PHILIPP AGRIC SCIENTIST
Vol. 104 No. 3, 259-276
September 2021

ISSN 0031-7454

Optimization of Ethanol Production from Enzymatically Saccharified Biomass of Acid-Pretreated Rice Straw

Muzammal Mateen Azhar[1], Qurban Ali[1,*], Arif Malik[1], Elham Khalili[2], Muhammad Arshad Javed[3], Shinawar Waseem Ali[4], and Muhammad Saleem Haidar[4]

[1]Institute of Molecular Biology and Biotechnology, University of Lahore, Lahore, Pakistan
[2]Department of Plant Science, Faculty of Science, Tarbiat Modarres University, Iran
[3]Department of Plant Breeding and Genetics, University of the Punjab Lahore, Lahore, Pakistan
[4]Department of Food Science, University of the Punjab Lahore, Lahore, Pakistan
[5]Department of Plant Pathology, University of the Punjab Lahore, Lahore, Pakistan

[*]Author for correspondence; E-mail: saim1692@gmail.com

Received: January 10, 2021/ Revised: June 01, 2021/ Accepted: August 05, 2021

The substrate of rice (*Oryza sativa*) straw employed in this study was procured from local market and stored in polyethene bags until needed. The study was conducted to find out the effects of process optimization of ethanol production from enzymatically saccharified biomass of acid-pretreated rice straw. The results indicated that acid pretreatment has reduced lignin content about 50%. Resultantly, pretreatment raised cellulose contents up to 52% making it suitable for enzymatic saccharification. Optimization of saccharification illustrated that parameters such as biomass, enzyme concentration, temperature, incubation time and agitation indeed affected the action of cellulase on pretreated biomass releasing sugars up to 31.0g/L. The results clearly indicate the need for optimization at every point in the ethanol production process for efficient use of resources and maximum yield without wastage. Among various operational modes of fermentation studied, such as batch, semi-continuous and continuous, the semi-continuous process gave better ethanol yield of 23.4 g/L. This implies potential use of the process in industrial grade ethanol production. Collectively the study has shown that rice straw can serve as a lignocellulosic substrate, utilizing acid pretreatment for cellulose exposure and enzymatic saccharification to release fermentable sugars for production of ethanol.

Keywords: *Oryza sativa*, enzymatic saccharification, lignin, cellulose, ethanol, fermentation

Abbreviations: CCD - central composite design, DNS - 3,5-dinitrosalicylic acid, RSM - response surface methodology, PBD - Plackett-Burman design

INTRODUCTION

With the advent of mechanization, man has been forced to opt for various forms of vehicular transport. With the growing population, an increase in need for transport is necessary to support flow of people and goods between and within areas. The flow is currently maintained with a system of different modes of transport all heavily dependent on one thing: petroleum based fuel. Since these fuels were abundant, so it prompted a boost in increase in vehicle sales and promotion in the motor vehicle sector. A direct linkage of this increase in motor vehicle usage and population has manifested itself in a predicted shortage in fuels, particularly oils. Though the fuel shortage is expected or theoretical to occur in another ten years, critics speculate a major physical and political crisis to occur in the coming years unless oil production is increased to meet worldwide demand (Bentley 2002; Bejan et al. 2014; Campbell et al. 2013: Razzaq et al. 2019). The prediction is supported by analysts that countries such as Canada, Venezuela and Russia will have to start increasing production in shale oil and oil sands to meet worldwide demand (Greene et al. 2004). Oil demand peaks prior to 2020 and falls to 86.5 million b/d in 2020 and falls to 74.6 million b/d by 2030, under this high tech, high penetration scenario that also includes pro-renewables policies that promote growth in wind and solar electricity to 16% of global electricity generation, up from 3% currently (WEF 2020). So an alternative source is required for fulfilling fuel requirements in an environmental friendly perspective. Lignocellulosics are the currently being employed in the industry for bioethanol production (Rosales-Calderon and Arantes

2019). They are prime candidates for second generation biofuels, whereas first generation biofuels required direct use of molasses for production of fuels (Moniruzzaman 1996). They include plant biomass that majorly consists of cellulose with a smaller part designated to lignin and hemicellulose, which depends upon physiological characteristics of the plant, hence the term lignocellulosic substrate. A main source for such substrate is being tapped from agricultural wastes of cereal crops and weeding activities on farmland, as well as industrial wastes from processing of crops. Major agricultural wastes being employed are of wheat, rice, corn, sugarcane alongside weeds and grasses which have no food value but can serve as substrates for fermentation (Itelima et al. 2013; Wi et al. 2013; Kim et al. 2013). The study was conducted to find out the effects of process optimization of ethanol production from enzymatically saccharified biomass of acid-pretreated rice straw. To evaluate the use of specific substrates for optimized fermentation.

MATERIALS AND METHODS

Substrate

The substrate of rice straw (*Oryza sativa*) employed in present study was procured from local market and stored in polyethene bags until needed.

Physical Treatment

The substrate of 100 g rice straw was crushed using a hammer-beater mill to a mesh size of 2 mm and oven dried at 60 - 80°C for 15 min. Then crushed substrate was stored in a polythene bag at 37°C room temperature for further use (Supplementary material Figure S1).

Chemical Treatment

Chemical treatment was carried out by soaking physically treated substrate in 2% H_2SO_4 solution in the ratio 1:10 (w/w) in an 1000 ml Erlenmeyer flask up to 24 hrs. The flask was covered with aluminum foil during processing.

Physiochemical Treatment

After soaking, the substrate was autoclaved at 121°C for 90 mins and then filtered through muslin cloth. The treated sample was washed thoroughly with tap water till neutrality. The washed substrate was then allows to dry in a hot air oven at 100°C for 30 min. The filtrate obtained during washing was also stored for further analysis (Supplementary material Figure S2).

Saccharification

Response surface methodology was used to work out optimization for saccharification conditions in rice straw. The process was implemented by using technique described by Pervez et al. (2014). *Saccharomyces cerevisiae* ML-6 used in the present study for ethanol production was procured from microbiology lab, Food Biotechnology Research Centre, PCSIR Laboratories, Lahore (Supplementary material Figure S3). The strains were re-cultured and inoculation media was prepared for fermentation (Supplementary material Figure S4 and S5). Three modes of fermentation were studied, differing in medium composition from one another and carried out at 30°C, the ambient temperature for *Saccharomyces cerevisiae* activity. The types studied were as followed: Batch Fermentation (Supplementary material Figure S6), Semi continuous Fermentation (Supplementary material Figure S7) and Continuous Fermentation (Supplementary material Figure S8).

Estimation of Sugars

The 3,5-dinitrosalicylic acid (DNS) method outlined by Miller (1959) was used to determine reducing sugars in filtrate(s) collected from the washings of the substrate. The phenol-sulfuric acid method by Dubois et al. (1956) was used for measuring total sugar content of filtrate(s). The method of Sanz et al. (2005) was used for measuring total phenol content in filtrate(s) from pretreatment.

Total phenol % = (Absorbance ofsample)/(Absorbance of standard) × concentration of standard Van Soest's (1991) method was used for estimating NDF (Nutrient detergent fibre), ADF (Acid detergent fibre), hemicellulose content in substrate.

(i) %NDF = (Y-X)/W × 100

(ii) %ADF = (Y-X)/W × 100

Hemicellulose was calculated by the formula given below:

(iii) %Hemicellulose = %NDF - %ADF

(iv) % cellulose = (Y-L)/W×100

(v) %lignin = (L-A)/W×100

where, Y = weight of ADF + crucible, W = weight of sample, L = weight of lignin + crucible, A = weight of ash + crucible

Standard Curve for Reducing Sugars

The standard curve for reducing sugars was made according to the method outlined by Miller (1959) (Supplementary material Figure S9). The method outlined by Sanz et al. (2005) was used to plot a standard curve for phenolic compounds (Supplementary material Figure S10). The method used for standard curve for total sugars was from Dubois et al. (1956) (Supplementary material figure S11). The method outlined by Caputi et al.

(1968) was used to formulate a standard curve for ethanol (Supplementary material Figure S12).

Enzyme Assay

An filter-paper cellulase (FPase) and endo-glucanase (CMCase) assay highlighted by Zhang et al. (2009) was done to find out activities for the cellulase employed in saccharification.

Estimation of Ethanol

The method by Caputi et al. (1967) was used to estimate the ethanol in the fermentation broth. High Performance Liquid Chromatography was used to estimate ethanol content in distillates from different fermentation processes.

Statistical Analysis

The data was recorded for various chemicals obtained from fermentation which was subjected to statistical analysis by using Statistica v. 10.0.2 software. For the evaluation of interactions among various factors including biomass concentration (X1), temperature (X2), Incubation period (X3), enzyme concentration (X4) and agitation speed (X5), linear regression analysis was carried out.

RESULTS AND DISCUSSION

Effect of Pretreatment on Composition of Rice Straw

After pretreatment of rice straw with 2% H_2SO_4, proximate analysis of both untreated and pretreated samples revealed 34% and 64.4% cellulose respectively, giving an increase of 52.79% in cellulose content as shown in table 1. Lignin content decreased from 9.66% to 4.8% showing delignification of 49.6%. A decrease in hemicellulose was also recorded from 27% to 15%. The filtrate contained a total sugar content of 0.202 g/L compared to reducing sugar content of 0.291 g/L as shown in table 1a. Phenolic compounds were also generated which were at a concentration of 4.0 mM. The control substrate contained 34.0% cellulose, 9.66% lignin and 27% hemicellulose content. Results for percentage composition are in agreement with those obtained by Roberto et al. (2004) and Maiorella (1985) who have stated the range of cellulose as 32-47%, hemicellulose 19-27% and lignin 5-24%. Pretreatment of substrate revealed a boost in cellulose content i.e. 64.4% whereas a decrease in both lignin and hemicellulose contents at 4.80% and 15% respectively.

Similar results were obtained by Anwar et al. (2012) who found out by RSM that treatment with 1.5% H_2SO_4 at 100°C for 15 mins was found most useful for maximal sugar release, especially lignin removal. Li et al. (2012) found similar cellulose contents i.e. 35.4% and 8.3% lignin with different treatments using microwave-assisted lye pretreatment. Though Li's team used microwave treatment at high firepower with 1% lye for 1hr to achieve 46.8% cellulose, the current study gave higher cellulose contents from treatment with 2% H_2SO_4 for 90 mins. This might be due to the fact that the control sample already contained 34.0% cellulose to begin with and that acid pretreatment under milder conditions for a longer time produced efficient solubilization of hemicellulose fraction as well as lignin, thereby increasing ratio of cellulose. The difference can be attributable to the variability in chemical use and type of pretreatment where the former used microwaves and NaOH whereas current study involved dilute H_2SO_4 and steam pretreatment. Gupta and Sharma (2008) reported that use of higher pressures of 20 psi and 25 psi resulted in solubilization of cellulose and hemicellulose but produced more furfurals revealing hydrolysis of liberated sugars.

Effect of Various Parameters on Saccharification of Pretreated Rice Straw

To study the effects of potential yet important factors on the enzymatic saccharification process, various parameters were tested using statistical designs generated via Statistica v.10.2.2. Potential factors studied were biomass concentration (X1), temperature (X2), Incubation period (X3), enzyme concentration (X4) and agitation speed (X5).

Saccharification of Pretreated Rice Straw by Using PB Design Under Various Factors at Two Levels

The Plackett-Burman design (PB) was used to determine significance of the factors chosen. The design featured 8 runs with each factor having two levels. In all eight runs, experiment 1 stood out with maximum reducing sugars released i.e. 14731.46 μg/ml after 24 hrs with an enzyme loading of 2.5 g at 55°C at 150 rpm (Maheshwari et al.

Table 1. Composition of untreated and pretreated rice straw on dry mass basis.

Percentage Composition (%)			
Sample	Cellulose	Lignin	Hemicellulose
Untreated	34.0±1.50	9.66±0.40	27.0±1.05
Acid Pretreated	64.40±1.88	4.80±0.19	15.0±0.52

Mean value ± standard deviation among triplicates.

Table 1a. Analysis of filtrate produced during acid pre-treatment from rice straw.

Composition of Filtrate		
Reducing Sugars (g/L)	Total Sugars (g/L)	Total Phenols (mM)
0.291±0.010	0.202±0.007	4.0±0.16

Mean value ± standard deviation among triplicates.

2000; Tong et al. 1980) as shown in Table S2. The smallest p value of 0.004599 and larger t-value of 14.69557 for phenols suggests that the model is significant in terms of phenols (Table S3: Supplementary material). These results indicate that optimization of various factors are very much important to obtain maximum yield through saccharification process. Hence, optimization was done to seek out the degree of significance of the chosen parameters (Table S4: Supplementary material). Response surface methodology (RSM) was used to screen for significant parameters and optimize chosen parameters. The screening process involved testing for significance of five factors: biomass (X1), temperature (X2), incubation time (X3) and enzyme loading (X4) and agitation speed (X5). This was accomplished by using a design generated by Statistica v.10.0.1: a Plackett Burman design (PBD) featuring two levels: +1 and -1 with 8 runs. Screening gave correlation coefficients (Multiple R) for reducing sugars (RS), total sugars (TS) and phenolic compounds (phenols) at 0.842, 0.796 and 0.997 respectively, indicating the goodness of fit for the model chosen for independent parameters RS, TS and phenols. The factor showing significance in the model was X4 supported by p value of $0.0046 < p = 0.05$ and t-coefficient of 14.697. Maximum reducing sugars were observed for experiment 1 where RS reached 14731.46 µg/ml at reaction conditions of 5g biomass, 2.5g enzyme loading at 55°C with agitation at 150 rpm for 24 hrs.

Effect of Various Factors on Reducing Sugars in Saccharification of Pretreated Rice Straw

A central composite design was generated using Box-Behnken model with the five factors previously selected for the PB design at five levels. As shown in Table 5, the highest observed value of reducing sugars was attained in experiment 13 with 302.3 µg/ml after 2 days at 5.0 g biomass concentration, 1.0 g enzyme loading and temperature at 50°C and agitation at 250 rpm compared with the expected or theoretical value at 316.46 µg/ml. Although the expected or theoretical value for experiment 15 seemed more promising with 334.11 µg/ml, the experiment yielded 300.78 µg/ml reducing sugars after 4 days at 5.0 g biomass concentration, 1.0 g enzyme concentration at 50°C at 250 rpm. Similar conditions for experiment 16, with the exception of change in agitation to 150 rpm and enzyme concentration at 2.5 g, a slightly lower yield at 290.11 µg/ml, which was more than 238.02 µg/ml. The results indicated that agitation had a formidable effect on the amount of reducing sugars released during the saccharification of pretreated biomass. The different sugars behave differently in different assays, so if have a mixture of different monosaccharides they will not all respond to the same degree. Most likely have something in our sample interfering with the type of assay. Any other aldehydes or ketones in sample would certainly be an issue for lower concentration of reducing sugars.

Optimization was done using central composite design (CCD) specifically the Box-Wilson design with five levels: -α, -1, 0, +1, +α and 31 runs for five parameters as chosen for PBD. The correlation coefficients (Multiple R) for RS, TS and phenols were 0.912705, 0.936 and 0.937 respectively showing that the goodness of fit for the design used, thus the model was significant for the three output parameters. The model is further supported by the corresponding F values of 3.815198, 5.427 and 5.494 and respective p values of 0.009, 0.0018 and 0.002 where $p <$ 0.05. The Pareto charts showed significant parameters and their interactions where agitation speed, enzyme loading-temperature and biomass emerged as significant for the design implemented. From the optimized conditions of biomass 124 g in 2 L of citrate buffer at pH 4.8 and enzyme loading of 35 g/L, incubated at 58°C with agitation at 240 rpm for 115 hrs, saccharification of the pretreated rice straw gave a reducing sugar content of 31 g/L, 3.1% sugar content. The reason behind such an output has been described by Gupta et al. (2014) in context of cellulases.

The Pareto chart for the reducing sugars (Fig. 1) shows the variable X5 (agitation speed) with t-value 3.939457 to be significant among the rest of the parameters, thus highlighting the role of agitation in the saccharification process for releasing maximum reducing sugars. The highest F value 15.5192 and lowest p value of 0.001695 further supports the model for reducing sugars in terms of agitation speed (Table S6 Supplementary material).

The interaction between enzyme concentration (X4) and agitation speed (X5) is visible from the response surface plot shown in Fig. 2. Increase in enzyme concentration as well as agitation speed show a shift towards desirable effects on reducing sugar yield. A

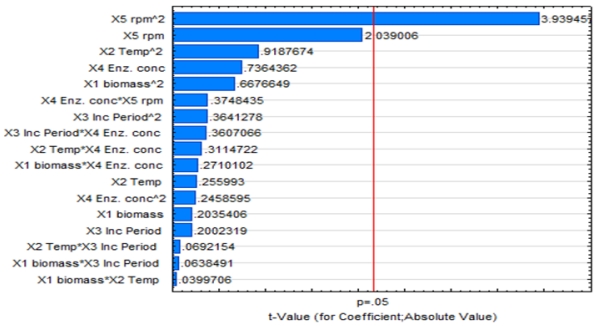

Fig. 1. Pareto chart showing t-value coefficients for reducing sugars at 0.05 sigma restriction generated by Statistica v.10.0.1.

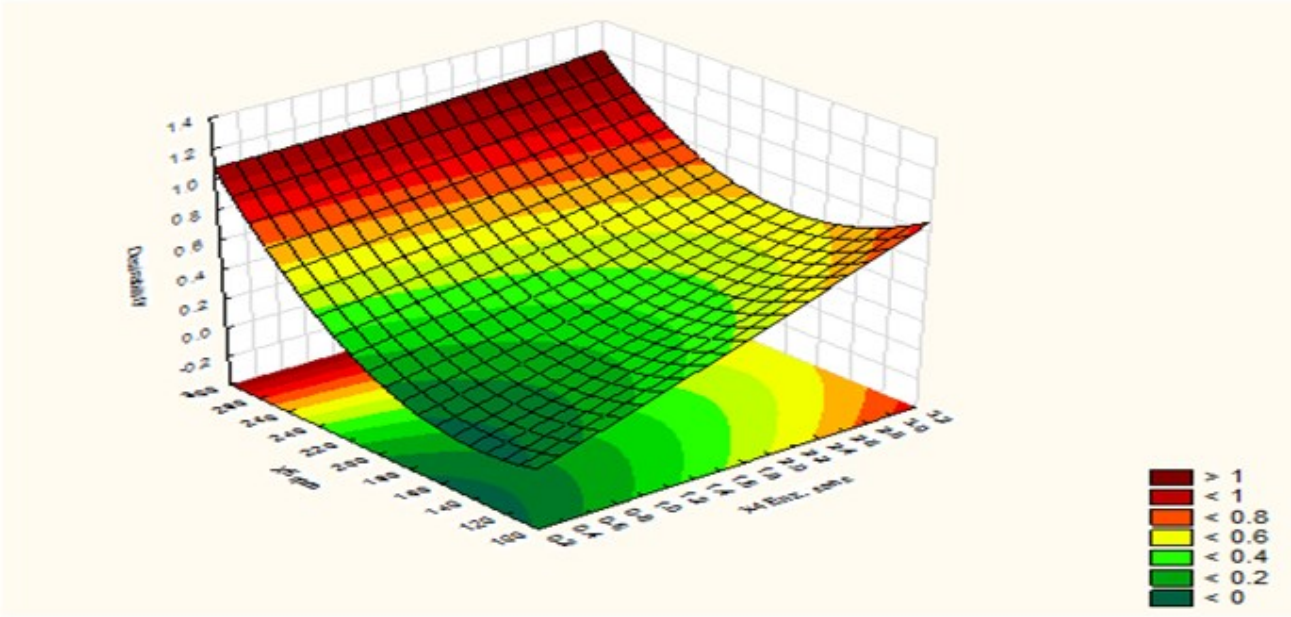

Fig. 2. Response surface plot showing effect of interaction between Enzyme concentration (X4) and Agitation speed (X5) on Reducing sugar yield generated by Statistica v.10.0.1.

decrease in both parameters beyond 1.8 g and 240 rpm respectively, results in decrease in desirability, where agitation speed is seen most significant.

Effect of Various Factors on Total Sugars During Saccharification of Pretreated Rice Straw

When total sugar yield is considered, the model yielded a maximum of 3645.33 µg/ml after 4 days which was considerably higher than the expected or theoretical value of 3568.740 µg/ml for experiment 15 with 5.0 g biomass loading, 1.0 g enzyme loading at 50°C at 250 rpm. A slightly lower yield was observed for experiment 13, similar to experiment 15, with 3423.976 µg/ml after 2 days, lower than the expected or theoretical yield of 3578.529 µg/ml. The lowest yield was observed for experiment 28 with 1027.590 µg/ml total sugars in comparison with expected or theoretical value of 1428.200 µg/ml after 3 days at 40°C, 3.5 g biomass concentration and 1.75 g enzyme concentration. The results obtained in table S7 (Supplementary material) depict the significance of enzyme concentration as well as biomass concentration and temperature. The saccharification process depends on two main factors among pH, temperature and nature of substrate i.e. DM or dry mass and enzyme concentration. Humbird et al. (2011) gives the average dosage in an industrial process in terms of g substrate per g cellulase as 20 mg per g substrate (DM) for well treated substrates when using state-of-the-art enzymes such as Novozymes. DM is the second important factor for the success of a reaction. If high DM is used, the process would be made difficult in terms of stirring and would require more enzymes for a good sugar output. But then again, a high DM content is required for a larger ethanol concentration and efficient distillation. In this regard, Wingren et al. (2008) pointed out that a DM near to 15-20% has been globally accepted for a feasible process. Gao et al. (2014) reports the Separate Hydrolysis and Fermentation process to be more feasible in terms of sugar production when using alkali pretreated sugarcane bagasse. Their study indicated that a high solid loading of 25% with 8.3 U/g substrate gave 79.53 g/L reducing sugars after 120 h into the saccharification when implemented using fed batch

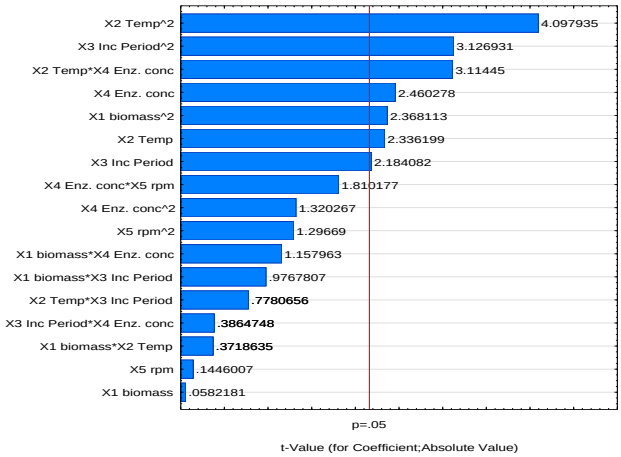

Fig. 3. Pareto chart showing *t*-value coefficients for total sugars at 0.05 sigma restriction generated by Statistica v.10.0.1.

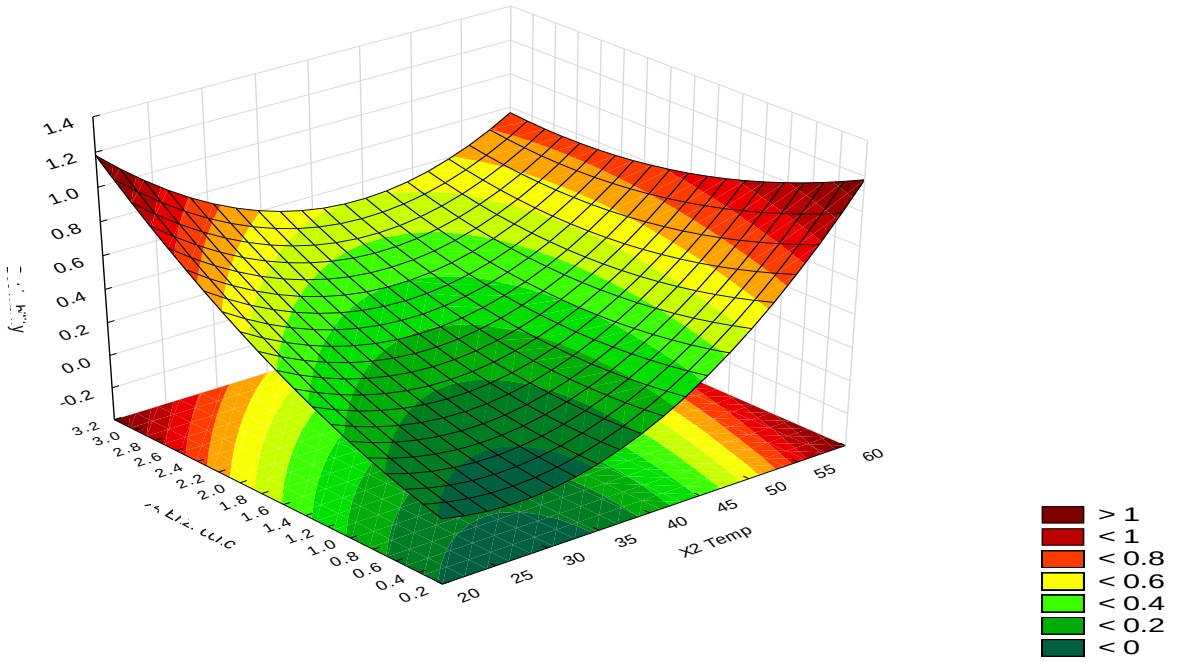

Fig. 4. Response surface plot showing effect of interaction between Enzyme Concentration (X4) and Temperature (X2) on Total sugar yield generated by Statistica v.10.0.1.

process. Song et al. (2011) proposed that presence of lignin may affect the activity of cellulose and hinder the enzymatic hydrolysis reducing sugar conversion rates. A higher DM may also cause issues with diffusion of enzyme due to lack of free water as stated by Ingesson et al. (2001).

The Pareto chart in Fig. 3 depicts the interaction between temperature and enzyme and significance of biomass concentration, temperature and enzyme concentration is illustrated by the highest t-values for temperature (4.097), then incubation period (3.127) following enzyme concentration (3.114) and biomass (2.368113). The significance is further supported by the high *F* value for temperature (16.793) and lowest *P* value recorded at 0.0013 for temperature alone and the enzyme-temperature interaction at 0.0082 (Table S4: Supplementary material). Both t-values and *P* values hence show the model was significant in terms of total sugars in saccharification. Ethanol production at the industrial level majorly uses the cheapest available yeast, Saccharomyces cerevisiae which has been used since history for its ability to ferment hexoses mainly glucose to ethanol. The ethanol fermentation process is driven by substrate concentration i.e. glucose which is linked with ethanol production. In the current study, batch fermentation using fermentation media gave 22.0 g/L ethanol (w/v). Since the process was run as batch mode, no additional medium was fed to the existing bioreaction. The drop in glucose levels was slow at 6hrs suggesting the adaptation phase of the yeast to the medium. The greatest drop was noted from 24 hrs to 72 hrs where residual indicating the shift from exponential phase to stationary phase with residual glucose at 1.89 g/L. The growth pattern suggests ethanol production is growth associated with biomass increasing from 0.21 g/L at 6hrs to 2.07 g/L at 72 hrs where ethanol yield ranged from 11.93 g/L at 24 hrs to 22.77 g/L at 72 hrs after which minimal change was noted. The meager changes after 72 hrs corresponds with Levenspiel's (1980) notion that very low substrate concentration result in exhaustion of carbon source as well as starvation of yeast cells resulting in low productivity. The results also help in establishing the characteristic of the ML-6 strain of consuming glucose within 72 hrs with maximum ethanol production.

The interaction X4^X2 had a *P* value 0.008 (Table 8: Supplementary material) hence was found significant. Increasing temperature and enzyme concentration had a profound effect on desirability of total sugar yield as shown by Fig. 4 whereas a decrease in both factors, especially temperature below 55°C has less desirable effect on total sugar yield.

Effect of Various Factors on Phenolic compounds During Saccharification of Pretreated Rice Straw

When studying the phenolic compounds during saccharification, the model gave the highest concentration

of phenols in experiment 24 at 32.129 mM rather than the predicted 33.130 mM, observed after 3 days of incubation at 40°C with 3.5 g biomass, 3.25 g of enzyme at 200rpm. In contrast, experiment 23 gave the lowest phenols at 0.406 mM which were produced in otherwise same conditions except with 0.25 g of enzyme. A slightly lower phenol count of 31.634 mM, compared to the expected or theoretical 27.057 mM was recorded with experiment 16 after 4 days of incubation at 50°C with a higher biomass loading of 5.0 g and 2.5 g of enzyme at 150 rpm. The results in table S9 (Supplementary material) indicated the connection of biomass and slightly of enzyme concentration with the phenol concentration during saccharification.

The Pareto chart in Fig. 5 for the variable phenols displays biomass with a higher t-value coefficient of 2.276 than enzyme concentration at 2.146 pushing toward more significance of biomass loading in the model. The notion is further strengthened by the lowest *P* value at 0.040 for biomass concentration (Table S10: Supplementary material), showing that the model was significant in terms of phenols.

The response plot in Fig. 6 shows interaction between Incubation period (X3) and Agitation speed (X5). An increase in rpm beyond 200 rpm brings about greater increase in desirability, as is the case for incubation period which has more desirable effects beyond 3 days.

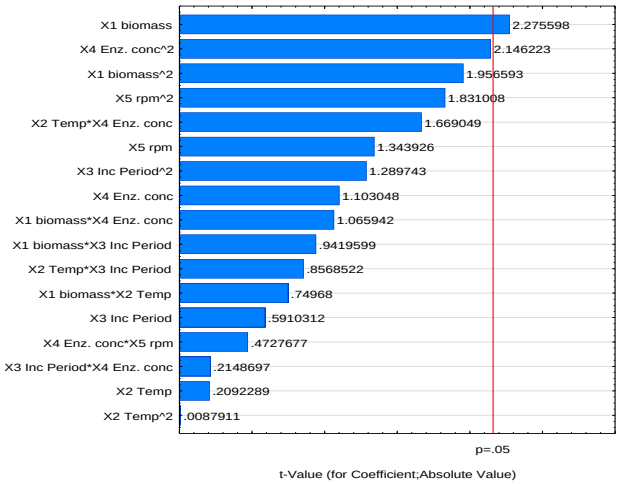

Fig. 5. Pareto chart showing t-value coefficients for phenolic contents at 0.05 sigma restriction generated by Statisitica v.10.0.1.

Decrease in both factors result in lower desirability below the aforementioned values in terms of phenolic compounds.

Comparison of Ethanol Contents and Sugar Utilization in Different Modes of Fermentation Batch Fermentation Utilizing Fermentation Medium

The batch fermentation process was carried out in fermentation medium for 10 days where ethanol content

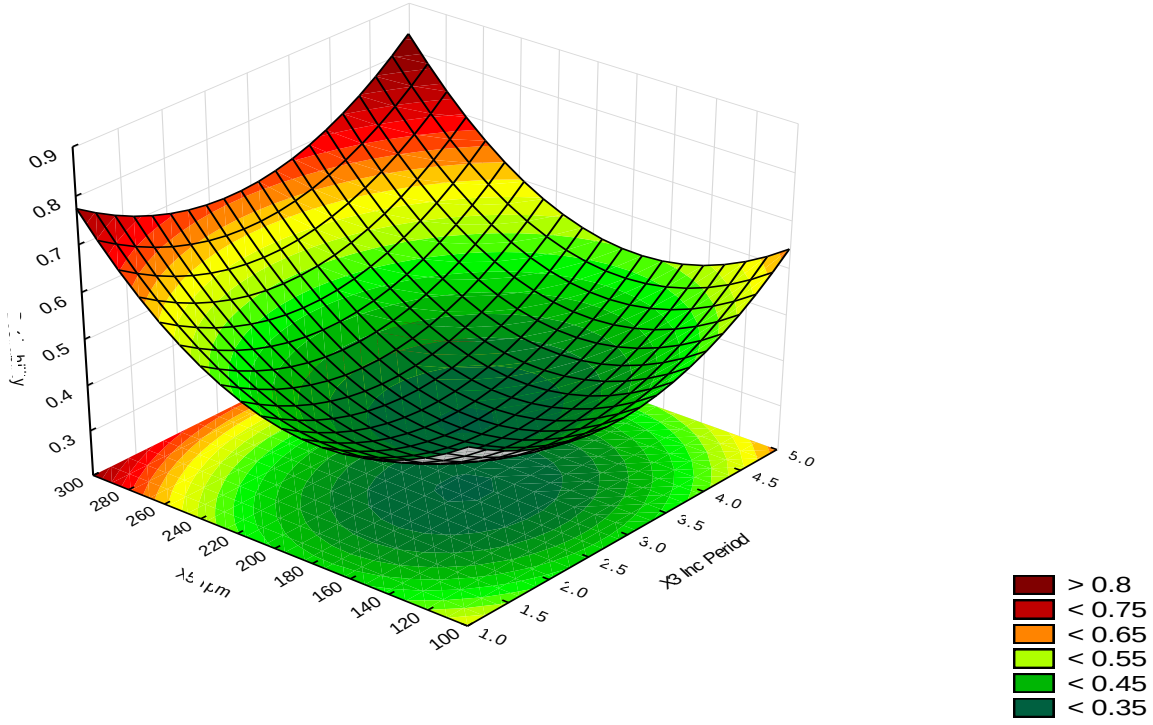

Fig. 6. Response surface plot showing effect of Incubation period (X3) and Agitation speed (X5) on phenolic compounds generated by v.10.0.1.

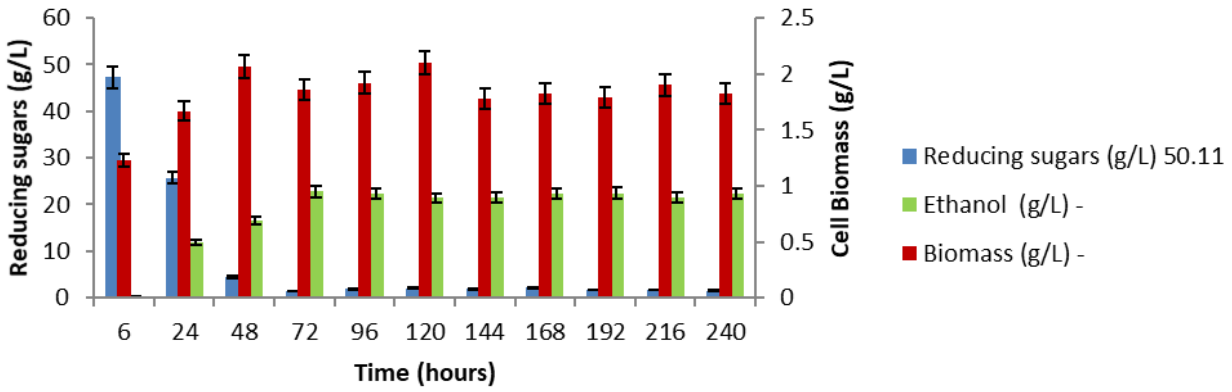

Fig. 7. Batch fermentation process for bioethanol production using *Saccharomyces cerevisiae* ML-6 in fermentation medium.

reached maximum value of 22.77 g/L at 72 hours. The cell biomass showed an gradual increase from 0.21 g/L at 6hrs reaching maximum at 2.07 g/L at 72 hrs after which minimal change was observed (Fig. 7). A drop in glucose consumption was observed from 6 hrs at 47.25 g/L to 24 hrs where glucose concentration had decreased to almost 54% with the lowest concentration recorded at 72 hrs i.e. 1.89 g/L, after which consumption was minimal. Ethanol production from lignocellulosic biomass requires hydrolysis of pretreated biomass releasing fermentable sugars in the resultant hydrolyzate. Batch fermentation conducted using hydrolyzate from pretreated rice straw saccharification gave 16.7 g/L ethanol (w/v). Although the medium was supplemented with nutrients, the low production could have resulted from the presence of low sugar levels in medium i.e. 31 g/L (Tahir et al. 2010). Biomass growth and ethanol production were relatively slow as compared to previous experiment, with biomass gradually increasing from 1.31 g/L and peaking off at 1.83 g/L at 96 hrs where highest ethanol concentration was recorded at 16.07 g/L. As revealed from batch experiment with fermentation medium, the tendency of the yeast to rapidly consume glucose in the hydrolyzate resulted in exhaustion of resources for further ethanol production. The addition of nutrients such as yeast extract strongly is supported by Johansson et al. (2014) which rejuvenates and maintains cell viability and enhances ethanol production levels.

Batch Fermentation Utilizing Saccharified Rice Straw Hydrolyzate

A change in medium was brought about by using saccharified rice straw hydrolyzate containing 3.1% reducing sugar content, which was fermented in batch mode for 5 days. Ethanol concentration was noted to increase gradually from 6hrs at 0.18 g/L, to 5.43 g/L at 48 hrs reaching a maximum value of 16.07 g/L at 96 hrs after which no change was observed. Biomass showed an increase from 1.31 g/L at 6hrs to a maximum of 1.76 g/L at 24 hrs after which it hovered around 1.7 g/L. Consumption of glucose was accelerated from 6 hrs to 24 hrs where reducing sugars were at 27.11 g/L and 4.13 g/L after which sugar consumption decreased to a minimum 1.02 g/L (Fig. 8). Since some enzymatic hydrolyses do not favor in terms of glucose yields, additional glucose may have to be added to achieve a higher ethanol titer. In the current study, the hydrolyzate from rice straw was supplemented to a final concentration of 75.0 g/L glucose (7.5% w/v). Batch fermentation using hydrolyzate from saccharification of pretreated rice straw supplemented with glucose gave 22.9 g/L ethanol. When compared to the previous experiment, supplementation of glucose showed a positive effect in both biomass growth and ethanol production which peaked in 72 hrs. Biomass was recorded as 2.14 g/L with corresponding value for ethanol as 22.96 g/L which was achieved relatively earlier than for the previous experiment. A decline in glucose from 12.31 g/L at 24 hrs to 1.23 g/L at 72 hrs shows that the exponential phase is restored as it were in the batch experiment with fermentation media. This suggests that the yeast was able to use the additional glucose in medium to boost its growth and initiate ethanol production before the additional reserves were tapped out, after which the yeast shifted to the sugars existing in the hydrolyzate.

Batch Fermentation Utilizing Saccharified Rice Straw Hydrolyzate Supplemented with Glucose

A similar experiment was carried out using saccharified rice straw hydrolyzate supplemented with glucose for which the ethanol concentration came up gradually from 7.23 g/L at 24 hrs to a maximum of 22.96 g/L at 72 hrs. Glucose consumption was relatively slow till 6hrs at 71.64 g/L but then consumption gradually elevated,

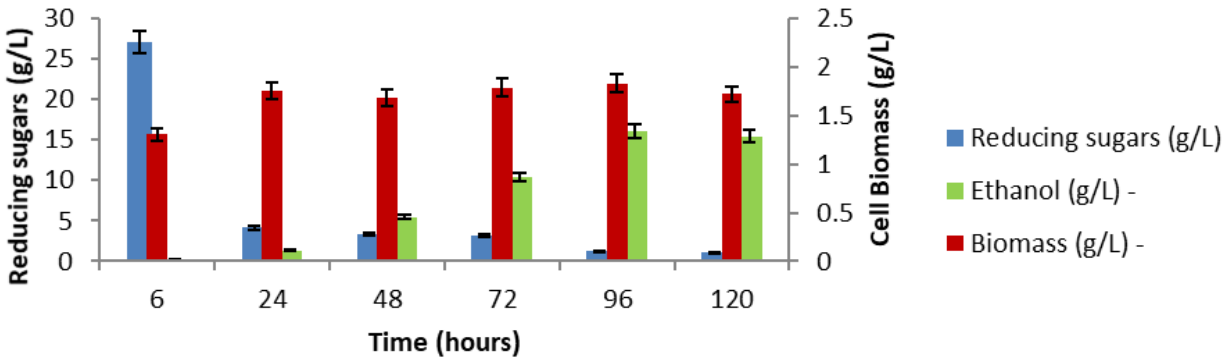

Fig. 8. Batch Fermentation for bioethanol production from Saccharified Rice Straw Hydrolyzate using *Saccharomyces cerevisiae* ML-6.

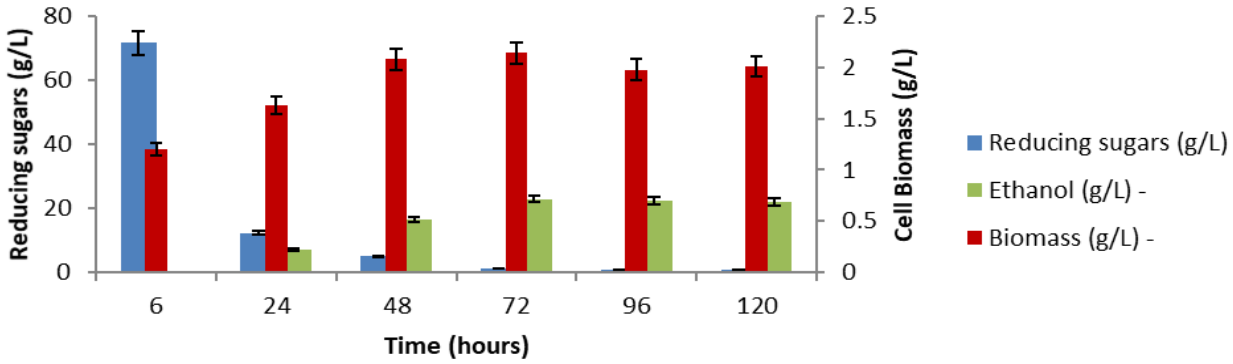

Fig. 9. Batch Fermentation for bioethanol production from Saccharified Rice Straw Hydrolyzate supplemented with Glucose using *Saccharomyces cerevisiae* ML-6.

decreasing the sugar content from 24 hrs at 12.31 g/L to 72 hrs at 1.23 g/L reaching to a minimum of 0.66 g/L at 120 hrs. This correlates with the increase in biomass from 1.2 g/L at 6 hrs to 2.14 g/L at 72 hrs after which minimal change was observed (Fig. 9). Since batch processes revealed the rapid glucose consumption intervals beyond 6 hrs and 24 hrs, the experiment was set with fermentation medium that was supplemented with drop-wise addition of glucose solution for about 5 days. The batch fermentation using pure glucose solution process yielded 23.0 g/L ethanol (w/v). The glucose concentration dropped after 6hrs where it was recorded at 21.45 g/L after 24 hrs from an initial 41.34 g/L. The glucose consumption further increased but gradually to 10.23 g/L at 72 hrs where ethanol concentration was recorded at 26.11 g/L, denoting a peak in ethanol production. Whereas ethanol yield fluctuated between 25-26% after 72 hrs, residual glucose concentration surprisingly showed an increase from 96-120 hrs where values were recorded at 13.76 g/L and 18.55 g/L respectively. The trend of peaking ethanol concentration maintained till 72 hrs as in the batch process. The increase in remaining glucose in broth can be attributable to a secondary effect caused by higher sugar concentration, resulting in cessation of fermentation, as described by Haukeli et al. (1971) which includes oxidative pathways being blocked through catabolite repression. A possible reason for this stoppage is defined by Wang et al. (2013) when using ethanol and high gravity medium containing glucose during continuous fermentation. The study speculated that a high concentration of xylose (non-fermentable) did produce osmotic stress conditions but the significant stress was produced when 30 g/L ethanol was added which produced changes in growth of *S. cerevisiae* as well as glucose consumption.

Hydrolyzate Supplemented with Glucose Using *Saccharomyces cerevisiae* ML-6 Semi Continuous Fermentation

In Semi continuous fermentation, the fresh medium was added after removal of a small portion of culture medium. This process was repeated at appropriate intervals. Usually lag phase and other non-productive phases are shortened and the product output is higher. After a period of 7 days of fermentation, harvesting one-third medium after 24 hrs, the ethanol content started off

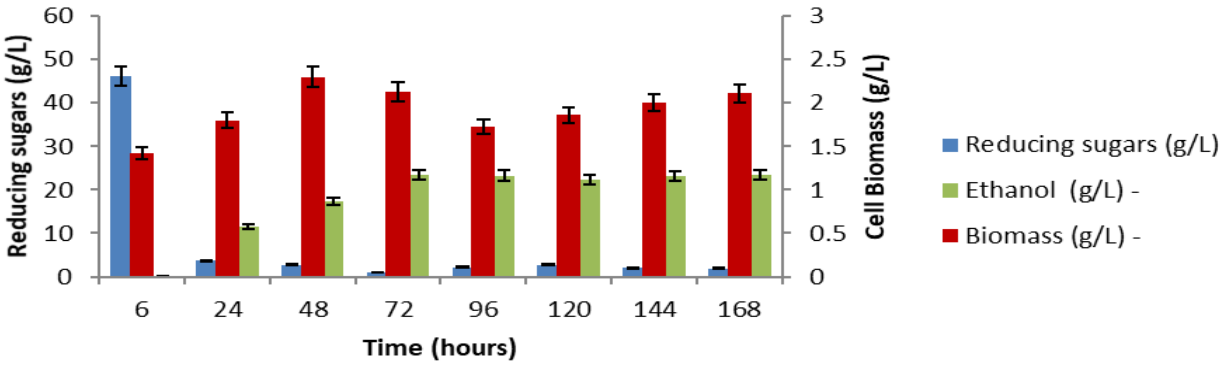

Fig. 10. Semi continuous Fermentation for bioethanol production in fermentation medium using *S. cerevisiae* ML-6.

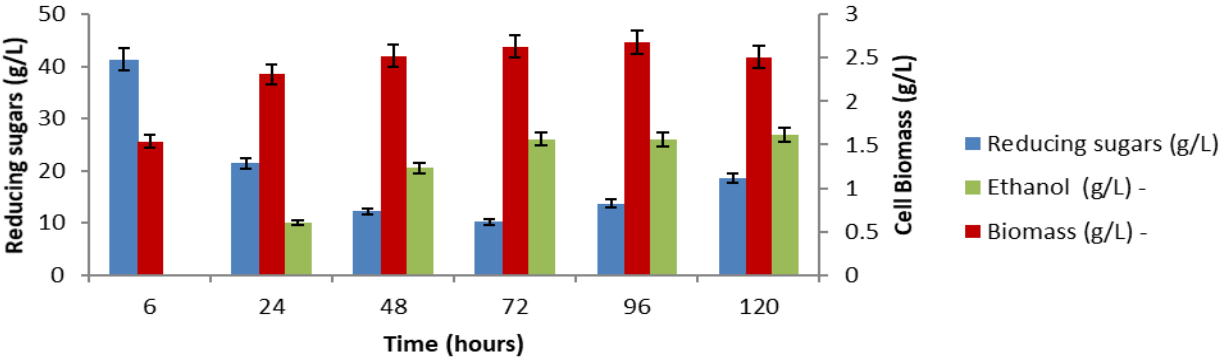

Fig. 11. Continuous Fermentation in fermentation medium supplemented with Glucose solution using *S. cerevisiae* ML-6.

from 11.52 g/L at 24 hrs to 17.36 g/L at 48 hrs peaking off at 23.3 g/L after 72 hrs. Consumption of sugar reached to 3.67 g/L at 24 hrs from 46.14 g/L at 6hrs, reducing to a minimum of 2.03 g/L around 96 hrs. Growth of cells after 6 hrs was 1.42 g/L which leveled off at 2.3 g/L at 48 hrs, after which minimal change was observed (Fig. 10).

Continuous Fermentation

Drop-wise addition of pure glucose solution to avoid any chance of chemical contamination as well as for the optimization of continuous fermentation, starting from 6 hrs after inoculation, for a period of 5 days in continuous mode of fermentation, initiated ethanol production at 10.06 g/L on 24 hrs to 20.56 g/L on 48 hrs leading to a maximum of 26.92 g/L after 120 hrs (Fig. 11). Levels of glucose were almost reduced to 51% from 41.34 g/L at 6 hrs to 21.45 g/L at 24 hrs. After 48 hrs, the glucose concentration started to show gradual increase from 10.23 g/L at 72 hrs to 18.55 g/L at 120 hrs. Cell growth after 6 hrs i.e. 1.54 g/L increased to 2.52 g/L at 48 hrs, leveling off at 2.68 g/L after 96 hrs. A different approach; a hybrid of both batch and continuous process was conducted: the continuous process with fermentation medium of same composition as in the batch experiment. Continuous fermentation in synthetic media gave 23.4 g/L ethanol (w/v). The process followed the trends of glucose consumption as the batch process where residual glucose reached 3.67 g/L after 24 hrs from an initial 46.14 g/L. Medium replacement after 24hrs showed biomass had boosted from 1.42 g/L to 1.8 g/L and ethanol production had initiated at 11.52 g/L. The periodic removal of medium maintained the cell biomass around 2.0 g/L whereas ethanol after peaking at 23.37 g/L at 72 hrs hovered between 22-23 g/L but not more than the maximum value. This change in ethanol content and biomass can be attributed to the dilution effect. The fermentation process is affected by end product and substrate concentration. Tahir et al. (2010) attributes substrate inhibition through a study pointing out a specific level of sugars is tolerated by *S. cerevisiae* after which inhibition sets which drastically affects ethanol production. Amenaghawon et al. (2012) demonstrated that as ethanol concentration increases, biomass decreases progressively. This biomass decrease is accompanied by a gradual stoppage in ethanol production as ethanol concentration in medium reaches a point where cells fail to produce fresh ethanol. Hence, removal of ethanol while maintaining cell viability and density is vital to the fermentation process.

CONCLUSION

The study showed that acid pretreatment has reduced lignin content about 50%. Resultantly, pretreatment raised cellulose contents up to 52% making it suitable for enzymatic saccharification. Optimization of saccharification illustrated that parameters such as biomass, enzyme concentration, temperature, incubation time and agitation indeed affected the action of cellulase on pretreated biomass releasing sugars up to 31.0 g/L. The results clearly indicate the need for optimization at every point in the ethanol production process for efficient use of resources and maximum yield without wastage. Among various operational modes of fermentation studied, such as batch, semicontinuous (Fed-batch fermentation) and continuous, the semicontinuous process gave better ethanol yield of 23.4 g/L. This implies potential use of the process in industrial grade ethanol production. Collectively the study has shown that rice straw can serve as a lignocellulosic substrate, utilizing acid pretreatment for cellulose exposure and enzymatic saccharification to release fermentable sugars for production of ethanol. A sustainable and low-cost saccharification is necessary for continued growth of the industrial biotech sector which can be realized by retrofitting existing dry-grind ethanol facilities. Lignocellulosic biomass has been considered as a potential resource for saccharification however, the lack of industrial process data and varying processing assumptions make it difficult to determine accurate glucose consumption and cost profile from past studied processing technologies.

RECOMMENDATIONS

Saccharification should be studied and optimized extensively to get maximum activity from cellulase and fermentable sugars. The saccharification process requires washing of pretreated substrate to remove inhibitors, in the process flushing out vast amounts of sugars. Efforts should be made to utilize the sugar-rich fractions from pretreatment for fermentation to increase the overall ethanol yield obtained from the fermentation process. Lignin residues generated after pretreatment can be utilized for growth of lignin degrading fungi for production of lignin degrading enzymes whereas lignin derivatives can be harnessed by the pharmaceutical industry.

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Supplementary Material Figures

Figure S1. Physically treated (crushed) sample of 2 mm mesh size rice straw.

Figure S2. Rice straw sample after physiochemical pretreatment with 2% H_2SO_4 at 121oC for 90 mins.

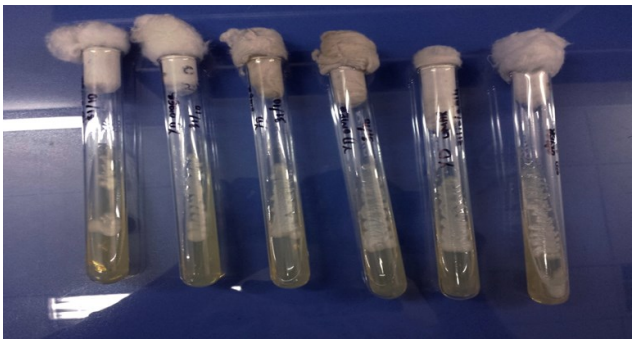

Figure S3. Revival of Saccharomyces cerevisiae ML-6 in PDA slants.

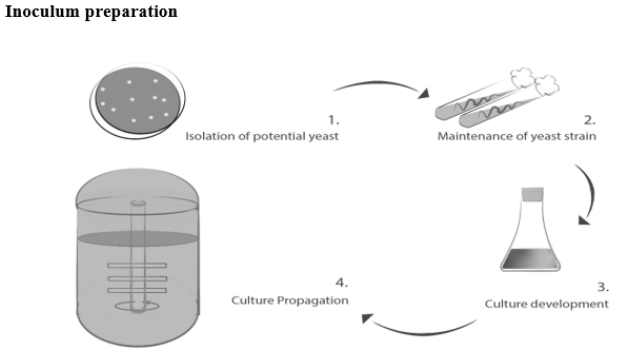

Figure S4. Sequential steps for inoculum preparation for fermentation.

Fermentation medium preparation

Figure S5. Inoculum of *Saccharomyces cerevisiae* ML-6 (left) and fermentation medium (right).

Batch fermentation:

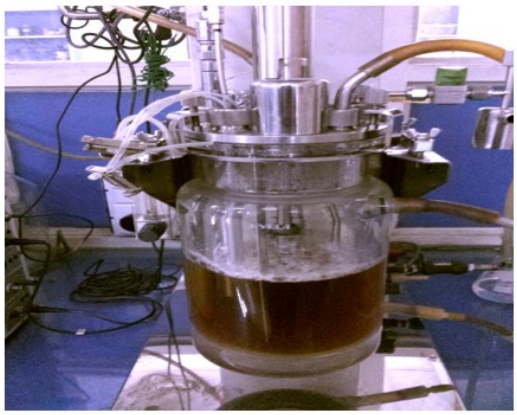

Figure S6. Batch fermentation using *Saccharomyces cervisiae* ML-6 in fermentation medium.

Semi continuous Fermentation:

Figure S7. Semi continuous process using *Saccharomyces cerevisiae* ML-6 in fermentation medium.

Continuous Fermentation:

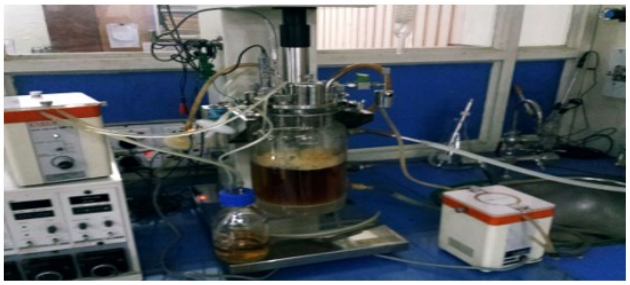

Figure S8. Continuous process using *Saccharomyces cerevisiae* ML-6.

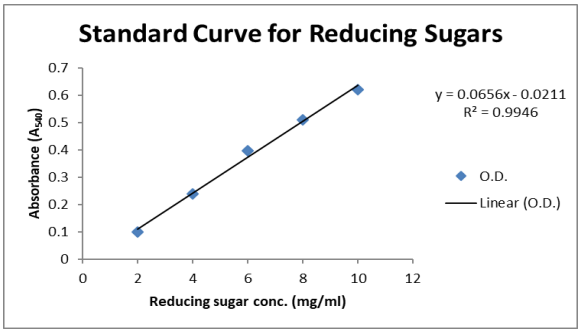

Figure S9. Standard curve for reducing sugars.

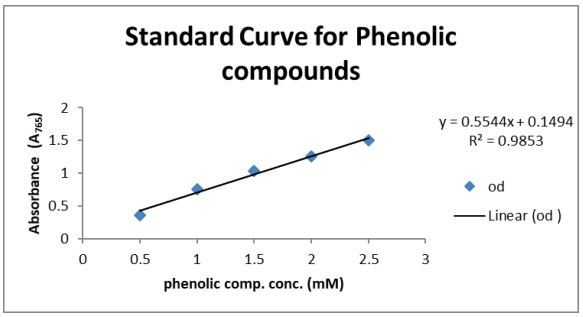

Figure S10. Standard curve for phenolic compounds.

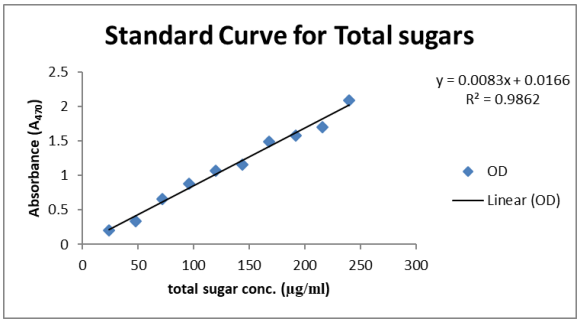

Figure S11. Standard curve for total sugars.

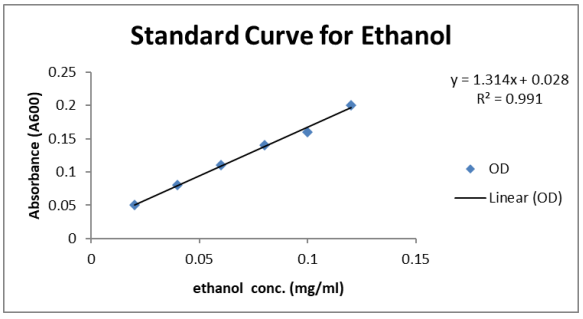

Figure S12. Standard curve for ethanol.

Table S2. Plackett Burman (PB) experimental design for screening of variables for saccharification of rice straw.

Experiment	X1	X2	X3	X4	X5	RS	TS	Phenol
1	5	55	24	2.5	150	14731.46**	3592.12	1.438
2	0.5	55	6	2.5	150	7632.37	4108.32	1.591
3	5	25	24	0.25	150	733.73	1237.58	0.262
4	0.5	25	6	2.5	25	14557.48	4407.87	1.703
5	0.5	55	6	0.25	150	3381.24	2520.31	0.216
6	0.5	25	24	0.25	25	1259.45	697.57	0.252
7	0.5	25	6	2.5	25	8827.53	4262.61	1.576
8	5	25	6	0.25	150	11675.49	4434.14	0.137
RS	1070.32	3.865	47.061	3165.43	6.617			
TS	180.3	17.355	-79.009	790.996	-3.18	Estimates		
Phenols	-0.15003	-0.18842	0.013175	0.704433*	0.006827			
RS	0.37613	0.008662	0.091746	1.396888	0.041995			
TS	0.212784	0.130836	-0.518052	1.17397	-0.072828	*t- values*		
Phenols	-2.49241	-1.9965	1.21419	14.69557*	2.19742			
RS	0.742972	0.993875	0.935262	0.297265	0.970318			
TS	0.851214	0.907878	0.656034	0.361275	0.948571	*p values*		
Phenols	0.130255	0.18398	0.348588	0.004599*	0.159098			

**Maximum Reducing sugars from corresponding experiment, *Significant values for corresponding columns, -RS: Reducing Sugars, TS: Total sugars & Phenol: Total phenolic compounds, -X1: Biomass concentration (g), X2: Temperature (°C), X3:Incubation period (days), X4: Enzyme concentration (g) & X5: Agitation speed (rpm).

Table S3. Test of SS Whole model vs. SS Residual for reducing sugars, total sugars and phenolic compounds.

Variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	Df Model	MS Model	SS Residual	Df Residual	MS Residual	F	p
RS	0.912705*	0.83303	0.614685	396453.54	17	23320.79	79463.83	13	6112.603	3.815198	0.009223
Variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	Df Model	MS Model	SS Residual	Df Residual	MS Residual	F	p
TS	0.936210*	0.87649	0.714977	15681249	17	922426.4	2209712	13	169977.8	5.426745	0.001783
Variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	Df Model	MS Model	SS Residual	Df Residual	MS Residual	F	p
Phenol	0.936919*	0.877817	0.718039	1722.789	17	101.3405	239.795	13	18.44577	5.493973	0.001678

*Significant values for corresponding row,
-RS: reducing sugars, TS: total sugars and Phenols: Phenolic compounds,

Table S4. Saccharification of Pretreated Rice Straw by using Box-Wilson Design under various factors at 5 levels.

Factors	Range	Optimum values
X1 (Biomass)	0.5-6.5	6.2
X2 (Temperature)	20-60	58
X3(Incubation Period)	01-May	4.8
X4(Enzyme conc.)	0.25-3.25	1.75
X5(rpm)	100-300	240

Table S5. Box Wilson Design for optimization of saccharification of rice straw for reducing sugars.

Experiments	X1	X2	X3	X4	X5	Observed RS	Predicted RS
1	2	30	2	1	250	85.076	119.3014
2	2	30	2	2.5	150	112.44	96.951
3	2	30	4	1	250	73.644	126.549
4	2	30	4	2.5	150	108.02	75.9974
5	2	50	2	1	250	281.8	270.0752
6	2	50	2	2.5	150	257.7	223.2549
7	2	50	4	1	250	287.97	282.7162
8	2	50	4	2.5	150	246.05	207.8127
9	5	30	2	1	250	106.49	119.0486
10	5	30	2	2.5	150	131.65	162.5785
11	5	30	4	1	250	140.34	174.827
12	5	30	4	2.5	150	73.491	103.0869
13	5	50	2	1	250	302.3	316.4683
14	5	50	2	2.5	150	283.63	248.5755
15	5	50	4	1	250	300.78	334.1192
16	5	50	4	2.5	150	290.11	238.0273
17	0.5	40	3	1.75	200	52.226	86.207
18	6.5	40	3	1.75	200	193.69	159.7076
19	3.5	20	3	1.75	200	70.137	-3.4655
20	3.5	60	1	1.75	200	208.63	282.2307
21	3.5	40	5	1.75	200	93.384	104.7535
22	3.5	40	3	1.75	200	112.82	101.5408
23	3.5	40	3	0.25	200	59.39	-44.7225
24	3.5	40	3	3.25	200	131.34	235.4542
25	3.5	40	3	1.75	100	33.552	137.6645
26	3.5	40	3	1.75	300	640.4	536.2836
27	3.5	40	3	1.75	200	120.52	79.2385
28	3.5	40	3	1.75	200	53.064	79.2385
29	3.5	40	3	1.75	200	76.768	79.2385
30	3.5	40	3	1.75	200	71.662	79.2385
31	3.5	40	3	1.75	200	74.405	79.2385

Table S6. Analysis of variance for optimization of saccharification of rice straw using Box Wilson design in terms of reducing sugars (RS).

Variable	Analysis of Variance				Parameter Estimates		
	SS	df	MS	F	Estimates	*t values*	*P values*
Intercept					362.9327	0.38912	0.703483
X1		0			-18.0578	-0.20354	0.841864
X1^2	2724.85	1	2724.85	0.44578	4.8526	0.66766	0.516026
X2		0			-4.045	-0.25599	0.801962
X2^2	5159.85	1	5159.85	0.84413	0.1502	0.91877	0.37495
X3		0			-28.4263	-0.20023	0.844398
X3^2	810.46	1	810.46	0.13259	5.9546	0.36413	0.721616
X4		0			220.6882	0.73644	0.474546
X4^2	369.49	1	369.49	0.06045	7.1477	0.24586	0.80963
X5		0			-7.6569	-2.03901	0.062319
X5^2	94863.43	1	94863.43	15.51932	0.0258	3.93946	0.001695*
X1*X2	9.77	1	9.77	0.0016	0.0521	0.03997	0.968724
X1*X3	24.92	1	24.92	0.00408	0.832	0.06385	0.950062
X2*X3	29.28	1	29.28	0.00479	0.1353	0.06922	0.945872
X1*X4		0			-4.7085	-0.27101	0.790639
X2*X4		0			-0.8117	-0.31147	0.76038
X3*X4		0			-9.4004	-0.36071	0.724112
X1*X5		0			0		
X2*X5		0			0		
X3*X5		0			0		
X4*X5	858.87	1	858.87	0.14051	-0.3758	-0.37484	0.713821

*Significant variables with calculated values

Table S7. Box Wilson Design for optimization of saccharification of rice straw in terms of total sugars (TS).

Experiments	X1	X2	X3	X4	X5	Observed TS	Predicted TS
1	2	30	2	1	250	1608.916	1735.055
2	2	30	2	2.5	150	2969.759	2954.061
3	2	30	4	1	250	1928.795	1807.193
4	2	30	4	2.5	150	2928.795	2866.862
5	2	50	2	1	250	3292.048	2858.499
6	2	50	2	2.5	150	2840.843	2793.468
7	2	50	4	1	250	3252.892	3251.421
8	2	50	4	2.5	150	2958.313	3027.053
9	5	30	2	1	250	2769.759	2608.398
10	5	30	2	2.5	150	3255.904	3349.995
11	5	30	4	1	250	2137.831	2277.826
12	5	30	4	2.5	150	2519.157	2860.085
13	5	50	2	1	250	3423.976	3578.529
14	5	50	2	2.5	150	3007.108	3036.089
15	5	50	4	1	250	3645.663	3568.74
16	5	50	4	2.5	150	2900.482	2866.963
17	0.5	40	3	1.75	200	1645.06	1888.433
18	6.5	40	3	1.75	200	2845.06	2601.687
19	3.5	20	3	1.75	200	2446.867	2276.586
20	3.5	60	1	1.75	200	3236.627	3406.908
21	3.5	40	5	1.75	200	2428.193	2555.301
22	3.5	40	3	1.75	200	2585.422	2458.314
23	3.5	40	3	0.25	200	1037.229	1224.338
24	3.5	40	3	3.25	200	2730	2542.891
25	3.5	40	3	1.75	100	1661.928	1474.819
26	3.5	40	3	1.75	300	2089.036	2276.145
27	3.5	40	3	1.75	200	2482.41	1428.2
28	3.5	40	3	1.75	200	1027.59	1428.2
29	3.5	40	3	1.75	200	1127.59	1428.2
30	3.5	40	3	1.75	200	1205.94	1428.2
31	3.5	40	3	1.75	200	1297.47	1428.2

Table S8. Analysis of variance for optimization of saccharification of rice straw using Box-Wilson design in terms of total sugars (TS).

Variable	Analysis of Variance				Parameter Estimates		
	SS	df	MS	F	Estimates	*t values*	*P values*
Intercept					2611.49	0.53097	0.604395
X1		0			-27.24	-0.05822	0.95446
X1^2	953229	1	953229	5.60796	90.76	2.36811	0.034056*
X2		0			-194.66	-2.3362	0.036143*
X2^2	2854450	1	2854450	16.79307	3.53	4.09794	0.001258*
X3		0			-1635.08	-2.18408	0.047876*
X3^2	1661992	1	1661992	9.7777	269.65	3.12693	0.008020*
X4		0			3887.87	2.46028	0.028654*
X4^2	296289	1	296289	1.7431	202.41	1.32027	0.20952
X5		0			2.86	0.1446	0.887244
X5^2	285802	1	285802	1.68141	0.04	1.29669	0.217282
X1*X2	23505	1	23505	0.13828	-2.56	-0.37186	0.715985
X1*X3	162176	1	162176	0.9541	-67.12	-0.97678	0.346505
X2*X3	102902	1	102902	0.60539	8.02	0.77807	0.45046
X1*X4		0			-106.09	-1.15796	0.267711
X2*X4		0			-42.8	-3.11445	0.008215*
X3*X4		0			-53.11	-0.38647	0.705397
X1*X5		0			0		
X2*X5		0			0		
X3*X5		0			0		
X4*X5	556973	1	556973	3.27674	-9.57	-1.81018	0.093434

*Significant variables with their calculated values.

Table S9. Box Wilson design for optimization of saccharification of pretreated rice straw in terms of phenol.

Experiments	X1	X2	X3	X4	X5	Observed Phenol	Predicted Phenol
1	2	30	2	1	250	5.758123	8.30103
2	2	30	2	2.5	150	14.75632	16.60044
3	2	30	4	1	250	7.075812	9.54765
4	2	30	4	2.5	150	19.02527	16.92423
5	2	50	2	1	250	8.258123	8.04043
6	2	50	2	2.5	150	28.70036	23.50816
7	2	50	4	1	250	9.765343	5.60699
8	2	50	4	2.5	150	16.06498	20.15189
9	5	30	2	1	250	5.415162	3.36233
10	5	30	2	2.5	150	14.11552	16.23981
11	5	30	4	1	250	5.49639	8.65452
12	5	30	4	2.5	150	18.3574	20.60917
13	5	50	2	1	250	6.254513	6.32149
14	5	50	2	2.5	150	26.80505	26.36729
15	5	50	4	1	250	7.743682	7.93363
16	5	50	4	2.5	150	31.63357	27.05659
17	0.5	40	3	1.75	200	14.24188	14.60364
18	6.5	40	3	1.75	200	16.93141	16.56965
19	3.5	20	3	1.75	200	10.61372	5.49414
20	3.5	60	1	1.75	200	6.561372	11.68096
21	3.5	40	5	1.75	200	11.56137	12.22247
22	3.5	40	3	1.75	200	14.81949	14.15839
23	3.5	40	3	0.25	200	0.406137	-0.59431
24	3.5	40	3	3.25	200	32.12996	33.13041
25	3.5	40	3	1.75	100	10.98375	11.9842
26	3.5	40	3	1.75	300	19.287	18.28655
27	3.5	40	3	1.75	200	12.61733	8.55596
28	3.5	40	3	1.75	200	11.7148	8.55596
29	3.5	40	3	1.75	200	8.962094	8.55596
30	3.5	40	3	1.75	200	5.036101	8.55596
31	3.5	40	3	1.75	200	4.449458	8.55596

Table S10. Analysis of variance for optimization of saccharification of rice straw using Box-Wilson design in terms of phenolic contents.

Variable	Analysis of Variance				Parameter Estimates		
	SS	df	MS	F	Estimates	*t values*	*P values*
Intercept		0			64.7414	1.2636	0.228564
X1		0			-11.0903	-2.2756	0.040447*
X1^2	70.6151	1	70.61511	3.828256	0.7812	1.95659	0.072225
X2		0			-0.1816	-0.20923	0.837513
X2^2	0.0014	1	0.00143	0.000077	0.0001	0.00879	0.993119
X3		0			-4.6093	-0.59103	0.564635
X3^2	30.6833	1	30.68335	1.663436	1.1586	1.28974	0.219613
X4		0			-18.1582	-1.10305	0.290008
X4^2	84.9662	1	84.96623	4.606273	3.4276	2.14622	0.051309
X5		0			-0.2772	-1.34393	0.201959
X5^2	61.8411	1	61.84107	3.352589	0.0007	1.83101	0.090109
X1*X2	10.3669	1	10.36689	0.56202	0.0537	0.74968	0.466798
X1*X3	16.3667	1	16.36671	0.887288	0.6743	0.94196	0.363389
X2*X3	13.5428	1	13.5428	0.734196	-0.092	-0.85685	0.407045
X1*X4		0			1.0173	1.06594	0.305847
X2*X4		0			0.2389	1.66905	0.118998
X3*X4		0			-0.3076	-0.21487	0.833203
X1*X5		0			0		
X2*X5		0			0		
X3*X5		0			0		
X4*X5	4.1228	1	4.1228	0.223509	0.026	0.47277	0.644218

* Significant variables with calculated value.