Bioethanol production from agro-waste by pretreatment methods and optimization using Response surface methodology.

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Abstract: Bioethanol is an alternative renewable fuel that can be produced from cellulosic biomass by hydrolysis and fermentation processes, for which Sugarcane bagasse (SCB) and Rice straw have been used in this study as they are cheap, readily available feedstock and possess high sugar content. For biological production of ethanol, agro-waste is enzymatically hydrolyzed to fermentable sugars. To increase this sugar content from substrate and to boost bioethanol production, for which along with enzymes, different pretreatment methods were studied. In this study three commercial enzymes, cellulase, xylanase and pectinase were used along with chemical pretreatment of NaOH and H_2SO_4 and then were optimized using response surface methodology for efficient hydrolysis. Further induction of bioethanol production was studied using fresh leaf, stem and fruit dried powder of *Moringa oleifera*. Heat, pressure and time were maintained constant during all pretreatments. Bioethanol production from treated biomass was performed by simultaneous scarification and fermentation (SSF) with different enzymes and their cocktail using yeast *Saccharomyces cerevisiae*. Optimal condition for SSF-27°C, 144h, Enzyme loading 1%, yeast inoculum 10 % (v/v) and substrate concentration 5% (w/v) were maintained. 5.2g/l bioethanol concentration was obtained using sugarcane bagasse (SCB) pretreated with 2% NaOH along with cocktail of all enzyme. In rice straw ethanol concentration of 3.36g/l was achieved with Xylanase enzyme and 2%NaoH pretreatment. Around 28.8% and 36.6% increase in production was induced using fresh leaf powder stem powder of *Moringa oleifera* in SCB and Rice straw respectively.

Index Terms **- SCB, Rice straw, Bioethanol, Pretreatment, response surface modelling.**

I. INTRODUCTION

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Crude oil has been the major resource to meet the increased energy demand. As energy demand increases the global supply of fossil fuels cause harm to human health and contributes to the greenhouse gas emission. At the same time, increasing waste generation linked to rising population and living standards is a worldwide challenge to waste management systems. World energy consumption is predicted to increase by 50% to 2030 according to the United States Energy Information Agency (Berhe et al., 2017). To solve this problem, renewable energy can help. Many alternative fuels have been suggested including bioethanol.

Bioethanol produced from lignocellulosic biomass is kept attracting global attention as alternative energy for oil fuel for sustainable energy society, since depletion of fossil fuel, global warming, and reduction of natural resources has been increased concern. Lignocellulosic biomass arises from agricultural, forestry, and industrial waste. Bioethanol is an alternative renewable fuel that can be produced from cellulosic biomass by hydrolysis and fermentation processes, for which Lignocellulosic biomass Sugarcane bagasse (SCB) and Rice straw have been used in this study as they are renewable, cheap, readily available feedstock and possess high sugar content. Rice straw is one of the agricultural waste caused from rice cropping and is a hopeful feedstock for bioethanol production, because cellulose and hemicellulose content is more than 50% (Takano et al., 2018). After consuming sugarcane for sugar production, sugar factories yield a huge amount of solid by product known as sugarcane bagasse (SCB), i.e., a retained fibrous material after crushing and squeezing the sugarcane (Thite et al., 2019).

Bioethanol production depend on the two sequential fundamental steps of biomass saccharification followed by fermentation of the released sugars. The saccharifying enzymes that act on different plant polysaccharides are categorized as core and accessory enzymes where cellulases and xylano-pectinolytic enzymes belong respectively. Though, this involvement alone is not sufficient for obtaining best enzymatic saccharification (Nerukar et al., 2019). In the bioconversion to ethanol from lignocellulose, pre-treatment by physical and/or chemical method is necessary to break the strong structure and obtain fermentable sugars easily by biocatalyst such as cellulase, xylanase, ligninase, etc. (Hoshino et al., 2018). The pre-treatment methods extensively used at present include dilute acid hydrolysis, alkaline or ammonia extraction, steam explosion and wet oxidation delignification (Zou et al., 2012). In this study, the physical treatment like Temperature and Pressure along with chemical treatment like H_2SO_4 and NaOH were used. This method are referred as Physico-chemical pre-treatment. Bioethanol production depend on the two sequential fundamental steps of biomass saccharification followed by fermentation of the released sugars. The saccharifying enzymes that act on different plant polysaccharides are categorized as core and accessory enzymes where cellulases and xylano-pectinolytic enzymes belong respectively (Thite et al., 2019).

In this research, simultaneous saccharification and fermentation were performed for bioethanol production from SCB and rice straw. They were pre-treated with alkali and acid at autoclavable temperature and pressure. Different commercial available enzyme were used to hydrolyze the substrate to fermentable sugars. The fermentable sugars were fermented to bioethanol using yeast *Saccharomyces cerevisiae*.

II. RESEARCH METHODOLOGY

1. RAW MATERIAL COLLECTION

Fresh Sugarcane Bagasse (SCB) were collected from sugarcane juice parlour near Tithal road in Valsad district, Gujarat. Rice straw were collected from Dived village near Valsad district, Gujarat. The straw was harvested in the month of December 2019. Samples were collected in a clean plastic bag. Collected samples were washed in hot (~60°C) distilled water in shaking condition at 120rpm for 1hour. After washing samples were filtered and then air dried in hot air oven at 60°C. Dried samples were then cut into small pieces of around 1cm with the help of scissors. The samples were stored in an airtight bag at 4°C until used. Fresh *Moringa Oleifera* leaf, stem and fruit were collected from charvada village near Valsad district, Gujarat. Leaf, stem and fruit were sun dried and then crushed using grinder in powder form and stored in air tight container at room temperature until use. Samples were collected in a clean plastic bag.

2. MEDIA AND CHEMICAL COMPONENTS

Baker's yeast, Inoculum medium, and fermentation medium were used. The other chemicals such as H_2SO_4, NaOH, DNSA, potassium dichromate, sucrose, yeast extract, $MgSO4{\cdot}7H_2O$, K_2HPO_4, and peptone were used. All these media were procured from Merck Ltd., ThermoFisher Scientific India Pvt. Ltd., Yashvi Fine Chem and Finar Ltd. The saccharification of SCB and rice straw were carried out using commercially available enzymes. Total of three enzymes used in hydrolysis are cellulase, xylanase and pectinase. The activity of enzymes reported by the supplier was considered in this study. All the enzymes used in this study were purchased from HiMedia Laboratories Pvt. Ltd.

3. ORGANISM

A commercial available baker's yeast *Saccharomyces cerevisiae* was purchased from a local store and kept in refrigerator until use. Baker's yeast were directly used without cultivation.

4. INOCULUM PREPARATION

Sterile Growth medium (100 ml) comprised of glucose, 10; peptone, 0.5; yeast extract, 0.5; $MgSO_4{\cdot}7H_2O$, 0.15; K_2HPO_4, 0.15 was prepared in 250 ml Erlenmeyer flask. The pH of the medium was adjusted to 4.0 using 1N NaOH or 1N HCL. The medium after sterilization in autoclave, and then was inoculated with one gram of Baker's yeast purchased from local market. This was incubated in shaking condition at room temperature on a gyratory shaker at 150 rpm for 48 h.

5. PRE-TREATMENT OF RAW MATERIAL

Biomass soaked in 100 ml distilled water at room temperature for 20 min without any pre-treatment was used as control and referred to as raw or untreated biomass control. Alkali and acid pre-treatment of SCB and rice straw were individually carried out with NaOH and H_2SO_4. Three different concentration of each chemical agent (1.0, 2.0 and 3.0%) with SCB and Rice straw loading of 5.0% (w/v) were performed individually in 250 ml Erlenmeyer flask. The physical parameters such as heat, pressure and time were maintained constant at all pre-treatment. Steam Pre-treatments were performed at autoclavable temperature i.e. 121°C and pressure i.e. 15 psi for 20 min. After pre-treatments, all samples were cooled at room temperature.

6. SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

SSF experiment were performed on the whole pre-treated slurry in 250ml Erlenmeyer flask with weight of 5% w/v. The sterile fermentation broth (100 ml) comprised of (% w/v), peptone, 5; yeast extract, 5; $MgSO_4{\cdot}7H_2O$, 1.5; K_2HPO_4, 1.5 and the pre-treated raw material; 5g/l as the fermenting sugar was used. The pH of medium was adjusted to 5.5. The sterile fermentation medium was inoculated with 10% v/v of yeast inoculum. Simultaneously the enzyme cellulases, xylanase and pectinase enzyme at concentration of 1% were inoculated individually and in cocktail. The bottles were sealed with the aid of an adhesive tape and incubated at room temperature for a period of up to 120hr. Bottles were removed at every 24 h intervals to determine the amount of ethanol produced using the Dipotassium chromate method (Patel R J & Patel K R, 2004) and the residual sugar in the medium after each period of fermentation was determined following the DNSA method of Miller (1959). Amount of reducing sugar was read off a curve of glucose standard and expressed as mg ml^{-1} Alcohol was produced through the distillation process.

7. DISTILLATION OF ALCOHOL

After SSF, the fermentation broth were filtered using cheese cloth and then centrifuged at 6000 rpm for 20 min. The collected supernatant was distilled at 70°C to get ethanol by simple distillation unit at laboratory level.

8. INDUCTION OF BIOETHANOL PRODUCTION USING *MORINGA OLEIFERA*

To increase the bioethanol production from rice straw and sugarcane bagasse, fresh *Moringa oleifera* was used. The SCB and rice straw were chemically pre-treated with 2% NaOH and steam pre-treatment at autoclavable temperature and pressure for 20 min. Then it was inoculated with fresh *Moringa oleifera* leaf, stem and fruit dried powder in each flask at concentration of 1% (w/v). The medium was also inoculated with cocktail of enzymes i.e. Cellulase, Xylanase and pectinase in SCB and only Xylanase enzyme in Rice straw for saccharification. The simultaneous saccharification and fermentation was carried out in 250ml Erlenmeyer flask containing 100ml of sterile medium by inoculating 10ml of previously prepared yeast inoculum.

9. EXPERIMENTAL DESIGN AND STATISTICAL ANAYLSIS

To determine the optimum concentration of ethanol produced in this study, a statistical tool, Response Surface Methodology (RSM) was used. To perform RSM Design of expert 11 statistical software was used. It was conducted through the central composite design, which is one of the most common design used for analysis. In central composite design two variable were added i.e. chemical pre-treatment and enzyme. The variables were coded to prevent scale effects from influencing the modelling. The coding was based on centering so that the zero value was assigned to the values of the variable at the centre points. The analysis of variance (ANOVA) and regression analysis were performed to evaluate the effectiveness of the model as a whole, i.e., to determine whether at least one

of the term is significant or not. To check whether the terms in model are fitted correctly, a test for the lack of fit and p-value was performed. Model and all the statistical analysis was performed using software Design expert 11 (Kovacs *et al*., 2019).

III. RESULTS AND DISCUSSION

1. Pre-treatment of Raw material

As mentioned in materials and methods section, collected raw materials were pre-treated with alkali and acid at different concentration in order to increase the yield of enzymatic hydrolysis. Each pre-treatment method was followed by enzymatic hydrolysis performed under the same condition. Thus, the difference in final yield was concluded to be due to the pre-treatment method. The pre-treated Sugarcane bagasse and rice straw are shown in following figures 1(A) and 1(B) respectively.

(A) **(B)**

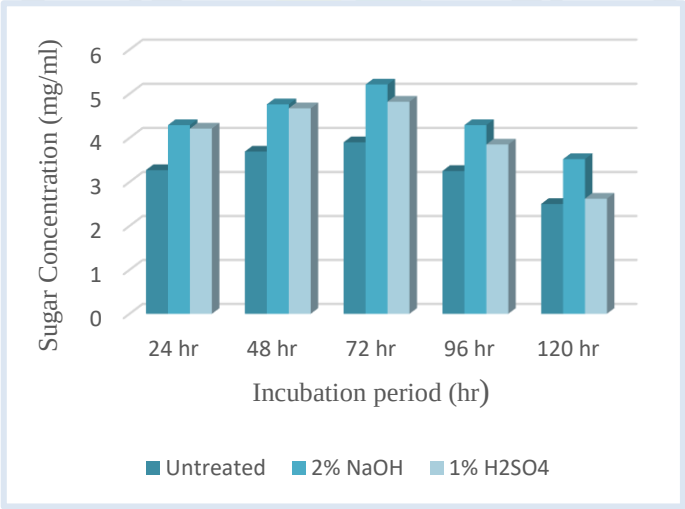
Figure 1: Pre-treated raw material

2. Simultaneous Saccharification and fermentation of raw material:

After pre-treatment method, the raw material i.e. Sugarcane bagasse and rice straw were hydrolyzed using different enzymes along with their cocktail in equal amount to produce fermentable sugars. The hydrolyzed sugar were then utilized by yeast to produce ethanol. The produced sugar were estimated using DNSA method.

2.1 Sugarcane bagasse:

The maximum yield of 5.21 mg/ml of sugar was produced on hydrolysis of sugarcane bagasse treated with 2% NaOH after 72hrs of incubation which was followed 4.82 mg/ml of hydrolyzed sugar produced by SCB treated with 1% H_2SO_4 after 72hrs of incubation. Both the biomass was saccharified using cocktail of enzymes i.e. Cellulase, Xylanase and pectinase in equal amount after pre-treatment of biomass. Compare to this the biomass which was not pre-treated with any chemical, was when saccharified using cocktail of enzymes i.e. Cellulase, Xylanase and pectinase yields 3.9 mg/ml sugar after 72hrs of incubation. This result indicated that 2% NaOH pre-treatment can be used to yield higher ethanol. The results are presented as follow in graph 1.



Graph 1: Graphical presentation of hydrolyzed sugar in SCB

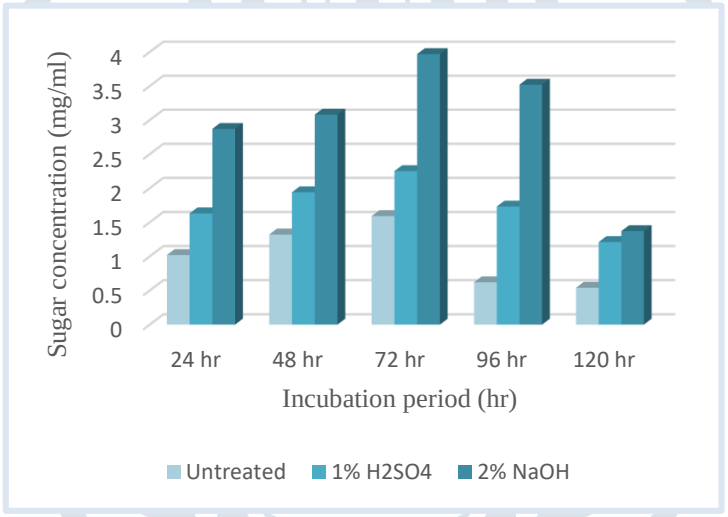
Using an ethanol producing yeast Saccharomyces cerevisiae and enzymes, the maximum amount of bioethanol of 5.2g/L was produced by SCB pre-treated with 2% NaOH and saccharified using cocktail of enzymes i.e. xylanase, pectinase and cellulase. While, SCB pre-treated with acid at 1% H_2SO_4 concentration and saccharification using cocktail of enzymes yields 3.12g/L of bioethanol. Maximum bioethanol was recovered at room temperature after 120hr. The results of bioethanol production are shown in below table 1.

Table 4: Bioethanol Production in SCB

Pre-treatment	Saccharifying Enzymes			
	Xylanase	Pectinase	Cellulase	Cocktail
Untreated SCB	1.6g/L	1.8g/L	2.1g/L	2.8g/L
Alkali Pre-treated SCB				
1% NaOH	3.5g/L	2.5g/L	2.6g/L	3.7g/L
2% NaOH	4.48g/L	4.04g/L	3.80g/L	5.2g/L
3% NaOH	3.1g/L	2.0g/L	1.9g/L	4.25g/L
Acid pre-treated SCB				
1% H_2SO_4	2.7g/L	2.4g/L	2.0g/L	3.12g/L
2% H_2SO_4	2.2g/L	2.3g/L	2.5g/L	3.0g/L
3% H_2SO_4	1.25g/L	0.5g/L	1.6g/L	2.5g/L

2.2 Rice straw

Upon the hydrolysis of rice straw using cocktail of enzymes i.e. Cellulase, Xylanase and pectinase in equal amount after pre-treatment with 2% NaOH, the maximum yield of 3.97 mg/l of sugar was produced. Whereas 2.25 mg/ml of sugar was produced by rice straw treated with 1% H_2SO_4 saccharified using xylanase enzyme. Compare to this the biomass which was not pre-treated with any chemical, was when saccharified using cocktail of enzymes i.e. Cellulase, Xylanase and pectinase yields 1.59 mg/ml of sugar.



Graph 2: Graphical presentation of hydrolyzed sugar in Rice straw

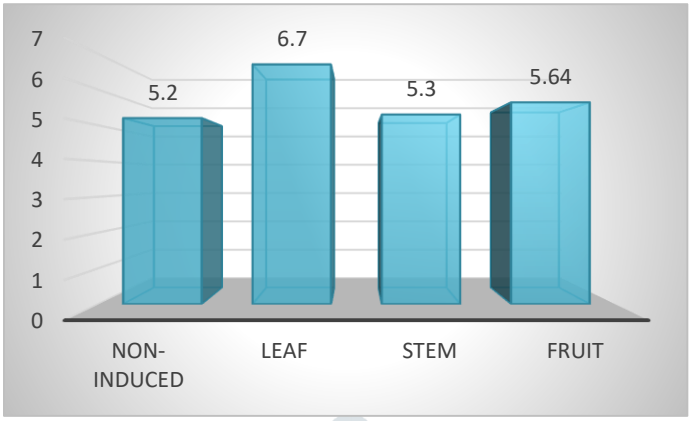
After hydrolysis of rice straw using cocktail of xylanase, cellulase and pectinase enzyme and pre-treatment using 2% NaOH yields highest amount of bioethanol i.e. 3.36g/L. While maximum of 2.16g/L of bioethanol was produced by acid pre-treated rice straw at concentration of 1% H_2SO_4 and saccharified using cocktail of enzyme. This results are comparable to the earlier study by author (Takano et al., 2018) where with H_2SO_4 6.83g/L and with NaOH 28.6g/L of bioethanol was produced. The bioethanol yield obtained in each case is given in table 2.

Table 5: Bioethanol Production in rice straw

Pre-treatment	Saccharifying Enzymes			
	Xylanase	Pectinase	Cellulase	Cocktail
Untreated rice straw	1.26g/L	0.5g/L	1.3g/L	1.4g/L
Alkali Pre-treated rice straw				
1% NaOH	2.6g/L	1.3g/L	2.32g/L	1.88g/L
2% NaOH	2.92g/L	3.28g/L	2.96g/L	3.36g/L
3% NaOH	1.68g/L	1.88g/L	2.1g/L	1.56g/L
Acid pre-treated rice straw				
1% H_2SO_4	1.92g/L	1.64g/L	1.84g/L	2.16g/L
2% H_2SO_4	1.48g/L	1.5g/L	1.28g/L	1.7g/L
3% H_2SO_4	1.24g/L	1.04g/L	0.8g/L	1.5g/L

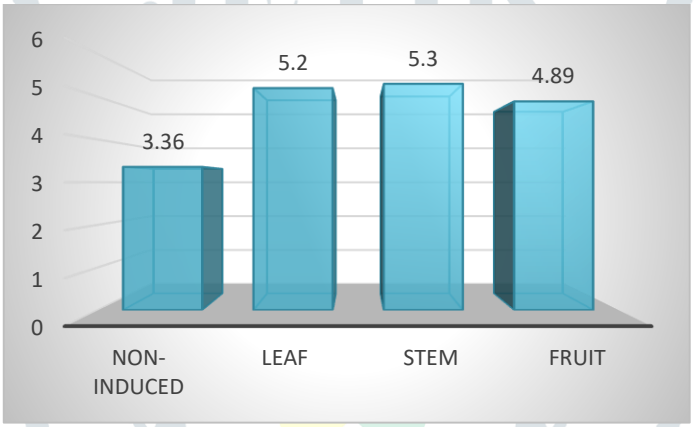
3. Induction of bioethanol production using *Moringa oleifera*:

The fermentable sugar produced during saccharification are utilized by yeast to produce ethanol in accordance of the sugar produced. As observed in below graph 3, in SCB highest amount of ethanol was produced when induced with dried *M.oleifera* leaf powder was 6.7g/L. Therefore, according to this study the induction with *M.oleifera* leaf powder significantly increased the bioethanol production by approximately 28.8% to that of non-induced SCB biomass.



Graph 3: Induction of bioethanol in SCB using *M.oleifera*

While in Rice straw, the highest amount of bioethanol of 5.3g/L was produced when induced with *M.oleifera* stem powder, followed by 5.2g/L with dried leaf powder. There was significant increase of approximately 36.6% in bioethanol production was observed in compare to that of non-induced rice straw. The results are presented in graph 4.



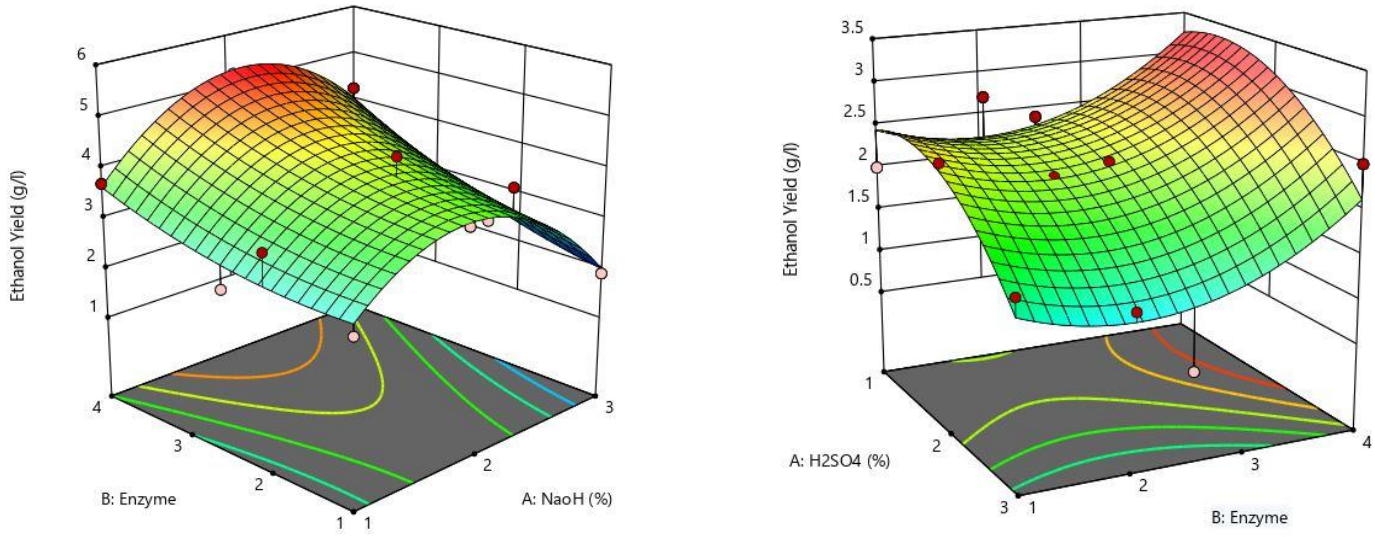
Graph 4: Induction of bioethanol in rice straw using *M.oleifera*

4. EXPERIMENTAL DESIGN AND STATISTICAL ANAYLSIS

To investigate the effect of chemical pre-treatment and enzyme on the ethanol yield from Sugarcane bagasse and rice straw design expert software were used. Two types of chemical pre-treatment i.e. alkali and acid with three different concentrations (1%, 2% and 3%) and three saccharifying enzyme cellulase, pectinase and xylanase along with their cocktail were assessed for maximum ethanol yield. Four models were built two of SCB and rice straw each. In each model, enzyme variable are coded as 1 for cellulase; 2 for xylanase; 3 for pectinase and 4 for cocktail of all enzymes. The experimental design and results are shown in below models.

4.1 Response for pre-treated SCB:

To observe the interactive effect of pre-treatment and enzyme on ethanol yield from SCB biomass, 3D response curve was drawn. The responses surface graphs of ethanol yield from SCB treated with alkali (a) and acid (b) are shown in graph 5. The graphs were plotted by considering the effects of two factors at a time. It is observed that the maximum amount of ethanol was produced with pre-treatment of 2% NaOH concentration and cocktail of enzyme. The amount of ethanol produced during the pre-treatment of NaOH ranges from 1.9 to 5.2g/L. The maximum amount of ethanol produced with pre-treatment of 1% H_2SO_4 concentration and cocktail of enzyme was 3.12g/L. It was observed that about 0.5 to 3.12g/L of ethanol was released from the SCB biomass matrix treated with acid.

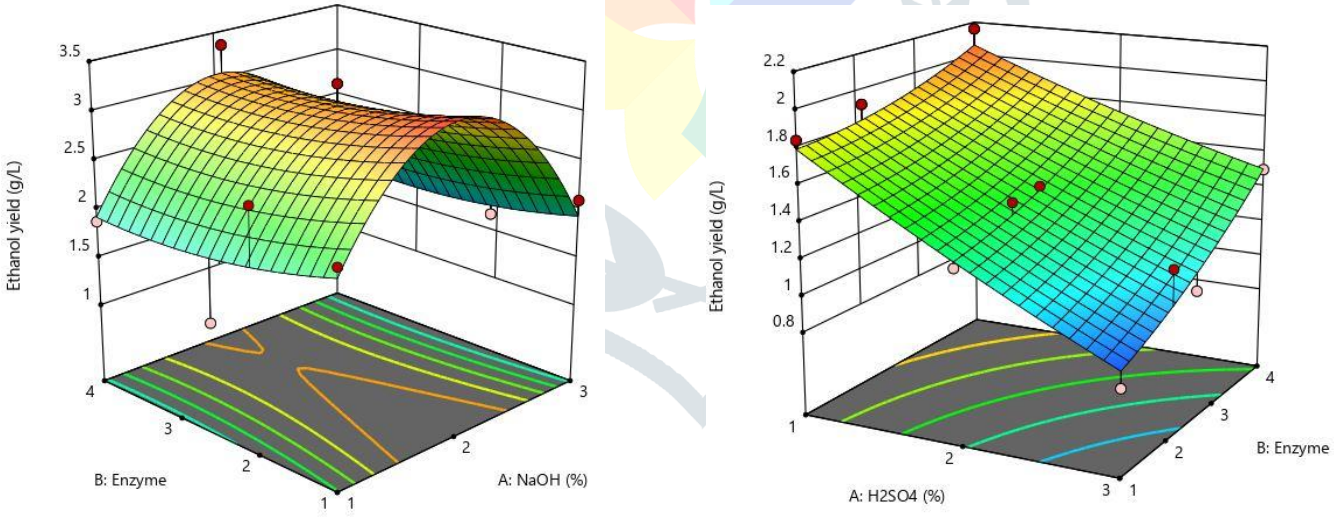


a **b**

Graph 5: Response surface plot of ethanol yield from SCB. a) alkali pre-treated; b) acid Pre-treated

4.2 Response for pre-treated rice straw:

The response surface plots of ethanol yield were generated by feeding the experimental result into design expert software version 11 to study the effect of process parameters on ethanol yield. The 3D responses surface graphs of ethanol yield from rice straw treated with alkali and acid was drawn and given in graph 6(a) and 6(b) respectively. Ethanol recoveries ranged from 1.3 to 3.36 g/L. Higher yield was obtained under milder conditions i.e. 2% NaOH concentration and saccharified using cocktail of enzyme. The amount of ethanol produced during the pre-treatment ranges from 0.8 to 2.16g/L. The maximum ethanol was obtained with 1% H_2SO_4 chemical pre-treatment and cocktail of enzymes. Both extreme, too high and too low H_2SO_4 concentration have adverse effects on ethanol yield. Too high concentration for prolonged pre-treatment time solubilizes the released reducing sugar and yields lower ethanol. Too low concentration of pre-treatment is inefficient to depolymerize the biomass components. The amount of ethanol produced during the acid pre-treatment ranges from 0.8 to 2.16g/L. The maximum ethanol was obtained with 1% H_2SO_4 chemical pre-treatment and cocktail of enzymes.



a **b**

Graph 5: Response surface plot of ethanol yield from rice straw. a) alkali pre-treated; b) acid Pre-treated

4.3 Statistical analysis of response models:

The influence of variables on the ethanol yield was further investigated by performing ANOVA to determine the significance of model. The model for all the responses are significant as noticeable from p-value which is < 0.05 in all the four cases. The lower p-value indicates that the model is more significant. The high R squared value and high adjusted R squared value for ethanol yield are showing that the models are adequate. These suggest that the experimental data are adequately fitted to the design model. Moreover, the factor pre-treatment have a significant impact on the responses (disruption of the rice straw and SCB biomass). These can be best described by comparing the F-values. The high F-values implies that the process parameter has a high impact on the response parameter.

Table 6: ANOVA for response models

	Response 1	Response 2	Response 3	Response 4
P-value	0.0263	0.0377	0.05	0.0038
Std. Deviation	0.5718	0.4456	0.4408	0.1521
R^2	0.8300	0.8063	0.7806	0.9135
Adjusted R^2	0.6884	0.6448	0.5977	0.8414
F-value				
Model	5.86	4.99	4.27	12.67
Pre-treatment	16.82	11.95	20.26	45.56
Enzyme	0.5619	4.64	0.2110	13.01

V. CONCLUSION

This study purposes the production of bioethanol from sugarcane bagasse and rice straw using SSF experiment pre-treated with alkali and acid. The highest amount of bioethanol was produced from SCB when pre-treated using 2% NaOH and saccharified using cocktail of enzyme cellulase, xylanase and pectinase i.e. 5.2g/L. When rice straw was pre-treated with 2% NaOH and hydrolyzed using cocktail of enzymes produces 3.36g/l of bioethanol. Further increase in production was carried out using fresh *Moringa oleifera* leaf, stem and fruit dried powder. In which, highest of bioethanol was produced with dried *M.oleifera* leaf powder i.e. 6.7mg/ml. Approximately 28.8% increase in production was observed. While in Rice straw, the highest amount of bioethanol of 5.3mg/ml was produced when induced with *M.oleifera* stem powder. The significant increase of approximately 36.6% in bioethanol production was observed. As mentioned the best results was obtained using alkaline pre-treatment i.e. 2% NaOH in both sugarcane bagasse and rice straw. According to the (Tsegaye et al., 2020) study, the reason of this can be that the cellulose and hemicellulose content degraded in too extreme condition and too low process conditions were insufficient.

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