Effect of amphiphilic lignin derivatives (A-LD) surfactant addition on the fermentation process of sorghum bagasse kraft pulp for bioethanol production

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Abstract. This research was intended to study the effect of Amphiphilic Lignin Derivatives (A-LD) surfactant addition on the bioethanol production by using simultaneous saccharification and fermentation (SSF) system. A-LD synthesis was obtained from single-step lignin isolation from black liquor as a by-product of the Acacia mangium kraft process. This synthesis was carried out by reacting single-step lignin isolation with the polyethylene glycol diglycidylether (PEDGE), which was heated at 60 °C for one h. SSF was performed by using Saccharomyces cerevisiae InaCC Y93, cellulase enzyme of 50 FPU/g, yeast inoculum in 10% and 20%, and a 15 mg/mL A-LD added into 5.37 g (wet weight) of sweet sorghum bagasse (SSB) kraft pulp. The concentration of reducing sugar and the ethanol contents were analyzed every 24 h for 72 h and also at 89 h. The ethanol concentration, ethanol yield, and the highest percentage of theoretical ethanol yield were 4.91 ± 0.10 g/L, 0.06 ± 0.001 g/g, and 11.29 ± 0.22%, respectively. They were obtained by 20% of yeast inoculum for 24 h fermentation on the SSF system and the addition of A-LD surfactant. The addition of A-LD on the SSF process increased ethanol production and shortened the fermentation time.

1. Introduction

Amphiphilic Lignin Derivatives (A-LD) is a bio-surfactant that can improve the performance of the enzymatic hydrolysis in reducing sugar production before bioethanol production. A-LD surfactant can significantly increase the reducing sugar from the enzymatic hydrolysis of wood pulp and oil palm fronds [1]. A-LD surfactants will regulate enzyme activity during the saccharification process to maintain high and significantly enhance the enzyme recovery process after saccharification [2]. Cheng et. al. [3] has synthesized two types A-LD from soda lignin of Japanese cedar, namely DOPEG-SL and EPEG-SL. A-LD can reduce enzyme adsorption and feedback inhibition; thus, the bioethanol production increases. The addition of A-LD increases the maximum concentration of ethanol by 3-5% v/v, and the ethanol production efficiency based on theoretical content increases by 40.7-64.1%.

Studies have been done on the effects of the isolating lignin method from black liquor of A.mangium kraft pulping. The amount of A-LD was added in enzymatic hydrolysis performance of sorghum bagasse pulp [4] and also on the optimization of the A-LD synthesis processes [5]. This study was conducted to study further the effects of A-LD addition in the fermentation of SSB kraft pulp to produce bioethanol by analysing the reducing sugar and ethanol content produced after simultaneous hydrolysis and fermentation (SSF).
Sorghum bagasse contains high cellulose content and low lignin content. The kraft pulping of SSB has a high delignification selectivity [6]. Previously, Solihat et al. [7] reported that kraft pulp with 17% active alkaline and 20% sulfidity resulted in 64.65% cellulose content, acid 0.68%, soluble lignin (ASL), and 0.81% acid-insoluble lignin (AIL). Besides, it resulted in the highest reducing sugar yield (45.57%) compared to other kraft pulping conditions. Kraft pulping with an active alkali of 19% and sulfidity of 20% showed higher levels of cellulose, which was 73.83%, with AIL and ASL levels of 0% and 0.79%, respectively [7]. It underlies the use of pulping conditions of SSB as the SSF substrate used in this study.

Enzyme activity of lignocellulose hydrolysis decreases with the presence of non-productive bonds formed between enzymes and cellulose and enzymes and lignin bonds (non-specific hydrophobic interactions) [8]. An additive to reduce the two bond types formed can be added in the hydrolysis process for increasing the enzyme activity. A-LD surfactants obtained from several types of isolated lignin, such as kraft, soda, and organosolv lignin were reacted with polyethylene glycoldiglycidylethers (PEDGE) [9]. Uraki et al. [2] show that A-LD can increase the enzymatic hydrolysis process, as indicated by high enzyme activity during the hydrolysis process. A-LD surfactants are bound to cellulase enzymes. Thus, those non-specific hydrophobic interactions between cellulase and lignin enzymes are not formed. The interaction between cellulase enzymes and A-LD causes the cellulose to hydrolysis more effectively. It increases the chances of cellulase enzymes to work again by minimizing non-productive bonds that might form [2]. A-LD increases the stability of the enzymes during hydrolysis; therefore, bioethanol production improves [10].

SSF is a method that combines enzymatic hydrolysis and fermentation in one reaction simultaneously [11]. Final products such as glucose and other simple sugar monomers in the saccharification process can inhibit the activity of enzymes, causing a decrease in the efficiency of these enzymes [12]. In the process of enzymatic reactions, the final product can be a negative effect or that is bound to the allosteric site of the enzyme so that it inhibits enzyme activity [13]. Inhibition of enzyme activity due to the formation of final saccharification products can be avoided by using the SSF method. It is because the sugar produced simultaneously is used by fermenting organisms to form ethanol [14].

In our previous study, the effect of A-LD addition in enzymatic hydrolysis of SSB kraft pulp has been evaluated [4]. Surfactants can increase the production of reducing sugars in the process of biomass hydrolysis and also improve bioethanol production [2, 10]. However, the effect of A-LD in fermentation has not been carried out yet. Therefore, in this study, we focused on the evaluation of A-LD addition from A.mangium lignin on the SSF performance of SSB kraft pulp.

2. Materials and methods

This research was conducted at the Microbiology and Biomass Conversion Laboratory, Research Center for Biomaterials, LIPI. The material used in this research was black liquor of kraft pulping of A. mangium wood. Sweet Sorghum bagasse (SSB) kraft pulp was prepared by using an active alkali of 19%, and sulfidity of 20% at 170 °C for four h was used as a SSF substrate. The enzyme and yeast for SSF were a commercial cellulase (meicellase), while Saccharomyces cerevisiae InaCC Y93 was obtained from Indonesia Culture Collection (InaCC) LIPI. The chemicals used were Potato Dextrose Agar (PDA) (Merck), yeast extract (Merck), glucose (Merck), peptone (Merck), HCl (Merck), NaOH (Merck), PEDGE (Polyethylene glycol diglycidylether), and CH3COOH (Merck).

2.1. One step lignin isolation

One hundred grams of black liquor was prepared in 2 L beaker for one step lignin isolation. The black liquor was dissolved in 1L of distilled water and then filtered to obtain a black liquor filtrate with a pH of 12. It was then titrated with 1M HCl to obtain a pH 2 of lignin suspension [3]. The pH 2 of lignin suspension was allowed to stand until complete deposition occurred, and two layers were formed. The water layer was separated from lignin deposits. The formed lignin precipitation was then washed fivetimes with distilled water, followed by vacuum filtering to obtain wet lignin. Wet lignin was dried in a 45°C oven for 24h. Dried lignin was finalized and sieved to obtain some 60 mesh of one step lignin isolation.
2.2. Crude A-LD synthesis and preparation of 15 mg/mL A-LD solution
The crude A-LD synthesis was carried out based on the modified method of Cheng et al. [3]. A1 g of one step lignin was prepared in a 50 mL beaker glass, and 10 mL of 1 M NaOH solution was added then stirred until dissolved and homogeneous. A 2 mL of the lignin solution was added with 0.6 g of PEDGE with Mn 500 and then reacted at 60°C for one h. The reaction process was stopped by adding 3 mL of CH₃COOH solution then shaken until homogeneous. A 15 mg/mL A-LD solution was prepared with 0.75 g A-LD dissolved in 10 mL 0.05 M citrate buffer solution. The solution was put into a 50 mL measuring flask, then 0.05 M citrate buffer was added to make a 50 mL solution and shaken until homogeneous.

2.3. Preparation of inoculum
Saccharomyces cerevisiae InaCC Y93 was preserved on Potato Dextrose Agar (PDA) 2% slant and incubated for 1-2 days at room temperature (28-32°C). A loop, which was full of S. cerevisiae InaCC Y93, was inoculated into a 300 mL Erlenmeyer containing 100 mL of 5% YPD (Yeast Peptone Dextrose) medium. The inoculum was then agitated at 120 rpm for 24h at 30°C using an incubator (Bio-Shaker BR-300). The inoculum was centrifuged at 10,000 rpm, 20 °C for 10 min. The supernatant was decanted. The cells were then re-suspended in one-tenth sterile distilled water of the initial volume of the medium. The suspension was used as inoculum for the SSF process.

2.4. Simultaneous saccharification and fermentation (SSF)
SSF was carried out by mixing SSB kraft pulp samples, cellulase enzyme, citrate buffer solution, yeast peptone (YP) medium, inoculum solution, crude A-LD solution, and distilled water with the treatment conditions, as shown in Table 1. SSF was performed by using Saccharomyces cerevisiae InaCC Y93 in the variation of 10% and 20% inoculum.

As much as 5.37 g (dry weight) sample was added into a 300 mL Erlenmeyer flask, then sterilized by using autoclave for fifteen min. SSF process was carried out by adding 0.05 M citrate buffer (pH 4.8), cellulase enzyme 50 FPU/g, YP medium consisting of 100 g/L yeast extract, 200 g/L peptone, yeast inoculum, and 15 mg/mL of A-LD solution into SSB kraft pulp. Sterile distilled water was added to the flask until the mixture reached a total weight of 30 g. The flask was then sealed with bubble traps and incubated in a shaker incubator of 150 rpm at 38°C for 89h. The SSF time was conducted based on the optimization of the hydrolysis time of SSB kraft pulp with the addition of A-LD that had been done before [4]. Samples were taken at 0, 24, 48, 72, and 89h during the fermentation process for reducing sugars and ethanol content analysis.

### Table 1. Number of samples and reagents in the SSF process.

| Flask   | Sample (g) | Meicellase (mL) | Citrate Buffer (mL) | YP (mL) | Inoculum (mL) | A-LD (mL) | Distilled water (mL) |
|---------|------------|----------------|---------------------|--------|---------------|-----------|---------------------|
| A I-10% | 5.37       | 8.13           | 2.5                 | 3      | 3             | 4.35      | 3.65                |
| A I-20% | 5.37       | 8.13           | 2.5                 | 3      | 3             | 4.35      | 0.66                |
| B I-10% | 5.37       | 8.13           | 2.5                 | 3      | 3             | 0         | 8.01                |
| B I-20% | 5.37       | 8.13           | 2.5                 | 3      | 3             | 0         | 5.01                |
| KS-A I-10% | 0       | 8.13           | 2.5                 | 3      | 3             | 4.35      | 9.02                |
| KS-B I-10% | 0       | 8.13           | 2.5                 | 3      | 3             | 0         | 13.37               |
| KS-A I-20% | 0       | 8.13           | 2.5                 | 3      | 6             | 4.35      | 6.02                |
| KS-B I-20% | 0       | 8.13           | 2.5                 | 3      | 6             | 0         | 10.37               |

A = Sample with A-LD; B = Sample without A-LD; KS-A = Control with A-LD; KS-B = Control without A-LD

### 2.5. Reducing sugar and ethanol content analysis
Sample after the SSF process was centrifuged at 10,000 rpm for 10 min. The supernatant was measured for reducing sugar and ethanol concentration. Reducing sugar was determined using DNS
Method [15], and absorbance measurements were carried out with a UV-Vis Shimadzu UV-1800 spectrophotometer at $\lambda = 540$ nm.

Ethanol content was analyzed by gas chromatography (Shimadzu GC-2010) with the Rtx-Wax column as a stationary phase, a mixture of He and H gas as a mobile phase, and a flame ionization detector (FID) detector. The temperature of the column when running was maintained at 80 °C as the boiling point of ethanol. At the same time, the injector and detector temperatures were maintained at 200°C and 210°C, respectively. The sample injected was 1 μL with a total flow of 83.5 mL /min and split ratio 40. The concentration of ethanol was calculated using Eq. 1 [16]:

$$\text{Ethanol concentration } (\frac{g}{L}) = \text{ethanol content } (\frac{L}{V}) \times 10 \times 0.789 \quad (1)$$

The ethanol yield and percentage of theoretical ethanol were determined by Eq. 2 and Eq. 3 [16]:

$$\text{Ethanol yield } (\frac{g}{g}) = \frac{\text{g of ethanol produced}}{\text{g cellulose in working volume } (\frac{Ax100}{B}) \times C} \quad (2)$$

where : A: initial dry weight of biomass (g), B: pulp recovery, and C: cellulose fraction of dry biomass (g).

$$\text{Percentage of theoretical ethanol}= \frac{\text{Ethanol yield } (\frac{g}{g})}{\text{Theoretical of ethanol yield } (\frac{g}{g}) \times 1.111 \times 0.51} \times 100 \quad (3)$$

3. Results and discussion

Crude A-LD surfactant was added in the SSF process to determine its effect on the fermentation process of reducing sugar and ethanol production. The process of cellulose saccharification and reducing sugar fermentation was carried out simultaneously. Thus, the reducing sugar concentration obtained was the residual reducing sugar that was not fermented into ethanol at a certain time. Figure 1 shows that the reducing sugar concentration increases significantly at 24-48h of incubation time; after that, the remaining sugar concentration is stable at the concentration between 12-15 mg/mL. From the result, the yeast starts to consume sugar and converts it to ethanol after 24-48 h of incubation time. By using 10% (v/v) yeast inoculum, the SSF with the addition of A-LD surfactant shows that the maximum ethanol concentration is faster than without A-LD surfactant. The maximum ethanol concentration with the A-LD addition is 3.03 ± 0.59 g/L at 48h incubation time. While in the SSF without A-LD, the maximum ethanol concentration is 3.46 ± 1.11 g/L at 89h incubation time.

**Figure 1.** Reducing sugar and ethanol concentration during fermentation by using 10% inoculum.

Figure 2 shows that ethanol yield and percentage of theoretical ethanol have the same pattern as ethanol concentration. The maximum ethanol yield and percentage of theoretical ethanol are 0.04 g/g
and 6.97%, respectively, and obtained on the SSF with the addition of A-LD. Those values are not significantly different in the SSF without A-LD.

![Ethanol yield and percentage of theoretical ethanol on 10% inoculum variation.](image)

**Figure 2.** Ethanol yield and percentage of theoretical ethanol on 10% inoculum variation.

By using 20% (v/v) yeast inoculum, the reducing sugar concentration increases sharply at 24h incubation time and decreases until the end of incubation time. The maximum ethanol concentration with the addition of A-LD is 4.91 ± 0.10 g/L at 24h incubation time. While in the SSF without A-LD, the maximum ethanol concentration obtained at 48h incubation is as much as 2.99 ± 0.07 g/L (Figure 3). The maximum ethanol yield and percentage of theoretical ethanol with the addition of A-LD are 0.06 g/g and 11.29%, respectively (Figure 4).

![Reducing sugar and ethanol concentration during fermentation by using 20% inoculum.](image)

**Figure 3.** Reducing sugar and ethanol concentration during fermentation by using 20% inoculum.

All of those results explain that the addition of A-LD on the SSF process gives some effects on ethanol production. In this study, the ethanol production with A-LD is higher and faster than without A-LD. It shows that the surfactant A-LD affects the SSF process. The hydrolysis process is more effective with the addition of A-LD so that the amount of reducing sugar formed and converted to ethanol is higher. Uraki et al. [17] have reported the proposed mechanism of A-LD as a cellulase aid agent for improving bioethanol production that can maintain the cellulase work. The action of A-LD is
as a water-soluble, immobilization support of cellulase to be used repeatedly and to prevent the interaction between cellulase and substrate lignin. These results are in line with Cheng et al. [3] reporting that the SSF fed-batch system on cedar wood pulp using cellulase and yeast, as well as the addition of A-LD, increased the efficiency of the hydrolysis and fermentation processes.

![Ethanol yield and percentage of theoretical ethanol on 20% inoculum variation.]

According to Eckard et al. [18], some factors influence the efficiency of amphiphilic surfactants derived from lignin, such as the presence of lignin, which causes enzyme deactivation. Increasing surfactant concentration can increase the amount of surfactant adsorbed on the hydrophobic surface of the biomass, causing irreversible adsorption of the enzyme and deactivation. SSB kraft pulp used in this study has a 100% percent delignification after the kraft pulping with an active alkali of 19% and sulfidity of 20% [7]. Hence, there is no lignin in the substrate that affects the efficiency of A-LD. The efficiency of A-LD might be influenced by the relatively high A-LD concentration causing deactivation of the enzyme so that the fermentation process becomes less optimal. Besides, the purity of A-LD might affect the fermentation process because, in the study, the A-LD synthesized is still in the form of crude A-LD without purification. However, this effect of A-LD purity needs to be studied further.

4. Conclusion

The ethanol concentration, ethanol yield, and highest percentage of theoretical ethanol yield were 4.91 ± 0.10 g/L, 0.06 g/g, and 11.29%, respectively, and obtained by SSF with the addition of A-LD and 20% of yeast inoculum at 24h fermentation. The addition of A-LD on the SSF process of SSB kraft pulp can increase ethanol production and also shorten the fermentation time.

Acknowledgments

This study was supported by LIPI through competitive programs in Material and Energy Development, and Manufacturing Program, Research Center for Physic, Indonesian Institute of Sciences (LIPI) in the Fiscal Year of 2016-2017. The authors thank the Research Center for Biomaterials, LIPI, for providing the equipment and testing facilities to support this study.

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