CHEMICAL ENGINEERING TRANSACTIONS
VOL. 89, 2021
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Jeng Shiun Lim, Nor Alafiza Yunus, Jiří Jaromír Klemeš

**ISBN** 978-88-95608-87-7; **ISSN** 2283-9216
DOI: 10.3303/CET2189008

# The Combination of Acidic and Alkaline Pretreatment for a Lignocellulose Material in Simultaneous Saccharification and Fermentation (SSF) Process

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This study investigates the combination of acidic and alkaline for pretreatment to enhance the efficiency of cellulose enrichment for a lignocellulosic material, herein, rubber sawdust. Treating the material with acid followed by alkaline treatment resulted in higher efficiency, leading to an increased hydrolysability of the material in Simultaneous Saccharification and Fermentation (SSF) Processes. The pretreatment of rubber sawdust with an $H_2SO_4$ 1.0 wt% solution for 5.5 h, followed by NaOH 2.75 wt% solution for 24 h, gave the best response to a factorial experimental design. Under these conditions, the efficiency was 69.25 %with a cellulose crystallinity index of 50.31 %. The SSF conversion of different pretreated materials to bioethanol also confirmed that increasing the amorphous cellulose content led to higher efficiencies.

## 1. Introduction

Lignocellulose is a raw material for second-generation bioethanol production technology intended to replace food source materials such as rice, potatoes, and cassava of the first-generation bioethanol (Dias et al., 2014). Herbaceous and woody lignocellulose are abundant biomass with low cost and high utility as agro-forestry by-products. The conversion of these raw materials into bioenergy - bioethanol addresses environmental issues and sustainability implications in a circular economy (Balat, 2011).

In bioethanol production from lignocellulose, a pretreatment stage is indispensable to overcome its tight material structure, made from intricately linked lignin, cellulose, and hemicellulose (Pandey et al., 2014). Pretreatment removes lignin and disrupts the lignocellulose structure, facilitating enzymatic hydrolysis and fermentation (Vargas et al., 2015). The cellulosic structure changes under different chemical agents. Pretreatment with an alkaline solution significantly reduced the crystallinity (CrI) and increased the porous structure of lignocellulose by swelling the fibres (Xu and Sun, 2016). Pretreatment with an acidic solution increased the crystallinity index owing to the partial hydrolysis of the amorphous cellulose regions. Enzymes can readily hydrolyse the amorphous cellulose regions with unstable structures into sugars (Mittal et al., 2011).

Another method that combines acid and base has emerged for quick treatment time and higher efficiency (Lee et al., 2016). This combined method also saves chemical usage as acid and base solutions can neutralise each other in the process before discharging or lignin recovery (Nguyen et al., 2019). In this method, the direct factors influencing the crystallinity and the efficiency are the acid pretreatment time (Kargarzadeh et al., 2012) and base concentration (Kassaye et al., 2017). This study investigated and optimised the mentioned factors above to maximise amorphous cellulose in lignocellulose for second-generation bioethanol conversion.

Paper Received: 25 June 2021; Revised: 10 August 2021; Accepted: 15 November 2021
Please cite this article as: Tan N.T.L., Dam Q.P., Mai T.P., Nguyen D.Q., 2021, The Combination of Acidic and Alkaline Pretreatment for a Lignocellulose Material in Simultaneous Saccharification and Fermentation (SSF) Process, Chemical Engineering Transactions, 89, 43-48 DOI:10.3303/CET2189008

## 2. Materials and methods

### 2.1 Materials

In this experiment, the source of lignocellulose used was rubberwood sawdust from Binh Duong province, Vietnam. The received sawdust was mechanically processed and screened to have sizes smaller than 1 mm, and then dried and stored at a humidity below 15 % by weight. Before determining the lignocellulose composition, the material was dried at 110 °C to a constant mass.

### 2.2 Pretreatment with different agents

Pretreat rubberwood sawdust by the following methods: with only NaOH 2.0 wt% solution for 48 h, with only $H_2SO_4$ 2.0 wt% solution for 48 h, and with a combination of both acid and base solution but carried out in different order:
Experiment 1: First with $H_2SO_4$ 2.0 wt% solution for 24 h and then with NaOH 2.0 wt% solution for 24 h.
Experiment 2: First with NaOH 2.0 wt% solution for 24 h and then with $H_2SO_4$ 2.0 wt% solution for 24 h.
All experiments were performed at room temperature with a solid/liquid ratio of 1/10 and constant shaking at 120 rpm.

### 2.3 Response Surface Methodology (RSM) model to survey acid-base combined pretreatment

From the experimental results in Section 3.2, a high acid concentration increases cellulose crystallinity. To limit the hydrolysis of cellulose by high concentration acid, first used acid $H_2SO_4$ 1.0 wt% solution and then filtered the solution to collect the solid part and treated it with NaOH [1.0 - 3.0] wt% solution for 24 h.
A survey model for the pretreatment performance of rubberwood sawdust was designed using response surface methodology (RSM) with the Design Expert v.11 software. The two factors of $H_2SO_4$ acid pretreatment time and NaOH concentration are a two-level design with lever arm $\alpha$ = 1. The number of repeated experiments at center $N_0$ = 3. The total number of experiments is according to the following Eq(1)

$$N = 2^k + 2.k + N_0 = 2^2 + 2.2 + 3 = 11 \quad (1)$$

### 2.4 Chemical analysis

The composition of sawdust rubberwood was analysed by NREL Laboratory Analytical Procedure (Sluiter et al., 2008). Sawdust was hydrolysed with concentrated $H_2SO_4$ 72 wt% for 30 min, then distilled water was added for secondary hydrolysis with 4.0 wt% diluted acid. Cellulose and hemicellulose components that hydrolysed to glucose and xylose were analysed by high-performance liquid chromatography (HPLC) using a Shimazu CTO-20A HPLC, column SUGAR SH101 (Shimadzu HPLC analyser, 2014). Lignin composition was determined using a UV-Vis analyser model NiR V770 (Japan).

### 2.5 Determination of crystallinity index (CrI) of cellulose

X-ray diffraction method was applied to determine the crystallinity of cellulose. The Burker D8 X-ray diffractometer uses a copper anode with voltage sources of 40 kV and 25 mA. The X-ray spectrum is scanned $2\theta$ from 5 to 50 with the scanning step of 0.02 and the speed of 0.25 s/step. The crystallinity was calculated according to the Eq(2) (Karimi and Taherzadeh, 2016):

$$\mathrm{CrI}(\%) = \frac{[(\mathrm{I}_{200} - \mathrm{I}_{\mathrm{non}})]}{\mathrm{I}_{200}} \cdot 100 \quad (2)$$

where $\mathrm{I}_{200}$ is the diffraction intensity at $2\theta \approx 22°$ (200 is the crystalline surface in cellulose) and $\mathrm{I}_{\mathrm{non}}$ is the diffraction intensity at $2\theta \approx 18°$ (amorphous region of cellulose).

### 2.6 Pretreatment efficiency

The pretreatment efficiency depends on the amount of enriched cellulose per lignin amount and ignoring the other components. The process removes mainly lignin and has no effect or minimal removal of the remaining component. The pretreatment process efficiency is calculated according to Eq(3) (Tran et al., 2020):

$$\mathrm{H}_1(\%) = 100(\%).\left(1 - \frac{\mathrm{Y}_2}{\mathrm{Y}_1}\right) \quad (3)$$

where $Y_1$ and $Y_2$ are the mass ratios of lignin/cellulose of the material before and after the pretreatment process. A corrected version for efficiency from Eq(3) accounts for the change of amorphous cellulose content after pretreatment. The adjustment is to evaluate whether the impact of pretreatment processes on the crystalline structure of cellulose would affect the hydrolysis of cellulose during SSF. The corrected efficiency is according to the Eq(4):

$$H_2(\%) = 100(\%).(1 - \frac{Y_2'}{Y_1'}) \quad (4)$$

where $Y_1'$ and $Y_2'$ are the mass ratio of lignin/ amorphous cellulose of the material before and after pretreatment. The amorphous cellulose content was calculated as show in Eq(5):

$$\text{Cellulose amorphous} = \text{Cellulose}.(1 - \text{CrI}) \quad (5)$$

### 2.7 Yeast and culture media

Before fermentation, we activated dry yeast (*Saccharomyces cerevisiae*, Ethanol ReD TM) in SDB liquid medium (sucrose and peptone in distilled water), autoclaved at 121 °C for 10 min. Yeast culturing occurred in a shaking incubator (120 rpm, 35 °C, within 48 h). Yeast density was measured by optical method (OD) at wavelength 610 nm using a UV instrument – Vis Hach DR 5000.

### 2.8 Simultaneous Saccharification and Fermentation

Rubberwood sawdust after pretreatment was loaded into 250 mL Erlenmeyer flask, adding water to a solid/liquid ratio of 0.1 and 2.0 wt% peptone nutrient. The solution was autoclaved at 121 °C for 10 min and then cooled to 35 °C. Yeast 5.0 vol% and Acremonium Cellulase enzyme (Meiji Seika Co.) 5.0 vol% with an activity of 100 IU/mL were added. The fermentation medium remained in the shaking incubator at 35 °C and 120 rpm during the SSF process. Sealing the flasks prevents $CO_2$ from escaping and contaminating the environment with other microorganisms.

Bioethanol conversion was calculated based on the theoretical amount of ethanol converted from the cellulose of the original rubberwood ($[EtOH]^*$ is 2.785 vol% in this study) according to the following Eq(6):

$$Bioethanol\ conversion\ (\%) = \frac{[EtOH]}{[EtOH]^*}\ 100(\%) \quad (6)$$

where [EtOH] is the highest ethanol concentration generated during the fermentation (vol%).

## 3. Results and discussion

### 3.1 Components of rubberwood

The analyses of the results showed that the main components included cellulose (41.20 wt%), hemicellulose (23.19 wt%), lignin (23.56 wt%), ash (4.42 wt%), and other components (7.63 wt%). The high composition of cellulose shows the conversion potential of rubberwood into ethanol.

### 3.2 Results of pretreatment survey with different chemical agents

Results of the change in Crystallinity index (CrI) and pretreatment efficiency (based on Eq(3)) are shown in the Table 1.

*Table 1: Pretreatment efficiency and crystallinity when treated with different agents*

| Pretreatment | I200 (Intensity) | Inon (Intensity) | CrI (%) | Pretreatment efficient (%) |
|---|---|---|---|---|
| Starting material | 1527 | 590 | 61.36 | - |
| With only NaOH 2.0 wt% | 1055 | 437 | 58.58 | 42.90 |
| With only $H_2SO_4$ 2.0 wt% | 1175 | 404 | 65.62 | 13.46 |
| Exp 1 | 1250 | 490 | 60.80 | 62.61 |
| Exp 2 | 1321 | 428 | 67.60 | 34.21 |

Table 1 showed that the pretreatment method strongly affects the pretreatment performance. The combined pretreatment with the order of acid before base gave the highest pretreatment efficiency of 62.61 %, while the reverse order gave the efficiency of only 34.21 %. This is due to the mechanisms when using acid in different order. The initial acidic solution stripped lignocellulose of impurities, leading to a more efficient treatment with the alkaline solution. Subsequent acid treatment will facilitate hydrolyzation of a portion of cellulose, yielding lower cellulose content at the end.

Compared with single processing, the combined method brings higher efficiency. NaOH seemed to reduce the crystallinity of the sawdust materials after pretreatment. This phenomenon is due to the alkali-inducing swelling of the structure of lignocellulose that causes the break of the crystalline cellulose, increasing the amorphous

region (Xu and Sun, 2016). The acidic agent will hydrolyse the amorphous cellulose increasing the crystallinity and cellulose content. An effective pretreatment method is to choose a combination of acid first and then base pretreatment.

Scanning Electron Microscope (SEM) images in Figure 1 show the change of starting material and after pretreatment with different methods (Tran et al., 2019). Compared with the starting material (Figure 1a), pretreatment agents had damaged the wood fibres. When using NaOH (Figure 1b) and combining in the order of acid-before-base (Figure 1c), the structure of lignocellulose greatly expanded, breaking the wood fibres smaller, revealing the inner cellulose core. When pretreatment is in the reverse order of base-before-acid, the breaking happens on the surface and the inside. The initial treatment with NaOH causes the fibre to swell, allowing the acid to hydrolyse the inner cellulose.

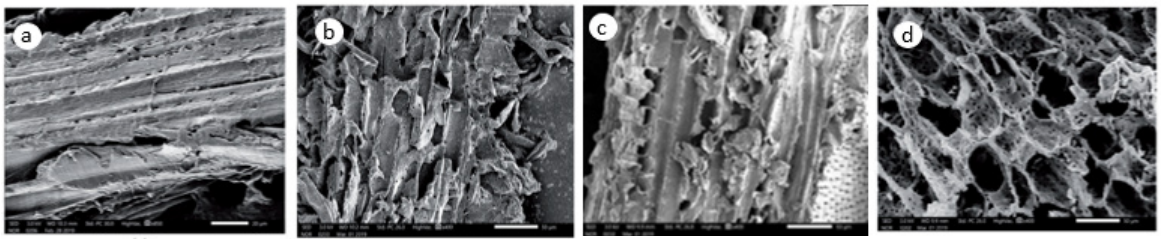


*Figure 1: SEM images of rubberwood (a) starting material (b) pretreated with NaOH 2.0 wt.% solution (c) Pretreated with a combined method in the order of (c) acid before base (d) base before acid.*

### 3.3 Results of RSM with the combination of acidic and alkaline pretreatment

Two influencing factors, pretreatment time (t) for acid $H_2SO_4$ 1.0 wt% solution in the range [3 – 9] (h) and the NaOH solution concentration, [C] with [1 – 3] wt%, gave a 3D surface response graph collected by RSM shown in Figure 2.

From Eq(3), the efficiency has the values in the range 41.47 % – 63.61 %. Acid pretreatment time and NaOH concentration are proportional to the process efficiency as characterised by the linear model (Figure 2a). The efficiency of the pretreatment process is higher if the time and concentration of NaOH increased and vice versa. This calculation does not account for the change in cellulose crystal content.

Accounting for factors affecting the crystallinity during pretreatment gives a quadratic model (Figure 2c) having crystallinity values in the range [47.76 % - 62.70 %]. During 3 to 9 h of acid treatment, the crystallinity index decreased from 3 h to 6 h and increased from 6 h to 9 h. The reason is that the initial period of acid treatment has the effect of removing impurities, but a long time will affect regions of amorphous cellulose and increase the crystallinity. As for the NaOH concentration factor, there is an inverse linear correlation with the crystallinity, but at a concentration of about 3.0 wt% solutions, this factor has little effect. Combine the change in crystallinity and lignin removal capacity forms an adjusted pretreatment efficiency formula that symbolises amorphous cellulose enrichment capability and lignin removal as characterised by the lignin/ratio amorphous cellulose of the starting material and after pretreatment. This efficiency is also correlated with the above two factors as described by the quadratic model (Figure 2b). The correction efficiency ($\mathrm{R_{H_2}}$) ranges from 40.93 to 69.25 %. The mode for corrected efficiency is significant (p-value < 0.05). This model reflects a relationship between the efficiency with lignin removal and crystallinity: efficiency is higher the more lignin removal and the less cellulose crystallinity.

The adjusted pretreatment performance in RSM is a second-order polynomial that describes this surface under the actual parameters of $R^2 = 0.9221$.

Eq(7) and Eq(8) were used to optimise the pretreatment. The most suitable pretreatment condition for the adjusted pretreatment efficiency with the highest of 69.25 % and the lowest crystallinity index of 50.31 % was pretreatment with acid $H_2SO_4$ 1.0 wt% for 5.5 h then proceed to treat with NaOH 2.746 wt% solution using the above conditions to experiment with the combined method in Section 3.4.

$$\mathrm{H_2} = -17.84 + 17.28\mathrm{t} + 20.82[\mathrm{C}] - 0.14\ [\mathrm{C}].\mathrm{t} - 1.33\mathrm{t}^2 - 2.09[\mathrm{C}]^2 \quad (7)$$

Combine with the equation describing the crystalline surface ($R^2 = 0.8527$):

$$CrI = 97.67 - 9.77t - 15.78[C] + 0.54\ [C].t + 0.78t^2 + 2.39[C]^2 \quad (8)$$

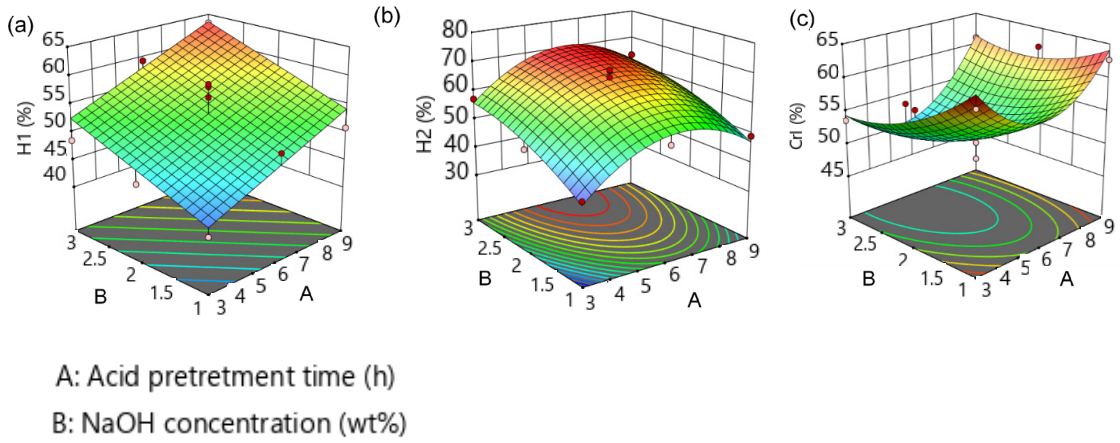


*Figure 2: 3D graphs of the response surface of (a) pretreatment efficiency $R_{H_1}$ (b) adjusted pretreatment efficiency $R_{H_2}$and (c) crystallinity index $R_{CrI}$.*

### 3.4 Results of Simultaneous Saccharification and Fermentation process (SSF)

The results showed that the combined method with the optimal parameters (in Section 3.3) produced the highest ethanol amount of 1.55 vol% with the bioethanol conversion of 55.75 %, higher than that of single-substance treatments: treated with only acidic solution is 0.79 vol% and with only alkaline solution is 1.27 vol% (Figure 3) with bioethanol conversions are 28.51 and 45.62 %. Since rubberwood with acids and bases combined before treatment has a low crystalline cellulose content of 50.31 %, enzymatic hydrolysis is more favorable with the materials treated with acid only (CrI: 65.62 %) and with base only (CrI: 58.58 % ) (Table 1). Increased hydrolysis will increase bioethanol conversion efficiency.

With the evaluation of the removed lignin content and the change in crystallinity, the new efficiency calculation method has correctly reflected the influence of the pretreatment process on the efficiency of the enzymatic hydrolysis and fermentation process.

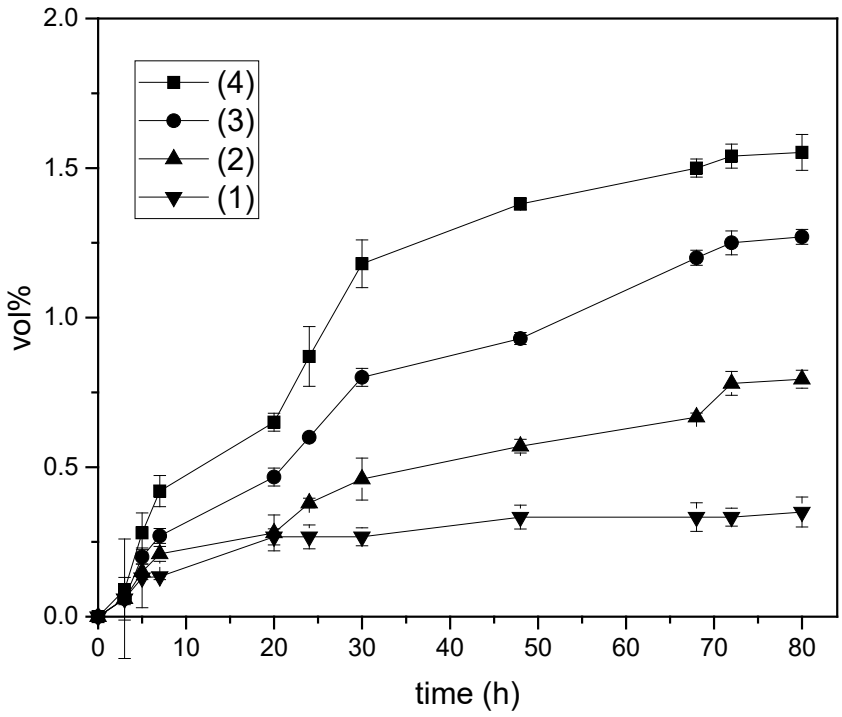


*Figure 3: Ethanol concentration of SSF process for rubberwood (1) starting material, pretreated with (2) acid (3) base and (4) pretreatment with a combined method of acid before base.*

## 4. Conclusion

The optimal conditions for rubberwood sawdust pretreatment are to pretreat with acid $H_2SO_4$ 1.0 wt% solution for 5.5 h first, followed by NaOH 2.746 wt% solution for 24 h, solid/liquid ratio is 1/10, 120 rpm at room temperature, can reduce the crystallinity index of cellulose to 50.31 % (compared to 61.36 % of the starting material) and give a pretreatment efficiency of 69.25 %. The enzymatic hydrolysis of SSF fermentation also demonstrated that the rubberwood sawdust pretreated with the above conditions had a higher bioethanol conversion than the other methods. In order of crystallinity index (CrI) of sawdust treated by combination method, base pretreatment method and finally acid pretreatment were: 50.31, 58.58 and 65.62 %, bioethanol conversions are 55.75, 45.65 and 28.51 %, proving the hypothesis of increased conversion efficiency as amorphous cellulose content increases.

**Acknowledgments**

We acknowledge the support of time and facilities from Ho Chi Minh City University of Technology (HCMUT), VNU – HCM for this study.

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