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POTASSIUM HYDROXIDE PRETREATMENT OF NAPIER GRASS: CONDITIONS FOR ENHANCED REDUCING SUGAR AND BIOETHANOL PRODUCTION

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ABSTRACT

The recalcitrance of lignocellulosic feedstock needs to be altered for to produce fuels and chemicals. Pretreatment is used to enhance the reactivity of cellulose and the digestibility of biomass, resulting in the effective generation of fermentable sugars. Potassium hydroxide (KOH) is particularly effective at selectively removing lignin from biomass without excessively degrading cellulose and hemicellulose. Moreover, KOH is generally less corrosive than sodium hydroxide (NaOH), leading to lower maintenance costs for pretreatment equipment. In present study, Napier grass was utilized as the substrate for reducing sugar production. Proximate analysis indicated that Napier grass contains approximately 28.50±0.12% hemicellulose, 34.15±0.08% cellulose and 26.41±0.04% lignin. With the use of the Box-Behnken Design (BBD) method, pretreatment conditions were improved. The ideal conditions for KOH pretreatment of Napier grass were determined to be 6% KOH, 180 °C temperature, and a pretreatment time of 120 min. Higher yields of reducing sugars (43.29 g/L) were achieved by this optimized condition. By analyzing the experimental data, ANOVA helps in developing a reliable model that predicts the ideal conditions for increasing reducing sugar yield. Desirability functional studies were employed in optimization to identify ideal conditions that satisfy multiple criteria simultaneously, and the design was validated by trial experiments to ensure accuracy. Desirability studies confirmed that the optimal yield of reducing sugars, approximately 43.72 g/L, was achieved with 6.79% KOH concentration, at a temperature of 178.4°C, and a pretreatment duration of 119.6 min. The results are closely resembles the experimental values predicted by the response surface model. Substrates pretreated with higher KOH concentrations yielded more ethanol (72 g/L) from *Saccharomyces cerevisiae* in SSF tests compared to those pretreated with lower KOH concentrations.

Keywords: Napier grass, potassium hydroxide, Box-Behnken design, pretreatment, reducing sugars, bioethanol

INTRODUCTION

Using lignocellulosic substrates such as forest residues, agricultural by-products, and energy-producing crops shows significant potential for bioenergy production. These resources are abundant globally and address concerns about a connection between food shortages and first-generation biofuels derived from edible sources. Elephant grass, also named Napier grass, is a versatile and productive forage grass native to Southeast Asia and Africa. Its substantial yield makes it popular for livestock feed and bioenergy purposes. With an energy output-to-input ratio of approximately 25:1, it is among the most beneficial energy crops for creating cost-effective and efficient bioenergy systems **(Jain, 2023)**. In India, Napier grass yields an output per acre each year ranging from 100 to 200 tons **(Bhakar and Ram, 2019)**, surpassing other energy grasses such as *miscanthus* and switchgrass, which typically yield 25 to 35 tonnes per hectare. Napier grass is a renewable biomass resource that helps reduce reliance on fossil fuels and contributes to sustainable energy production. The composition of typical Napier grass is as follows: cellulose, 35–39%; silane, 19–23%; lignin, 15–19% (on a dry weight basis) **(Narinthorn et *al*., 2019)**.

Lignocellulosic biomasses are renewable bioresources abundantly available on earth and are carbon-neutral. However, their widespread utilization is hindered by strong bonds among their constituents—cellulose, hemicellulose, and lignin. Several pretreatment procedures are available and employed to effectively separate these interconnected components, maximizing lignocellulosic biomass potential, particularly for bioethanol production **(Nauman Aftab *et al*., 2019)**. Second-generation bioethanol is derived from lignocellulosic feedstocks through saccharification, microbial fermentation, and subsequent product recovery. Biomass resources worldwide can be separated into four main groups: municipal solid waste, wood product industry wastes, agricultural residues, and dedicated energy crops. Agricultural residues, which are abundant and rich in lignocellulose, are a significant renewable biomass resource for bioethanol production, often generated as by-products during or after agricultural crop processing **(Saini *et al*., 2015)**.

Pretreatment leads to physical, biological, and chemical changes in the structure of biomass, highlighting the importance of choosing the right pretreatment method. This initial phase is crucial for breaking down the strong cell wall barrier, increasing sugar production through enzymatic hydrolysis, while decreasing the degradation of carbohydrates and the creation of inhibitory compounds **(Rasid *et al*., 2021)**. Important considerations for a successful pretreatment process include the use of cost-effective chemicals, minimal chemical consumption, and preservation of cellulose and hemicellulose integrity, low energy demand, cost-effective size reduction, and the creation of reactive cellulosic fibers. According to **Nauman Aftab *et al*. (2019)**, primary pretreatment techniques include chemical, physical, thermophysical, thermochemical, and biological procedures. Alkali pretreatment is a method that involves the utilization of alkaline chemicals such as sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$), and ammonia hydroxide (NH_4OH) to disrupt the lignocellulosic biomass structure **(Fuertez *et al*., 2021)**. Typically conducted under high temperatures and pressures, this process enhances the accessibility of hemicellulose and cellulose to enzymes, facilitating their conversion into biofuels or other valuable products **(Patrick *et al*., 2015)**. Alkaline pretreatment is widely accepted as a leading method for processing lignocellulosic substrate for bioethanol generation because of its ease and high efficacy **(Nguyen *et al*., 2019)**. A crucial aspect of practical ethanol production technology is the efficient conversion of inexpensive lignocellulosic biomass into fermentable sugars. Alkali pretreatment is commonly used to extract hemicelluloses and lignin from lignocellulosic biomass **(Kim *et al*., 2016)**. Researchers discovered that the yield of reducing sugars from alkali-pretreated cotton stalks was appreciably higher compared to acid-pretreated stalks and control samples, with a twofold increase (**Malik *et al*., 2020**). **Anuradha and Sampath (2022)** explored various pretreatment methods, including acids (H_2SO_4 and HCl), alkalis (NaOH and KOH), and organic solvents (ethanol and methanol), for sugar production from rice husk. Their study revealed that a 4% NaOH pretreatment effectively breaks down the biomass, leading to a high sugar recovery. In another study, **Lee *et al*. (2021)** used response surface methodology (RSM) to investigate the optimum KOH pretreatment parameters to enhance the enzymatic digestibility of chestnut shells (CNS). The results showed that KOH pretreatment significantly increased the glucan content and enzymatic digestibility of CNS by 1.8-fold and 3.8-fold, respectively, emphasizing the substantial impact of KOH pretreatment on improving enzymatic hydrolysis. Furthermore, **Premkumari *et al*. (2019)** observed that pretreating ground cotton stalk with 2% KOH for 1 h at 120°C resulted in a higher sugar yield, demonstrating that even low KOH concentrations are effective for hydrolysis, with carbohydrate retention of 71.51% under these conditions.

Design of Experiments (DOE) is a statistical methodology used to systematically plan, conduct, analyze, and interpret experiments to obtain valid and reliable results. DOE is essential for controlling input factors or variables to determine their relationship with outputs (responses), thereby ensuring product or process quality. This methodology helps researchers identify optimal experimental conditions **(Lamidi *et al*., 2024)**. The most commonly used DOE method is RSM based on the Box-Behnken Design (BBD). BBD combines a two-stage factorial plan with an incomplete block plan, utilizing coded variables at three levels (−1, 0, and +1). One advantage of BBD is that it avoids combinations where all variables simultaneously take on extreme edge values, preventing experiments under extreme conditions. Additionally, BBD offers a more straightforward way to organize and analyze results **(Gadomska-Gajadhur *et al*., 2020; Gundogdu *et al*., 2016)**, reducing both time consumption and the number of experimental runs required. RSM consists of mathematical and statistical techniques designed to fit a polynomial equation to experimental data **(Aydar, 2018)**. The benefit of the BBD is that it works to prevent unsatisfactory outcomes in extreme situations by avoiding combinations where all elements are at their maximum or lowest values. The BBD is particularly suitable for RSM because it allows for: (i) estimation of quadratic model parameters; (ii) construction of sequential designs; (iii) detection of model lack of fit; and (iv) use of blocks **(Ferreira *et al*., 2007)**.

Numerous studies have examined diverse chemical pretreatment procedures for converting lignocellulosic biomass, like Napier grass, into biofuels and other valuable products. Using techniques such as RSM and BBD, previous researchers **(Chinwatpaiboon *et al*., 2023; Kang and Haslija, 2019)** have investigated the interactions between various factors. However, there is a lack of information on the application of RSM for optimizing the conditions of KOH pretreatment to maximize the production of reducing sugars from Napier grass. This study aims to assess the effectiveness of KOH pretreatment in improving the processing of Napier grass and increasing the yield of reducing sugars. Additionally, a mathematical model based on BBD and RSM was developed and applied to predict reducing sugar yields and determine the optimal pretreatment conditions. The use of KOH in a pretreatment method is advantageous because of its environmental friendliness, cost-effectiveness, and the fact that it does not require the neutralization of raw materials.

MATERIALS AND METHODS

Collecting and preparing Napier grass biomass

Napier grass was gathered from local agricultural fields around Tirupati, India in May month. The grass was chopped into small pieces, air-dried, and then further dried in an oven at 60°C for 12 h. The dried Napier grass was then ground into a fine powder using a mixer grinder. The resulting biomass powder was stored in an airtight polyethylene bag to prevent moisture absorption.

Analysis of chemical composition

The primary components of the biomass such as lignin, cellulose, and hemicelluloses were assessed using the acid detergent fiber (ADF) and neutral detergent fiber (NDF) methods. The Association of Official Analytical Chemists (AOAC) standard methods 973.18 **(AOAC, 1990)** and 992.16 **(AOAC, 1990)** were used to determine lignin, ADF, and NDF. The percentages of cellulose and hemicellulose were indirectly calculated from the ADF and lignin percentages, as outlined by **Mani *et al*. (2006)**.

Box-Behnken method

We used Response Surface Methodology (RSM), a statistical technique, to optimize the conditions for reducing sugar production. Specifically, we employed the BBD of RSM to refine the variables considered in this study. We conducted experiments based on the design in 250 mL conical flasks, using Napier grass biomass as the solid substrate. After reviewing the literature, we identified that three factors were the most important: KOH concentration (A), temperature (B), and pretreatment time (C). Each variable was examined at three levels as presented in **Table 1**. Using the BBD, the software planned a total of 17 experiments to represent each variable at these three levels **(Table 2)**. We utilized a quadratic equation to model the correlation between reducing sugar yield and the independent variables, as presented in Equation 1.

$$Y = b_0 + b_1A + b_2B + b_3C + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{12}AB + b_{13}AC + b_{23}BC \quad (1)$$

In above equation, Y represents the predicted response, b_0 is the intercept, b_1, b_2, and b_3 are the linear coefficients; b_{11}, b_{22}, and b_{33} represent the squared coefficients, and b_{12}, b_{13}, and b_{23} are the interaction coefficients. The results of the trial were examined and interpreted by Design-Expert software **(Design-Expert, 2023)**.

Sample extraction and analysis

After the experiment was finished, the pretreated blend was spun at 10,000 rpm for 8-10 min to separate the solid and liquid parts. After that, the liquid portion was collected and neutralized using 1M H_2SO_4 solution. After neutralization, the liquid was filtered through a 0.2 μm membrane filter, and the resulting filtrate was used to examine the reducing sugars.

Model validation studies

Experiments should be carried out to confirm the accuracy of the theoretically determined models under ideal conditions. Model validation involves calculating the experimental error between the theoretical predictions and the actual experimental results.

Enzyme source

In our investigation of biomass saccharification, we used commercially available *Aspergillus niger* cellulase (≥0.3 units/mg solids) and *Trichoderma reesei* xylanase (100-300 units/mg protein) purchased from Sigma Aldrich, India.

Preparation of yeast inoculum

Saccharomyces cerevisiae NCIM 3455 was cultivated at 30°C for 24 h in a basic medium with an initial pH of 5.5. The medium consisted of glucose (20.0 g/L), polypeptone (1.0 g/L), yeast extract (1.0 g/L), KH_2PO_4 (1.0 g/L), and $MgSO_4$ (3.0 g/L). After the 24 h incubation, the suspension of *S. cerevisiae* cells was collected. The cultured *S. cerevisiae* showed a cell density of 6.5×10^7 cells per milliliter.

Simultaneous saccharification and fermentation (SSF)

To carry out the SSF, separate experiments were conducted using high and low KOH pretreated Napier grass. Both substrates went through the following process: 5g of pretreated substrate was added to 100 ml of distilled water in 250-ml conical flasks, with the pH adjusted to 5.0 using 0.01 M H_2SO_4. Next, 1 ml of cellulase and 1 ml of xylanase were added to the reaction solution as a pre-hydrolysis step, and the mixture was continuously shaken at 350 rpm for 24 h at 45 to 50 °C. The enzyme mixture's suggested loading range provided by the manufacturer, Sigma Aldrich, India, was used as the basis. After the pre-hydrolysis stage, 1 ml of yeast inoculum and 0.2 ml of a 24% urea solution were added. Nitrogen gas was injected into the flask's headspace for 1 min to ensure anaerobic conditions and allow carbon dioxide (CO_2) emission. The fermentation was then conducted at 32 °C with continuous agitation at 300 rpm for 5 to 7 days. There was no further pH control. The flasks were weighed daily to monitor fermentation by measuring the weight loss caused by CO_2 release. Each SSF experiment was performed in duplicate, and an enzyme-free reference experiment was conducted in parallel. The total amount of ethanol produced at the end of fermentation was determined along with the concentration of reducing sugars using gas chromatography (GC) and Dinitrosalicylic Acid (DNSA) methods.

Reducing sugar estimation

To test for reducing sugars, mix about 0.3 mL of DNSA reagent with 0.3 mL of the test solution. Then, heat this combination by placing it in hot water for 5 to 10 min. If there are reducing sugars present, the yellow solution will change to orange or red. After heating, dilute the mixture by adding 3 mL of distilled or deionized water. Next, transfer the diluted solution into a cuvette and use a green light or filter to measure the absorbance at a wavelength of 500–560 nm, ideally at 540 nm **(Miller, 1959)**.

Ethanol estimation by gas chromatography (GC)

The Agilent 6890 system, installed with a flame ionization detector (FID), was employed. To detect ethanol, the following conditions were set for column chromatography: a glass-packed GC Column (compatible with Agilent, Configuration "A"), with a 5% Carbowax 20M phase, 80/120 Carbopack B AW support matrix, and dimensions of 6.0 ft (1.8 m) x 1/4 in. x 2.0 mm. A consistent flow rate of 20 mL/min of nitrogen was utilized as the carrier gas, and a steady flow rate of about 40 mL/min of hydrogen was utilized as the fuel gas. An internal standard was prepared using secondary butyl acetate **(Anthony, 1984)**.

RESULTS AND DISCUSSION

Proximate analysis of substrate

Untreated Napier grass was evaluated for its chemical composition, revealing the following percentages: 86.04±1.20% moisture, 28.50±0.12% hemicellulose, 34.15±0.08% cellulose, 26.41±0.04% lignin, and 13.22±0.16% ash content **(Table 3)**. The present achieved values differ from those reported in other studies. **Kamarullah *et al*. (2019)** conducted a proximate analysis of untreated Napier grass and reported 34.14% hemicellulose, 46.58% cellulose, and 22.25% lignin. Meanwhile, **Montipo *et al*. (2018)** found the grass to contain 20.62% hemicellulose, 33.60% cellulose, 18.42% lignin, and 12.25% ash content. These variations in composition could be influenced by factors such as harvesting age, nutritional inputs, and cultivation location, as noted by **Kataria *et al*. (2017)** and **Sladden *et al*. (1991)**. Although hemicellulose and cellulose contents generally align with previous studies, these components may fluctuate over time; while the overall carbohydrate content remains relatively stable **(Takara and Khanal, 2015)**. Due to its high hemicellulose and cellulose content and low lignin and ash percentages, Napier grass is a promising option for bioethanol production.

Table 1 Chemical composition of Napier grass

	Consequents	Percentage (%)
1	Moisture	86.04±1.20
2	Ash	13.22±0.16
3	Cellulose	34.15±0.08
4	Hemicellulose	28.50±0.12
5	Lignin	26.41±0.04

*Cellulose, hemicelluloses, lignin and ash was determined on dry weight basis.

Box-Behnken design

The experimental design and the variables examined at three levels are presented in **Table 1**. Experiments were performed to determine the theoretical and experimental yield of reducing sugars (g/L) according to the experimental design is given in **Table 2**. The RSM based BBD was employed to predict the optimal levels of key factors and their combined effects. The highest reducing sugar yield of 43.29 g/L was achieved at the optimal conditions of 180 °C temperature, 6% KOH concentration, and a pretreatment time of 120 min. In compare, the lowest reducing sugar yield of 10.24 g/L was observed when the conditions were 60 °C temperature, 2% KOH concentration, and a pretreatment time of 75 min.

The analysis of variance produces a regression equation that depicts the response level based on three independent factors. A quadratic model was used to examine the data using the least squares method, incorporating all terms regardless of their statistical significance. The resulting equation is as follows:

$$\text{Reducing sugars (g/L)} = 22.17+6.95A+7.37B+5.00C-2.06A^2+0.82B^2+2.85C^2+3.31AB+2.11AC+3.56BC \quad (2)$$

In above equation, Y denotes the determined response (Reducing sugar, g/L), while A, B, C, and D are coded independent variables. Predictions for the response at specific levels of each factor can be made using the coded factors equation. The high levels of the factors are typically denoted as +1, and their low levels as −1. By comparing the factor coefficients, the coded equation can be used to verify the relative effect of the components.

Table 2 Actual level of variables tested with the Box-Behnken Design (BBD)

Factor	Name	Units	Minimum	Mean	Maximum	Coded Low	Coded middle	Coded High	Std. Dev.
A	KOH concentration	% w/v	2.00	6.00	10.00	−1	0	+1	2.83
B	Temperature	°C	60.00	120.00	180.00	−1	0	+1	42.43
C	Pretreatment time	min	30.00	75.00	120.00	−1	0	+1	31.82

Table 3 Experimental design in term of actual factors and results of the BBD model

Std.	A:KOH concentration (% w/v)	B: Temperature (°C)	C:Pretreatment time (min)	Reducing sugars (g/L)
1	2	60	75	10.24 (9.93)
2	10	60	75	18.06 (17.21)
3	2	180	75	17.21 (18.06)
4	10	180	75	38.26 (38.57)
5	2	120	30	14.32 (13.12)
6	10	120	30	23.47 (22.81)
7	2	120	120	18.25 (18.91)
8	10	120	120	35.82 (37.02)
9	6	60	30	15.53 (17.04)
10	6	180	30	24.32 (24.67)
11	6	60	120	20.26 (19.91)
12	6	180	120	43.29 (41.78)
13	6	120	75	22.43 (22.17)
14	6	120	75	22.12 (22.17)
15	6	120	75	21.86 (22.17)
16	6	120	75	22.43 (22.17)
17	6	120	75	22.02 (22.17)

* Values without brackets are actual values and values in the brackets are predicted values.
*Std: Standard run order. *Values in ( ) represents model predicted values.

It is important to evaluate model fitting to ensure that the model accurately represents the real-world application. Though, it is crucial to verify the model fitting approach, as the investigated and optimized response surface might not consistently produce precise findings. Generating an actual versus predicted values plot is essential for validating and interpreting the effectiveness of prediction models, especially when aiming to reduce sugar yield. This plot provides both visual and quantitative insights into the model's accuracy and highlights areas for potential improvement. It helps in understanding the model's effectiveness and identifying any mistakes in the predictions. **Figure 1** shows the plot of predicted versus actual values for the yield of total reducing sugars. According to **Figure 1**, there is a strong association between the actual and predicted results for the reducing sugar yield (g/L) system, signifying that the second-order regression model is satisfactory. The fitted model's R^2 value is 99%, indicating a high level of accuracy.

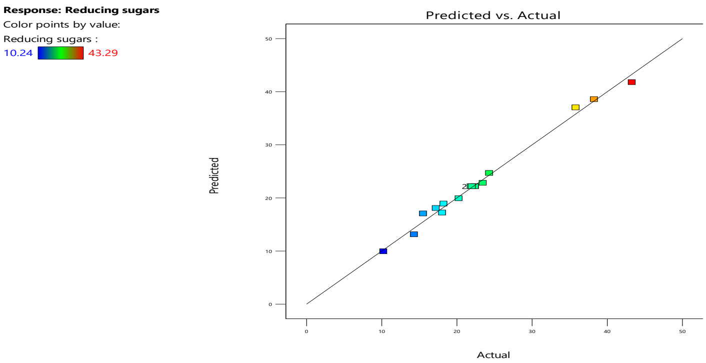

Figure 1 Box–Behnken design plot for predicted versus actual values for reducing sugar yield

Influence of independent factors and their interactions

When using design expert software, one variable in the polynomial equation is held constant in order to generate response surfaces. This allows for the visualization of a three-dimensional image that illustrates how two independent factors are related to each other and the resulting response. Response surface graphs present useful observations into experimental domains, helping to optimize process parameters efficiently to achieve desired outcomes.

The combination of a 6% KOH concentration, a temperature of 180 °C, and a 75-min pretreatment time **(Figure 2)** is essential for maximizing the yield of reducing sugars in biomass hydrolysis. This combination influences the efficiency of converting biomass into fermentable sugars. The 6% KOH acts as a potent alkaline catalyst, breaking down composite polysaccharides, like hemicellulose and cellulose, into simpler reducing sugars. A temperature of 180 °C speeds up the hydrolysis process by increasing the reaction rates, promoting the breakdown of cellulose and hemicelluloses for maximum sugar yield. Higher temperatures ensure the disruption of hydrogen bonds and crystalline structures in cellulose, which is vital for effective hydrolysis **(Wei *et al*., 2018)**. Research has showed that this combination can appreciably enhance the production of reducing sugars compared to lower temperatures or lower KOH concentrations.

The combination of an optimal 6% KOH concentration, a pretreatment time of 120 min, and a high temperature of 180 °C have a important impact on the yield of reducing sugars **(Figure 3)** in biomass hydrolysis processes. Increasing the pretreatment time at high temperature and optimal KOH concentration can greatly affect the amount of reducing sugars produced. Extending the pretreatment time to 120 min allows for more thorough interaction between KOH and the biomass, potentially leading to improved hydrolysis. However, prolonged exposure to high temperature and alkaline conditions can lead to thermal and alkaline degradation of the produced sugars, which can lower the overall yield of reducing sugars. Study has revealed that while increasing the pretreatment time initially leads to higher yields of reducing sugars, beyond a certain point, further increases in time can result in diminishing returns or even a decrease in yield due to sugar degradation **(Fileto Perez *et al.*, 2013)**. Extended pretreatment time at higher temperature can result in the formation of degradation by-products such as furfural and hydroxymethylfurfural (HMF), which can inhibit subsequent fermentation, processes **(Alimny *et al.*, 2019)**.

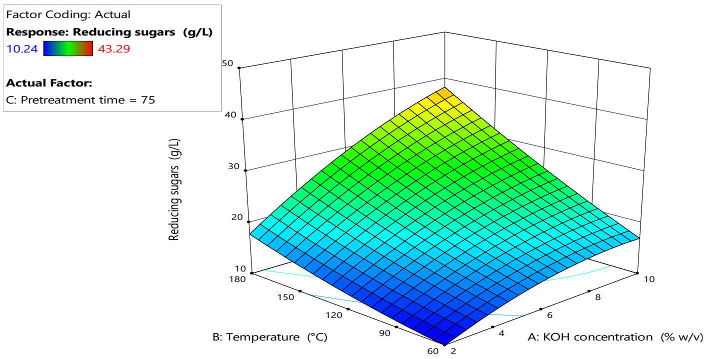

Figure 2 Surface graph of reducing sugar yield showing interaction of KOH concentration and temperature

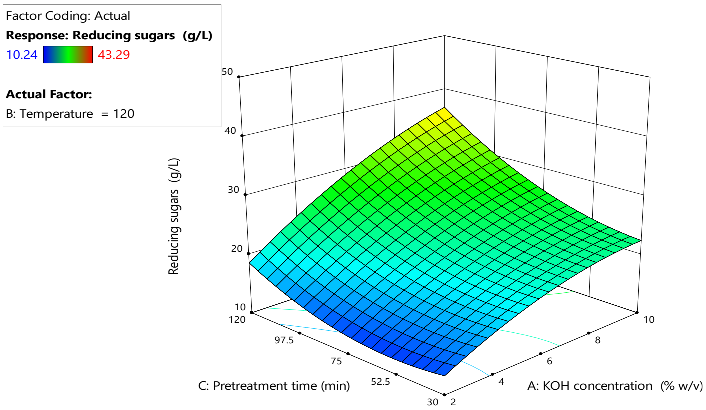

Figure 3 Surface graph of reducing sugar yield showing interaction of KOH concentration and time

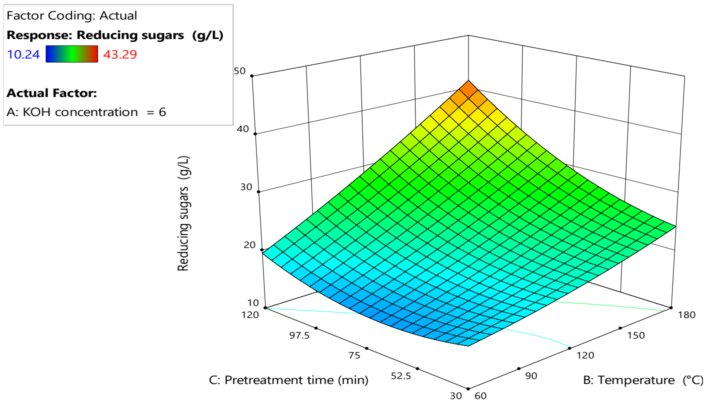

Figure 4 Surface graph of reducing sugar yield showing interaction of temperature and time

The effectiveness of the reducing sugar yield during biomass hydrolysis depends on the interaction of high temperature (180°C), extended pretreatment time (120 min), and an optimal KOH concentration (6%), as shown in **Figure 4**. Elevated temperature help break down the cellulose's crystalline structure, making it more susceptible to chemical hydrolysis. Longer pretreatment times ensure thorough breakdown of the biomass, enhancing the reducing sugar yield. The combination of 6% KOH concentration, 180°C temperature, and 120 min pretreatment time maximizes the breakdown of hemicellulose and cellulose into reducing sugars. This combination significantly enhances the reducing sugar yield compared to lower temperatures or shorter pretreatment times under optimal conditions. However, extending the pretreatment time or increasing the temperature too much may lead to diminishing returns or reduced yield due to excessive sugar degradation. Studies shows that higher yields of reducing sugars are frequently acquired with high temperature in combination with optimal KOH concentration and extended pretreatment times **(Lukajtis *et al.*, 2018)**. Understanding the interaction among these factors is important for creating more effective designs and cost-effective biomass conversion processes.

ANOVA for BBD

The effect of a specific factor is defined as the change in response resulting from a change in the level of that factor. Two factors are considered to interact when their individual effects depend on each other's levels. To further assess the polynomial model (Equation 2) and take into account the interaction between factors, a statistical analysis of variance (ANOVA) was conducted by design expert statistical software. The statistical importance of the factors influencing the response (Y) of the process was determined by performing Fisher's F-test **(Sureiman and Mangera, 2020)**.

Table 4 ANOVA of BBD of KOH pretreatment

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1186.02	9	131.78	88.54	< 0.0001	significant
A-KOH concentration (% w/v)	386.28	1	386.28	259.52	< 0.0001	significant
B-Temperature (°C)	434.98	1	434.98	292.24	< 0.0001	significant
C-Pretreatment time (min)	199.80	1	199.80	134.24	< 0.0001	significant
AB	43.76	1	43.76	29.40	0.0010	significant
AC	17.72	1	17.72	11.91	0.0107	significant
BC	50.69	1	50.69	34.06	0.0006	significant
A^2	17.82	1	17.82	11.97	0.0105	significant
B^2	2.88	1	2.88	1.94	0.2065	---
C^2	34.21	1	34.21	22.98	0.0020	significant
Residual	10.42	7	1.49			
Lack of Fit	10.16	3	3.39	52.87	0.0011	significant
Pure Error	0.2563	4	0.0641			
Cor Total	1196.44	16				
Fit Statistics						
Std. Dev.	1.22		R^2		0.9913	
Mean	22.93		Adjusted R^2		0.9801	
C.V. %	5.32		Predicted R^2		0.8638	
Press	163.0		Adeq Precision		34.0425	

The coefficient of determination, R^2, was calculated to assess the model's fit. R^2 measures the amount of variance that can be ascribed to natural variation. It is a summary metric used in regression studies to indicate how well the independent factor accounts for difference in the dependent factor. The R^2 shows how well the regression line fits the data by measuring the percentage of the overall variation in the dependent factor that is described by the independent factor **(Romeo, 2020)**. In addition, R^2 quantifies the proportion of variability in the identified response variables that is clarified by the model, including the interactions between variables. The closer R^2 is to 1, the better the fit of the design. R^2 represents the percentage of total deviation in the dependent variable defined by the independent variable. An R^2 of 1.0 indicates a perfect fit to the linear model, while any value below 1.0 indicates that some variability in the data is not accounted for by the design **(Hamilton *et al.*, 2015)**. Table 4 shows that the response surface models developed in the current study for estimating reducing sugar production were

sufficient. P-values, or probability values, were employed as a measure to confirm the importance of the model.
In addition, the ANOVA and a high R^2 value of 0.99 demonstrate the model's appropriateness and its alignment with the experimental data, explaining 99% of the differences in responses. This high R^2 value also validates the model's strong predictability, with a recommended minimum R^2 of 0.80 **(Montgomery, 1991)**. ANOVA and the R^2 were used to evaluate the model fit's appropriateness. **Table 4** presents the ANOVA results for both the linear and quadratic models proposed to elucidate the response of reducing sugar yield, emphasizing significant terms identified through variance analysis. ANOVA with 99% confidence and a p-value less than 0.05 (Montgomery, 1991) verified the quadratic model's predictive ability for reducing sugar yield.
The model's F-value of 88.54 indicates high significance. The probability of obtaining such a high F-value from random variation is approximately 0.01%. Model terms A, B, C, AB, AC, BC, A^2, and C^2 are statistically significant with p-values below 0.0500, while terms with values exceeding 0.1000 are considered not important. If many terms are found to be insignificant, simplifying the model could enhance its accuracy, excluding those necessary for model hierarchy. The lack of fit F-value of 52.87 suggests a significant lack of fit, with a probability of about 0.11% that this arises from random noise. The Predicted R^2 of 0.8638 closely aligns with the Adjusted R^2 of 0.9801, with a difference of less than 0.2. The Adequacy Precision, measuring the signal-to-noise ratio, is satisfactory at 34.043, indicating a robust signal compared to noise. This model is suitable for effective exploration of the design space **(Design Expert, 2023)**.

Determination of optimal pretreatment conditions

Based on the experimental result and the developed model, design expert software identified the ideal conditions for maximizing reducing sugar production from Napier grass using KOH pretreatment. As per **Table 3**, the criteria for three variables were aligned with achieving high reducing sugar yields. The model predicted that the highest reducing sugar yield would be achieved at 6% KOH concentration, 180°C temperature, and 120 min of pretreatment duration. The model demonstrated good accuracy in predicting reducing sugar yields across the investigated parameter ranges.

Optimizations using the desirability function (DF)

The study of polynomial equations demonstrates the roles of independent and dependent variables. Optimization is carried out using design expert software with the desirability function (DF), a recognized method for identifying optimal conditions. The DF transforms each response into a function, and then constructs a global function to achieve the desired responses. This process selects the ideal values for variables while taking into account their interactions **(Roosta *et al*., 2014)**. Through numerical optimization, optimal values for each input factor and the response variable can be determined. Input optimizations include specifying ranges, maximum values, minimum values, target values, or none (for responses), aiming to achieve an optimized output under specific conditions. In this study, input variables were assigned specific ranges, while the response aimed to maximize reducing sugar production. According to the model, reducing sugar might have a maximum output of 43.72 g/L **(Figure 5)** at an initial KOH concentration of 6.79 %, temperature of 178.49°C, and time of 119.6 min. Under optimal conditions, the reducing sugar production from confirmatory studies was 43.72 g/L, which was extremely near to the expected value 43.56 g/L, demonstrating the model's accuracy in predicting optimal process parameters and maximizing reducing sugar yield.

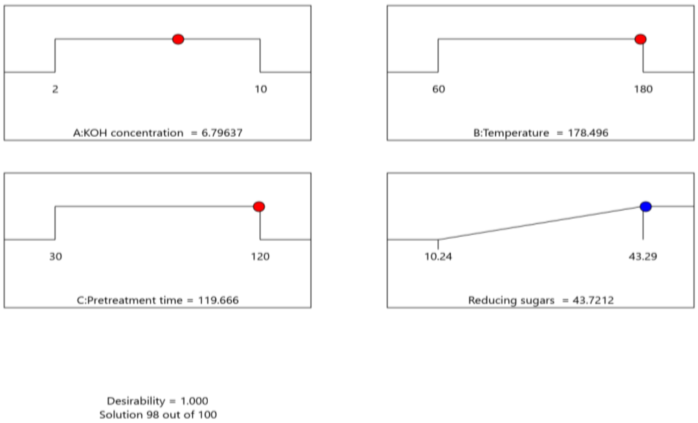

Figure 5 Desirability ramp plot for optimization

Optimization Analysis
Researchers have utilized a range of optimization techniques to enhance product quality and productivity **(Gutema *et al*., 2022; Patel and Brahmbatt, 2018)**. For estimate the desirability of responses, it is required to find the desirability function (DF) for each response. This includes factors like KOH concentration, temperature, and pretreatment time. Desirability is measured on a scale from 0 to 1, where zero indicates an undesirable outcome and one indicates a highly desirable outcome **(Trautmann and Weihs, 2006)**. This scale helps to create a comprehensive function that is optimized by effectively selecting and adjusting the variables. **Table 5** lists the criteria for optimizing all factors investigated based on the results. For every independent variable, the upper and lower bound values were determined using the BBD levels (**Table 2**). The DF for different responses aims to maximize the reducing sugar yields within the observed values. The BBD optimization was done employing design expert software, resulting in 100 solutions, each with a desirability score of 1.000 (**Table 6**). Only 6 solutions were mentioned in the table.

Table 5 Range of input parameters and response

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:KOH concentration (% w/v)	is in range	2	10	1	1	3
B:Temperature (°C)	is in range	60	180	1	1	3
C:Pretreatment time (min)	is in range	30	120	1	1	3
Reducing sugars (%)	maximize	10.24	43.29	1	1	3

Table 6 Iterative determination of optimum conditions

Number	KOH concentration (% w/v)	Temperature (°C)	Pretreatment time (min)	Reducing sugars (%)	Desirability	
1	**6.796**	**178.496**	**119.666**	**43.721**	**1.000**	**Selected**
2	7.196	177.622	119.508	44.596	1.000	
3	7.685	174.189	116.692	44.205	1.000	
4	8.333	175.000	110.750	44.086	1.000	
5	9.324	162.768	115.142	44.712	1.000	
6	9.751	179.793	109.638	47.926	1.000	

The **Figure 6**, bar graph in the present model illustrates the estimated geometric mean as the maximum overall desirability (D = 1.000) for the first solution (**Table 6**) along with the individual desirability functions (di) for each response. The desirability function for the independent variables (KOH concentration, temperature, and time) was set to 1 because they fell within the optimization range. The desirability functions for KOH concentration, temperature, and time were all 1.000.
To obtain the optimal specific value for each response to solution number 1 **(Table 6)**, we used this desirability function and pre-selected a target for each factor. The results are shown in **Figure 5**. The optimal values for the independent variables to achieve a higher reducing sugar yield of 43.72 g/L were a KOH concentration of 6.79 % w/v, temperature of 178.4 °C, and pretreatment time of 119.6 min. Additionally, Table 6 recommends 6 ideal options (out of 100 solutions) for achieving appropriate reducing sugar yield values. Experimental runs were conducted using the ideal conditions of solution 1 in duplicate to validate the model. The optimal parameters for reducing sugar production are presented in **Table 7**.

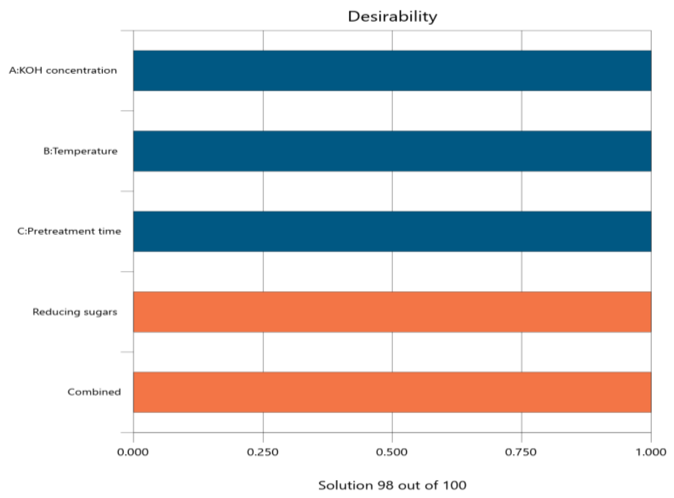

Figure 6 Bar graph for desirability

Table 7 Optimum values during reducing sugar production

S. No	Name	Goal	Optimum values
1	A:KOH concentration (% w/v)	is in range	6.79
2	B:Temperature (°C)	is in range	178.4
3	C:Pretreatment time (min)	is in range	119.6
4	Reducing sugars (%)	maximize	43.72
5	Overall desirability		1.000

Model validation

The experiment was conducted twice under the ideal conditions outlined in **Table 8**. The average sugar yield obtained was 43.29 g/L, which closely matches the predicted value of 43.20% from **Table 8**, with an error of 0.20%. When comparing the actual and predicted values, it was observed that they agreed fairly well, indicating the model's validity. The correlation between the actual and predicted data confirmed the model's accuracy in predicting reducing sugar yield from Napier grass. This approach reduces the number of experiments required and allows for the utilization of lower concentrations of KOH to achieve reliable results.

Table 8 Model validation studies

Trail	KOH concentration (% w/v)	Temperature (°C)	Pretreatment time (min)	Reducing sugars (g/L)		Error (%)
				Actual	Predicted	
1	6	180	120	43.29	43.20	0.20

Ethanol production through SSF

The findings of SSF are displayed in **Figure 7**. The ethanol yield was 60 % in the SSF of non-treated Napier grass using *S. cerevisiae* 3594, cellulase and xylanase mixture. Maximum ethanol yield was 72% in SSF of high KOH-pretreated Napier grass using *S. cerevisiae* 3594, cellulase, and xylanase for 4 days. Ethanol yield increased consistently for both substrates, peaking at 4 days of fermentation. After this point, a gradual decline in ethanol yield was observed for both substrates. This decrease is attributed to depletion of nutrients which are essential for yeast growth and the inhibitory effects of byproducts from pretreatment process, higher sugars, ethanol and presence of salts in the fermentation medium **(Taherzadeh and Karimi, 2011)**. The conditions mentioned above could potentially affect the yeast's fermentative ability.

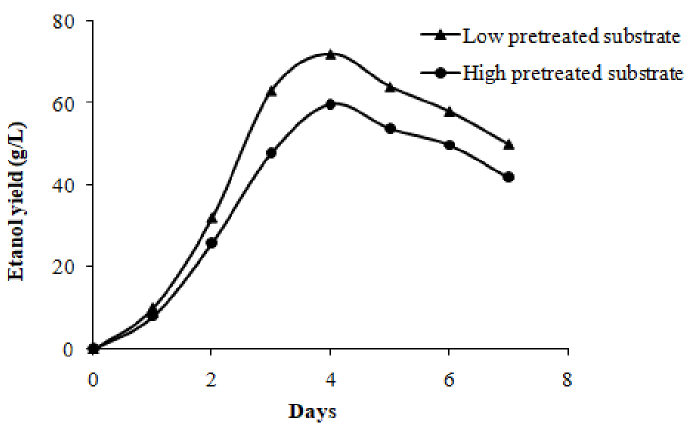

Figure 7 Ethanol fermentation by *S. cerevisiae* on high and low KOH pretreated Napier grass

Comparative studies on sugar production

In current study, the yield of reducing sugar is 43.29 g/L **(Table 9)**, compared to other research listed in the literature. **Remli *et al*. (2014)** found that the highest quantity of sugars released at 120 °C after pretreating rice straw with 2% KOH was 59.90 g/L. In contrast, the minimum sugar release was 37.00 g/L in the sample pretreated with 2% KOH at 30 °C. The findings indicate that a high concentration of KOH is necessary to release increased amounts of sugars from the substrate. According to **Li *et al*. (2012)**, the maximum glucose sugar release of 49.91 ± 0.18 g/L was achieved when the bamboo substrate was pretreated with 12% KOH for 1 h. The findings specify that the substrate pretreated with microwave-assisted KOH had a significantly higher sugar yield compared to the untreated samples. A recent study by **Anuradha *et al*. (2022)** reported that the highest yield of reducing sugars, 1.906 ± 0.2 g/L, was achieved when the rice husk substrate was pretreated with 4% NaOH for 6 h. This study demonstrates that 4% NaOH pretreatment effectively disintegrates rice husk biomass, resulting in high sugar recovery. According to a study by **Tanangteerapong *et al*. (2017)**, the pretreatment of Napier grass with hydrochloric acid produced the greatest quantity of reducing sugar: 44.24 g/L at 90 min. This yield was somewhat higher than that achieved with sulfuric acid, which was 41.83 g/L at 150 min. **Pensri *et al*. (2016)** conducted a study on the possibility of converting NPG residue into fermentable sugar. They treated the substrate with different concentrations of sodium hydroxide (NaOH) and then used enzymatic hydrolysis for saccharification. At 10% (w/v) total solids and with an enzyme loading volume of 2.0 ml/g of substrate, they achieved glucose and reducing sugar generation of 43 g/L and 64 g/L, respectively, from the NPG residue. The study's sugar yield of 43.29 g/L closely aligns with the results of **Li *et al*. (2012), Pensri *et al*. (2016), and Tanangteerapong *et al*. (2017)**, suggesting that the alkali treatment effectively improved the yield. The usage of higher temperatures and a longer pretreatment duration are involved in the high sugar production. By utilizing RSM, the study successfully optimized the process factors and determined the individual, cumulative, and combined effects on the response variable in the pretreatment method **(Veza *et al*., 2023)**. The RSM approach helped identify the best conditions for pretreating Napier grass to increase reducing sugar production and improve ethanol yield.

Table 9 Comparative table on reducing sugar production

Biomass substrate	Pretreatment conditions	Reducing sugar yield(g/L)	References
Rice straw	2% KOH, 120 °C	59.90	Remli *et al*. (2014)
Bamboo substrate	12% KOH for 1 h	49.91	Li *et al*. (2012)
Rice husk	4% NaOH for 6 h	1.906	Anuradha *et al*. (2023)
Napier grass	HCl, 90 min	44.24	Tanangteerapong *et al*. (2017)
NPG residue	3 % NaOH, 120 °C for 1 h	43.00	Pensri *et al*. (2016)
Napier grass	8% KOH, 60 min, 180 °C	43.26	Present study

CONCLUSION

The study examined untreated Napier grass and found that it consisted of 86.04±1.20% moisture, 28.50±0.12% hemicellulose, 34.15±0.08% cellulose, 26.41±0.04% lignin, and 13.22±0.16% ash. These values may vary depending on factors such as the age at which the grass is harvested, nutritional inputs, and location. The high hemicellulose and cellulose content, coupled with low ash and lignin content, make Napier grass a promising material for bioethanol production. The study optimized the pretreatment parameters using a BBD of RSM to enhance sugar production. The highest yield of 43.29 g/L was achieved at a 6% KOH concentration, 180°C temperature, and 120 min pretreatment time. The quadratic regression model showed a high R^2 value of 99%, signifying strong predictability. The ANOVA confirmed the model's statistical significance, with a high F-value and significant p-values. The coefficient of determination (R^2) was 0.99, indicating the model's fitness. The optimal conditions predicted a yield of 43.72 g/L at 6.79 % KOH, 178.49 °C, and 119.6 min, and experimental validation actual value closely matched this at 43.29 g/L. The desirability function scored the ideal conditions at 1.000. Using high KOH-pretreated Napier grass, the highest ethanol yield produced by SSF was 72%. In conclusion, optimizing pretreatment conditions using a BBD and RSM improves the yield of reducing sugar from Napier grass, making it a viable bioethanol feedstock. The model's accuracy and validation underline the potential for efficient and cost-effective conversion of biomass.

Conflict of interest: The authors have disclosed no competing interests.

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