--------------|---------|
| 5.41 | 7.49 | 4.89 | 401.65 | 415.18 | 3.37 |
| 10.66 | 5.44 | 2 | 223.33 | 230.18 | 3.07 |
| 7.48 | 7.41 | 3.1 | 287.99 | 281.44 | -2.28 |
| 11.74 | 6.84 | 3.41 | 234.70 | 252.08 | 7.40 |

Enzymatic saccharification of different lignocellulosic biomasses, such as food waste [24], rice straw [25], rice hulls [26], sunflower stalks [27], waste paper [28], water hyacinth [23], etc, were carried out. Among them 32% yield was achieved from rice hulls [26], 57.8% saccharification was yielded from sunflower stalks [27] and 30.3% yield was recorded by Huan et al. [25] during saccharifying of rice straw.

IV. CONCLUSION

This study showed that the after statistical optimization applying the FCCCD under RSM of enzymatic saccharification of EFB, maximum yield is 534.53mg/gm of EFB of reducing sugar with 4% (w/v) of EFB, 10% (v/v) of enzyme after 120 hours of incubation. After the validation study, it was confirmed that the yield (53.45%, w/w) of sugar from the saccharified EFB is consistent under the above mentioned process condition.

ACKNOWLEDGMENT

The authors thank to the Ministry of Higher Education (MOHE) for approval a research grant under Fundamental Research Grant Scheme (FRGS 0207-49). The co-operation of the Sime-darby, Banting, by supplying the experimental samples is acknowledged as well.

REFERENCES

- [1] Chiari, L. and Zecca A. (2010). Constraints of fossil fuels depletion on global warming projections. *Energy Policy* 39(9):5026–34.
- [2] Hoel, M.K., verndokk, S. (1996). Depletion of fossil fuels and the impacts of global warming. *Resource and Energy Economics* 18(2):115–36.
- [3] Nel, W.P., Cooper, C.J. (2009). Implications of fossil fuel constraints on economic growth and global warming. *Energy Policy* 37(1):166–80.
- [4] Boer, K. D., Bahri, P. A. (2011). Supercritical methanol for fatty acid methyl ester production: A review. *Biomass and Bioenergy* 35:983-991.
- [5] Kosugi, A.; Tanaka, R.; Magara, K.; Murata, Y.; Arai, T.; Othman Sulaiman; Rokiah Hashim; Zubaidah Aimi, A. H.; Mohd Khairul, A. Y.; Mohd Nor, M. Y.; Wan Asma Ibrahim; Mori, Y. (2010). Ethanol and lactic acid production using sap squeezed from old oil palm trunks felled for replanting. *Journal of Bioscience and Bioengineering* VOL. 110 No. 3, 322–325.
- [6] Fangfang, L., Li, L., Xiansh, F. (2005). Separation of acetone–butanol–ethanol (ABE) from dilute aqueous solutions by pervaporation. *Separation and Purification Technology* 42:273–282.
- [7] Zahira, Y., Masita, M., Mohammad, A., Zahangir, A., Kamaruzaman S. (2013). Overview of the production of biodiesel from waste cooking oil. *Renewable and Sustainable Energy Reviews* 18:184–193.
- [8] Syafawati, A. K., Jamaliah, M. J., Nurina, A., Osman, H., Wan, R.W.D., Mariatul, F.M., Rashid, S. S. (2011). Pre-Treatment Effect of Palm Oil Mill Effluent (POME) during Hydrogen Production by a Local Isolate *Clostridium butyricum*. *International Journal on Advanced Science, Engineering and Information Technology*, Vol. 2 No. 4, pages: 54-60.
- [9] Islam, M.R., Mekhilef, S., Saidur, R. (2013). Progress and recent trends of wind energy technology. *Renewable and Sustainable Energy Reviews* 21:456–468.
- [10] Kianoush, K., Alaleh, Z. (2008). Comparison of pretreatment strategies of sugarcane bagasse: Experimental design for citric acid production. *Bioresource Technology* 99:6986–6993.
- [11] Xuejing, W., Huiquan, L., Yan, C., Qing, T. (2011). Cellulose extraction from wood chip in an ionic liquid 1-allyl-3-methylimidazolium chloride (AmimCl). *Bioresource Technology* 102:7959–7965.
- [12] Megawati, A., Wahyudi, B., S., Hary, S., Muslikhin, H. (2011). Kinetics of sequential reaction of hydrolysis and sugar degradation of rice husk in ethanol production: Effect of catalyst concentration. *Bioresource Technology* 102:2062–2067.
- [13] Hsu, T.A. (1996). Pretreatment of Biomass. In: Wyman, C.E. (Ed.), *Handbook on Bioethanol, Production and Utilization*. Taylor & Francis, Washington, DC.
- [14] Millett, M.A., E.and, M.J., Caul.eld, D.P. (1979). Influence of grinding on the hydrolysis of cellulosic materials—acid versus enzymatic. *Advances in Chemistry Series* 181, 71–89.
- [15] Rivers, D.B., Emert, G.H. (1987). Lignocellulose pretreatment: a comparison of wet and dry ball attrition. *Biotechnology Letters* 9 (5), 365–368.
- [16] Sidiras, D.K., Koukios, E.G. (1989). Acid saccharification of ballmilled straw. *Biomass* 19 (4), 289–306.
- [17] Tassinari, T., Macy, C., Spano, L., (1982). Technology advances for continuous compression milling pretreatment of lignocellulosics for enzymatic hydrolysis. *Biotechnology and Bioengineering* 24, 1495–1505.
- [18] Rashid, S.S., Alam, M.Z., Karim, M.I.A. and Salleh, M.H. (2011). Development of pretreatment of empty fruit bunches for enhanced enzymatic saccharification. *African Journal of Biotechnology* Vol. 10(81), pp. 18728-18738.
- [19] Rashid, S.S., Alam, M.Z., Karim, M.I.A., Hamzah, M.S. (2009). Management of palm oil mill effluent through production of cellulases by filamentous fungi. *World Journal of Microbiology and Biotechnology* 25(12), 2219-2226.
- [20] Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 31, 426-428.
- [21] Datta, R. (1981). Acidogenic fermentation of lignocellulose - acid yield and conversion of components. *Biotechnology and Bioengineering*. 23, 2167-2170
- [22] Imandi, S.B., Bandaru, V.V.R., Somalanka, S.R., Bandaru, S.R., Garapati, H.R. (2008). Application of statistical experimental designs for the optimization of medium constituents for the production of citric acid from pineapple waste. *Bioresource Technology* 99, 4445–4450.
- [23] Aswathy, U.S., Sukumaran, R.K., Devi, G.L., Singhania, R.R., Pandey, A. (2010). Bio-ethanol from water hyacinth biomass: An evaluation of enzymatic saccharification strategy. *Bioresource Technology* 101, 925–930.
- [24] Jung, K.K., Baek, R.O., Hyun-Jae, S., Chi-Yong, E. and Si, W.K. (2008). Statistical optimization of enzymatic saccharification and ethanol fermentation using food waste. *Process Biochemistry* 43(11), 1308-1312
- [25] Huan, M., Wei-Wei L., Xing, C., Yue-Jin, W., Zeng-Liang, Y. (2009). Enhanced enzymatic saccharification of rice straw by microwave pretreatment. *Bioresource Technology* 100, 1279–1284.
- [26] Badal, C.S., Michael, A.C. (2008). Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol. *Biomass and Bioenergy* 32, 971 – 977.
- [27] Sharma, S.K., Kalra, K.L., Grewal, H.S. (2002). Enzymatic saccharification of pretreated sunflower stalks. *Biomass and Bioenergy* 23, 237–243.
- [28] Wyk, J.P.H.V. (1999). Hydrolysis of pretreated paper materials by different concentrations of cellulase from *Penicillium funiculosum*. *Bioresource Technology* 69, 269-273.