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RESEARCH

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Optimisation of combined acid and enzymatic hydrolysis of paddy straw to produce fermentable hydrolysate

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Abstract

Paddy straw (PS), a by-product of rice production, has a large volume, low economic value, and environmental impact due to burning, contributing to pollution and health hazards. This manuscript highlights the combined effect of acid treatments and enzymatic hydrolysis of paddy straw to produce fermentable hydrolysate, a potential biofuel. This study uses response surface methodology (RSM) with a Box–Behnken design to optimize process parameters (acid concentration, temperature, and duration of hydrolysis), thereby improving the efficiency of converting paddy straw into fermentable sugars. The efficacy of pretreatment was evaluated based on cellulose content and lignin reduction. The optimal conditions of 1% H_2SO_4, 80 °C, and 20 min resulted in effective cellulose enrichment (95.4%) and lignin reduction (38.2%), promoting efficient enzymatic hydrolysis. The enzymatic hydrolysis used cellulase from *Trichoderma reesei*, yielding high glucose concentrations of 225.2 mg glucose ml^{-1} g^{-1} paddy straw. Using Brunauer–Emmett–Teller (BET) analysis and morphology of pretreated and raw PS samples, the surface modification was validated for the optimized hydrolysis conditions. Surface area and pore volume for optimized condition decreased by 58.6% and 25% respectively. However, mean pore diameter increased by 87.9%. Herein, this study offers a more efficient, optimized, and sustainable pathway for converting paddy straw into biofuel using cellulase, with broader implications for agricultural waste management and renewable energy production.

Keywords Enzymatic hydrolysis, Fermentable sugars, Lignocellulosic biomass, Paddy straw, Pretreatment methods

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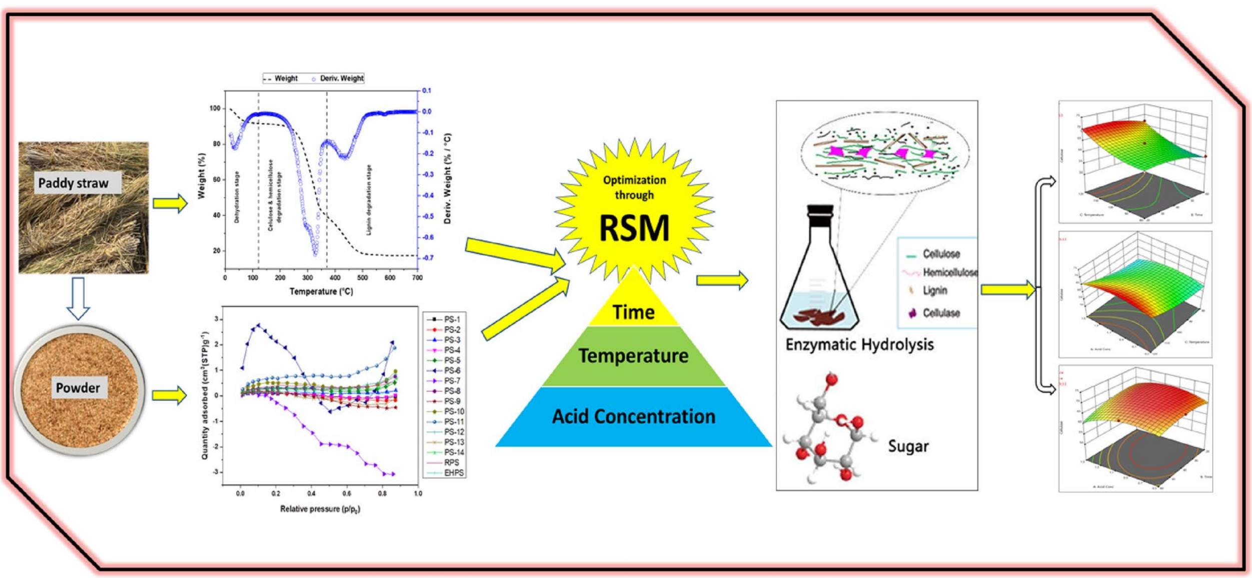
Graphical Abstract



Introduction

Rice is a crucial pillar of global food security [1], and it is a staple food crop in many regions, including Asia [2]. Alongside rice grain production, a substantial amount of straw, approximately one billion tons is annually generated [3]. This straw has historically been a valuable resource used for various purposes in India, but still the major part of paddy straw is open burned in field itself [4]. When this straw is burned, numerous pollutants, such as polycyclic aromatic hydrocarbons (n-alkanes), carbon dioxide, traces of sulfur dioxide, and carbon monoxide, are released into the environment. These pollutants are associated with serious human diseases, including asthma and cancer [5]. Carbon dioxide emissions from open-air burning also pose a significant threat to the ecosystem and contribute substantially to global warming [6]. Additionally, the widespread practice of discarding paddy straw in fields has led to considerable environmental damage and financial loss [7]. Managing crop residues, particularly in the rice–wheat cropping system prevalent in India, poses a significant challenge due to the short interval between harvesting rice and sowing wheat [8]. Biofuel production from paddy straw is a recent alternative to burning, and numerous research efforts are currently underway [9, 10].

The quest for sustainable and renewable energy sources has fueled intensive research into the utilization of lignocellulosic biomass, such as paddy straw, for the production of biofuels. In this context, the pretreatment of biomass stands out as a crucial initial step, playing a pivotal role in removing and isolating hemicellulosic and cellulosic polysaccharides [11]. Once released, these polysaccharides can be transformed into valuable products, such as fermentable sugars that are crucial for biofuel production [12]. Lignocellulosic biofuel production involves a multi-step process encompassing pretreatment, enzymatic hydrolysis, and fermentation. The efficacy of the pretreatment step is paramount in determining the success of subsequent stages. A successful pretreatment method should not only facilitate efficient enzymatic hydrolysis and fermentation but also address challenges, such as minimizing inhibitory compounds, optimizing enzyme loading, preventing sugar losses, and ensuring the recovery of lignin and other compounds for further conversion. This paper contributes to the growing body of knowledge surrounding lignocellulosic biomass pretreatment, offering valuable insights into the optimization of conditions for enhanced sugar release and lignin reduction. The findings presented herein hold the promise of advancing the development of sustainable and economically viable pathways for biofuel and biochemical production from agricultural residues like paddy straw.

Materials and methods

Feedstock preparation

The paddy straw was obtained from the research field of ICAR-Central Institute of Agricultural Engineering, Bhopal (M.P.) after the harvesting of the paddy crop. The raw paddy straw (RPS) material was ground to the particle size of 2–3 mm. The ground paddy straw was then dried at 45 °C for 24 h to achieve the moisture content below 5% w/w.

Experimental design for pre-treatment of RPS

The efficiency of acid hydrolysis is heavily influenced by factors, such as the type of acid used, the pH, the hydrolysis temperature, and the duration of hydrolysis. Strong acids and high temperatures tend to increase the rate of hydrolysis but also promote the formation of degradation products. However, lowering the hydrolysis temperature or reducing the acid concentration can help minimize the formation of these by-products [13, 14]. Hence, hydrolysis temperature levels of 80 °C, 100 °C, and 120 °C and acid concentration levels of 0.5%, 1%, and 1.5% were selected for this study.

The response surface methodology (RSM) applied in this study is Box–Behnken design (BBD) along with three variable factors as stated above. Three variables (acid concentration, hydrolysis temperature, and duration of hydrolysis) with three replicates at the center point were performed to optimize the operating conditions for acid hydrolysis to maximize the cellulose conversion, and lignin reduction using Design-Expert 13 software. The experimental design consisted of 15 runs, including three center points. The experimental run was given the nomenclature of PS-1 to PS-15 for all 15 runs. The operational condition for experiments is given in Table 1. The experimental data for maximum lignin reduction of paddy straw were fitted using a quadratic polynomial function model. The analysis of variance (ANOVA) was determined using Design-Expert 13 (trial version) software to evaluate the statistical significance of the model. The response was obtained by designing the contour plots by setting two parameters (at three different levels each) and their interactions on the total reducing sugar yield and lignin reduction.

Pre-treatment processes of RPS

The pre-treatment of paddy straw was performed with dilute sulfuric acid (H_2SO_4) treatment under different temperature conditions at varied time durations. Five grams of paddy straw was put into a 500 mL Erlenmeyer flask, and 50 mL of dilute sulfuric acid with varying concentrations was added to maintain a 1:10 w/v ratio. After incubating at a specific temperature and a pre-treatment time, the flasks were cooled to room temperature, and the samples were filtered using Whatman no. 1 filter paper. Filtrate samples were neutralized by 1 N sodium hydroxide (NaOH), and sugar content was determined by the DNS method. The recovered pre-treated solids were washed thoroughly in deionized water (6–10 volumes) to a neutral pH and then dried in an oven at 50 °C for 48 h. The dried samples were placed and sealed into ziplock bags and stored at a temperature of 4 °C until enzymatic hydrolysis. Dilute acid pre-treatment experiments were conducted in duplicate.

Table 1 Details of experimental run suggested by Design expert

Run	Sample name	Acid concentration (%)	Time (min)	Temperature (°C)
1	PS-1	1	20	120
2	PS-2	1	20	80
3	PS-3	0.5	40	80
4	PS-4	1.5	40	80
5	PS-5	1	40	100
6	PS-6	0.5	40	120
7	PS-7	1	60	80
8	PS-8	1.5	20	100
9	PS-9	0.5	60	100
10	PS-10	1.5	40	120
11	PS-11	1	60	120
12	PS-12	0.5	20	100
13	PS-13	1	40	100
14	PS-14	1	40	100
15	PS-15	1.5	60	100

Analytical analysis of RPS and pretreated RPS

Biochemical composition of RPS and pretreated RPS

The biochemical composition of RPS was determined using the detergent fiber analysis method described by van Soest (1963), which has also been used by other researchers in similar studies [15]. The paddy straw was then stored under room temperature conditions for further analysis. Carbohydrates (cellulose and hemicellulose), lignin, and ash contents of raw paddy straw fractions and recovered paddy straw fractions from pretreatment were also determined using method described by van Soest (1963).

The analysis procedure involves several steps to determine the composition of plant material. First, the NDF analysis separates soluble and insoluble fiber, revealing the cell wall components like cellulose, hemicellulose, and lignin. Subsequently, the remaining residue undergoes ADF analysis to identify cellulose and lignin content. ADL estimation determines the lignin content, enabling the calculation of cellulose content by the difference between ADF and ADL. Hemicellulose content is then calculated by subtracting ADL from NDF. The ash content, representing inorganic material, is determined by burning a sample in a muffle furnace. Each analysis is conducted thrice for accuracy. The impact of pretreatment techniques on lignin and hemicellulose removal, as well as biomass weight, was assessed.

Thermogravimetricanalysis of RPS

Thermogravimetric (TGA) analysis is a prominent technique to study the thermal behavior of polymer or biomass by monitoring the loss of mass over temperature or time [16]. Before conducting the TGA analysis, the RPS was well dried and converted into powder form,having a particle size from 250 to 300 µm [17]. The powdered sample of 10 ± 5 mg was subjected to TGA analysis in TGA-55, Discovery series, available at the Bio-fuel laboratory of Agricultural Energy and Power Division at ICAR-CIAE, Bhopal.

BET surface area analysis of RPS and pretreated RPS

The specific surface areas and the mean pore diameters of the samples, including RPS were determined using nitrogen adsorption/desorption with a BET BELSORP mini X instrument. Nitrogen molecules, being sufficiently small, can enter the pores of adequate size during the analysis, making the BET surface area and the mean pore diameter reliable estimates of the enzyme-accessible surface area [13, 18]. Before the analysis, the samples undergo degasification at 120 °C for 2 h to remove any adsorbed contaminants, such as water or gasses, ensuring accurate measurements. This preparation step is crucial to obtaining precise data on the surface properties of the samples.

Enzymatic hydrolysis

The hydrolysis step was carried out in a 250 mL Erlenmeyer flask. It was performed using the pre-treated paddy straw recovered after the acid hydrolysis step. The paddy straw obtained after acid pretreatment was added to 5 mL of 50 mm citrate buffer (pH 4.8) with cellulase enzyme from *Trichoderma reesei* (Make: Sigma Aldrich). The paddy straw sample with enzyme load was placed in an incubator shaker at 50 °C, 150 rpm for 72 h. After the completion of the incubation period, the paddy straw hydrolysate (PSH) was filtered to separate the solid paddy straw residue from the liquid fraction. The collected liquid fraction was then subjected to sugar analysis by employing the DNS method.

Enzymatic activity and glucose content in hydrolysates

Cellulase activity was evaluated using the DNS method, a technique outlined by Miller, 1959, which is commonly adopted in similar research endeavors [13, 14]. This method measures the filter paper unit (FPU), representing the enzyme quantity required to release 1 µmol of glucose from a Whatman no. 1 filter per minute.

In the experimental setup, a strip of Whatman no. 1 filter paper (1.0 × 6.0 cm, weighing approximately 50 mg) was placed into a test tube containing 1.0 mL of 0.05 M Na–citrate buffer (pH 4.8). After incubation at 50 °C for 60 min, the mixture was cooled to room temperature. Subsequently, 2.0 mL of DNS solution was added, followed by boiling in a water bath at 95 °C for 5 min to halt the enzymatic reaction. The absorbance of the solution was measured at 540 nm using a UV spectrophotometer. A standard curve correlating absorptivity to glucose concentration facilitated the determination of glucose levels in each sample. The absolute amount of glucose released in the FPU assay at the critical dilution is 2.0 mg (2 mg glucose = 2/0.18 µmol). This amount of glucose is released by 0.5 mL enzyme in 60 min. The FPU reaction is:

$$\frac{2\,\mathrm{mg\,Gulcose} \Big/ 0.18016\,\mathrm{mg}\,\frac{\mathrm{glucose}}{\mu\mathrm{mol}}}{0.5\mathrm{ml\,enzyme\,dilution} * 60\ \mathrm{min}} = 0.37\,\mu\mathrm{mol\,min}^{-1}\,\mathrm{ml}^{-1} \tag{1}$$

Therefore, the estimated amount of enzyme that releases 2.0 mg glucose in the FPU reaction contains 0.37 units, and FPU:

$$\text{Filter paper unit (FPU)} = \frac{0.37}{\text{Concentration of enzyme releasing 2 mg of glucose}}\ \text{units/ml} \tag{2}$$

Similarly, the concentration of reducing sugar (glucose) in the hydrolysate of pretreated paddy straw fractions was determined using the DNS method [18]. The process resembled the determination of filter paper activity, except that 4 mL of hydrolysates underwent centrifugation (at 4000 rpm for 25 min) before 1 mL of each sample was mixed with 2 mL of DNS reagent, followed by boiling and cooling. Standard curves, prepared from known glucose concentrations, were employed to extrapolate the reducing glucose content in the samples.

Morphology analysis

Optical microscopy was used to analyze the overall structural changes in the paddy straw at a cellular level. In this study, microscopic analysis examined the structural changes in paddy straw before and after pretreatment and hydrolysis. For the analysis of fiber structure and cellular integrity, optical microscopy was performed. Thin sections of paddy straw with and without treatment were used to highlight the cellular components, including cellulose, hemicellulose, and lignin. The samples were examined under a light microscope (Ultracam X64) at 10X magnification.

Results and discussion

Biochemical analysis of RPS and pretreated RPS

The composition of the rice straw employed herein was initially compared with those of previous studies (Table 2), and in this case, it was found to contain mainly hemicellulose (23.5 wt%), cellulose (35.4 wt%), lignin (18.73 wt%), and ash (13.6 wt%). However, a slightly higher cellulose content (i.e., 35.8%) was reported by Imman et al. 2015 [19] for the rice straw sample containing hemicellulose (21.5 wt%), lignin (24.4 wt%), and ash 15 wt%). Agbagla-Dohnani et al. [20] implied that different compositions were reported in different studies [20]. Indeed, such variation is common in lignocellulosic biomass as the biochemical composition is influenced strongly by soil type, nitrogen fertilization, the time of harvest, and origin and breeding history (ancestor/parental lineage). Jin and Chen, 2007 conducted an in-depth analysis and concluded that the structural properties of rice straw, including chemical composition, cell proportion, and fiber characteristics, significantly affect enzymatic hydrolysis [21]. Considering such diversity, our focus was directed toward the effects of the structural features of selected biomass including the detailed breakdown of hemicellulose, cellulose, lignin, and ash content, which provides a clear understanding of the primary components that, will influence the hydrolysis process. This specificity helps in identifying which factors need to be optimized for efficient hydrolysis.

Table 2 Composition of rice straw (wt %)

Component	Present study	[34]	[19]	[35]	[36]
Cellulose	35.4	28	35.8	31.1	35.3
Hemicellulose	23.5	55	21.5	22.3	23.8
Lignin	18.73	11	24.4	13.3	17.5
Ash	14.46	6	15	14.5	11.3

The acid pretreatment of paddy straw resulted in notable compositional alterations, particularly a decrease in lignin and hemicellulose, alongside an increase in cellulose content. Cellulose emerged as the predominant component, acting as the main source of fermentable sugars for bio-butanol production. Figure 1 illustrates the compositional alterations in paddy straw biomass specifically cellulose, hemicellulose, and lignin under different acid pretreatment conditions (PS1–PS15) in comparison to raw paddy straw. After acid treatment, the cellulose fraction was found to be enriched in all pretreated paddy straw and the cellulose enrichment was found to

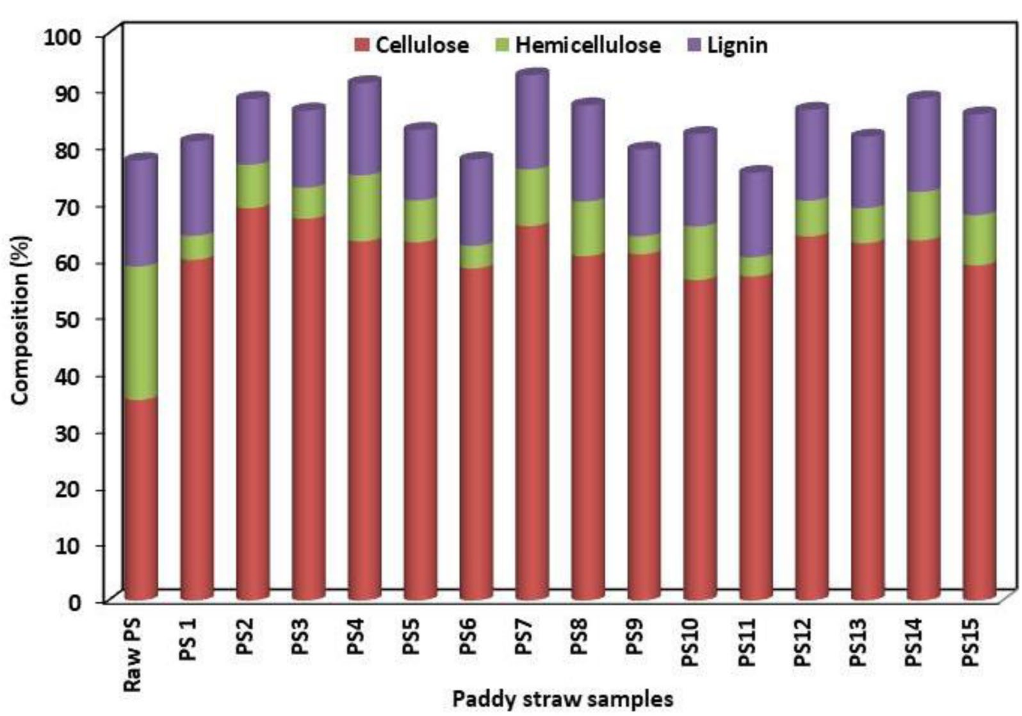


Fig. 1 Bio-chemical analysis results for paddy straw and various acid treatments of PS

be in the range of 59.5–95.4%. The highest cellulose content was observed in treatment PS2 having 69.17% cellulose followed by PS3 sample having 67.32% cellulose content which is comparable to raw paddy straw having 35.4% cellulose content. On the other hand, the reduction was observed in hemicelluloses and lignin content in treated paddy straw samples. The raw paddy straw consists of higher hemicelluloses content as compared to pre-treated biomass samples. The percentage reduction in hemicelluloses content was observed in the range of 50.4–86.5% depending on acid concentration and temperature. The hemicelluloses reduction data have shown that at higher acid concentrations, i.e., 1.5% sulfuric acid, the hemicelluloses' removal was lower. This may attributed to the fact that higher acid concentrations could lead to lignin polymerization, making it more difficult for hemicellulose to be accessed and degraded. Lignin is hardly dissolved in most cases, but is disrupted to a high degree, thus leading to increased susceptibility of the cellulose to the enzymes. In our study, a lower fraction of lignin was solubilized when compared with hemicelluloses. The highest lignin removal of 38% was achieved in the treatment PS2 where biomass was treated with 1% dilute sulfuric acid at 80 °C for 20 min. Dilute acid pretreatment also leads to a partial redistribution and removal of lignin, making the remaining cellulose more accessible to enzymatic hydrolysis in subsequent steps.

Thermal behavior of RPS

Thermal behavior of paddy straw was explored using a thermogravimetric analysis at 10 °C/min. Figure 2 delineated weight loss (Thermogram; TG) and derivative weight loss (Deferential thermogram; DTG) with respect to temperature. The whole degradation process was divided into three major stages in accordance with the degradation of their compositions at different temperature ranges as shown in Fig. 2. The first stage of degradation is associated with the devolatilization of moisture and some lighter volatile materials exist in paddy straws [22]. The dehydration stage was observed up to 120 °C, a peak of the moisture can be noticed under this stage. Second stage was started from 120 to 360 °C. This temperature range is linked with the degradation of hemicellulose and cellulose composition of the biomass [23]. The peak appeared at the right shoulder is related to hemicellulose and on the other hand, the highest peak of degradation is related to cellulosic composition. The third stage of degradation is associated with lignin devolatilization from 360 to 600 °C [24]. According to Durga et al., 2022, the escape of moisture (up to 150 °C), and degradation of hemicellulose, cellulose (175–375 °C), and lignin (379–600 °C) occurred in paddy straws at a heating rate 10 °C/min [25]. The maximum temperature of 120 °C for acid pretreatments of paddy straw has been strategically chosen based on the TGA results. It ensures an effective hydrolysis by maximizing the exposure of cellulose and hemicellulose while avoiding excessive thermal degradation and preserving enzymatic activity. This temperature strikes a balance between process efficiency, energy consumption, and structural integrity of the paddy straw components.

BET analysis of RPS and pretreated PS

The nitrogen adsorption isotherms of raw paddy straw (RPS), enzymatic hydrolyzed paddy straw (EHPS), and treated samples are explicated in Fig. 3. The absorbed quantity of nitrogen is small in amount, which reflects the existence of typical mesoporous structures. A trifling variation in the adsorption among the samples was noticed. Table 3 elucidates the textural properties, such as BET surface area (m^2/g), mean pore diameter (nm), and pore volume (m^3/g), for all the samples. The surface

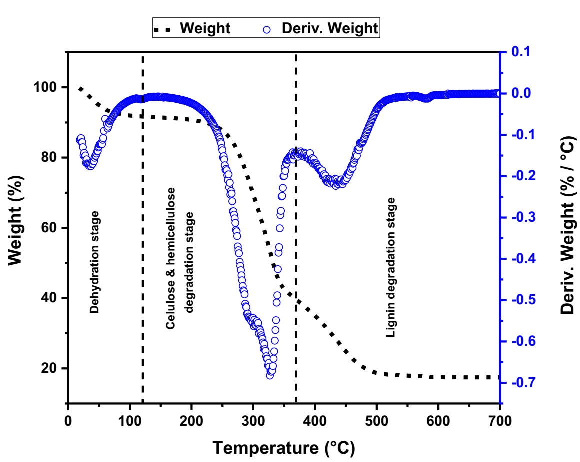


Fig. 2 Thermogravimetric analysis of raw paddy straw

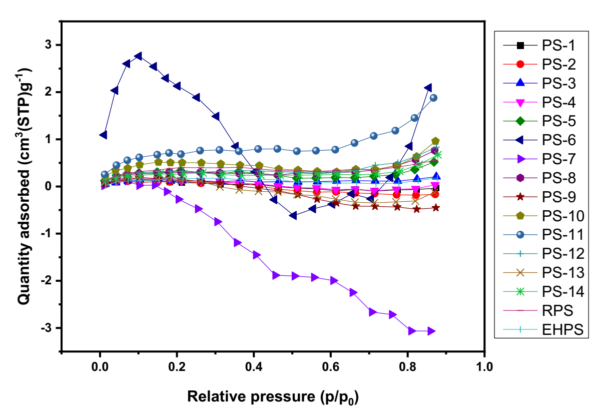


Fig. 3 Adsorption characteristics trend

Table 3 Textural properties of raw paddy straw

Run	Surface area (m^2/g)	Mean pore diameter (nm)	Pore volume (m^3/g)
PS-1	0.49	39.32	0.010
PS-2	0.55	34.72	0.009
PS-3	0.82	23.90	0.010
PS-4	0.64	32.22	0.010
PS-5	1.19	13.55	0.008
PS-6	1.81	13.58	0.073
PS-7	0.81	30.35	0.012
PS-8	1.26	20.63	0.013
PS-9	0.75	27.16	0.010
PS-10	2.17	10.95	0.012
PS-11	0.75	12.28	0.017
PS-12	2.77	21.13	0.014
PS-13	1.31	27.62	0.011
PS-14	1.05	24.04	0.013
EHPS	0.51	41.69	0.011
RPS	1.33	18.48	0.012

area ranges of 0.49–10.89 m^2/g and pore volume varied from 0.008 to 0.073 m^3/g. However, it can be noticed that all the samples have the mesoporous structure with a mean pore diameter in the range of 12.28–41.69 nm.

The pore size of the RPS is 18.48 nm which is lower than most of the pre-treated samples. Increment in the pore size reflected a swelling effect in paddy straw due to pre-treatment. Also the specific surface area was found to decrease slightly for the treated samples. A similar kind of trend was observed by Momayez et al. [26]. A notable increase in mean pore diameter was observed for treated and EHPS. In the process of enzymatic hydrolysis of cellulose, the first essential step involves the binding of celluloses to the cellulose surface. This initial interaction is critical as it initiates the breakdown of cellulose into fermentable sugars. The distribution of pore sizes within the cellulose structure is important. Enzymes can access pores with diameters exceeding 5.4 nm, influencing their ability to penetrate cellulose matrix and catalyze the hydrolytic reactions effectively. Together, these factors significantly impact efficiency and speed of enzymatic cellulose hydrolysis.

A study conducted by Chen et al., 2018 reported the existence of mesoporous structure in the paddy straw and the decrease in BET surface area and pore volume with an increase in nickel and cerium (Ni/Ce) molar ratios [27]. They mentioned that the decrease in pore volume and BET surface area was attributed to Ni nanoparticles partially blocked the pores of the support, which explained the effect of Ni/Ce molar ratios on hydrogenation of rice straw.

Optimization of pretreatment conditions using response surface methodology

Pretreatment of biomass leads to the hydrolysis of hemicelluloses into their monomeric constituents which causes disruptions in lignin–hemicellulose–cellulose bonds. Thus, the purpose of pretreatment is to remove and separate hemicellulose from cellulose, disrupt and remove the lignin component, increase cellulose accessible surface area, and increase cellulose pore size to facilitate the penetration of hydrolysis agents (enzymes). The most effective pretreatment for obtaining fermentable sugars for the production of biofuels is to remove lignin while preserving as much cellulose and hemicellulose as possible, which can increase their fermentable sugar yields [28].

Optimization of process parameters for cellulose content

The dilute acid pretreatment was conducted to ensure the maximum cellulose preservation and reduction of lignin. This allows the easy accessibility of cellulase enzyme toward cellulose during hydrolytic step. Response surface methodology (RSM) is an effective and fast approach for optimization of pretreatment conditions for biomass. Three independent variables viz., acid concentration, incubation time, and temperature were coded and used to determine the dependent variables i.e., cellulose and lignin reduction. The quadratic equation in terms of coded variables for TRS is as follows:

Cellulose = +89.15875 + 5.40 * Acid Conc −0.089625*Time −0.271125* Temperature + 0.038250* Acid Conc * Time + 0.047250* Acid Conc * Temperature + 0.000119* Time * Temperature-7.26500* Acid Conc^2−0.000353* Time^2 + 0.000047* Temperature.^{2}

After the analysis of all the results of cellulose by analysis of variance (ANOVA) for quadratic model is given in Table 4. The model F value of 92.63 implies the model is significant. There is only a 0.01% chance that an F value this large could occur due to noise. P values less than 0.0500 indicate model terms are significant. In this case, A, B, C, and A^2 are significant model terms. A lack of fit with an F value and a P value of 5.03 and 0.1703, respectively, indicates that the lack of fit is not significant relative to the pure error. According to Amenaghawonet al., 2016, a non-significant lack of fit is actually desirable [13]. This shows that the model could be used in theoretical predictions of the acid hydrolysis of paddy straws. The model summary for regression coefficients (R^2 = 99.4%, adjusted R^2 = 98.3% and predicted R^2 = 91.42%) shows that the quadratic model fits into the experimental data.

Table 4 ANOVA results for the Response Surface Model (RSM) of cellulose content in acid pre-treated paddy straws

Source	Sum of squares	df	Mean square	*F* value	*p* value	
Model	185.76	9	20.64	92.63	< 0.0001	Significant
A-Acid Conc	16.53	1	16.53	74.19	0.0003	
B-Time	14.69	1	14.69	65.92	0.0005	
C-Temperature	140.78	1	140.78	631.83	< 0.0001	
AB	0.5852	1	0.5852	2.63	0.1660	
AC	0.8930	1	0.8930	4.01	0.1017	
BC	0.0090	1	0.0090	0.0405	0.8484	
A^2	12.18	1	12.18	54.66	0.0007	
B^2	0.0737	1	0.0737	0.3306	0.5902	
C^2	0.0013	1	0.0013	0.0058	0.9421	
Residual	0.11	5	0.2228			
Lack of fit	0.9837	3	0.3279	5.03	0.1703	Not significant
Pure error	0.1304	2	0.0652			
Cor. Total	186.88	14				

The analysis indicates that the quadratic model is appropriate for describing the relationship between the factors studied and the response of cellulose content. Temperature is the most critical factor, followed by acid concentration and time. The non-significant interaction terms suggest that the combined effects of the factors are not substantial, and each factor primarily influences the response independently. The non-significant quadratic terms (except for acid concentration) imply that the effects of acid concentration and time are primarily linear within the range studied. The lack of fit test further supports that the model is a good fit for the data as there is no significant lack of fit.

The three-dimensional response surface plots are graphical representations of a regression model equation which represents an infinite number of combinations of two test variables with the third variable maintained at a center point. The contours were plotted to observe the interaction of different variables. The 3D surface plots obtained by the interaction of time and acid concentration (Fig. 4a), temperature and acid concentration (Fig. 4b), and temperature and time (Fig. 4c). Acid concentration and time significantly affect the cellulose content, but their influence is relatively moderate compared to the temperature. The response of cellulose content increases significantly with an increase in temperature. This aligns with the ANOVA results, where temperature had a highly significant effect ($F = 631.83$, $p < 0.0001$). Acid concentration is less pronounced but still shows a noticeable effect on cellulose content. The increase in cellulose content response was observed as acid concentration increases up to a certain extent and then the cellulose content decreased. The milder conditions seem to be more favorable for hydrolysis reaction. The strong acids may lead to increase the risk of sugar breakdown into inhibitory compounds like furfural and hydroxyl methyl furfural. Temperature has a substantial and significant effect on the cellulose content response as indicated by the steep slope and the color gradient.

The lack of significant interaction between acid concentration and temperature is evident from the parallel contour lines and the relatively flat surface in the direction of acid concentration. This graphical representation supports the conclusion that temperature is the most influential factor affecting cellulose content, while acid concentration has a secondary effect (Fig. 4b). The total cellulose content decreased with an increase in temperature. This observation might be attributed to an increase in the rate of collision of molecules of the reacting species during the reaction. Time also affects the cellulose though to a lesser extent compared to temperature. The 3D surface plot visually confirms the findings from the ANOVA table. Time also affects the total cellulose content, but its influence is less pronounced compared to temperature. The lack of significant interaction between time and temperature is evident from the relatively flat surface in the direction of time and the parallel contour lines.

Optimization of process parameters for lignin reduction

The removal or degradation of lignin indicates the effectiveness of the pre-treatment method. As lignin reduction facilitates the easy access to cellulose and hemicellulose at enzymatic hydrolytic stage for glucose recovery for biofuels production, many studies stated that the dilute

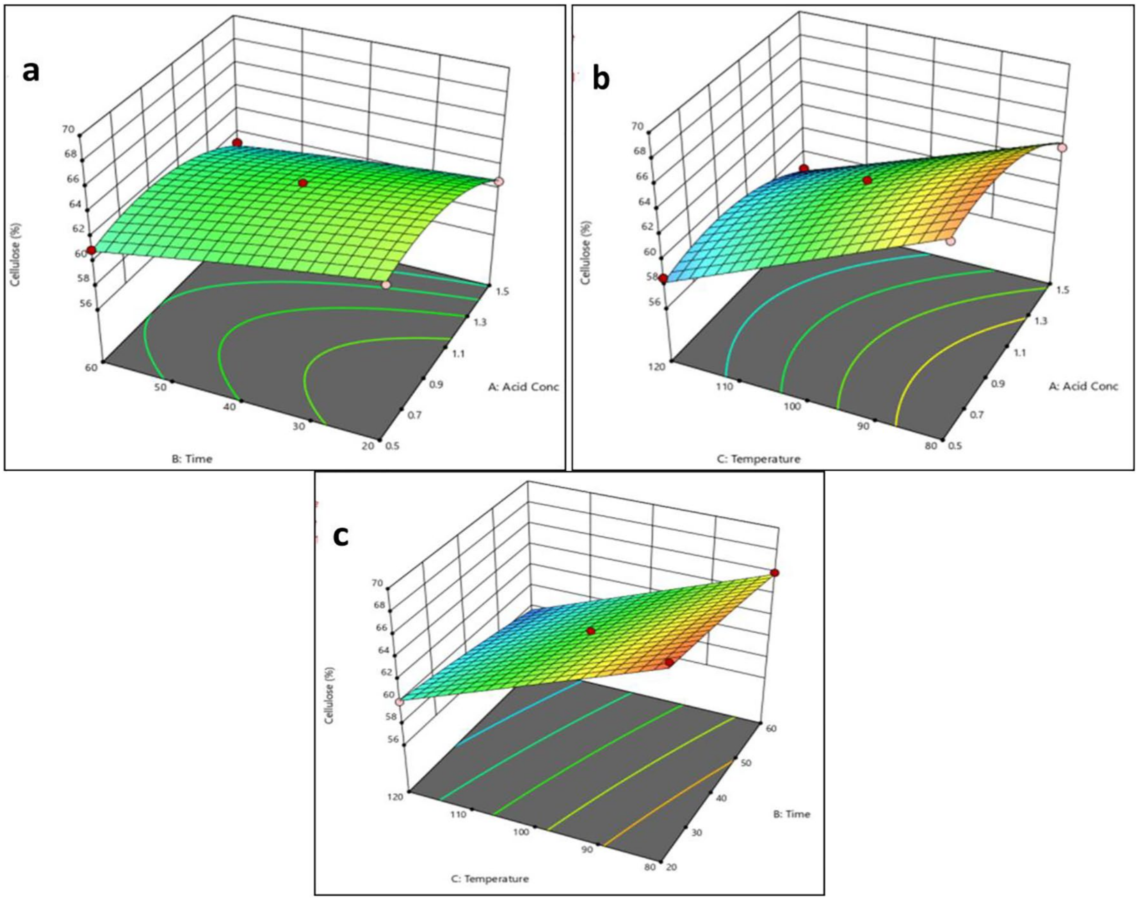


Fig. 4 Surface plot for TRS release **a** time and acid concentration, **b** temperature and acid concentration and **c** temperature and time

acid pretreatment led to the lowering of lignin content and it also converted the non-carbohydrate polymers into low molecular weight compounds [29].

The lignin content in raw paddy straw was 18.73%, which was reduced to some extent after the acid hydrolysis or pretreatment. The lignin content in acid treated paddy straw was found in the range of 12.44–17.8% as shown in Fig. 5. The highest reduction of lignin content was obtained in the PS2 sample treated with 1% H_2SO_4 at 80 °C for 20 min. The higher lignin content was observed in the samples treated with higher acid concentrations (0.5%), while the samples treated with 0.5% acid concentration showed a variable range of lignin content. As depicted from the graph, the lowest lignin reduction was observed in 1.5% acid treated paddy straw which was 4.97%. Increased acid concentration can lead to excessive degradation of hemicellulose and cellulose. This over-hydrolysis may also result in the formation of lignin–carbohydrate complexes, which are more resistant to further breakdown. Additionally, higher acid concentrations can lead to the re-condensation of lignin, making it less amenable to removal. The 1.5% acid pretreated paddy straw has the cellulose content of 59% which is lower than that of the 1% acid treated sample having cellulose content of 67%. After preliminary studies, the optimization runs were performed for determining the suitable temperature

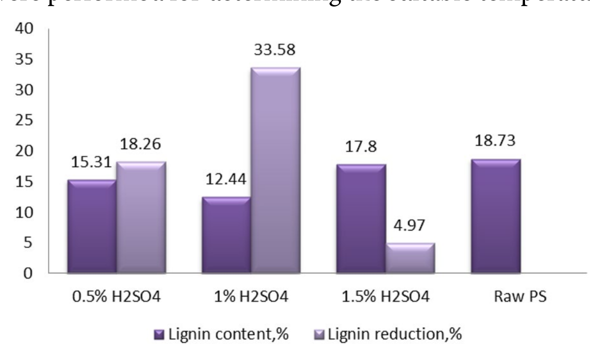


Fig. 5 Effect of acid concentration on lignin content and %lignin reduction

and time for acid pretreatment.

The experiments run a sample which has shown the highest lignin content which leads to the release of the lowest sugar concentrations after enzymatic hydrolysis since lignin hinders cellulase access to cellulose fibers and binds non-productively to cellulose. The pretreatment experiments were performed and the quadratic equation in terms of coded variables for lignin removal is shown below:

$$\begin{aligned} Y_{\text{lignin reduction}} = {} & 16.9633 + (1.34375 * A) + (-0.06 * B) \\ & + (3.25125 * C) + (1.22 * AB) \\ & + (-0.3075 * AC) + (-0.4 * BC) \\ & + \left(0.0720833 * A^2\right) + \left(0.709583 * B^2\right) \\ & + \left(-0.262917 * C^2\right) \end{aligned} \tag{3}$$

The results of lignin reduction in different pretreatment conditions were analyzed by analysis of variance (ANOVA) and the regression results are given in Table 5. The regression analysis shows that the model was significant due to an *F* value of 21.56 and a low probability value. The slight lower p value of acid concentration and time than temperature indicates that the change in acid concentration and time imparts a significant effect on response surface and lignin reduction. The model summary for regression coefficients (R^2=97.4%, adjusted R^2=92.7% and predicted R^2=73.9%) shows that the quadratic model fits into the experimental data.

Three-dimensional plots of response surfaces were constructed based on the model equation (Eq. 2) to investigate the interaction among factors and verify the optimum concentration of each variable for maximum lignin reduction. The response surface plots are shown in Fig. 6, which explain the interaction between the variables based on the final model equation. In these plots, one factor is constant at the optimum level, whereas the other two factors are varying within their experimental range.

The results indicate that both acid concentration and time significantly influence lignin concentration. The response surface plot shows that increasing either acid concentration or time leads to higher lignin concentrations i.e., lower lignin reduction. However, the relationship is not strictly linear, suggesting that optimal conditions involve a combination of both high acid concentration and extended time (Fig. 6a). The interaction between acid concentration and temperature is evident as shown by the curvature of the response surface (Fig. 6b). The response surface plot reveals that both temperature and time significantly impact lignin concentration, with lower levels of both parameters resulting in maximum lignin yields (Fig. 6c).

Enzymatic hydrolysis of treated paddy straw

The cellulose and hemicellulose contents of rice straw can be hydrolyzed chemically or enzymatically. Studies suggested that the hydrolysis of biomass should be carried out in two steps: first, pretreatment for hydrolysis of hemicellulose fraction and lignin removal under milder conditions, and second, the enzymatic hydrolysis of exposed cellulose fraction for higher yield of sugar in hydrolysate [30]. In the present study, the paddy straw was pre-treated with mild acid (dilute sulfuric acid) and small amount of glucose had been found to release after pretreatment step. The glucose liberated at mild hydrolysis conditions most likely originated from hemicellulose [31]. Dilute acid pretreatment is rather a typical method for rapidly converting hemicellulose to soluble sugars during pretreatment, and hemicellulose reduction can greatly increase enzymatic hydrolysis of pretreated biomass [32]. The pretreatment of lignocellulosic biomass plays an important role in enzymatic hydrolysis. The principal goal of the pretreatment is to enhance hemicellulose solubility and reduce/modify lignin as well as the degree of polymerization and crystallinity of cellulose, which increases pore size and surface area of the biomass for improved conversion to sugar [33, 34].

The cellulase enzyme from *Trichoderma reesei* (Make: Sigma Aldrich) was used for enzymatic hydrolysis of paddy straw. The cellulase activity was determined by the NREL method and it was found to be 42 filter paper units (FPU) mL^{-1}. In contrast to larger enzymatic loading used by other researchers in similar investigations, Axelsson, 2011 states that the low enzymatic loading is a more practical dose for industrial application. In our present study, the enzymatic loading of 8 FPU/g of biomass was maintained similar to the studies conducted by Aboagye et al. [15]. The hydrolysis of paddy straw samples generated after acid pretreatment optimization studies was performed. Figure 7 illustrates the impact of varying pretreatment conditions on total reducing sugar yield and saccharification efficiency (%).

The raw paddy straw has saccahrification rate of just 7.3% with a corresponding total sugar yield of 28.3 mg/g of raw paddy straw. The unprocessed paddy straw

Table 5 ANOVA results for the Response Surface Model (RSM) of lignin reduction in acid pre-treated paddy straws

Source	Sum of squares	df	Mean square	*F* value	*p* value	
Model	47.40	9	5.27	21.56	0.0017	Significant
A-Acid Conc	5.66	1	5.66	23.17	0.0048	
B-Time	2.39	1	2.39	9.77	0.0261	
C-temperature	1.92	1	1.92	7.86	0.0378	
AB	0.3136	1	0.3136	1.28	0.3086	
AC	2.61	1	2.61	10.68	0.0223	
BC	3.05	1	3.05	12.46	0.0167	
A^2	6.89	1	6.89	28.19	0.0032	
B^2	8.58	1	8.58	35.11	0.0020	
C^2	14.72	1	14.72	60.25	0.0006	
Residual	1.22	5	0.2443			
Lack of fit	0.7203	3	0.2401	0.9579	0.5472	Not significant
Pure error	0.5013	2	0.2506			
Cor Total	48.62	14				

exhibited the lowest saccharification efficiency, highlighting the significance of pretreatment in improving enzymatic hydrolysis. Pretreatment, on the other hand, greatly improved enzymatic breakdown by increasing porosity, which facilitated better enzyme penetration and enhanced saccharification yield. The highest sugar yield of 221.24 mg/g of paddy straw and saccharification efficiency of 56.25% was found in sample PS2 treated with 1% sulfuric acid for 20 min at 80 °C. The saccharification efficiency was found to decrease with the increase in temperature for all the acid treatments (0.5%, 1%, and 1.5% H_2SO_4). The higher acid concentrations (e.g., 1.5% H_2SO_4) negatively impacted enzymatic activity, likely due to the formation of inhibitory by-products that hinder enzyme performance. The lignin content also impacts the total reducing sugar yield and saccahrification efficiency as shown for the sample PS2 where lignin content in pretreated sample was the lowest i.e., 11.6% compared to 18.73% in raw paddy straws. This indicates that acid pretreatment reduces lignin, which disrupts the internal structure of straw, allowing enzymes to act more effectively, and enhancing the solubility of cellulose to produce reducing sugars in the hydrolysate.

Morphology of RPS and treated RPS samples

Quantitative analysis of surface area, pore size, and fiber alignment was conducted to evaluate the extent of structural changes during the pretreatment and hydrolysis process. Table 6 illustrates the comparison between the untreated and the treated samples to assess the efficacy of the combined acid and enzymatic hydrolysis in enhancing the biomass breakdown. From microscopic images, it was inferred that with higher duration and higher temperature, pretreatment results in higher losses in cellulose contents. In the untreated sample, the cellulose fibers appear relatively intact and dense, with minimal disruption to the cell walls. This shows the natural structure of the paddy straw with cellulose content largely preserved. In PS2 and PS4, the cellulose fibers begin to show some signs of disruption. There is a partial degradation in the hemicellulose and cellulose structure. Some pores or cracks may be visible on the fiber surface. This indicates that pretreatment at moderate temperature causes some breakdown, but significant cellulose remains. The loss of cellulose is still relatively low at this stage, whereas in case of PS8, PS12, and PS 15, combined effect of increased acid concentration at higher temperature shows more disrupted cellulose fibers, with noticeable surface erosion, large pores, and some fiber fragmentation. The cell wall structure becomes less distinct).

Furthermore, for PS 10 and PS11, a considerable damage to the cellulose fibers has been observed. The surface of the fibers is heavily eroded, with extensive pore formation and fiber fragmentation. The cellulose content appears significantly reduced. This phenomenon was observed due to prolonged exposure to high temperature which exacerbates the degradation of cellulose, resulting in a higher loss of cellulose content compared to shorter pretreatment times. Increased temperature accelerates the breakdown of cellulose and hemicellulose, leading to a more significant loss of cellulose content.

Optimized pretreatment conditions

The optimal pretreatment conditions of paddy straw were attained by Design-Expert software through graphical optimization. Taking both total cellulose content after

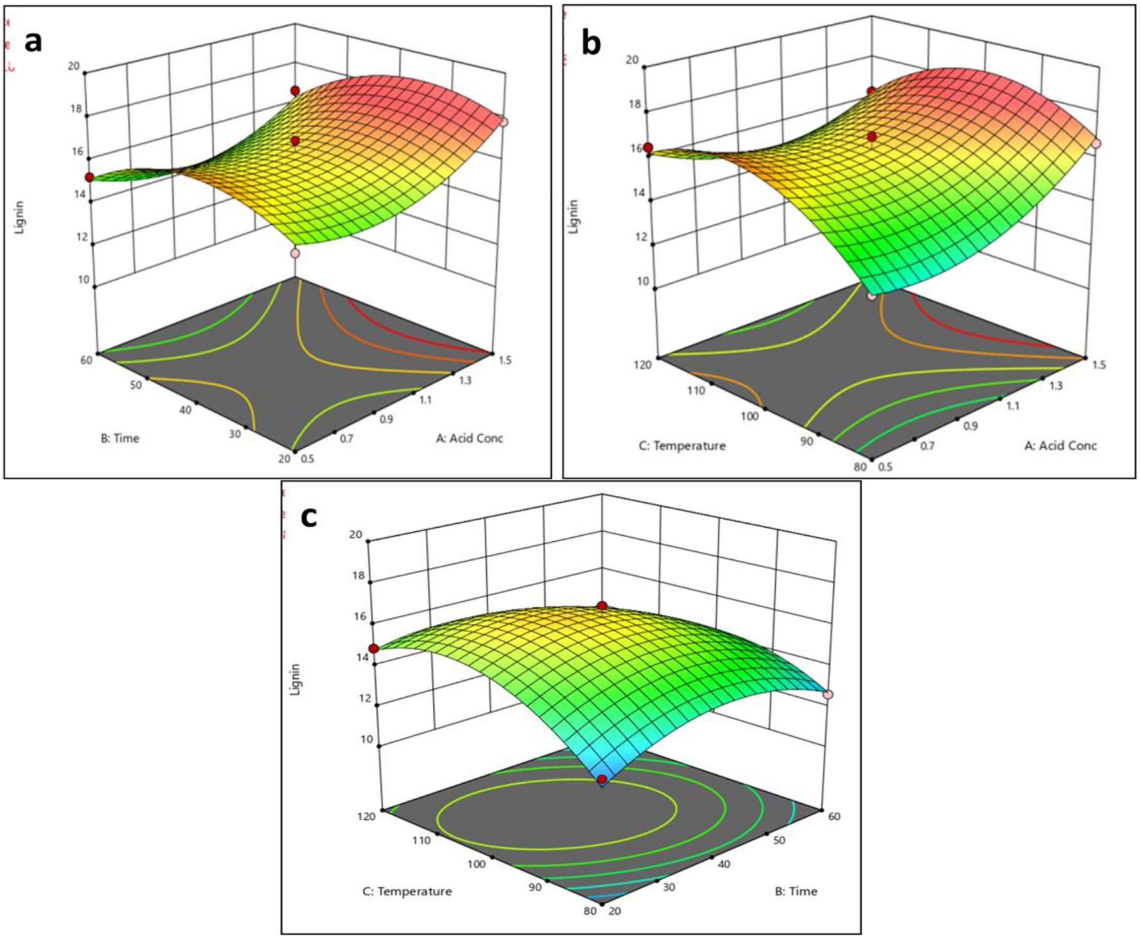


Fig. 6 Surface plot for lignin reduction **a** time and acid concentration, **b** temperature and acid concentration and **c** temperature and time

pretreatment and lignin reduction into consideration, the optimum operating parameters were found to be: acid concentration of 1% v/v H_2SO_4 for 20 min at 80 °C temperature with desirability index of 1.00. These conditions were predicted to yield the highest cellulose content and lignin reduction for releasing maximum reducing sugar yield after enzymatic hydrolysis.

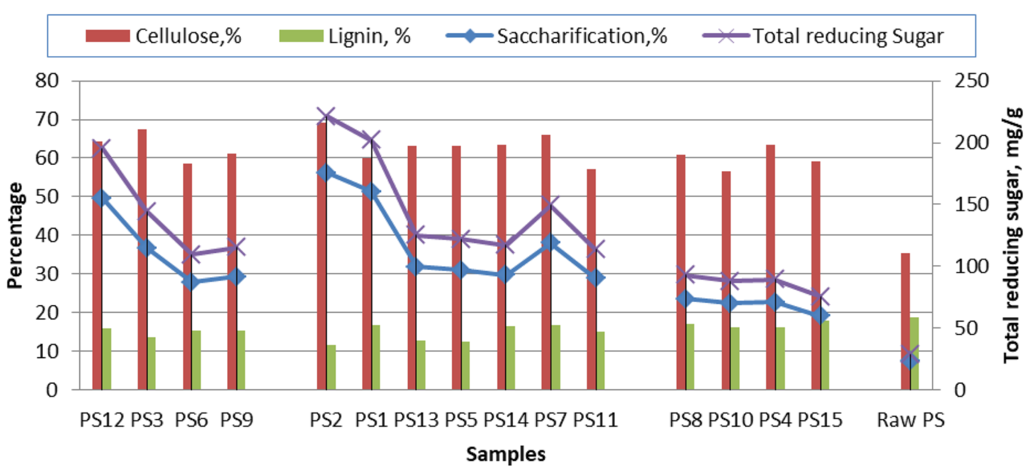


Fig. 7 Effects of acid pretreatment, time and temperature on total reducing sugar yield and saccharification efficiency of paddy straw

Table 6 Microscopic image of RPS and different pretreated PS with the enzymatic hydrolysis phase of PS

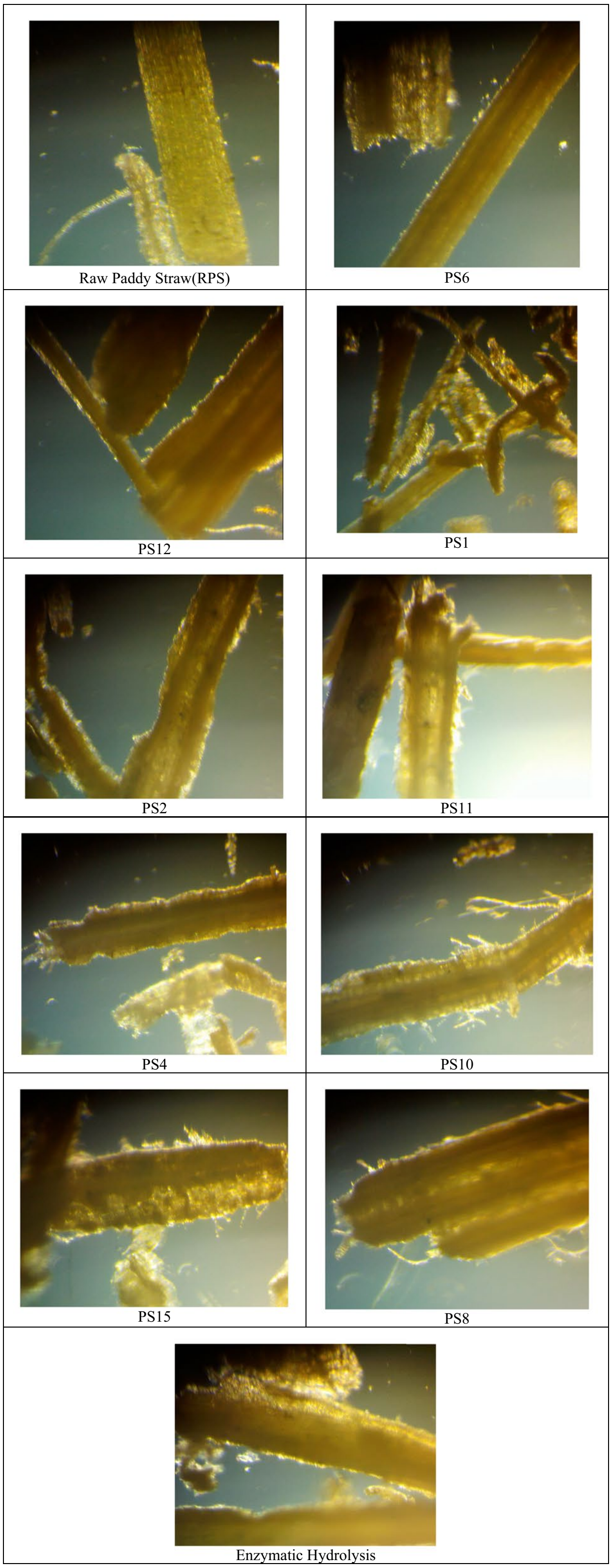


Table 7 Comparison of predicted and experimental yields of cellulose and lignin reduction under optimized pretreatment conditions

Optimized conditions	Temperature (°C)	Time (min)	Acid Conc. (%)	Yield %	
				Cellulose (%)	Lignin reduction (%)
Predicted	80	20	1	68.71	12.06
Experimental	80	20	1	68.38	12.12

The optimum pretreatment conditions predicted by the BBD experiment and RSM analysis have been verified by validation experiments of acid hydrolysis of paddy straw. Under optimized conditions, the validation experiment was conducted in triplicates to ensure the reproducibility of results. As shown in the Table 7 below, the experimental results closely matched the predicted values, with an average cellulose yield of 67.42% and lignin reduction of 12.12% under the optimal conditions with the yield of 220.89 mg/g of total reducing sugar. These results were well within the 95% confidence interval, confirming the model's accuracy and the effectiveness of the pretreatment process.

Conclusion

Dilute acid hydrolysis is an effective method for converting paddy straws into fermentable sugars for biofuel production, such as butanol and ethanol. The first-stage hydrolysis with dilute sulfuric acid efficiently depolymerizes xylan, the main sugar in rice straw hemicelluloses. The second-stage enzymatic hydrolysis yields a significant amount of glucose, resulting in 220 mg/g of reducing sugar. Response surface methodology successfully optimized the combined acid and enzymatic hydrolysis process, with regression models showing statistical significance ($p < 0.05$). The acid hydrolysis step achieved a cellulose content of 68%, and the subsequent enzymatic hydrolysis produced the maximum reducing sugar at 80 °C for 20 min with 1% acid concentration.

While acid hydrolysis is a quick and efficient method for breaking down lignocellulosic materials, it can raise environmental concerns. However, acids can be recovered and recycled, reducing waste. The high sugar yield also lowers biofuel production costs, making the process economically viable and scalable for industrial applications. Moreover, combining acid hydrolysis with bioprocesses like anaerobic digestion enhances sustainability by producing biogas. Overall, despite environmental considerations, the efficiency and the cost-effectiveness of acid hydrolysis position it as a promising method for utilizing paddy straws as a renewable resource.

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Author contributions
P K G: Conceptualization, Experimental and data analysis, Manuscript writing and editing M G: Experimental and data analysis, Methodology, Manuscript writing A R: Resources, Conducted BET experiments, Manuscript writing P S: Conducted TGA experiments, Manuscript writing P D: Data analysis, Manuscript writing G P: Experiments, MAnuscript writing S G: Conceptualization and guidance, Review and Editing.

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Availability of data and materials
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Declarations

Ethics approval and consent to participate
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Competing interests
The authors declare no competing interests.

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