Conversion of rice husk into fermentable sugar by two stage hydrolysis

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Abstract. Rice husks, a complex lignocellulosic biomass which comprised of high cellulose content (38-50%), hemicellulose (23-32%) and lignin (15-25%) possesses the potential to pursue as low cost feedstock for production of ethanol. Dilute sulfuric acid at concentration of 1, 2, 3 (% v/v) were used for pretreatments at varied hydrolysis time (15-60 min) and enzymatic saccharification at range of 45-60˚C and pH 4.5-6.0 were evaluated for conversion of rice husk's cellulose and hemicellulose to fermentable sugars. The maximum yield of fermentable sugars from rice husks by dilute sulfuric acid (2%, 60 minutes) was 0.0751 g/l. Total fermentable sugar was identified using dinitrosalicylic acid (DNS) method and expressed in g/l. Enzymatic hydrolysis for conversion of cellulose to fermentable sugar has been studied by applying response surface methodology (RSM) and Analysis of Variance (ANOVA). Two independent variables namely initial pH and incubation temperature were considered using Central Composite Design (CCD). The determination coefficient, R² obtained was 0.9848. This indicates that 98.48% capriciousness in the respond could be clarified by the ANOVA. Based on the data shown by Design Expert software, the optimum condition for total sugar production was at pH 6.0 and temperature 45˚C as it produced 0.5086 g/l of total sugar.

1. Introduction
Today, more than 90% of world’s energy supply came from fossil fuels [1]. As consequences from increasing fossil fuel consumption, depletion of fossil fuels and environment impacts has raised public awareness in shifting to new energy resources. The theory of supply and demand accounts that prices will rise as hydrocarbon sources diminish. Therefore, higher prices lead to development of alternative energy source which is more available and affordable.

Research reveals that one of the important energy alternatives is utilization of lignocellulosic feedstock [2]. Lignocellulosic resources can be predominantly processed to produce liquid or gaseous fuel which is known as biofuel. Examples of biofuel are such as bioethanol, methanol, biodiesel and gaseous fuels like hydrogen and methane [3]. Unlike fossil fuel, bioethanol is a form of renewable energy that can be made from any lignocellulosic feedstock containing sugar or carbohydrates. Bioethanol is a promising energy alternative due to the potential to replace gasoline, as it increases the fuel octane level of gasoline and burns cleaner than gasoline. In Brazil, most of the ethanol used as motor fuel is produced from sugar cane [4]. As compared to energy of non-renewable sources, energy of renewable sources are sustainable, clean and eco-friendly.

Among the lignocellulosic feedstocks, rice husk is one of the most abundant agriculture by-products worldwide where its production reaches million tons annually [5]. This inedible lignocellulosic material is the outermost layer of paddy grains, commonly removed in milling process.
as biowaste, which occupies up to 20-22% of total weight of milled paddy [2]. Rice husk is suitable for bioethanol production owing to high cellulose content. Significant improvement in engine performance, including brake power, torque, brake mean effective pressure, volumetric efficiency and fuel consumption by using ethanol produced from rice husk is observed when blends with gasoline [6]. Consequently rice husk is recommended to be exploited for low cost bioethanol production on a commercial scale, which eventually solve for disposal issue and reduce waste treatment cost [7].

Conversion of lignocellulosic materials to ethanol includes two processes: hydrolysis of cellulose into fermentable reducing sugar and fermentation of the sugars to ethanol. Commercial cellulase enzymes could be used in conversion of lignocellulose into fermentable reducing sugars. The conversion is carried out by highly specific cellulase enzyme involving mixture of endoglucanases, exoglucanases, β-glucosidases and cellobiohydrolases [8]. Various research have been completed on degradation of rice husk cellulose to yield monomeric sugars using enzymes [9]-[12].

The efficiency of enzymatic hydrolysis depends on various factors including type of pretreatment and catalytic action of enzymes [8]. Lignin content, availability of enzymatic site of cellulose fibers and crystallinity properties reduce the accessibility of cellulase enzyme to cellulose, thus affect the efficiency of enzymatic hydrolysis. Therefore, lignocellulosic materials require a pretreatment to solubilize the hemicellulose and enhance the cellulose accessibility, which can be carried out by different techniques such as alkalis, acids, heat, pressure, solvents, etc. [13]. In present study, methods were developed to optimize acid pretreatment and enzymatic hydrolysis of rice husk cellulose into fermentable sugars. The hydrolysis efficiency, in terms of total reducing sugar yields obtained from pretreatment and enzymatic hydrolysis were evaluated.

2. Experiment

2.1. Materials
Rice husks were collected from a rice processing mill, Padi Beras Nasional Berhad (BERNAS) located at Simpang Empat, Perlis. Rice husks were washed thoroughly with distilled water to eliminate adhering soil and dust. The husks were dried at 100˚C overnight. The dried husks were then grinded into powder form and sieved by passing through a 1 mm screen to standardize the particle size range of 1 mm. The sample was kept in a tightly closed container at room temperature.

2.2. Methodology

2.2.1. Sulfuric acid pretreatment. In optimization of acid hydrolysis of rice husk, grinded rice husk was pretreated with dilute sulfuric acid (1%, 2% or 3%, v/v) and the sample was soaked in water bath at 90˚C at different retention time (15-60 min). The liquid fraction was tested for glucose concentration while the solid residue was washed several times with distilled water to remove retaining acid solution. Then, the residue was dried in the oven at 50˚C overnight.

2.2.2. Enzymatic hydrolysis. The solid residue resulted from optimal condition in acid pretreatment was proceeded for enzymatic hydrolysis. Commercial enzyme cellulase produced by Aspergillus niger was used. Enzyme dose of 0.04 ml/g husk was added into the hydrolysis process. The solid residue was suspended in 100 ml sodium citrate buffer and incubated in a rotating shaker for 24 hour at agitation rate of 150 rpm. The incubation pH and temperature were adjusted according to the parameters selected for optimization.

2.3. Design of experiment
The optimization of sugar production from enzymatic hydrolysis was conducted by Design Expert 7.1.5 software by using Response Surface Methodology (RSM) through Central Composite Design (CCD). The objective of RSM is optimization [14]. In this case, variables tested were pH and temperature, thus response surface designed experiment is used to determine the optimal settings for each variables. A total of 13 experiment runs are required for this study and total sugar concentration
was taken as the response of the designed experiments. The effect of pH and temperature for hydrolysis of rice husks were studied in the range between pH 4.5 to pH 7.5 and 30˚C to 60˚C, respectively, shown in Table 1.

| Parameters | Minimum | Maximum |
|------------|---------|---------|
| pH         | 4.5     | 7.5     |
| Temperature (˚C) | 30     | 60     |

2.4. Model fitting and statistical analysis
The results obtained from CCD were fitted to a second order model equation to explain the reliance of total sugar concentration on the effect of pH and temperature in terms of coded values A and B, respectively. The experimental data was analyzed by using Design Expert software and further discussed in following regression analysis, ANOVA and graphical analysis sections.

2.5. Fermentable sugar analysis
The response variable in this research is concentration of glucose produced from each runs, which was determined by using DNS method [15]. Sample of 1.0 ml was withdraw and added with dinitrosalicylic acid (DNS) reagent (dilution factor will be used if needed), then the sample was place in hot water bath at 90˚C for 5 minutes. Absorbance reading was taken by using UV-Vis spectrophotometry once the sample cooled down to room temperature. Blank is prepared from mixture of DNS and distilled water.

3. Result and discussion

3.1. Optimization of acid pretreatment
The optimization of acid pretreatment of rice husks was done by manipulating two variables, including concentration of sulfuric acid and hydrolysis time duration. Purpose of this optimization is to examine the optimum condition to break down rigid lignin structure and matrix conformation of cellulose for accessible of enzymatic hydrolysis in next step. The target is to produce maximum yield of sugar. The sugar concentration produced from acid dose (1.0-3.0 %, v/v) on the pretreatment at 90˚C for 15 – 60 minutes were shown in Figure 1. Pretreatment with an acid dose of 2.0% (v/v) for 60 minutes generated 0.0751 g/l sugars, which is the highest yield among the acid dose up to 3.0%. The effects of acid dose and hydrolysis time are illustrated.

From Figure 1, each hydrolysis at specified acid concentration followed a trend where concentration of total sugar increased when the hydrolysis time increasing. This indicated that longer time is required by acid to carry out hydrolytic chemical reaction, which is the cleavage of β-1-4-glycosidic bond, before converting cellulose into glucose. However, it can be observed that the sugar production increased when the acid dose increased from 1.0% to 2.0% but decreased when the acid dose increased from 2.0% to 3.0%. This result is corresponded to the research of Saha et al. (2005) when the total sugar they obtained with pretreatment with 2, 3 and 4 (% v/v) sulfuric acid followed by enzymatic saccharification were decreased, respectively. Thus, the highest sugar recovery efficiency from the optimization of acid hydrolysis performed is 2.0% sulfuric acid and hydrolysis time of 60 minutes, where 0.0751 g/l sugar is produced. Therefore, this optimum condition is used to proceed enzymatic hydrolysis in the next step. The hydrolyzed material was washed with distilled water to eliminate retained acid and the solid residue is further processed for optimization enzymatic hydrolysis.
3.2. Optimization of enzymatic hydrolysis

3.2.1. Regression Analysis.

Quadratic model was selected as suggested by the software and was used to explain the mathematical relationship between the independent variables and the dependent response. The regression model for both coded and actual factors were obtained where positive sign represents synergistic influence while negative sign represents antagonistic influence.

Final equation in terms of coded factors:
\[
\text{Total sugar concentration} = 0.52 + 0.15A + 0.445B - 0.018AB - 0.16A^2 - 0.18B^2
\]  
(1)

Final equation in terms of actual factors:
\[
\text{Total sugar concentration} = -9.29736 + 1.94747\text{pH} + 0.15726\text{Temperature} - 0.07886\text{pH}\times\text{Temperature} - 0.14483\text{pH}^2 - 0.08127\text{Temperature}^2
\]  
(2)

3.2.2. ANOVA.

Analysis of variance (ANOVA) is a collection of statistical models used to analyze the differences among group means and their variation. The analysis of variance of the selected quadratic model was presented in Table 2 and Table 3.

The magnitude of p-value (prob>F) is used to determine statistical significance of a model. A p-value lower than 0.01 indicating only a 1.0% chance that a ‘model F-value’ could occurred. The model F value obtained in Table 2 is 90.80 with p-value less than 0.0001, indicates that the model generated was statistically significant with only 0.01% chance of noise occurrence. The significant of each coefficient can be distinguished from the F-test and p-value. In this study, the variables A, A^2 and B^2 had significant effect on the total sugar yield as their p-values are less than 0.0001. The variable B and AB were insignificant because their p-value is greater than 0.05. The p-value of lack of fit is less than 0.0001, indicates that this model term is significant. However, this can be explained by claiming that two factors interaction model or linear model normally features significant lack of fit [16].

The analysis of the coefficient of determinates is shown in Table 3. The coefficient of determination value (R^2) is very high at 0.9848 indicates that the equation was highly reliable. The "Predicted R-Squared" of 0.9046 is in reasonable agreement with the "Adjusted R-Squared" of 0.9740 because the difference between them is less than 0.2. The "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 22.650 indicates an adequate signal and this model can be used to navigate the design space.

The coefficient of variance (CV) is the degree of accuracy. The value of CV obtained in the study was 11.41%. CV is a function of standard deviation and normally standardizes the scale of data. In general, low CV value means the factors are highly reliable during optimization.

![Figure 1. Effect of treatment time (15 – 60 min) on the release of total sugar from rice husks by dilute H2SO4 pretreatment (1.00 – 3.00%) at 90˚C.](image-url)
Table 2. Analysis of variance (ANOVA) for Response Surface Quadratic Model.

| Source      | Sum of Squares | Degree of Freedom | Mean Square | F Value  | p-value Prob > F |
|-------------|----------------|-------------------|-------------|----------|-----------------|
| Model       | 0.55           | 5                 | 0.11        | 90.80    | < 0.0001        |
| A-pH        | 0.17           | 1                 | 0.17        | 143.21   | < 0.0001        |
| B-Temperature | 0.117         | 1                 | 0.117       | 0.53     | 0.4892          |
| AB          | 0.063          | 1                 | 0.063       | 1.06     | 0.3379          |
| A²          | 0.18           | 1                 | 0.18        | 153.80   | < 0.0001        |
| B²          | 0.23           | 1                 | 0.23        | 195.38   | < 0.0001        |
| Residual    | 0.418          | 7                 | 0.060       |          |                 |
| Lack of Fit | 0.356          | 3                 | 0.119       | 7.57     | 0.0399 significant |
| Pure Error  | 0.063          | 4                 | 0.058       |          |                 |

Table 3. R-squared value for model developed by Design Expert.

| Standard deviation | C. V. (%) | R-squared | Adjusted R-Squared | Predicted R-Squared | Adequate Precision |
|--------------------|-----------|-----------|--------------------|---------------------|--------------------|
| 0.035              | 11.41     | 0.9848    | 0.9740             | 0.9046              | 22.650             |

3.2.3. **Graphical Analysis.** Predicted versus Actual Plot reveals how well the model fit the data. The plot is also used to compare the fit against the null model. The actual value indicates the data obtained from the experimental run while the predicted value is the value evaluated from Design Expert software. Figure 2 shows a great correspondence between the predicted and actual sugar concentration. Most of the points in the Figure 2 are scattered along the straight line although some points shows slightly deviation. Points that vertically distant from the straight lines indicating possible existence of outliers. The result overall considered as good fitted to the quadratic model.

![Figure 2. Predicted versus the actual total sugar concentration.](image)

3.2.4. **Interaction between parameters.** The response surface was used to obtain the maximum response which is the total sugar production at optimum condition at each design variables. Variables pH and temperature were considered in this study for the interaction effect between the variables and effect on the total sugar concentration.

Two dimensional (2D) contour plot and three dimensional (3D) response surfaces are used as graphical tool to display the interaction between variables on fermentable sugar concentration. The shape of the response surface indicate the nature and extent of the interaction between factors. Figure 3 and Figure 4 demonstrate the correlation of the pH and temperature. In Figure 3, it shows egg-shaped contour plot which indicates that the associations between the related variables are vital. Figure 4 illustrating a surface with maximum point located inside the experimental region [17]. The red region in the contour represented the maximum predicted value.

According to the 2D contour plot and 3D response surface, total sugar increased when the pH value increased. However, the sugar concentration started to decline when pH approach neutral state, where
pH near 7 is considered as optimum for many enzymes, enzyme is less active at pH lower or higher than this optimum. This variation in enzyme activity with changing pH is due to the influence of pH on acidic and basic side chains in active site of enzyme which can cause substrate cannot bind to the active site or it cannot undergo catalysis [18].

From analysis of variance, effect of temperature is not significant to the sugar production, this has been proven by the plots where sugar is produced in the range between 34.39°C to 55.61°C. This might be due to the adaptability of cellulase complex for the temperature range from 30°C to 60°C which was selected in this study. However, there was slightly decreased in enzymatic hydrolysis efficiency at temperature beyond 50°C, this could partially be explained by the loss of enzyme activity due to thermal inactivation [19]. It is observed that the highest sugar concentration was produced at the range of pH 6 to pH 7 and 40°C to 50°C, where this result is also found in study of Ong et al. (2012) by using rice straw as substrate [20].

3.3. Total glucose production
Total production of fermentable sugar were retrieved by adding up sugar yield from acid hydrolysis and enzymatic hydrolysis. Table 4 showed the concentration of sugar for both hydrolysis. The values obtained mostly were contributed by enzymatic hydrolysis. Therefore, it implies that enzymatic hydrolysis is more effective in producing fermentable sugar than acid hydrolysis.

There is difference in amount of fermentable sugar from both acid hydrolysis and enzymatic hydrolysis. The sugar concentration in acid hydrolysis is almost the same for each run due to the no changes in variables addressed. The highest sugar produced from the acid hydrolysis was 0.0905 g/l which was obtained from sample 2 and 13. The lowest sugar produced in this hydrolysis is 0.0559 g/l obtained in run 6, which differed 0.0346 g/l from the highest value.

The sugar concentration in enzymatic hydrolysis shows significant difference for each runs where the highest and lowest amount of sugar produced were 0.5471 g/l and 0.0019 g/l, from run 6 and run 12 respectively. However, sugar produced from enzymatic hydrolysis is overall higher than that of acid hydrolysis, except for run 12 and 13. The difference in amount of sugar produced is affected by the variables manipulated in enzymatic hydrolysis, in this case, pH and incubation temperature.

The highest total sugar yield is obtained in run 6, where 0.6030 g/l of sugar is achieved. In this hydrolysis, the condition applied is pH 6.0 and 45°C. Meanwhile, similar condition but using different substrate, Ong et al. (2012) recovered 3.62 g/l sugar from rice straw [20]. The amount of sugar obtained in present study is apparently lesser than the research reported. However, the difference may due to the period of hydrolysis of rice straw is longer and variation of cellulose content in the substrate.
Table 4. Total sugar production from acid and enzymatic hydrolysis.

| Run | Acid Hydrolysis (g/l) | Enzymatic Hydrolysis (g/l) | Total Sugar Concentration (g/l) |
|-----|----------------------|-----------------------------|--------------------------------|
| 1   | 0.0616               | 0.3391                      | 0.4007                         |
| 2   | 0.0905               | 0.5009                      | 0.5914                         |
| 3   | 0.0867               | 0.5086                      | 0.5953                         |
| 4   | 0.0578               | 0.4103                      | 0.4681                         |
| 5   | 0.0655               | 0.5124                      | 0.5779                         |
| 6   | 0.0559               | 0.5471                      | 0.6030                         |
| 7   | 0.0732               | 0.2697                      | 0.3429                         |
| 8   | 0.0790               | 0.5163                      | 0.5953                         |
| 9   | 0.0751               | 0.1156                      | 0.1907                         |
| 10  | 0.0578               | 0.2138                      | 0.2716                         |
| 11  | 0.0674               | 0.0058                      | 0.0732                         |
| 12  | 0.0905               | 0.0019                      | 0.0925                         |
| 13  | 0.0809               | 0.0077                      | 0.0886                         |

3.4. Validation model
Validation is conducted to signify the capability of RSM through the predicted fermentable sugar concentration generated by the Design Expert software. Table 5 demonstrates the validation of data collected. According to the table, there were slightly difference between the value of predicted sugar concentration and the value of actual total sugar concentration by 1.64%.

\[
\% \text{ Error} = \frac{\text{Theoretical value} - \text{Experimental value}}{\text{Theoretical value}} \times 100\%
\]

Table 5. Result of validation for verification of optimization.

| Run | pH  | Temperature (˚C) | Predicted total sugar concentration (g/l) | Actual total concentration (g/l) | Percentage error (%) |
|-----|-----|------------------|-------------------------------------------|----------------------------------|----------------------|
| 1   | 6.00| 45               | 0.517073                                   | 0.5009                           | 3.1278               |
| 2   | 6.00| 45               | 0.517073                                   | 0.5086                           | 1.6386               |
| 3   | 6.00| 45               | 0.517073                                   | 0.5163                           | 0.1495               |
|     |     |                  |                                            | Average                          | 0.5086               | 1.64                 |

4. Conclusion
Rice husk is one of the most abundant agriculture wastes in the world. From a view point of the biomass energy, rice husks, comprises about 70% mass organics which could be effectively used as environmentally friendly fuels to supply electric power or biomass ethanol. This study has qualitatively and quantitatively discussed the production of total fermentable sugar by two steps hydrolysis. Rice husk was pretreated by using dilute sulfuric acid at different hydrolysis time. The optimized acid hydrolysis condition is obtained by using 2.0% (v/v) sulfuric acid at 60 minutes. The rice husk residue produced from this optimized condition undergo enzymatic hydrolysis with the presence of cellulase enzyme. The combined of both hydrolysis processes gave a high yield of total sugar production. Statistical study was performed by Response Surface Methodology (RSM) through Central Composite Design (CCD) for the enzymatic hydrolysis and result showed that the model is reliable and significant. The optimal condition was identified by the Design Expert software which conducted the enzymatic hydrolysis at pH 6 and at temperature of 45˚C. When the rice husk was hydrolysed at this optimum condition, approximately 0.50 g/l of sugar was recovered. The determination coefficient ($R^2$) obtained in this study was 0.9848 which correspond to 98.48% and the
standard deviation is 0.035. The magnitude of $R^2$ indicated there was a correlation between the experimental and predicted data. The model F value of 90.80 with p-value less than 0.001 imply that this model was statistically significant.

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6. References
[1] Miller G T and Spoolman S E 2016 Living in the Environment (Canada: Cengage Learning)
[2] Bazargan A, Bazargan M and McKay G 2015 Renew.Ener. 77 512-520
[3] Balat M and Balat H 2010 Appl.Ener. 87 1815-1835
[4] Oth Mullis 1993 Alcohol Fuels: Impacts from Increased Use of Ethanol Blended Fuels (United States: Diane Publishing)
[5] Yavari S, Malakhamad A and Sapari N 2016 Environ.Sci.Pollut.Res. 23 17928-17940
[6] Nyachaka C J, Yawas D S and Pam G Y 2013 Int.J.Engir.Res. Tech. 2 3516-3533
[7] Kumar S, Sangwan P, Dhankhar R Mor V and Bidra S 2013 Res.J.Chem.Env.Sci. 1 126-129
[8] Sartori T, Tibolla H, Prigol E, Colla L M, Costa J A and Bertolin T E 2015 Biomed.Res.Int. 2015:342716
[9] Saha B C, Iten L B, Cotta M A and Wu Y V 2005 Biotechnol.Prog. 21 816-822
[10] Saha B C, and Cotta M A 2007 Enz.Mic.Tec. 41 528-532
[11] Wei G-Y, Lee Y-J, Kim Y-J, Jin I-H, Lee J-H, Chung C-H. and Lee J-W 2010 Appl.Biochem.Biotechnol. 162 1471-1482
[12] Dutta N, Mukhopadhyay A, Dasgupta A K and Chakrabarti K 2014 Bior.Tech. 153 269-277
[13] Dagnino E P, Chamorro E R, Romano S D, Felissia F E and Area M C 2013 Ind.Crop.Prod. 42 363-368.
[14] Myers R H, Montgomery D C and Anderson C M 2009 Response Surface Methodology: Process and Product Optimization Using Designed Experiments 3rd ed (New York: Wiley)
[15] Mizutani S, Iijima S, Morikawa M, Shimizu K, Matsubara M, Ogawa Y, Izumi R, Matsumoto K and Kobayashi T 1987 J.Ferm.Tech. 65 325-331
[16] DeLoach R and Ulbrich N 2007 A Comparison of Two Balance Calibration Model Building Methods (Nevada: American Institute of Aeronautics and Astronautics) p 147
[17] Bezerra M A, Santelli R E, Oliveira E P, Villar L S and Escaleira L A 2008 Talanta 76 965-977
[18] Seager S L and Slabaugh M R 2010 Organic and Biochemistry for Today 7th ed (Belmont: Brooks/Cole Cengage Learning)
[19] Pandiyen K, Tiwari R, Singh S, Nain P K S, Sarika R, Arora A, Singh S B, Nain L 2014 Enzy.Res. 2014:764898
[20] Ong L G A, Chan C H and Chew A L 2012 J.Med.Bioeng. 1 14-16