Low-temperature and Low-concentration Sodium Hydroxide Pretreatment for Enhanced Enzyme Hydrolysis Rate from *Quercus variabilis* Blume

Si Young Ha, Ji Young Jung, Hyeon Cheol Kim, Woo Seok Lim, and Jae-Kyung Yang *

A surface response design was employed to develop a sodium hydroxide (NaOH) pretreatment method for *Quercus variabilis* Blume using low NaOH concentration at low temperature. Nevertheless, the persistent issues associated with alkaline pretreatment of lignocellulose, namely high-water consumption and wastewater generation, remain prevalent in this pretreatment process. To address these challenges, this study aimed to conduct enzymatic hydrolysis of NaOH-treated *Q. variabilis* Blume without the intermediary washing steps. The results revealed that, following pretreatment and solid-liquid separation, NaOH-treated *Q. variabilis* Blume could be directly subjected to cellulase-mediated hydrolysis with pH adjustment, eliminating the need for washing steps. The maximum enzymatic hydrolysis efficiency reached 95.9% under specific conditions (1.2% NaOH, 8.9 °C, 32.1 h). This approach offers a promising avenue to enhance the enzyme hydrolysis rate of NaOH-treated lignocellulose. Notably, the low-temperature and low-concentration NaOH treatment effectively removed a substantial portion of lignin and hemicelluloses, resulting in a higher crystallinity index of the cellulose-rich residue compared to substrates treated solely with steam explosion. The integration of direct pretreatment and alkaline treatment emerges as an environmentally friendly and economically viable method for producing glucose and high-purity lignin. The obtained lignin can be further transformed into high-value products within the biorefinery framework.

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Keywords: Alkaline treatment; Eco-friendly condition; Enzymatic hydrolysis; Oak; Steam explosion

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INTRODUCTION

Lignocellulosic materials stand out as the sole sustainable and renewable resources capable of offering alternatives to crude oil and fossil fuels (De Bhowmick *et al*. 2018). The conversion of abundant lignocellulosic sources into biofuels emerges as a practical solution to enhance energy security and mitigate greenhouse gas emissions (Lin and Lu 2021). Oak, an angiosperm within the Fagaceae family, belongs to the genus *Quercus*, comprising approximately 600 species distributed globally. The fundamental composition of oak remains relatively consistent across various species (Tantray *et al*. 2017). Oak woods primarily consist of cellulose (40%) and hemicellulose (25%), which form the structural framework and matrix, while lignin, a predominant polymer in cell walls, constitutes 20% of dried oak wood (Zhang *et al*. 2015).

With the substantial economic and technical potential of the biochemical conversion of lignocellulose into biofuels, these materials inherently resist enzymatic degradation due to various physicochemical factors. Factors, such as the presence of lignin and hemicelluloses, cellulose crystallinity, accessible surface area, and the covalent cross-linkages between lignin and hemicelluloses, contribute to this resistance (Brethauer and Studer 2015). Consequently, an effective pretreatment system is essential before enzymatic hydrolysis to overcome the recalcitrance of lignocellulose and enhance cellulase accessibility to pretreated substrates (Akhtar *et al*. 2016).

In recent years, several promising pretreatment technologies, including steam explosion, hydrothermal, and organosolv methods, have been developed (Ab Rasid *et al*. 2021). Among these, steam explosion pretreatment (SEP) stands out as the most widely employed approach (Sun *et al*. 2014). In SEP, chipped lignocellulosic materials undergo treatment under high saturated vapor pressure, followed by rapid pressure reduction. This process imparts a more expansive structure to the material, facilitating enhanced enzyme accessibility through explosive decompression (Kumar *et al*. 2009). The SEP process typically starts at pressures ranging from 0.69 to 4.83 MPa for several seconds to a few minutes before exposure to atmospheric pressure (Balat 2011). Additionally, the process induces hemicellulose degradation and lignin transformation at high temperatures, rendering cellulose more susceptible to enzymatic attack and thereby increasing the potential for biomass hydrolysis (Zheng *et al*. 2009). Furthermore, an influential factor in the effectiveness of SEP is the decision to presoak lignocellulosic materials before subjecting them to the pretreatment process.

While the SEP process stands as a promising technology for lignocellulosic materials, certain drawbacks, including only partial degradation and removal of hemicelluloses and lignin, along with incomplete disruption of the lignin-hemicelluloses matrix, pose challenges to the enzymatic digestibility of the pretreated substrate (Zhao *et al*. 2020). Consequently, an additional post-treatment becomes necessary to eliminate lignin and degraded hemicellulosic products from the steam-exploded substrates, thereby enhancing the enzymatic digestibility of the pretreated substrates (Yang *et al*. 2002). Existing literature suggests that alkaline treatment represents a promising technology for effectively removing hemicelluloses and lignin, leading to a significant improvement in the enzymatic digestibility of the substrate (Song *et al*. 2021).

Alkaline pretreatment is widely recognized as an effective method for lignin removal (Bali *et al*. 2015). Sodium hydroxide (NaOH) is a commonly employed alkali for treating lignocellulose because of its cost-effectiveness compared to potassium hydroxide, superior solubility relative to calcium hydroxide, and stronger alkalinity compared to ammonium hydroxide (Oriez *et al*. 2020). It exhibits remarkable lignin removal capabilities under non-pressurized conditions at low temperatures (< 100 °C) (Gandla *et al*. 2018). However, NaOH pretreatment faces two persistent challenges in alkaline pretreatment: the substantial water consumption required to wash alkali-treated lignocellulosic solid residue for subsequent enzymatic hydrolysis and fermentation (Xu *et al*. 2016), and the generation of considerable black liquor during the pretreatment process and waste washing water during the washing process (Shah *et al*. 2023).

Various efforts have been undertaken to mitigate black liquor generation and wastewater discharge (Goshadrou 2019). Recycling black liquor from the pretreatment process for the subsequent lignocellulose pretreatment has been explored (Triwahyuni *et al*. 2015). However, as the recycling proportion of black liquor was increased, the

enzymatic hydrolysis of treated lignocellulose deteriorated, albeit the loss being recoverable with the introduction of non-ionic surfactants (Wang *et al*. 2022). Wastewater from the washing process has been reused for washing alkali-treated lignocellulose, contributing to reduced water consumption (Madadi *et al*. 2023). Nevertheless, there remains some wastewater generated from the washing process due to the limitations of the recycling method. Further studies are warranted to optimize the current technology for wastewater recycling.

In this study, the authors tested a way to directly convert low concentration NaOH-treated *Q. variabilis* without washing steps as means to achieve water-saving, low wastewater, and enhanced enzyme hydrolysis rate. This work presents a promising way to produce cellulosic ethanol with low water consumption, little wastewater discharge, and good enzyme hydrolysis rate.

EXPERIMENTAL

Materials

The raw *Q. variabilis* wood chip was washed with tap water until the wastewater was clear. Then, it was dried at 60 °C in an oven to a constant weight and stored in a desiccator at room temperature.

Steam explosion pretreatment (SEP)

The raw *Q. variabilis* samples weighing 10 kg (dry weight) were cut into dimensions of 2.5 cm × 2.5 cm and subjected to steam explosion at 25 kg/cm^2 and 225 ℃ for 3 min individually, using saturated steam in a 100-L batch reactor (Youlim Hightech Co., Ltd., Korea). In these short pretreatment conditions, it is important to be sure about the reactor heating time. According to the variation of pressure in steam explosion reactor with time, the steam explosion process can be divided into following three continuous stages: pressure boost stage, holding pressure stage, and instantaneous decompression stage. The three-minute time presented in this work belongs to the holding pressure stage. The actual time spent in the pressure boost stage is about 2 hours.

Generally, steam explosion processes occur at temperatures between 200 and 280 °C for retention times varying from 2 to 10 min (Quiévy *et al*. 2010). At these conditions, thermal degradation of cellulose can take place. Jacquet *et al*. (2015) also found that, under relatively mild conditions (pressure, 15 to 25 bar; temperature, 200 to 220 °C; 1 to 5 min) that hemicellulosic fraction could be recovered as monomers and oligomers. The steam expansion process temperature was set to 225 °C with reference to these previous studies.

The costs of a process are the expenses made in order to set up and operate the process. Vasilakou *et al*. (2023) reported that the capital expenditures (CAPEX) was the lowest for steam explosion and liquid hot water among the pretreatment process. These two physicochemical methods require a simpler reactor design, with less expensive materials. Therefore, it is proposed that the steam expansion process will be economically feasible. The severity parameter (log10 Ro [min]) of steam explosion pretreatment (SEP), representing the extent of destruction and depolymerization, was determined as 3.0 (225 ℃ /5 min), using Eq. 1,

$$\text{Ro} = \int_0^t \exp\left[\frac{\text{T}-100}{14.5}\right] dt \quad (1)$$

where T is the temperature (°C) and t is the time (min). The exploded material was recovered in a cyclone and after cooling to about 40 °C. The liquid was filtered and the solid was recovered. The solid fraction was water-washed and then used for enzymatic hydrolysis after chemical pretreatment.

Alkaline post-treatment

Aqueous solution, 0.1 % NaOH was used for alkaline post-treatment. Steam exploded sample (5 g, dry basis) was dispersed in the extraction solution (100 mL). The post-treatment was performed using a 250-mL conical flask in a water bath shaker with a rotation speed of 200 rpm/min. Following the post-treatment, the slurry was filtered to isolate the solid residue (utilizing Whatman filter paper No. 2), which was not washed with water. The resulting solid residue was dried in an oven at 105 °C until a constant mass was achieved. Subsequently, it was placed in a desiccator at room temperature for subsequent chemical component analysis and enzymatic hydrolysis.

Enzymatic hydrolysis

The water-insoluble residue from steam-exploded *Q. variabilis*, both with and without chemical pretreatment, underwent enzymatic hydrolysis using a cellulolytic complex (Celluclast 1.5 L) generously provided by NOVO Nordisk (Denmark). Celluclast 1.5 L is a commercial multicatalytic enzyme with high cellulo-, xylanolytic, and mannanase activities intended for plant tissue breakdown (Wikiera *et al*. 2015). The cellulase enzyme loading was set at 65 Filter Paper Units/g substrate. To supplement ß-glucosidase activity, fungal ß-glucosidase (Novozyme 188, Novo Ltd.) was added with an enzyme loading of 24 cellobiase units/g substrate. Enzymatic hydrolysis was conducted in a 0.05 M sodium citrate buffer (pH 4.8) at 50 °C on a shaking incubator (IS–97IR from Jeio–Tech Co., Korea) operating at 150 rpm for 96 h, with a pretreated material concentration of 2% (w/v). Samples (1 mL) were extracted at the beginning and after 72 h. These samples underwent boiling water treatment for 10 min to halt enzymatic activity, followed by centrifugation at 3,000 rpm for 5 min using a Hanilmicro–12 (Hanil Science Industrial Co., Korea) centrifuge. The collected samples were then stored at 4 °C.

Analysis methods

After NaOH treatment, the slurry was filtrated to separate out the solid residue. The residue would be washed with water several times until its pH was neutral, as needed for analysis of chemical compositions and physical properties. The chemical compositions (%, w/w) of the substrates were analyzed following the standard analytical procedure established by the National Renewable Energy Laboratory (NREL). X-ray diffraction (XRD) in reflection mode was conducted using an XRD-6000 apparatus (Shimadzu, Japan) with Ni-filtered Cu Ka radiation (λ = 1.54 Å) generated at 40 kV and 30 mA. The scattering angle (2θ) ranged from 5° to 35° at a scanning speed of 2°/min. Biomass crystallinity, as expressed by crystallinity index (CrI), was determined from XRD data and calculated using the formula CrI (%) = $(I_{002} - I_{am})/I_{002} \times 100$, where I_{002} is the intensity for the crystalline portion of biomass (cellulose) at about 2θ = 22.5°, and I_{am} is the peak for the amorphous portion (*i.e*., cellulose, hemicellulose, and lignin) at about 2θ = 15.5°. Scanning electron microscopy (SEM) images were captured using a Hitachi S-3400N II instrument (Hitachi,

Japan) at 10 kV and 81 mA. Prior to examination, all substrates underwent gold coating. Brunauer–Emmett–Teller (BET) surface areas and Barrett–Joyner–Halenda (BJH) pore volumes of the substrates were measured through nitrogen adsorption analysis using a TriStar 3000 surface area analyzer (Micromeritics Ltd., Tokyo, Japan) following 8 h of degassing at 120 °C and 1 h of degassing at 150 °C.

Box-Behnken experimental design

The Box-Behnken model was used to design the response surface experiment of NaOH pretreatment including three factors of time, temperature, and NaOH concentrations as shown in Table 1, and one response value of enzyme hydrolysis rate. Seventeen experimental groups were obtained.

Table 1. Three-factorial Box-Behnken Design

Variable	Symbol	Coding Level		
		-1	0	1
NaOH concentration (%)	X_1	0.5	1	1.5
Temperature (°C)	X_2	-15	25	65
Time (h)	X_3	12	24	36

RESULTS AND DISCUSSION

Mass Changes of Steam Exploded *Q. variabilis* Blume during the Sodium Hydroxide Post-Treatment

Figure 1 provides a flow chart of the steam explosion process, followed by the NaOH treatment, including the temperatures and times of the latter treatment.

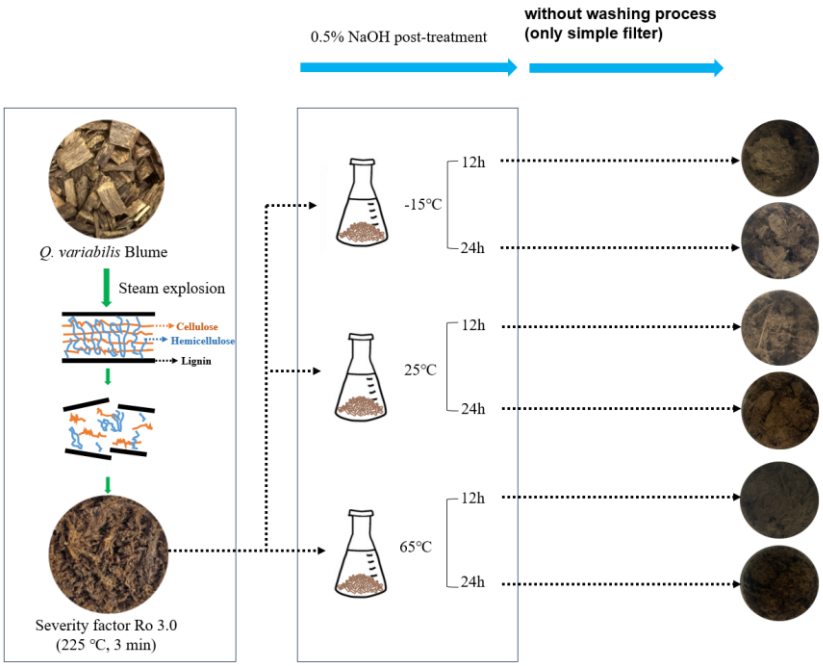

Fig. 1. Flow chart for *Q. variabilis* Blume prepared by NaOH after SEP

Table 2 shows the recovery yield of oak chip residues obtained through chemical pretreatment. It was confirmed that the recovery rate was affected by the concentration of chemicals, treatment time, and treatment temperature among the chemical pretreatment conditions, and particularly the recovery rate decreased as the treatment temperature was increased.

Table 2. Yields of the Steam-Exploded, and Further Alkali Post-Treated Substrates

	Concentration of NaOH (%)	Treatment Temperature (°C)	Treatment Time (h)	Yield (%)
Only-SEP [1)]	-	-	-	98
NaOH treated [2)]	0.5	-15	12	83
			24	80
		25	12	71
			24	68
		65	12	66
			24	62

[1)] 225 °C , 3 min (Ro 3.0)
[2)] Post-treatment after SEP (225 °C , 3 min (Ro 3.0))

Compositional Analysis

Figure 2 shows the results of chemical composition analysis according to the chemical treatment conditions of the steam-exposed oak chip (without bark). NaOH showed a decrease in lignin content when treated at 25 °C and 65 °C at concentrations of 2% and 3%. In general, NaOH was effective in dissolving hemicellulose and in promoting synergy in the delignification effect. Most of the lignin contained in wood is combined with hemicellulose components that are bound to cellulose like a binder, making the structure of wood complex and difficult to access (Fujita and Harada 2001). Difficulties in delignification and dissolution can be partly attributed to the binding between lignin and carbohydrates (Lawoko *et al*. 2005; Wang *et al*. 2009; Moigne and Navard 2010), and thus the removal of most hemicellulose and all lignin is still limited but can promote the dissolution of glucan. As shown in Fig. 2, NaOH mostly dissolves hemicellulose and has been reported to be effective for delignification. The steam expansion/NaOH system has been studied (Cai and Zhang 2005; Cai *et al*. 2007; Yang *et al*. 2011), and this method is attracting attention because it is inexpensive, has less pollution to the environment than some of the alternative, and it is simple to handle. Alkali treatment is reported to break down cell walls by dissolving hemicellulose, lignin, and swelling cellulose (Jackson 1977). It has been effective in removing lignin, especially when continuous chemical reactions were treated rather than single steam exposure treatment. Therefore, from the results of the chemical composition evaluation, NaOH had a relatively high cellulose content and a low hemicellulose and lignin contents, which was speculated to be effective in increasing the enzyme hydrolysis rate.

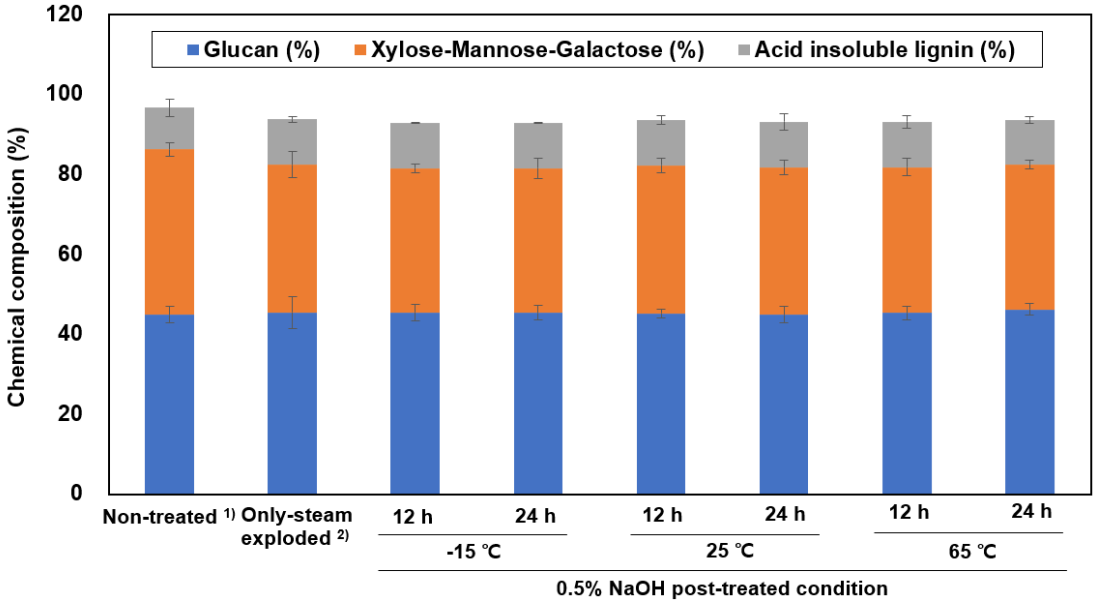

Fig. 2. Chemical compositions of the non-treated, steam-exploded or NaOH post-treated *Q. variabilis* Blume: [1)] 225 °C , 3 min (Ro 3.0); [2)] Post-treatment after SEP (225 °C , 3 min (Ro 3.0))

Crystallinity, Surface Morphology, and BET Surface Area of Substrate

Alkaline chemical pretreatment caused changes in the BET specific surface area and total pore volume of the steam-exposed oak chip (Table 3). In particular, oak chips reacted for 12 h to 24 h at a temperature of 25 to 65 °C using 2% to 3% NaOH and samples reacted for 12 h to 24 h using 0.5% NaOH showed high specific surface area values, which tended to be similar to the total pore volume. The increase in specific surface area and total pore volume has been reported to improve the enzyme hydrolysis rate by improving the enzyme's access to cellulose (Yang *et al*. 2008).

The present results showed a relatively higher surface area after alkali treatment compared to the steam expansion-treated oak chips. The trend of increasing specific surface area or total pore volume after NaOH chemical treatment can be attributed to the partial destruction of the microstructure of the sample particles. The presence of loose structures in steam expansion and chemically treated oak chips appeared to be efficient for enzymatic hydrolysis of biomass, as it allows cellulases' extensive access to the substrate surface. The specific surface area and pore volume of oak chips pretreated at -15 °C using 0.5% NaOH reached their maximum values simultaneously. Therefore, the high specific surface area and total pore volume of oak chips pretreated with 0.5% NaOH were speculated to be effective in increasing the enzymatic hydrolysis rate. Table 3 shows the results of the crystallinity analysis according to the chemical treatment of the steam exposure-treated oak chips. It was confirmed that the crystallization index of the steam expansion-treated oak chip differed according to the chemical treatment conditions, especially when 0.5% NaOH was mixed and reacted for 24 h at -15 °C. It is believed that the crystalline region of cellulose increased due to the decomposition of hemicellulose or lignin, which has been speculated to be a positive signal that can increase the effect on enzyme hydrolysis.

Table 3. Specific Surface Area and Total Pore Volume of Substrates Analyzed by BET Method and Crystallization Index

Chemical Treatment Condition			Specific Surface Area (m^2/g)	Total Pore Volume (cm^3/g)	Crystallinity Index (%)
Concentration	Temperature	Time			
SEP [1)]			0.77 ± 0.0b[3)]	0.65 ± 0.0a	67.2 ± 0.0b
NaOH [2)]					
0.5%	–15 °C	12 h	0.78 ± 0.0a	0.63 ± 0.0ab	67.5 ± 0.0b
		24 h	0.78 ± 0.0a	0.61 ± 0.0ab	67.3 ± 0.0b
	25 °C	12 h	0.79 ± 0.0a	0.60 ± 0.0b	68.9 ± 0.0ab
		24 h	0.78 ± 0.0a	0.60 ± 0.0b	70.2 ± 0.0a
	65 °C	12 h	0.79 ± 0.0a	0.60 ± 0.0b	71.1 ± 0.0a
		24 h	0.79 ± 0.0a	0.60 ± 0.0b	71.1 ± 0.0a

[1)] 225 °C , 3 min (Ro 3.0)
[2)] Post-treatment after SEP (225 °C , 3 min (Ro 3.0))
[3)] The p-values ($p < 0.05$) by the statistical analysis are presented in column

SEM Evaluation of Surface Microstructure and Pore Distribution

Steam explosion/chemical pretreatment can considerably change the shape of lignocellulosic biomass, which can also increase the accessibility of the material to enzymes. SEM imaging was used to evaluate the shape change due to the steam explosion and chemical treatment (Fig. 3). Non-chemically treated, only steam exploded single-treated oak chips exhibited a hard, rough surface morphology, which may interfere with the accessibility of cellulose to the enzyme. In contrast, the surface of oak chips reacted with 0.5% NaOH after steam explosion treatment was broken into separated fibers or fiber bundles and revealed some cracks and small particle-size debris. Specifically, oak chips reacted with 0.5% NaOH for 24 h at 25 °C after steam explosion treatment exhibited loose fiber structures due to wider separation of fibers, indicating that lignin and hemicellulose were removed by alkali treatment. These show that the fiber structure in the non-treated oak was obvious, rigid, and highly ordered, while the structure was blurred after pretreatment, especially in samples pretreated with steam explosion and 0.5% NaOH (25°C, 24 h). The oak pretreated with assisted steam explosion and 0.5% NaOH was fragile, distorted, and had less fiber structure than that pretreated with only steam explosion treatment. The steam explosion and 0.5% NaOH treatment of the oak increased the surface area of cellulose, making it accessible to enzymatic hydrolysis accessible. The changes in fiber structure generally increase the accessibility of enzymes and improve enzyme hydrolysis efficiency by generating a large amount of reaction sites on the fiber surface (Vahidi *et al*. 2021). Accordingly, oak chips reacted with 0.5% NaOH for 24 h at 25 °C were estimated to have a high enzyme hydrolysis rate. It was demonstrated that the combination of steam explosion and NaOH can destroy the surface structure of wood.

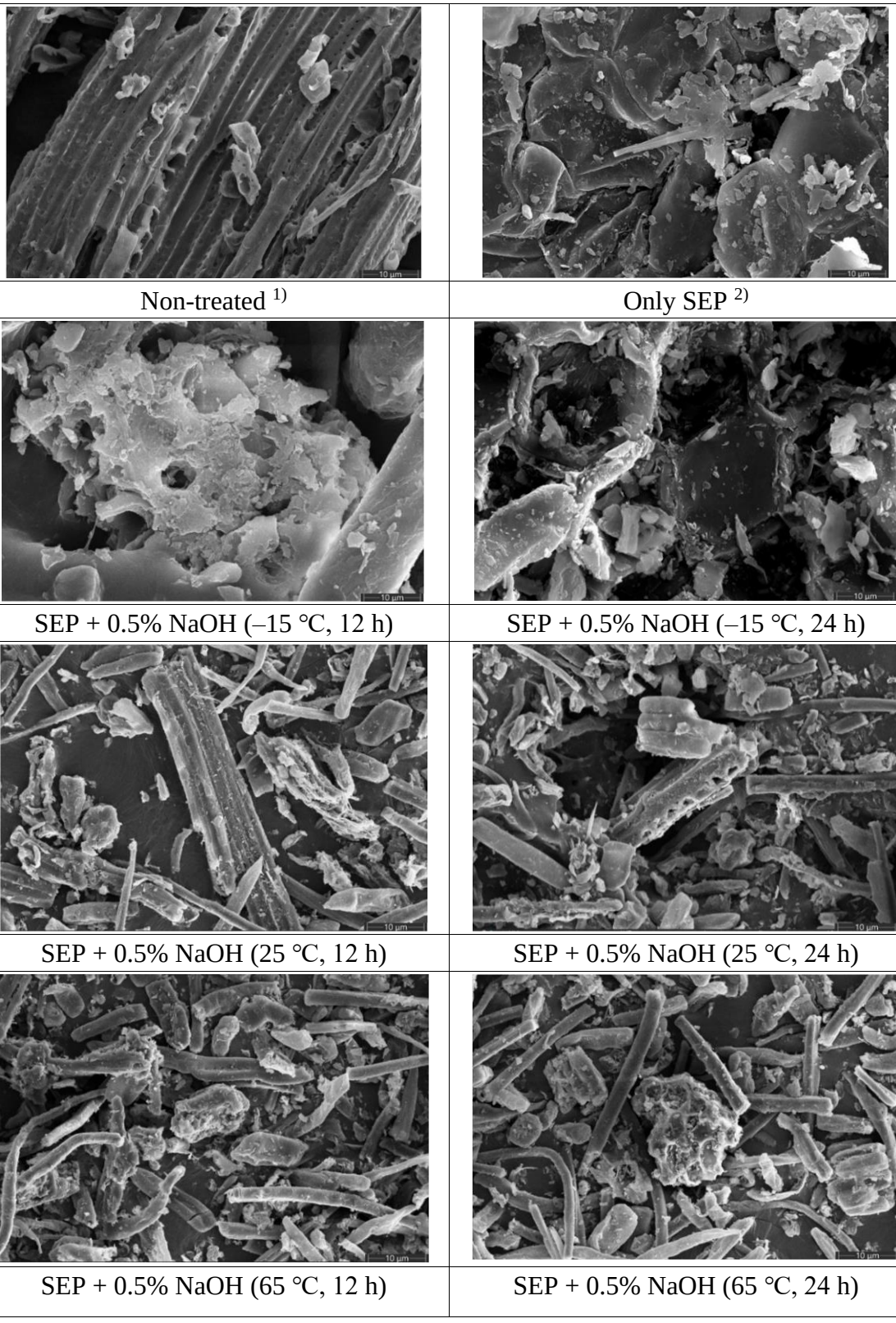

Fig. 3. SEM images of wood chips obtained from SEP treatment and chemical treatment: 1) 225 °C, 3 min (Ro 3.0); 2) Post-treatment after steam explosion (225 °C, 3 min (Ro 3.0)); SE: steam explosion

Enzymatic Hydrolysis

Table 4. Enzyme Hydrolysis Rate of Substrates After Steam Explosion and Chemical Treatment

Chemical Treatment Condition			Enzyme Hydrolysis Rate (%)
Concentration	Temperature (°C)	Time (h)	
SEP [1)]			35.60 ± 0.5c[3)]
NaOH[2)]			
0.5%	–15 °C	12 h	59.38 ± 1.3b
		24 h	60.82 ± 0.1b
	25 °C	12 h	79.27 ± 0.2ab
		24 h	80.06 ± 1.7a
	65 °C	12 h	82.10 ± 2.1a
		24 h	82.11 ± 0.7a

[1)] 225 °C , 3 min (Ro 3.0)
[2)] Post-treatment after steam explosion (225 °C , 3 min (Ro 3.0))
[3)] The p-values ($p < 0.05$) by the statistical analysis are presented in column

Table 5. 17 Experimental Trials on Box–Behnken Design of Enzyme Hydrolysis Rate

Run	NaOH Concentration (%)	Temperature (°C)	Time (h)	Enzyme Hydrolysis Rate (%)
1	0.5	-15	24	84.908
2	1.5	65	24	59.994
3	1	25	24	95.569
4	1	25	24	90.354
5	1	25	24	94.714
6	1.5	-15	24	85.073
7	1.5	25	12	84.384
8	0.5	25	12	73.326
9	1	65	12	54.747
10	1	65	36	64.681
11	0.5	65	24	59.404
12	1	-15	36	85.007
13	1	-15	12	83.886
14	1.5	25	36	93.887
15	0.5	25	36	89.765
16	1	25	24	94.350
17	1	25	24	92.963

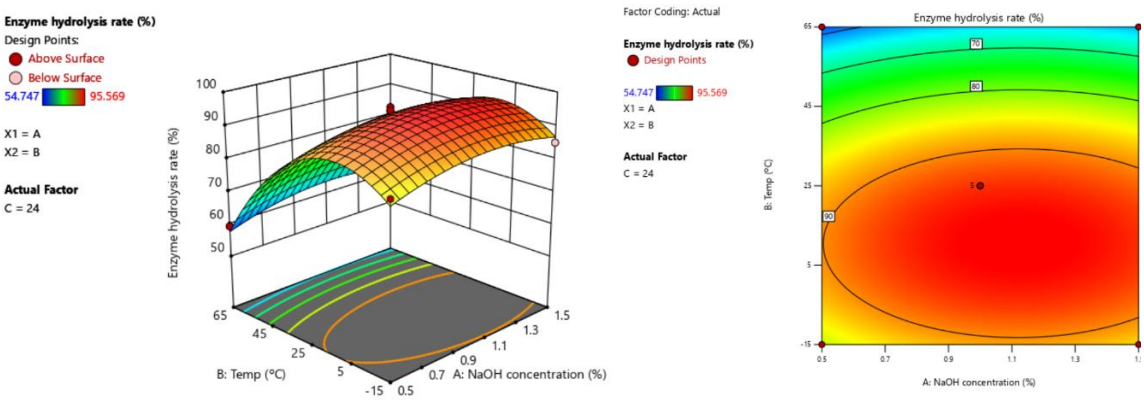

Fig. 4. 3D surface graph in NaOH concentration and extraction temperature for enzyme hydrolysis rate

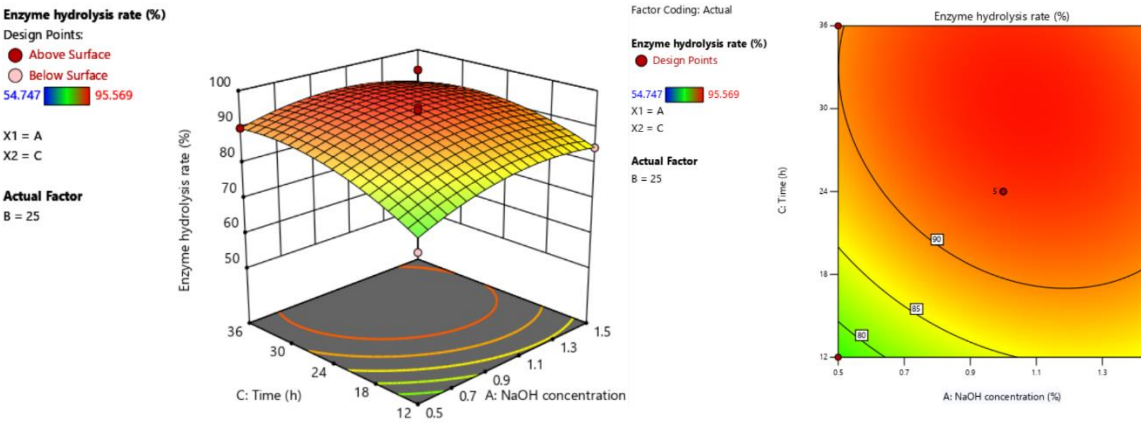

Fig. 5. 3D surface graph in NaOH concentration and extraction time for enzyme hydrolysis rate

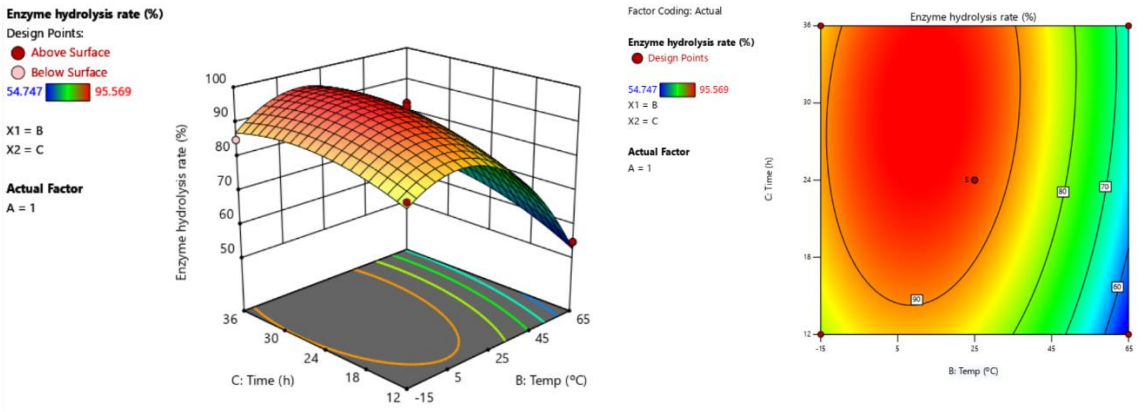

Fig. 6. 3D surface graph in NaOH concentration and pretreatment time for enzyme hydrolysis rate

The curves in the 3D surface plots showed the greatest slope at the treatment temperature, meaning that the temperature had the greatest effect in the variables for enzyme hydrolysis. A curve on a 3D plot means an influence on the enzyme hydrolysis rate.

A gentle curvature means a low influence, whereas a strong curvature means a high influence. Therefore, it is judged that the treatment temperature will have a higher effect on the enzyme hydrolysis rate than the treatment time or the concentration of the treatment drug. As a result of ANOVA analysis, it was confirmed that among the three variables (NaOH concentration, treatment temperature, and treatment time), the treatment temperature affected the enzyme hydrolysis rate at $p < 0.0001$, which is the same trend as the curve of the 3D plot. In the ANOVA analysis results, "Lack of Fit" means that the variation caused by the multinomial regression model derived through the experiment is not suitable for explaining the response, making it difficult to trust the regression model because the variation is so large that it cannot be ignored.

Table 6. ANOVA for Quadratic Model

Source	Sum of Squares	df	Mean Square	F-Value	p-Value	
Model	2965.43	9	329.49	32.72	< 0.0001	Significant
X[1)]	31.74	1	31.74	3.15	0.1191	
B[2)]	1251.20	1	1251.20	124.25	< 0.0001	
C[3)]	171.10	1	171.10	16.99	0.0044	
AB	0.0452	1	0.0452	0.0045	0.9485	
AC	12.03	1	12.03	1.19	0.3106	
BC	19.42	1	19.42	1.93	0.2075	
A^2	67.12	1	67.12	6.66	0.0364	
B^2	1253.29	1	1253.29	124.46	< 0.0001	
C^2	76.30	1	76.30	7.58	0.0284	
Residual	70.49	7	10.07			
Lack of Fit	53.87	3	17.96	4.32	0.0957	Not significant
Pure Error	16.62	4	4.16			
Corrected Total	3035.92	16				

[1)] NaOH concentration (%)
[2)] Temperature (°C)
[3)] Time (h)

The reliability of the model can be verified through Fig. 7, and the predicted and measured values were projected to be similar within a certain range along the trend line. From the results derived from the response surface methodology (RSM), the enzyme hydrolysis rate of the steam explosion/chemical treatment of oak chip can be predicted using the value of the variable, and the prediction formula is shown in Eq. 2. Figure 8 shows the optimal steam explosion/chemical treatment conditions for the maximum enzyme hydrolysis rate, and as a result, it was confirmed that NaOH concentration 1.2%,

extract temperature 8.9 °C, and extract time 32 h were the optimal conditions for the maximum enzyme hydrolysis rate. The treatment temperature was lower than that of general alkaline reaction conditions, which is believed to be the result of mixing NaOH with urea reacting at a low temperature. When treated under optimal conditions for maximum enzyme hydrolysis, it was estimated to reach a maximum of 95.9%.

To validate this optimization, several experiments were conducted at 1.2% NaOH, 9 °C, and 32 h. The obtained enzyme hydrolysis rate was 94% of the experimental data. In the present work, both values (predicted value and actual value) were very close to each other, showing the accuracy of the model and thereby implying on the suitability of the RSM model to forecast the enzyme hydrolysis rate data. A study was carried out to maximize the enzyme hydrolysis rate, which is a pre-fermentation step. Based on the optimized data in this study, the authors are planning also conduct a study in the fermentation stage. In addition, in the present work, a small amount of black liquid was present due to the fact that a low concentration of NaOH was used and the washing step was omitted. However, the issues related to treatment of a small amount of black liquid will have to be solved in the future.

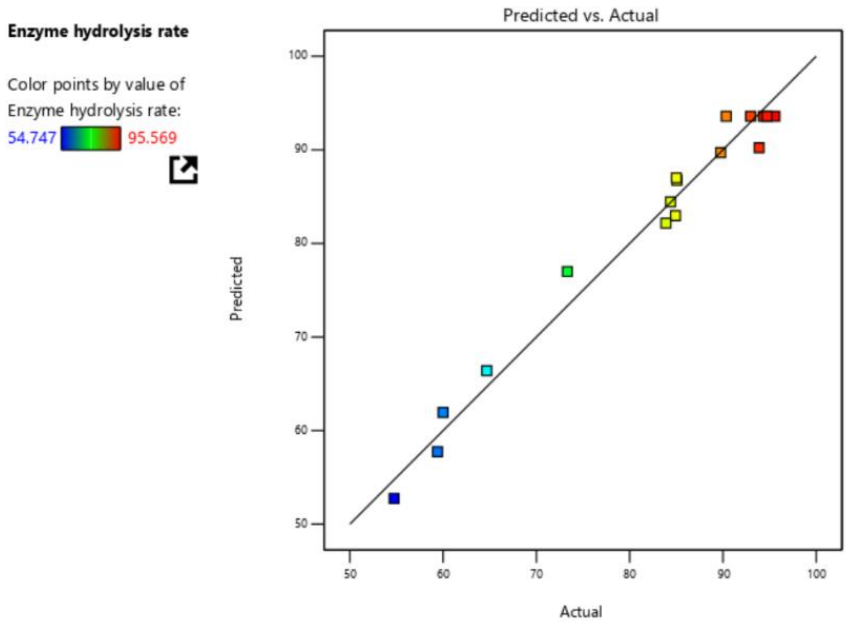

Fig. 7. Predicted *vs* Actual graph of RSM model for enzyme hydrolysis rate of two step treated oak wood

Enzyme hydrolysis rate (%) = -44.38677+42.72694A+0.111023B+0.19863C+0.005313AB- 0.289000AC+0.004590BC $-15.97000A^2-0.010783B^2-0.029563C^2$ * A: NaOH concentration (%) * B: Temperature (°C) * C: Time (h)	Eq. 2

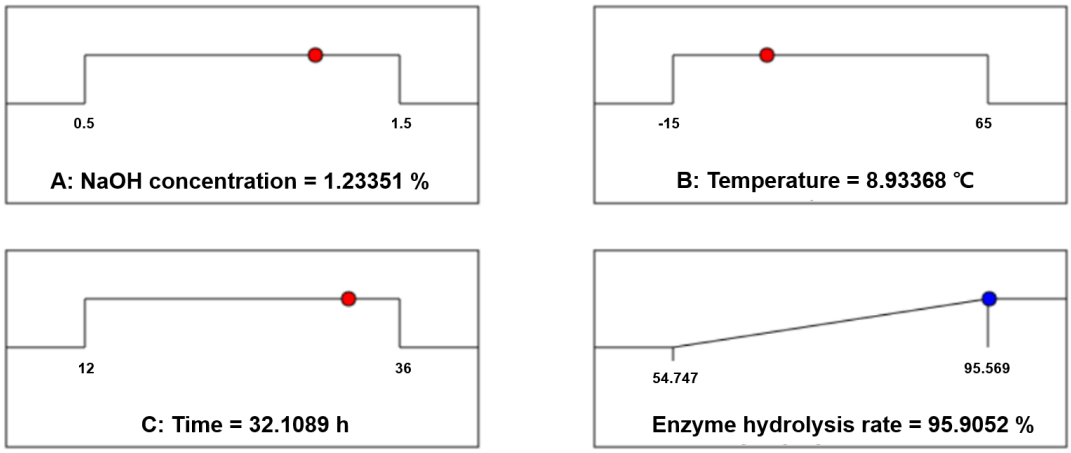

Fig. 8. Ramp function graph for enzyme hydrolysis rate

CONCLUSIONS

1. In this study a combined system, comprising steam explosion pretreatment (SEP) followed by low-temperature and low-concentration alkaline post-treatment, to enhance the enzymatic digestibility of *Q. variabilis* was explored.

2. Post-SEP, the morphological structure of *Q. variabilis* was observed to have undergone fragmentation, exposing the cellulose bundle surface. These results, influenced by pretreatment pressure and duration, proved conducive to cellulase enzyme absorption on cellulose. Subsequent alkaline treatment resulted in substantial cellulose hydrolysis by cellulase, producing glucose for bioethanol production, while lignin was recovered for further utilization.

3. Achieving an enzymatic digestibility of cellulose at 95.9% (1.2% NaOH, 8.9 ℃, 32.1 h) was possible after direct SEP and subsequent alkaline treatment.

4. This approach provides insights into converting low-temperature and low-concentration NaOH-treated lignocellulose, ensuring a high enzyme hydrolysis rate with minimal water consumption and wastewater generation.

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Data Availability

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

REFERENCES CITED

Ab Rasid, N. S., Shamjuddin, A., Rahman, A. Z. A., and Amin, N. A. S. (2021). "Recent advances in green pre-treatment methods of lignocellulosic biomass for enhanced biofuel production," *Journal of Cleaner Production* 321, article ID 129038. DOI: 10.1016/j.jclepro.2021.129038

Akhtar, N., Gupta, K., Goyal, D., and Goyal, A. (2016). "Recent advances in pretreatment technologies for efficient hydrolysis of lignocellulosic biomass," *Environmental Progress and Sustainable Energy* 35(2), 489-511. DOI: 10.1002/ep.12257

Balat, M. (2011). "Production of bioethanol from lignocellulosic materials *via* the biochemical pathway: A review," *Energy Conversion and Management* 52(2), 858-875. DOI: 10.1016/j.enconman.2010.08.013

Bali, G., Meng, X., Deneff, J. I., Sun, Q., and Ragauskas, A. J. (2015). "The effect of alkaline pretreatment methods on cellulose structure and accessibility," *ChemSusChem* 8(2), 275-279. DOI: 10.1002/cssc.201402752

Brethauer, S., and Studer, M. H. (2015). "Biochemical conversion processes of lignocellulosic biomass to fuels and chemicals–A review," *Chimia* 69(10), 572-572. DOI: 10.2533/chimia.2015.572

Cai, J., and Zhang, L. (2005). "Rapid dissolution of cellulose in LiOH/urea and NaOH/urea aqueous solutions," *Macromolecular Bioscience* 5, 539-548. DOI: 10.1002/mabi.200400222

Cai, J., Zhang, L., Zhou, J., Qi, H., Chen, H., Kondo, T., Chen, X., and Chu, B. (2007). "Multifilament fibers based on dissolution of cellulose in NaOH/urea aqueous solution: Structure and properties," *Advanced Materials* 19, 821-825. DOI: 10.1002/adma.200601521

De Bhowmick, G., Sarmah, A. K., and Sen, R. (2018). "Lignocellulosic biorefinery as a model for sustainable development of biofuels and value-added products," *Bioresource Technology* 247, 1144-1154. DOI: 10.1016/j.biortech.2017.09.163

Fujita, M., and Harada, H. (2001). "Ultrastructure and formation of wood cell wall," in: *Wood and Cellulosic Chemistry*, D. N. S. Hon, N. Shiraishi, and M. Dekker (eds.), Taylor & Francis, Boca Raton, FL, USA, pp. 1-50.

Gandla, M. L., Martín, C., and Jönsson, L. J. (2018). "Analytical enzymatic saccharification of lignocellulosic biomass for conversion to biofuels and bio-based chemicals," *Energies* 11(11), article ID 2936. DOI: 10.3390/en11112936

Goshadrou, A. (2019). "Bioethanol production from Cogon grass by sequential recycling of black liquor and wastewater in a mild-alkali pretreatment," *Fuel* 258, article ID 116141. DOI: 10.1016/j.fuel.2019.116141

Jackson, M. G. (1977). "The alkali treatment of straws," *Animal Feed Science and Technology* 2(2), 105-130. DOI: 10.1016/0377-8401(77)90013-X

Jacquet, N., Maniet, G., Vanderghem, C., Delvigne, F., and Richel, A. (2015). "Application of steam explosion as pretreatment on lignocellulosic material: A review," *Industrial & Engineering Chemistry Research* 54(10), 2593-2598. DOI: 10.1021/ie503151g

Kumar, P., Barrett, D. M., Delwiche, M. J., and Stroeve, P. (2009). "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production," *Industrial and Engineering Chemistry Research* 48(8), 3713-3729. DOI: 10.1021/ie801542g

Lawoko, M., Henriksson, G., and Gellerstedt, G. (2005). "Structural differences between the lignin-carbohydrate complexes present in wood and in chemical pulps," *Biomacromolecules* 6, 3467-3473. DOI: 10.1021/bm058014q

Lin, C. Y., and Lu, C. (2021). "Development perspectives of promising lignocellulose feedstocks for production of advanced generation biofuels: A review," *Renewable and Sustainable Energy Reviews* 136, article ID 110445. DOI: 10.1016/j.rser.2020.110445

Madadi, M., Nazar, M., Shah, S. W. A., Li, N., Imtiaz, M., Zhong, Z., and Zhu, D. (2023). "Green alkaline fractionation of sugarcane bagasse at cold temperature improves digestibility and delignification without the washing processes and release of hazardous waste," *Industrial Crops and Products* 200, article ID 116815. DOI: 10.1016/j.indcrop.2023.116815

Moigne, N. L., and Navard, P. (2010). "Dissolution mechanisms of wood cellulose fibres in NaOH–water," *Cellulose* 17, 31-45. DOI: 10.1007/s10570-009-9370-5

Oriez, V., Peydecastaing, J., and Pontalier, P. Y. (2020). "Lignocellulosic biomass mild alkaline fractionation and resulting extract purification processes: Conditions, yields, and purities," *Clean Technologies* 2(1), 91-115. DOI: 10.3390/cleantechnol2010007

Quiévy, N., Jacquet, N., Sclavons, M., Deroanne, C., Paquot, M., and Devaux, J. J. P. D. (2010). "Influence of homogenization and drying on the thermal stability of microfibrillated cellulose," *Polymer Degradation and Stability* 95(3), 306-314. DOI: 10.1016/j.polymdegradstab.2009.11.020

Shah, T. A., Khalid, S., Nafidi, H. A., Salamatullah, A. M., and Bourhia, M. (2023). "Sodium hydroxide hydrothermal extraction of lignin from rice straw residue and fermentation to biomethane," *Sustainability* 15(11), article ID 8755. DOI: 10.3390/su15118755

Song, W., Peng, L., Bakhshyar, D., He, L., and Zhang, J. (2021). "Mild O_2-aided alkaline pretreatment effectively improves fractionated efficiency and enzymatic digestibility of Napier grass stem towards a sustainable biorefinery," *Bioresource Technology* 319, article ID 124162. DOI: 10.1016/j.biortech.2020.124162

Sun, S. L., Wen, J. L., Ma, M. G., and Sun, R. C. (2014). "Enhanced enzymatic digestibility of bamboo by a combined system of multiple steam explosion and alkaline treatments," *Applied Energy* 136, 519-526. DOI: 10.1016/j.apenergy.2014.09.068

Tantray, Y. R., Wani, M. S., and Hussain, A. (2017). "Genus *Quercus*: An overview," *International Journal of Advance Research in Science and Engineering* 6(8), 1880-1886.

Triwahyuni, E., Hendarsyah, H., and Abimanyu, H. (2015). "Reuse black liquor of alkali pretreatment in bioethanol production," *Energy Procedia* 68, 236-243. DOI: 10.1016/j.egypro.2015.03.252

Vahidi, G., Bajwa, D. S., Shojaeiarani, J., Stark, N., and Darabi, A. (2021). "Advancements in traditional and nanosized flame retardants for polymers—A review," *Journal of Applied Polymer Science* 138(12), article ID 50050. DOI: 10.1002/app.50050

Vasilakou, K., Nimmegeers, P., Billen, P., and Van Passel, S. (2023). "Geospatial environmental techno-economic assessment of pretreatment technologies for bioethanol production," *Renewable and Sustainable Energy Reviews* 187, article 113743. DOI: 10.1016/j.rser.2023.113743

Wang, Q., Tan, X., Wang, W., Miao, C., Sun, Y., Yuan, Z., and Zhuang, X. (2022).

"KOH/urea pretreatment of bagasse for ethanol production without black liquor or wastewater generation," *Industrial Crops and Products* 178, article ID 114567. DOI: 10.1016/j.indcrop.2022.114567

Wang, Z., Yokoyama, T., Chang, H. M., and Matsumoto, Y. (2009). "Dissolution of beech and spruce milled woods in LiCl/DMSO," *Journal of Agricultural and Food Chemistry* 57, 6167-6170. DOI: 10.1021/jf900441q

Wikiera, A., Mika, M., Starzyńska-Janiszewska, A., and Stodolak, B. (2015). "Application of Celluclast 1.5 L in apple pectin extraction," *Carbohydrate Polymers* 134, 251-257. DOI: 10.1016/j.carbpol.2015.07.051

Xu, H., Li, B., and Mu, X. (2016). "Review of alkali-based pretreatment to enhance enzymatic saccharification for lignocellulosic biomass conversion," *Industrial and Engineering Chemistry Research* 55(32), 8691-8705. DOI: 10.1021/acs.iecr.6b01907

Yang, B., Boussaid, A., Mansfield, S. D., Gregg, D. J., and Saddler, J. N. (2002). "Fast and efficient alkaline peroxide treatment to enhance the enzymatic digestibility of steam-exploded softwood substrates," *Biotechnology and Bioengineering* 77(6), 678-684. DOI: 10.1002/bit.10159

Yang, Q., Fukuzumi, H., Saito, T., Isogai, A., and Zhang, L. (2011). "Transparent cellulose films with high gas barrier properties fabricated from aqueous alkali/urea solutions," *Biomacromolecules* 12, 2766-2771. DOI: 10.1021/bm200766v

Zhang, B., Cai, J., Duan, C. Q., Reeves, M. J., and He, F. (2015). "A review of polyphenolics in oak woods," *International Journal of Molecular Sciences* 16(4), 6978-7014. DOI: 10.3390/ijms16046978

Zhao, Y., Shakeel, U., Rehman, M. S. U., Li, H., Xu, X., and Xu, J. (2020). "Lignin-carbohydrate complexes (LCCs) and its role in biorefinery," *Journal of Cleaner Production* 253, article ID 120076. DOI: 10.1016/j.jclepro.2020.120076

Zheng, Y., Pan, Z., and Zhang, R. (2009). "Overview of biomass pretreatment for cellulosic ethanol production," *International Journal of Agricultural and Biological Engineering* 2(3), 51-68. DOI: 10.3965/j.issn.1934-6344.2009.03.051-068

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