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MODESTUM

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Design of experiment-driven optimization of alkaline pretreatment and enzymatic hydrolysis for *parthenium hysterophorus*

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ABSTRACT

The goal of this study is to find the best pretreatment conditions and look into how alkali pretreatment affects the amount of C5 sugar produced when *parthenium hysterophorus* (PH) is broken down by enzymes. This method made it easier to look at several factors at the same time that affect alkaline pretreatment (substrate concentration: 2-4%, NaOH concentration: 1.00-1.75%, autoclave duration: 20-40 min) and enzymatic hydrolysis (enzyme loading: 0.40-0.80 ml). We evaluated 30 experimental scenarios and attained the maximum C5 sugar production (0.219 g/g) after 12 hours of enzymatic hydrolysis under ideal conditions. The settings were a substrate concentration of 3%, a NaOH concentration of 1.37% w/v, autoclave duration of 30 minutes, and an enzyme loading of 0.60 ml. The examination of biomass composition indicated that the pretreatments achieved a substantial reduction in lignin content, with a delignification rate of 61.30%. This thorough experimental design achieved a high enzymatic hydrolysis efficiency of 73.08% for PH. This suggests that PH is a viable non-conventional lignocellulosic source for C5 sugar synthesis. Thus, alkali pretreatment has become an effective method for biomass valorization within the biorefinery context.

Keywords: enzymatic hydrolysis, delignification, response surface methodology, alkali pretreatment, *parthenium hysterophorus*

INTRODUCTION

Parthenium hysterophorus (PH) is classified as one of the seven most detrimental weeds globally. This annual plant grows quickly and has a long primary root that can reach two meters in height. Additionally, PH seed can also be transported by flooding, water currents, cars, animals, feed, and wind, but to a lesser degree (Kumar et al., 2022b). Moreover, due to its untamed and intrusive characteristics, PH thrives abundantly amidst indigenous flora and functions as a nuisance plant. The composition of PH consists of 47.64% cellulose, 15.31% hemicellulose, and 11.40% lignin, as reported by Kumar et al. (2022a). The first and most important step in lignocellulosic biorefineries is to use an appropriate pretreatment technique to overcome the biomass's recalcitrance tendency (Maibam & Maiti, 2020). Any pretreatment technique's main goal is to increase the exposure of cellulosic fractions by dissociating lignin molecules and breaking down the lignocellulosic matrix's recalcitrance, which will increase the accessibility of enzymes towards their substrate. Numerous studies have been conducted on the various pretreatment techniques used for different lignocellulosic biomasses. In particular, the alkaline pretreatment has shown efficacy in decreasing lignin and improving the pore size and interior surface area of the biomass. Asghar et al. (2015) found that 2.5 percent sodium hydroxide at 121 °C significantly influenced the delignification of wheat straw. The first occurrences and reactions that take place inside the carbohydrate-based materials during alkaline treatment are called solubility and saponification, and they frequently because the treated material to become bloated (Lehto et al., 2015). During the alkali treatment procedure, lignin's alkali-labile bonds with carbohydrates or other molecules may be disrupted, significantly lowering its large molecular size. Therefore, lignin reduction or elimination is crucial to boosting the efficiency of enzyme hydrolysis. The pretreatment factors were improved because process variable optimization is an excellent technique to increase method effectiveness. When optimizing the process, temperature, autoclave time, enzyme loading, and substrate concentration are all crucial considerations. Response surface methodology is a statistical tool used in factor analysis, experiment design and development, and the search for the best conditions to elicit desired responses. These methods also enable the prediction of interactions among various process elements (Zheng et al., 2021). Central composite design (CCD) tool was

Table 1. Range of four independent variables used in experimental design

Symbol	Independent variables	Level		
		+1	0	-1
A	Substrate (%)	2	3	4
B	NaOH (%)	1.00	1.25	1.75
C	Autoclave time (min)	20	30	40
D	Enzyme loading (ml)	0.40	0.60	0.80

applied in this investigation. This study used statistical design techniques to investigate the effects of steam assisted alkaline pretreatment on pH in order to obtain optimal enzymatic saccharification of the alkali pretreated biomass.

MATERIAL AND METHODS

Materials and Reagents

PH biomass was acquired from the Kurukshetra, Haryana, India. Prior to use, it underwent a meticulous cleansing process using water to remove any remaining impurities. The collected PH was dried for 48 hours at 60 °C in a hot air oven to remove any remaining moisture. Once the material was cleaned and dried, it was pulverized using a Sujata laboratory grinder with a size of less than 1 mm. The resulting powder was then kept in polybags for further analysis.

NaOH, CH_3COOH, HNO_3, H_2SO_4, and HCl used were analytical grade from Himedia Pvt. Ltd., India.

Design Experiment (RSM)

In order to improve saccharification and evaluate the effects of various parameters on the reaction, we constructed an experimental model in this work using CCD. Eq. (1) examines how variables affect the reaction to experimental RSM values using a second-order polynomial model.

$$Y = \beta_0 + \beta_1 A_1 + \beta_2 B_2 + \beta_3 C_3 + \beta_4 D_4 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_1\beta_4 AD + \beta_2\beta_3 BC + \beta_2\beta_4 BD + \beta_3\beta_4 CD + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2. \quad (1)$$

In order to improve the NaOH pretreatment, RSM was utilized to examine the effects of several operational factors in CCD experiments, including substrate (A), NaOH (B), autoclaving time (C), and enzyme loading (D). To significantly delignify biomass and overcome its recalcitrance, careful fine-tuning of these essential parameters is needed. CCD utilized variables measured on a 4-point scale (**Table 1**). The overall design consisted of 30 possibilities, each with three copies at a central point, which were carried out randomly. Each expression was performed with 10% (w/v) consistency. The plant slurry was autoclaved at 121 °C for 30 minutes. The hydrolysates were filtered with Whatman No. 1 filter paper to isolate water-soluble components, including carbohydrates (Kumar et al., 2022a). A solid substance was employed to carry out compositional analysis.

Composition Analysis of Raw PH and Pretreated PH

For 20 minutes, 1 g of biomass that had been dried in an oven (w0), whether or not it had been treated, was mixed with a solution made up of 10 ml of 80% CH_3COOH and 1.5 ml of HNO_3 to measure the quantity of cellulose in the biomass. The lignin and hemicellulose components present in biomass were to be broken down using this technique (Updegraff, 1969). Gravimetric analysis was used to determine the amount of remaining cellulose in the solid fraction (Ahmed et al., 2010). After that, the solution was run through filtering crucibles (w_1) that had been previously weighed using a vacuum pump, namely a Rocker 300. After that, these crucibles were dried in an oven set to 105 °C until they reached a constant weight (w_2). Eq. (2) was used to estimate the percentage of cellulose (%w/w):

$$Cellulose\ content\ (\%) = \frac{w_2 - w_1}{w_0} \times 100. \quad (2)$$

A modified method based on Hauli et al. (2013) was used to extract Xylan from lignocellulosic biomass. First, the lignocellulosic powders (made from PH) were soaked continuously at 60 °C for an entire night in a 10% NaOH solution (1:10). After that, they received three hours of steam treatment at 100 °C. Following the alkaline treatment, the mixture was acidified to a pH of 5.0 using 12N HCl after the liquid phase was separated by centrifugation for 15 minutes at 10,000 rpm. The Xylan was then precipitated by adding 1.5 times the volume of 95% C_2H_5OH. After a second centrifugation cycle, the Xylan was first left to air dry before being dried in a hot air oven for four more hours at 55 °C. The combination was weighed, crushed into pellets with a mixer, and then allowed to sit at room temperature for additional analysis. Eq. (3) was used to calculate the actual Xylan content:

$$Xylan\ content\ (\%) = \frac{Dry\ weight\ of\ extracted\ Xylan\ (g)}{Weight\ of\ the\ sample\ (g)} \times 100. \quad (3)$$

The lignin content was determined using the Yao et al. (2010) method. In order to conduct this evaluation, the dried biomass (also known as w_0) was hydrolyzed for 2 hours at 20 °C with a bath ratio of 1:15 using 72% sulfuric acid. Both cellulose and hemicellulose were hydrolyzed during this process (Bhagia et al., 2016). The components were separated using glass crucibles, and their initial weight was noted as w_1. Once the solid residue that had accumulated in the crucible was cleansed with hot water and dried at 105 °C in an oven, it reached a consistent weight (w_2). The weight difference between the pre- and post-acid hydrolysis samples was used to determine the lignin content % (w/w):

$$Lignin\ content\ (\%) = \frac{w_2 - w_1}{w_0} \times 100. \quad (4)$$

Characterization of Pretreated PH

The ABB MB 300 IR spectrophotometer (Japan) was utilized to examine chemical modifications in the structure of both raw and processed PH biomass. We studied samples of PH via FTIR spectroscopy at a resolution of 4 cm^{-1} within 400-4,000 cm^{-1} range.

Enzymatic Hydrolysis

In order to enhance enzymatic hydrolysis, the pretreated biomass was mixed with 0.1 M sodium citrate buffer (pH 5.0) at the appropriate moisture content for the substrate. This

Table 2. CCD of experiment runs

Run	A	B	C	D	Response
1	2	1.00	40	0.80	120.5
2	3	1.37	30	0.60	218.1
3	3	1.37	10	0.60	191.4
4	2	1.75	20	0.40	140.1
5	3	1.37	30	0.60	216.6
6	4	1.75	20	0.40	144.4
7	4	1.00	20	0.80	147.5
8	4	1.00	40	0.80	138.3
9	3	1.37	30	0.60	218.4
10	4	1.75	40	0.80	155.1
11	2	1.00	20	0.80	164.2
12	3	1.37	30	1.00	110.5
13	4	1.75	40	0.40	156.3
14	4	1.00	20	0.40	168.4
15	3	2.12	30	0.60	153.5
16	3	1.37	30	0.20	139.8
17	2	1.00	40	0.40	122.4
18	3	0.62	30	0.60	119.6
19	4	1.75	20	0.80	148.2
20	5	1.37	30	0.60	154.6
21	2	1.75	40	0.80	171.8
22	3	1.37	30	0.60	198.2
23	2	1.75	40	0.40	179.4
24	3	1.37	30	0.60	219.6
25	3	1.37	30	0.60	212.4
26	3	1.37	50	0.60	186.1
27	1	1.37	30	0.60	136.4
28	4	1.00	40	0.40	122.9
29	2	1.75	20	0.80	144.9
30	2	1.00	20	0.40	140.6

Note. A: Substrate (%); B: NaOH (%); C: Autoclave time (min); D: Enzyme loading (ml); & Response: C5 sugar (mg/g substrate)

hydrolysis was done using the fungal crude xylanase enzyme (U/g) for twelve hours at 50 °C and 120 rpm. Under SSF conditions, *aspergillus niger* synthesized the xylanases used in this investigation. Environments of growth for enzyme activity (the culture was acquired from the department of microbiology, MDU, Rohtak, enzyme and fermentation laboratory; NCBI accession No. OP270219) (Kumar et al., 2023). The samples were centrifuged for 10 minutes at 10,000 rpm, and the amount of fermentable sugars in the supernatant was measured using Miller's (1959) DNS technique. A spectrophotometer set at 540 nm was used to determine the absorbance readings. All experiments were conducted three times. Eq. (5) was used to calculate the saccharification yield (Premalatha et al., 2015):

$$Saccharification\ yield\ (\%) = \frac{Reducing\ sugars\ released}{Xylan\ content\ in\ biomass} \times 0.9 \times 100. \quad (5)$$

RESULTS AND DISCUSSION

Experimental Design and CCD Optimization Study

Bhagwat et al. (2016) used RSM, a multivariate statistical method, to optimize and evaluate the collective impact of various factors associated with the specific process involved in LCB. This study enhances RSM's NaOH treatment by increasing sugar release. Under certain experimental circumstances, the liberated fermentable sugars varied from 119.6 mg/g of substrate to 219.6 mg/g of substrate (**Table 2**).

Table 3. ANOVA results for the optimization of liberation of C5 sugars (mg/g substrate)

Source	SS	df	MS	F	p	D
Model	29,861.13	14	2,132.94	19.75	< 0.0001	S
A-substrate	47.04	1	47.04	0.43	0.5193	
B-NaOH	1,398.43	1	1,398.43	12.95	0.0026	
C-autoclave time	74.20	1	74.20	0.68	0.4202	
D-enzyme loading	75.62	1	75.62	0.70	0.4159	
AB	237.16	1	237.16	2.20	0.1591	
AC	101.00	1	101.00	0.93	0.3488	
AD	29.70	1	29.70	0.27	0.6076	
BC	2,540.16	1	2,540.16	23.52	0.0002	
BD	16.81	1	16.81	0.15	0.6987	
CD	2.72	1	2.72	0.02	0.8760	
A^2	8,201.19	1	8,201.19	75.94	< 0.0001	
B^2	10,460.94	1	10,460.94	96.87	< 0.0001	
C^2	1,151.44	1	1,151.44	10.66	0.0052	
D^2	13,736.97	1	13,736.97	127.20	< 0.0001	
Residual	1,619.92	15	107.99			
Lack of fit	1,293.51	10	129.35	1.98	0.2332	NS
Pure error	326.41	5	65.28			
Cor total	31,481.05	29				

Note. SS: Sum of squares; MS: Mean square; F: F-value; p: p-value; D: Decision; S: Significant; & NS: Not significant

ANOVA was employed to examine the effects of enzymatic hydrolysis on NaOH pretreated PH biomass (**Table 3**).

The coefficient of determination (R^2) quantifies the degree of the linear relationship between the experimental and expected results (Sharmada et al., 2016). The model's F-value of 19.75 indicates that it is considered significant. The likelihood of noise being the cause of this type of large F-value is 0.01%. When p-values are less than 0.0500, model terms are deemed significant. Important model terms in this case are B, BC, A^2, B^2, C^2, and D^2. The model terms don't matter if the value is higher than 0.1000. When compared to the pure error, the lack of fitness is not statistically significant, as indicated by the 1.98 lack of fit F-value. The likelihood that noise is the cause of a major lack of fit F-value is 23.32%. Since we want the model to fit, a non-significant lack of fit is good. The difference between the adjusted R^2 of 0.9005 and the expected R^2 of 0.7484 is less than 0.2, indicating a reasonably satisfactory agreement. In terms of coded values, the generic second-order polynomial equation depicts the link between the variables and the quantity of fermentable sugar produced when enzymes break down treated PH.

$$Y = +213.8 + 1.40A + 7.63B - 1.76C - 1.78D - 3.85AB - 2.51AC - 1.36AD + 12.60BC - 1.02BD - 0.412CD - 17.29A^2 - 19.53B^2 - 6.48C^2 - 22.38D^2, \quad (6)$$

where Y is saccharification (%), A is substrate (%), B is NaOH (%), C is autoclave time (min), and D is enzyme loading (ml).

Validation of the Model

The point prediction tool in RSM program was used to create an experiment that verified the model. To produce the best C5 sugar synthesis, the model was created to alter the

Table 4. Yield of reducing sugar under the optimized conditions (mg/g substrate) at 50 °C, pH 5.0, with xylanase from fungus *A. niger*

Response variable	Optimized conditions			Enzyme loading	Fermentable sugar yield (mg/g)
	Substrate	NaOH	Autoclave time		
C5 sugar	3%	1.37 % w/v	30 min	0.60 ml	219

Note. Hydrolysis conditions: 50 °C temperature, pH 4.8, 120 rpm, enzyme loading 0.60 ml, & 3% solid loading

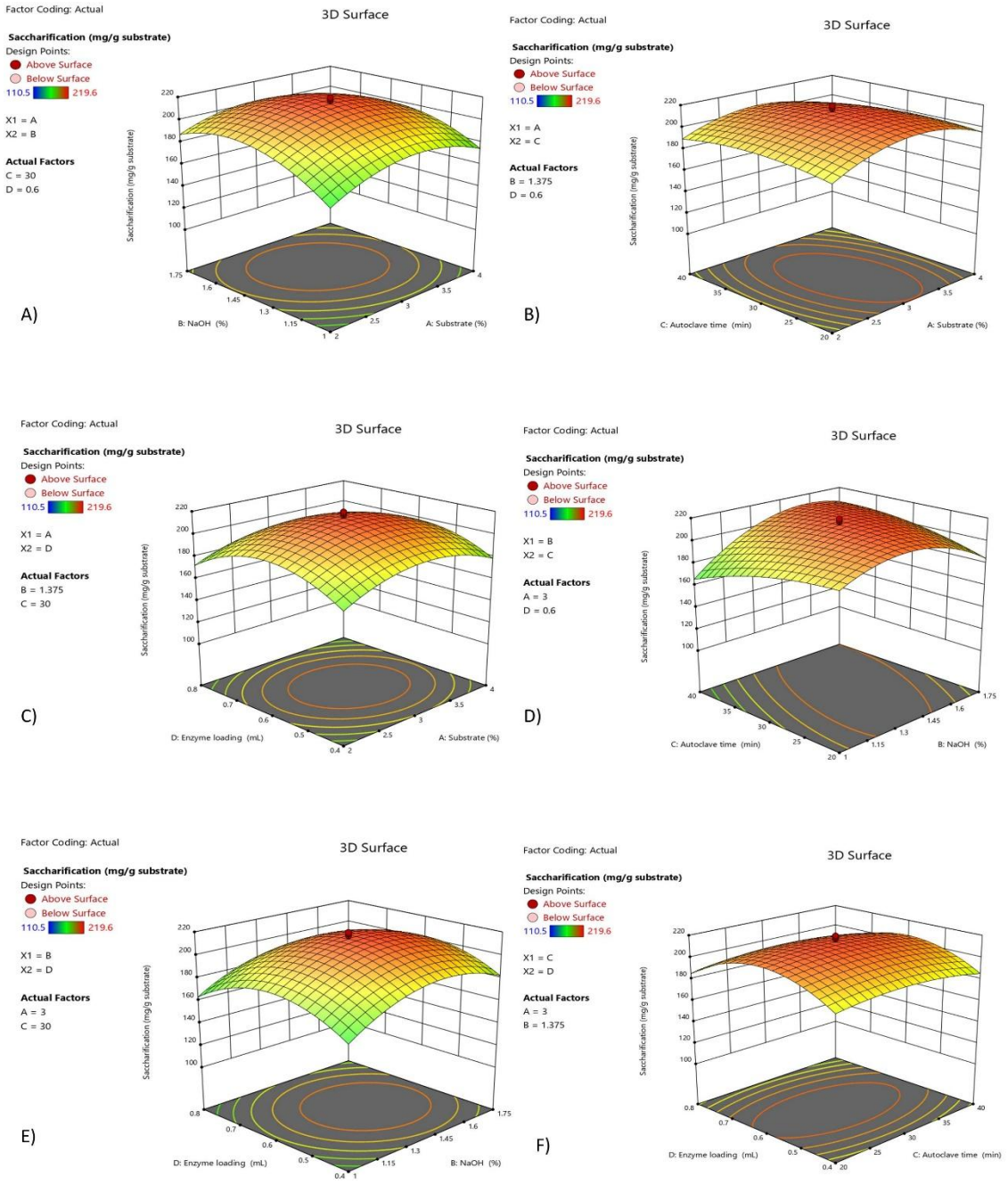

Figure 1. 3D plot diagrams depicting the influence and interaction of (A) sodium hydroxide and substrate concentration, (B) autoclave time and substrate, (C) enzyme loading and substrate, (D) autoclave time and sodium hydroxide, (E) enzyme loading and sodium hydroxide, and (F) enzyme loading and autoclave time on PH pretreatment for enzymatic saccharification (Source: Authors' own elaboration)

variable values simultaneously with enzymatic hydrolysis. In order to compare the experimental and expected results, the experiment was repeated under ideal release conditions for the reaction. The hydrolysis substrate was pretreated with pH, and the process involved 3% solid loading. The following parameters can be used to achieve a C5 sugar content of 219.6 mg/g: substrate (3%), NaOH (1.37% w/v), autoclave time (30 min), enzyme loading (0.60 ml), and a 12-hour enzymatic hydrolysis (**Table 4**).

Figure 1 shows 3D response graphs that show how substrate, NaOH, autoclave time, and enzyme loading affect the process of saccharification.

Part A in **Figure 1** illustrates the interaction between the substrate and NaOH concerning saccharification. It is clear that augmenting the quantities of substrate and NaOH results in elevated yields of C5 sugar. Nonetheless, when the concentrations of substrate and NaOH increase, the yield of C5 sugars diminishes. The optimal C5 sugar yield occurs at a substrate concentration of 3% and a NaOH concentration of 1.37% w/v. The maximum yield of C5 sugars is achieved with a substrate concentration of 3% and autoclave duration of 30 minutes (part B in **Figure 1**). In the case of the interaction between substrate and enzyme loading as illustrated in part C in **Figure 1**, a pattern akin to that obtained with substrate and NaOH is visible. Sugar yield peaks when substrate and enzyme loading are maintained at their central levels. The greatest sugar yield is observed when substrate concentration is at 3% and enzyme loading is 0.60 ml. beyond these numbers, there is a drop in sugar yield. The optimal sugar output occurs at a NaOH concentration of 1.37% and autoclave duration of 30 minutes (part D in **Figure 1**). Similarly, when studying the interaction between NaOH concentration and enzyme loading, as indicated in part E in **Figure 1**, the greatest yield occurs at NaOH concentration of 1.37% and an enzyme loading of 0.60 ml. Similarly, when investigating the relationship between autoclave duration and enzyme loading, as indicated in part F in **Figure 1**, the maximum yield occurs at an autoclave time of 30 minutes and an enzyme loading of 0.60 ml. Under these exact conditions, the maximal sugar output reaches 219 mg/g. To determine the optimal process factors and investigate the individual, cumulative, and interaction impacts of the test factors in saccharification, the statistical experimental design performed admirably. The fact that the experimental results closely matched the values predicted by the RSM model (Preetha & Viruthagiri, 2007) demonstrates this. The total fermentable sugar findings indicate that there was no significant difference between the experimental and projected outcomes ($p > 0.05$). The model can therefore be utilized to maximize the conditions for PH's reducing sugar liberation.

Component Analysis of Raw PH and Pretreated PH

According to a compositional analysis, the untreated PH biomass had a composition of 29% Xylan, 39% cellulose, and 23% lignin. The biomass had 49% cellulose, 27% Xylan, and 8.9% lignin after treatment with 1.37% NaOH+30-min autoclave (61.30% decrease compared to control) (**Figure 2**).

NaOH pretreatment releases ester bonds and breaks up lignin's alkyl and aryl connections, making it an efficient delignification method. **Table 5** displays comparisons of pretreatment conditions for PH biomass.

As shown in **Figure 3**, the observed bands near 1,415 cm^{1}, and 1,730 cm^{1} in the spectrum of untreated biomass shifted with the observation of new bands near 1,406 cm^{1} (C-OH vibration), and 1,620-1,610 cm^{1} (C-O, C = C stretching bonds) in the spectra of the treated biomass. Alkaline hydrolysis enhances cellulose's accessibility for enzymatic hydrolysis by removing lignin and reducing cellulose crosslinking and crystalline. It is the most advantageous and successful method, as evidenced by its success. Alkali treated biomass exhibits superior hydrolysis compared to acid treated biomass

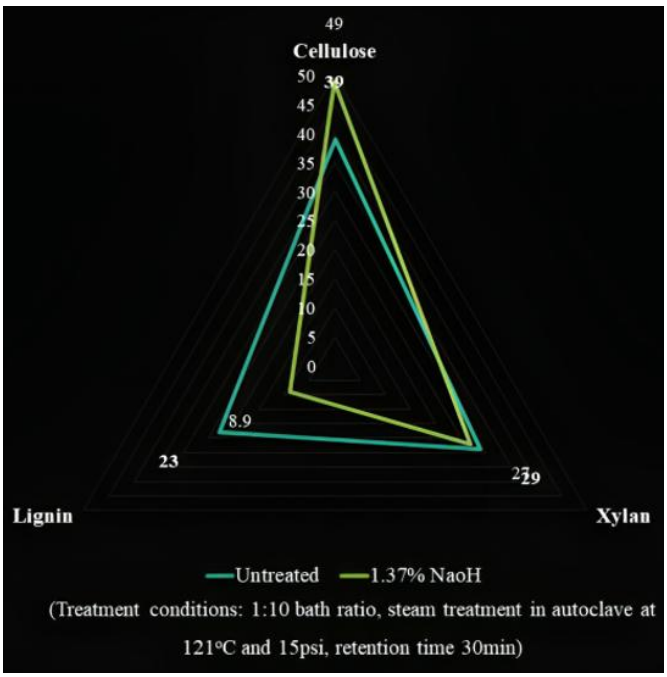

Figure 2. Effect of NaOH concentration on PH biomass (Source: Authors' own elaboration)

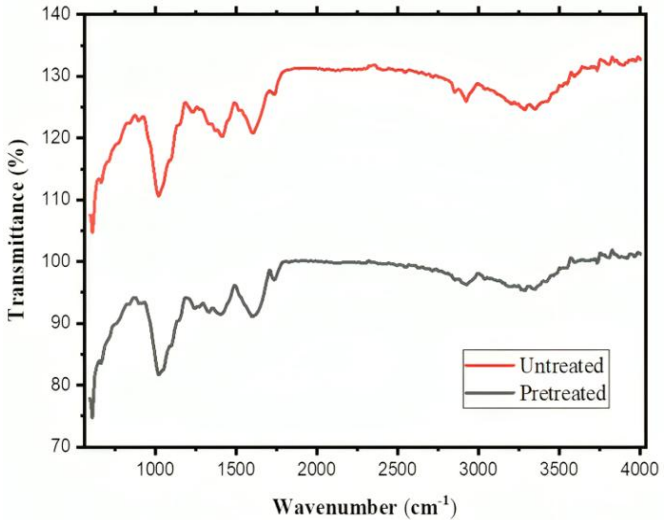

Figure 3. FTIR spectra of untreated and pretreated biomass (Source: Authors' own elaboration)

Table 5. Comparison of pretreatment conditions for PH biomass

Substrates	Pretreatment	Content (%, w/w)			References
		Cellulose	Hemicellulose/Xylan	Lignin	
PH	Sulphuric acid	29.70	21.71	26.60	Bhagwat et al. (2016)
PH	1.5% sulphuric acid	47.64	15.31	11.40	Kumar et al. (2022a)
PH	2.0% sulphuric acid	36.5 ± 1.3	27.7 ± 0.90	24.5 ± 1.20	Bharti et al. (2024)
PH	ChCl/sorbitol	58.78	11.32	13.94	Nargotra et al. (2020)
PH	1.37% NaOH + 30-min autoclave	49	27	8.90	Present study

Table 6. Saccharification efficiency of pretreated PH biomass using fungal xylanase enzyme preparations

Source of enzyme	Xylan content in pretreated biomass (g/g biomass)	C5 sugar (g/g of substrate)	C5 sugar (g/g of Xylan)	Saccharification value (%)
Crude xylanase from *A. niger*	0.27	0.219	0.812	73.08

due to the more effective removal of lignin. After lignin is removed, subsequent hydrolytic processes may benefit from the elimination of unproductive cellulolytic enzymes attached to lignin (Ying et al., 2018). Sharma et al. (2019) discovered that the lignin was reduced by 17.4% when 1% NaOH was used.

Table 6 shows that the fungus's crude xylanase enzyme hydrolyzed the pretreated PH biomass to the greatest extent (73.08%).

CONCLUSIONS

The use of food crops for fuel production has raised concern about competition between food and fuel. To address this issue, researchers have turned their attention to 2G ethanol, which is derived from non-edible lignocellulosic biomass. This type of biomass, found abundantly in various sources, offers an economic and sustainable feedstock for ethanol production. However, it is crucial to explore unconventional feedstock options to avoid over-reliance on a limited range of resources, which could negatively impact land management and biodiversity. In light of these considerations, the present study focuses on utilizing PH weed biomass as an unconventional feedstock for C5 sugar production. PH is a harmful weed known for its adverse effects on animal and human health, as well as biodiversity. By using PH biomass for C5 sugar production, the study aims to not only provide an alternative feedstock but also manage the weed's proliferation through large-scale utilization.

Author contributions: NK: conceptualization, data curation, formal analysis, methodology, software, validation, visualization, writing–original draft, and writing–review & editing & **NKA:** data curation, funding acquisition, investigation, project administration, resources, supervision, validation, and writing–review & editing. Both authors agree with the results and conclusions.

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Declaration of interest: No conflict of interest is declared by the authors.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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