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OPTIMIZATION OF DILUTE ACID PRETREATMENT USING RESPONSE SURFACE METHODOLOGY FOR BIOETHANOL PRODUCTION FROM CELLULOSIC BIOMASS OF RICE POLISH

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Abstract

Lignocellulosic biomass is abundant and a renewable resource for the production of biofuel (bioethanol) by using fermentative organisms (*Sacchromyces cervesiae* & *Fusarium oxysporum*). Rice polish is a cheaper agro-waste for bioethanol production. The conversion of biomass into maximum yield of glucose, an important step for the bioethanol production, requires optimum dilute acid treatment. Inhibitory compounds reduce the ethanol production; therefore an attempt has been made in the present study to select suitable dilution by using Response surface methodology (RSM) design and to minimize the effects of inhibitory compounds during sulphuric acid (H_2SO_4) dilution. The treatment of biomass with H_2SO_4 (1.5%) at 100°C for 30 minutes exhibited optimum results. During enzymatic hydrolysis 16.52 mg/mL glucose was obtained by using 1 mL of enzymatic load at 50°C after 72 hours of hydrolysis. The Glucose thereafter converted to 5.21g/L and 3.69 g/L of ethanol during fermentation process by using *Sacchromyces cervesiae* and *Fusarium oxysporum* respectively.

Introduction

A variety of lignocellulosic materials like agricultural residues, municipal and industrial wastes are being investigated for bioethanol production (Ying *et al*., 2009). Cellulose is an abundant biopolymer on the earth and is considered the best renewable energy resource (Rezaei *et al*., 2008). The major limitations exist for the productions of ethanol from agricultural wastes due to physical and chemical associations between lignin and polysaccharides of plant cell wall along with cellulose crystallinity. Lignin forms a protective covering around cellulose and hemicellulose, which restrict the enzymatic degradation (Dawson & Boopathy, 2008). Cellulose is separated from lignin by dilute acid treatment and then glucose is recovered from cellulose by enzymatic hydrolysis. However, economical production of ethanol depends on efficient conversion of cellulose to its monomeric sugars (Takagi *et al*., 1977). Saccharification technologies are necessary to liberate fermentable sugar for ethanol production. Therefore, efficient pretreatment method is needed to increase the efficacy of enzymatic hydrolysis to obtain maximum glucose yield (Teng-Chieh *et al.,* 2010).

Rice polish is a by-product of rice milling and is the cheapest source of cellulose material available in bulk quantity in major rice growing areas of the Pakistan (Ambreen *et al.,* 2006). The different strains of Thermoanaerobacter and Clostridium were used for the production of ethanol from different lignocellulosic biomass. However, *Saccharomyces cerevisiae* or *Zymomonas mobilis* has advantage on other strain due their efficiency for conversion of glucose into ethanol and also being ethanol tolerant (Sommer, *et al*., 2004). Rice polish has potential for the production of ethanol and there is no proper utilization of this agro-waste in Pakistan. The present study was conducted to select a better dilute acid treatment by using response surface methodology and also to determine the possibly designed factor which gives less loss of glucose, better solubilization of xylose and maximum degradation of lignin leading to a better recovery of glucose by enzymatic hydrolysis and ethanol production using *Sacchromyces cervesiae* (Badal *et al.,* 2005).

Materials and Methods

Sample collection: All chemicals used during these researches were of analytical grade manufacture by Merck (Germany). Rice polish samples were collected from a processing rice mill of Gujranwala (Pakistan). The samples were chopped, oven dried and ground to powder form (80 mesh size). Dried samples were stored in sealed plastic bags at room temperature.

Dilute acid pretreatment: The sulphuric acid concentration 0.5, 1.5, 3% (w/v) were used to pretreat 5g sample of rice polish. The treatment were performed at 100°C for a residence time of 15, 30, 45 minute and the coded values were noted in Table 1 and pretreatment conditions for dilute acid and residence time were preoptimized by using response surface methodology described in Table 2. The pretreated samples were filtered and residues were washed with distilled water to remove the acid and dried in an incubator at 40°C. The lignin loss was determined. The filtrate of each sample was centrifuged at 10,000 rpm for 10 minutes using (Centurion Scientific Ltd, UK) and the sample was used for glucose and xylose analysis.

Enzymatic hydrolysis: The enzyme (Celluclast) by Novo Nordisk (Bagsvaerd, Denmark) was used in this work. Enzymatic activities (FPU) were measured according to the methods described by Ghose, 1987. The activity of cellulase was determined 74 FPU/mL. The enzymatic hydrolysis was carried out in 250mL blue capped reagent bottles containing 50 mM citrate buffer (pH=4.8) and 2 gram substrate. Cellulose concentrations i.e., 0.25, 0.5, 0.75, 1mL/2g were loaded each sample bottle containing 25 mg/mL tetracycline and 62.5 mg/mL augmentum at 50°C and samples (1mL) were collected from each bottle after 0, 3, 6, 24, 48, 72 hours and heated in water bath for 10 minute which were centrifuged at 10,000 rpm for 5 minutes after cooling. Glucose concentration was determined and the sample containing maximum glucose was utilized for Yeast fermentation.

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Table 1. Value of independent variable with coded level.

	Coded level				
	-1.41421	**-1**	**0**	**1**	**-1.41421**
Dilute acid pretreatment (X_1)	0.25	0.5	1.5	3	3.25
Time of incubation (X_2)	10	15	30	45	50

Media and their composition: Baker Yeast (*Sacchromyces cervesiae*) were grown in complete YPD (Yeast Peptone Dextrose) growth media which contained medium (1% w/v yeast extract, 2% w/v peptone, 5% w/v glucose) while locally isolated strain *Fusarium oxysporum* is maintained on Chick pea media at 30°C for 72 hours and was stored at 4°C till further process.

Inoculum preparation: Inoculum medium Sabroud Dextrose Broth 100mL were: glucose, 70g/L; yeast extract, 10g/L, peptone, 50g/L (Yu *et al.,* 2007). Duplicate sample of inoculums medium was prepared in 500 ml Erlenmeyer flasks closed with cotton plug (pH= 4.5) and autoclaved at 121°C for 15 min after closing the flask with cotton plug. The flasks were placed in rotary shaker 120 rpm at 28 ± 0.5°C. The cell count of *Sacchromyces cervesiae* and *Fusarium oxysporum* of about $3.6x10^6$ was obtained in obtained after 48 h and used as inoculum for future experiments.

Fermentation: Fermentation was carried out in duplicate by adding 2mL *Sacchromyces cervesiae* and *Fusarium oxysporum* inoculum containing 10^7 cells/mL of fermentation medium in 250 mL Erlenmeyer flasks containing maximum glucose yield. After fermentation at temperature 28 C at 24, 36, 48, 72 hours 1 mL sample were collected for further study.

Biomass determination: Cell density was measured at 600 nm spectrophotometer (Humas Think HS 3300, Korea) absorbance (Summer and Somers, 1944).

Reducing sugars: Reducing sugar like glucose concentration was determined by using 3, 5-dinitrosalicylic acid (DNS) reagent as described by Miller, 1959. Xylose concentration was also determined by modified method (Miller, 1959). A sample 50 µL was taken from filtrate, 350 µL Citrate buffer (pH=6.5) and 600µL of DNS were added the sample was boiled for 5 minutes immediately to stop the reaction. The absorbance was measured for reducing sugar measured at 540nm using spectrophotometer (Humas Think HS 3300, Korea).

Lignin Measurement: The lignin content in treated and untreated samples was hydrolyzed by using 1.25% H_2SO_4 for two hours and 72% H_2SO_4 for four hours. The residues were filtered and washed with distilled water for ten minutes to neutralized H_2SO_4 and then oven dried at 105°C for 10 hours to a constant weight. The amount of lignin was expressed by using the following formula (Krisztina *et al.,* 2009).

$$\text{Lignin} = \frac{\text{Lignin Weight}}{\text{Biomass}} \text{ X } 100$$

Determination of raw cellulose: Raw cellulose was determined by Weendize method described by Henneberg-Stohmann (1975).

Determination of ethanol content by dichromate method: Acid dichromate solution (0.1 M $Cr_2O_7^{2-}$ in 5 M H_2SO_4) was prepared by using 7.5g of potassium dichromate in dilute H_2SO_4 and the final volume was adjusted to 250 mL with deionized water. Standard curve was made by adding 300 µL of ethanol solutions into beakers containing 3 mL of acid dichromate. The beakers were sealed with Para film and kept at room temperature for 30 minutes. The absorbance was measured by using spectrophotometer (Humas Think HS 3300, Korea) at 590 nm to determine ethanol (Bennett, 1971).

Experimental design and statistical analysis: The Response surface methodology (RSM) applied in the present study is a central composite design (CCD) along with two different factors. In (CCD), 14 experiments were required for this procedure (Table 8). The selected dependent variable was temperature and independent variables were incubation time (15-45) minutes and dilute acid pretreatment (0.5-3%). The responses were detected after glucose, xylose and lignin analysis, and its statistical significance was determined by the *F*-test and its significance was determined by using Student's t-test. The coefficients of the equation and Analysis of variance (ANOVA) were determined by employing MINITAB 13 software. The response was obtained by designing the contour plots by setting two parameters (at three different levels each) and their interactions on the glucose and xylose yield and lignin degradation. The response of dilute acid concentration and time period was explained in quadratic regression model and expressed by the second order polynomial.

$$Y=b_o + \sum b_i x_i + \sum b_{ii}x_i^2 + \sum b_{ij}x_i xj \qquad /1/$$

Whereas Y is the value of the response, X is the coded value of the factors where i and j are linear and quadratic coefficients, respectively and b is a regression coefficient respectively. Significance of the design was determined by coefficient of determination R^2 and F-test. The Analysis of variance (ANOVA) and significance of coefficient was determined using Student's t-test applying MINITAB 13 software. The response surface equation was optimized for maximum lignin degradation, minimum glucose loss and high xylose solublization by using STATISTICA 7.0 software. Contour plots were used to determine the effect of the levels of two parameters (at five different levels each) which and their interactions on the yield of glucose, xylose and lignin degradation (Table 1).

Result and discussion

Glucose released: The cellulosic biomass utilization, xylose solubilizing and degradation of lignin by dilute acid treatment are an important step for recovery of glucose to produce bioethanol. Response surface methodology (RSM) is an efficient and rapid technique for optimizing the pretreatment conditions of biomass. The experiments were performed by using2^2 full factorial central composite design with four star points ($\pm$1.414) and five replicates. The two independent variables like dilute acid pretreatment and Incubation time were coded and used to determine the dependent variables glucose, xylose and lignin (Table 1). The quadratic equation /2/ is shown below for glucose

$$Y=-0.2975+0.0147*X1+1.4933*X2+0.0012X^22-0.0137*X1*X2-0.2213X^22 \quad /2/$$

The results obtained after pretreatment shown in Fig. 1 result indicate that 1.5% dilute sulphuric acid treatment for 30 minutes of incubation time give minimum glucose release. The regression results are described in Table 2 while the ANOVA results from quadratic equation described in Table 3. ANOVA show that model was significant due to high *F*-Value of 27.51 and have a low Probability value (P model > F- test). The regression coefficients and *P*-values, for glucose loss are shown in Table 2. Table 3 showed that the quadratic coefficients of X2, X1*X1, X2*X2 and X2 were found to be significant at <1% (p<0.001) whereas interaction coefficient was least significant *p*-values <0.005 (Table 3). ANOVA and *p*-values <0.001 are also important to determine the significance of the model the results also indicated that model was significant. The Coefficient of determination (R^2) value was 0.985 and the adjusted R^2 was 0.976. This indicated that model was good and is capable of explaining 98.5% of the variation in response. The response surface graph (Fig. 1) for glucose indicated that pretreatment 1.5 % dilute H_2SO_4 for 15 minute gives the lowest loss. The maximum glucose released was observed with 1.5% dilute H_2SO_4 treatment for 50 minutes (Fig. 1).

Table 2. Regression analysis of optimization of glucose yield.

Term constant	Coef	SE Coef	t-Test	p-value
Intercepts	2.4332	0.05489	44.328	0.000
X1	1.2444	0.06345	19.613	0.000
X2	0.4641	0.05933	7.822	0.000
*X1*X1*	0.4763	0.09243	5.153	0.001
*X2*X2*	-0.4978	0.08941	-5.567	0.001
*X1*X2*	-0.4095	0.10283	-3.983	0.004

R-Sq = 0.98.5%
R-Sq(adj) = 97.6%

Table 3. ANOVA (fit model) for glucose release after response surface methodology.

Source	Degree of freedom (df)	Sum of squares Seq. SS	Adj SS	Adj mean square (MS)	F-value	p-value
Regression	5	9.00391	9.00391	1.80078	106.98	0.000
Linear	2	7.81066	7.50492	3.75246	222.92	0.000
Square	2	0.92625	0.92625	0.46313	27.51	0.000
Interaction	1	0.26700	0.26700	0.26700	15.86	0.004
Residual error	8	0.13466	0.13466	0.01683	-	-
Lack-of-fit	3	0.06246	0.06246	0.02082	1.44	0.335
Pure error	5	0.07220	0.07220	0.01444	-	-
Total	**13**	**9.13857**				

Xylose released: Experiment result of Xylose was obtained after pretreatment as shown in Fig. 2 while the quadratic equation for Xylose shown below.

$$Y=2.3187+0.2524*X1+1.9895*X2-0.0033*X^22+0.0192*X1*X2-0.9586*X^22 \quad /3/$$

Three-dimensional response surface Fig. 2 shows the effect of Pretreatment and incubation time while at Z-axis show xylose solublization which indicates that 1.5% of dilute acid treatment for 30 minutes give highest xylose solublization. The regression results from Table 4 and the ANOVA results from quadratic equation described in Table 5 showed that the model was significant due to *F*-Value of 49.20 and a low Probability value (P model > F-test). The Table 4 shows that the regression coefficients of quadratic coefficients of X1, X2, X1*X1 and X2*X2

were significant at <1% (p<0.001) while interaction coefficient is least significant *P*-values <0.005 which show that X1*X2 are least significant interaction due to high value from 0.005. The R^2 (Coefficient of determination) value was 0.97 and the adjusted R^2 is 0.951 which indicates that the model is reliable. This model is capable of explaining 97% of the variation in response.

Lignin reduction: The lignin degradation is an important indicator of better treatment. The results obtained after pretreatment are shown in Fig. 3. Quadratic equation is shown below.

$$Y = 33.1669-0.4771*X1-14.3872*X2+0.0086*X^2 1 -0.0097*X1*X2+4.0994*X^2 2 \quad /4/$$

The decrease in the lignin content indicated the dilute acid treatment not only reduce the lignin contents it's also converted the Non-carbohydrate polymers into lower molecular weight phenolic compounds (Lee, 2007). The three dimensional graph (Fig. 3) indicates that 1.5% dilute acid treatment for 30 minutes was optimum for better lignin reduction. The regression results from Table 6 and the ANOVA results from quadratic equation as shown in Table 7 show that model was significant due to *F*- Value of 49.20 and a low Probability value (P model > F-test).The Table 8 show that the regression coefficients of quadratic coefficients of X1*X1 and X2*X2 were significant at <1% (p<0.001) while interaction coefficient is least significant *p*-value <0.005 which show that and X1*X2 are least significant interaction due to high value from 0.005 and the coefficients of X1 and X2 is not significant due to high *p*-Value. The R^2 (Coefficient of determination) value was 0.987 and the adjusted R^2 is 0.97.8 shows that the model was good and capable of explaining 98.7% of the variation in response. The overall results from Glucose loss, Xylose solublization and lignin degradation indicated that the treatment with 1.5% dilute sulphuric acid for 30 minute is best treatment.

Table 4. Results of regression analysis of optimization of Xylose released.

Term constant	Coef	SE Coef	t-Test	p-value
Intercept	8.461	0.1586	53.351	0.000
X1	1.741	0.1833	9.498	0.000
X2	-1.182	0.1714	-6.898	0.000
*X1*X1*	-1.327	0.2671	-4.967	0.001
*X2*X2*	-2.157	0.2583	-8.349	0.000
*X1*X2*	0.577	0.2971	1.943	0.088

R-Sq = 97.0%
R-Sq(adj) = 95.1%

Table 5. ANOVA (fit model) for xylose released after response surface methodology.

Source	Degree of freedom (df)	Sum of squares Seq. SS	Adj. SS	Adj. mean square (MS)	F-value	p-value
Regression	5	36.0803	36.0803	7.21607	51.35	0.000
Linear	2	21.7234	19.3624	9.68121	68.90	0.000
Square	2	13.8264	13.8264	6.91320	49.20	0.000
Interaction	1	0.5306	0.5306	0.53058	3.78	0.088
Residual error	8	1.1242	1.1242	0.14052	-	-
Lack-of-fit	3	0.8066	0.8066	0.26885	4.23	0.077
Pure error	5	0.3176	0.3176	0.06352	-	-
Total	**13**	**37.2045**				

Table 6. Results of regression analysis of optimization of lignin reduction (%).

Term constant	Coef	SE Coef	t-test	p-value
Intercept	13.3223	0.2489	53.520	0.000
X1	0.4182	0.2877	1.454	0.184
X2	-1.0060	0.2691	-3.739	0.006
*X1*X1*	3.8702	0.4192	9.233	0.000
*X2*X2*	8.5595	0.4055	21.109	0.000
*X1*X2*	-0.2924	0.4663	-0.627	0.548

R-Sq = 98.57%
R-Sq (adj) = 97.68%

Table 7. ANOVA (fit model) for lignin reduction after response surface methodology.

Source	Degree of freedom (df)	Sum of squares Seq. SS	Adj. SS	Adj. mean square (MS)	F-value	p-value
Regression	5	191.486	191.486	38.2971	110.63	0.000
Linear	2	0.981	5.571	2.7853	8.05	0.012
Square	2	190.368	190.368	95.1841	274.96	0.000
Interaction	1	0.136	0.136	0.1361	0.39	0.548
Residual error	8	2.769	2.769	0.3462	-	-
Lack-of-fit	3	1.681	1.681	0.5604	2.57	0.167
Pure error	5	1.088	1.088	0.2177	-	-
Total	13	194.255				

Table 8. Dilute acid pretreatment and time of incubation (%).

	Coded level		Actual value	
	Time of incubation	Dilute acid pretreatment (%)	Time of incubation	Dilute acid pretreatment (%)
1.	-1	-1	15	0.5
2.	0	0	30	1.5
3.	0	0	30	1.5
4.	1	-1	45	0.5
5.	0	0	30	1.5
6.	-1	1	15	3
7.	1	1	45	3
8.	1.41421	0	50	1.5
9.	0	0	30	1.5
10.	0	-1.41421	30	0.25
11.	-1.41421	0	10	1.5
12.	0	1.41421	30	3.25
13.	0	0	30	1.5
14.	0	0	30	1.5

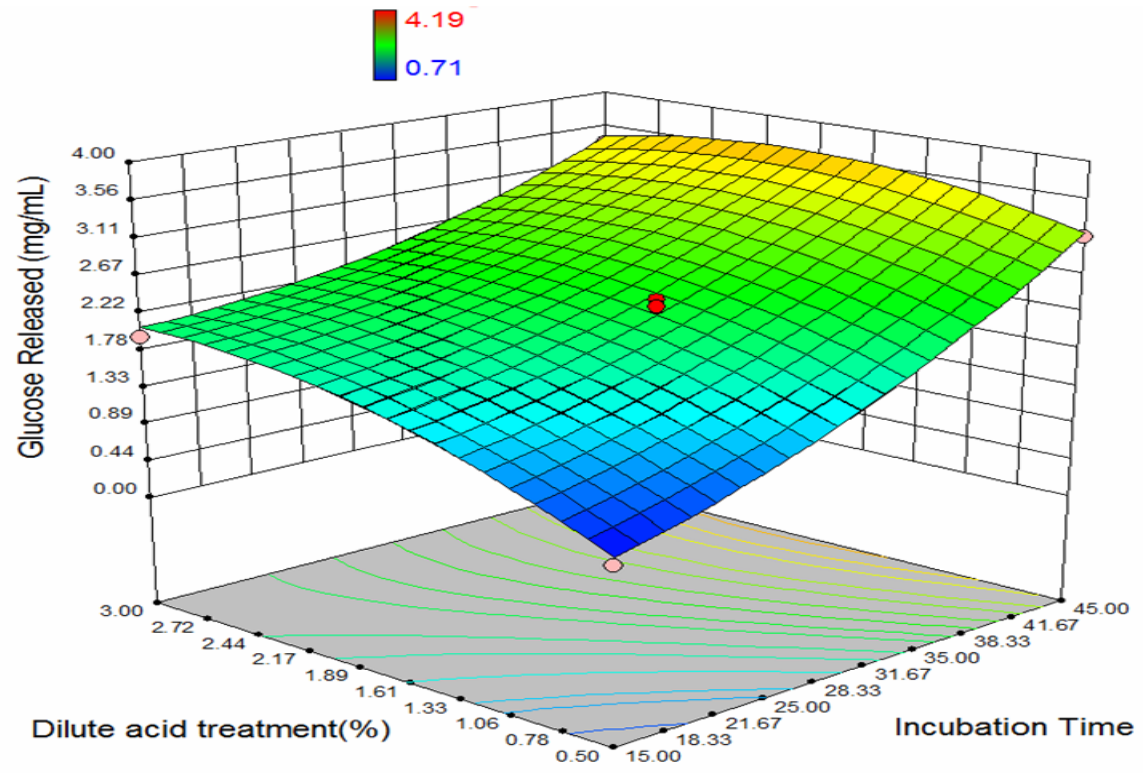

Fig. 1. Response surface graph of Glucose release from different treatment of acid at 100 ^{0}C with varying the residence time.

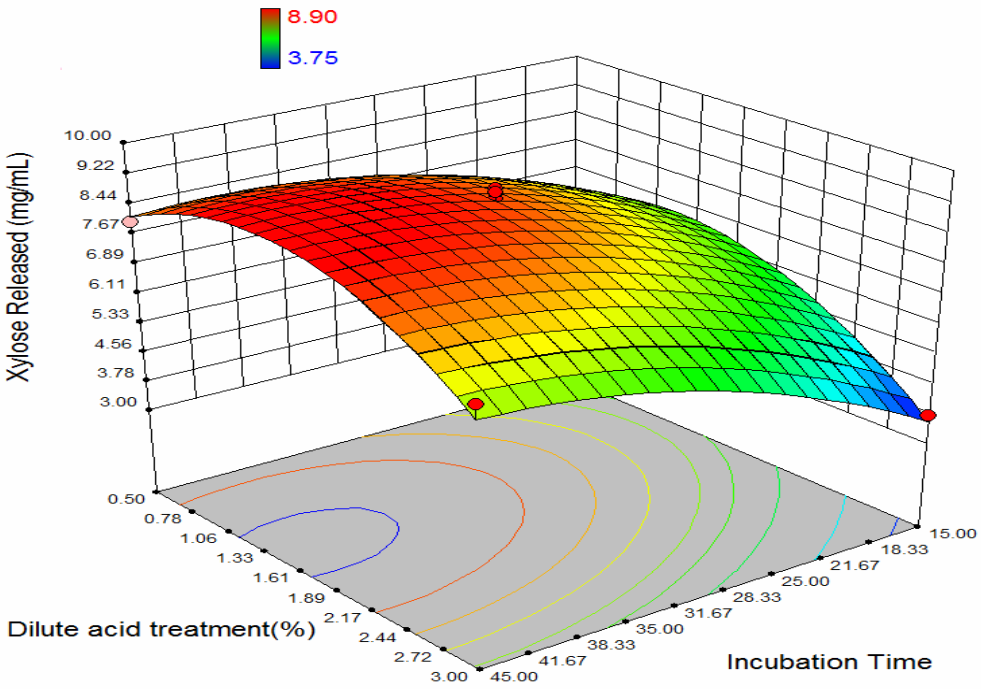

Fig. 2. Response surface graph of Xylose released from different treatment of acid at 100°C with varying the residence time.

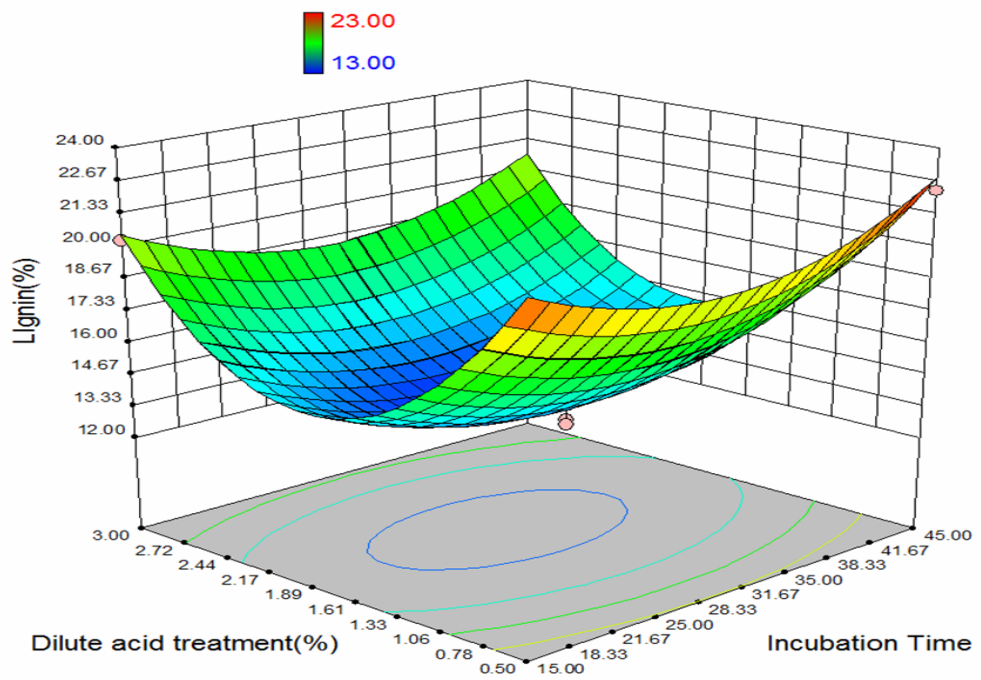

Fig. 3. Response surface graph of Lignin reduction from different treatment of acid at 100°C with varying the residence time.

Removal of inhibitory compounds: The cellulose content determined by Weendize method was 21.4% and the lignin content was 27%. Dilute acid pretreatment of rice polish influences the released of glucose and are also produced inhibitory compounds like furfural, hydroxymethyl furfural and phenolic compound. To avoid these inhibitory compounds, the slurry was filter and residue washed several time with distilled water and dried at 40°C for 24 hours. The remaining residue was selected for enzymatic hydrolysis.

Enzymatic hydrolysis: Higher concentration of glucose was obtained with cellulase (Celluclast) as reported by Krisztina *et al.*, (2009). Higher saccharification rate accepted in presence of higher enzyme concentration. The hydrolysis of cellulose in pretreated sample is key step for

production of ethanol. Cellulose hydrolysis by cellulose increased by increasing porosity and surface area of biomass. The high saccharification rate was expected in the presence of higher enzyme concentration (Latif *et al*., 1994; Wayman *et al*., 1992; Vancov & McIntosh, 2011). The cellulase in rice polish buffer medium had significant ($p<0.05$) impact on the conversion of cellulose to sugars. Fig. 4 revealed that an enzymatic load 1ml/2g substrate gave maximum glucose recovery. The Substrate concentration was optimized at 2% which give maximum released of glucose.

Our results are comparable with the results of Yu *et al,* (2007). The enzymatic hydrolysis is a more effective way to obtain the reducing sugars leading to the production of bioethanol (Wayman *et al*., 1992). The glucose released with 1 mL cellulase load was 16.52 mg/mL, whereas 14.32, 13.22 and 12.69 mg/mL of glucose obtained by using 0.75, 0.5 and 0.25 mL of enzyme load respectively. Enzymatic load 1 mL gave the highest conversion cellulose into glucose and the results obtained after saccharification were in line with previous finding (Rishen *et al*., 2007; Hari *et al*., 2001; Sedlak & Ho, 2004). At high substrate concentration, low saccharification rate has been reported even when high enzymatic load was used (Szczodrak, 1988). Results obtained after dry biomass indicated that the dilute acid pretreatment enhanced enzymatic saccharification. With the passage of time, the biomass production was also increased (Fig. 5) and an enzymatic load 1mL gave highest yield of ethanol, Moreover dry pretreated sample resulted high ethanol production as compared to slurry (Fig. 6).

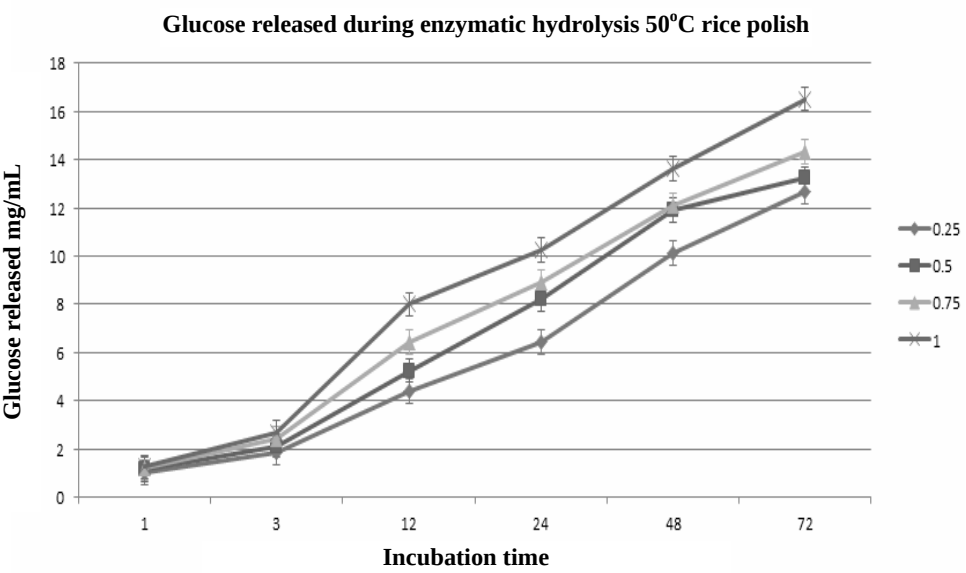

Fig. 4. Different concentration of cellulase load on 2g pretreated rice polish. Glucose are presented as mg/mL and presents averages of experiments.

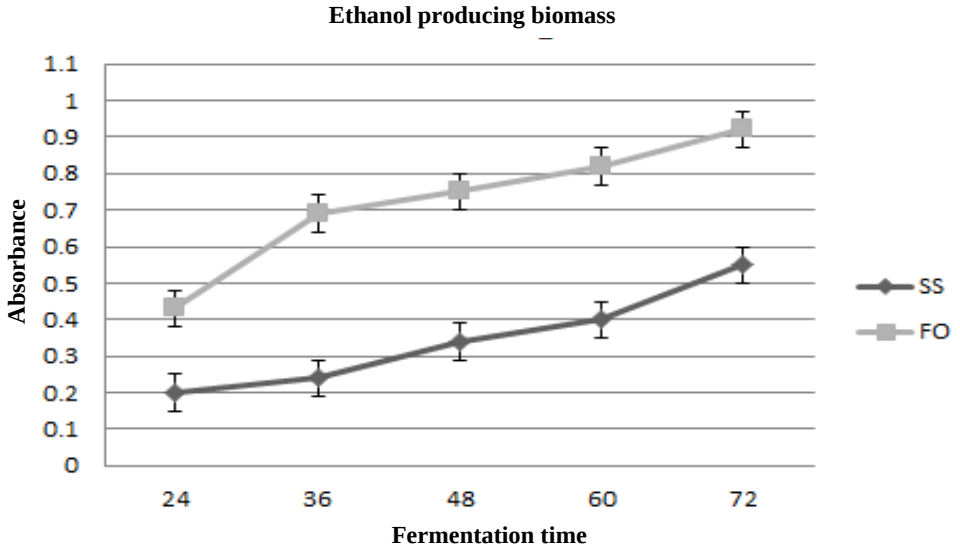

Fig. 5. Biomass growth at different time interval.

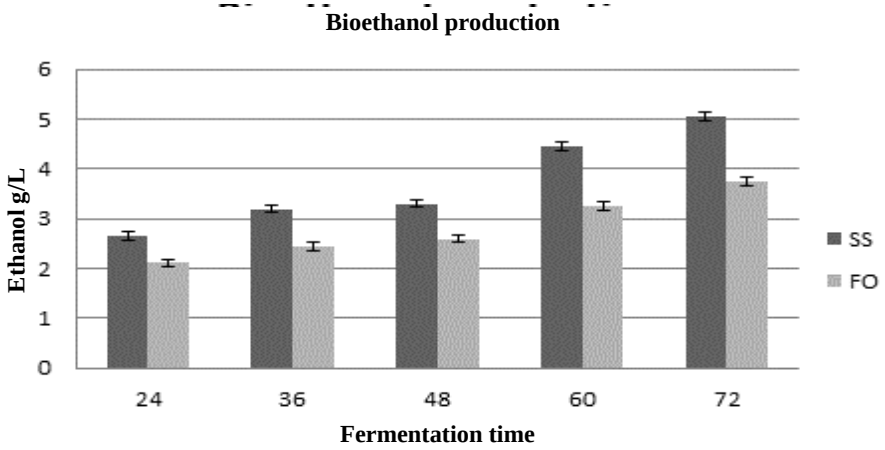

Fig. 6. Ethanol yield after different time interval.

Conclusions

This research was designed to utilize rice polish, a product of rice mills. The study showed that response surface methodology is reliable tool for optimizing the pretreatment of biomass for ethanol production. The enzymatic hydrolysis of dry pretreated samples gave better glucose recovery. Inhibitory compounds (furfural and hydroxymethyl furfural and phenolic compounds) were removed by filtering the pretreated samples and drying the biomass. Finding of this research work will be helpful in near future to utilized the rice polish for the ethanol production on industrial scale which may be used as cheaper fuel by blending ethanol with petrol to save foreign exchange.

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