

Microbial Conversion of Rice Straw Into Lactic Acid Through Simultaneous and Separate Hydrolysis and Fermentation

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Submitted: January 31, 2023; Revised: September 11, 2023, May 28, 2024, September 6, 2024;
Accepted: October 2, 2024; Published: May 28, 2025

ABSTRACT

Lactic acid is often produced from lignocellulosic materials through various steps, including pretreatment, hydrolysis, and fermentation. Therefore, this study aims to evaluate the conversion of lactic acid from rice straw using simultaneous and separate hydrolysis and fermentation. The process was initiated with and without pretreatment using $\text{Ca}(\text{OH})_2$ at 85 °C for 16 hours, followed by hydrolysis using *Trichoderma reesei* PK1J2 and fermentation with *Rhizopus oryzae* AT3. Hydrolysis and fermentation were performed simultaneously and separately. The results showed that pretreatment could reduce lignin content, but this process was not needed because *Trichoderma reesei* PK1J2 degraded lignin during hydrolysis. In addition, fermentation conditions in this study could not support the production of lactic acid by *Rhizopus oryzae* AT3. Based on biomass growth during the treatment, simultaneous hydrolysis and fermentation (108 mg/g dry substrate) was better compared to separate hydrolysis and fermentation (104 mg/g dry substrate).

Keywords: Fermentation; lactic acid; *Rhizopus oryzae*; rice straw; *Trichoderma reesei*

INTRODUCTION

Lactic acid ($\text{CH}_3\text{CHOHCOOH}$) and its derivatives are important chemicals in the food, chemical, and pharmaceutical industries. These compounds are used as a casein coagulant in fermented milk, a sour flavoring in bread, and a pH regulator in beer making. Meanwhile, pure L-lactate monomer can be used for the production of environmentally friendly biopolymers (polylactic acid). The production of lactic acid using lignocellulose

is very attractive because it can reduce environmental problems (Chen et al., 2021). Rice straw has been reported to be a lignocellulosic material abundantly available, reaching 731.34 teragrams per year (Kim et al., 2004). In addition, several studies have shown that approximately 75% of farmers in Indonesia burn their rice straw in the fields (Sumardi, 2015).

The production of lactic acid using straw is carried out in several stages, namely pretreatment, hydrolysis, and fermentation (Maas, 2008). Pretreatment serves to

reduce lignin, hemicellulose, and cellulose crystallinity (Sun et al., 2002). One of the pretreatments for lignocellulosic materials with lignin (such as straw) can be performed by heating at a temperature of 85 °C accompanied by the addition of Ca(OH)_2 (Chandel et al., 2013). Hydrolysis process serves to convert lignocellulose into simple sugar. *Trichoderma reesei* is widely used in the process because it has amylase enzymes and can produce high-reducing sugar (Singhania et al., 2010). Meanwhile, fermentation helps to convert simple sugar from hydrolysis into lactic acid. The microorganism often used in fermentation is *R. oryzae* which can produce L-lactate with a high purity level (Yamane et al., 2012).

Hydrolysis and fermentation can be carried out simultaneously or separately. Simultaneous hydrolysis and fermentation system minimizes inhibition due to the accumulation of metabolites from hydrolysis. However, the difference in conditions (temperature and pH) of hydrolysis and fermentation processes is one of the obstacles. The optimum temperature of hydrolysis process is 50 °C and pH below 5, while fermentation requires 37–42 °C and pH 5.5–6.5. These obstacles can be overcome by separate system, where both processes are carried out separately. However, this system also has weaknesses, namely the presence of cellobiose and glucose produced during hydrolysis process can inhibit cellulase activity and the time required is relatively long (Li et al., 2010). Although the study on the comparison of simultaneous and separate hydrolysis and fermentation systems for ethanol production has been conducted by Maas (2008), further investigations are required. Therefore, this study aims to compare simultaneous and separate hydrolysis and fermentation systems for lactic acid production from rice straw.

METHOD

Materials

This study used a material namely IR 64 rice straw, obtained from Moyudan, Sleman, Yogyakarta, Indonesia. The microbes used were *T. reesei* PK1J2 and *R. oryzae* AT3, from the Faculty of Agricultural Technology, Universitas Gadjah Mada (UGM), Indonesia.

The tools used included a centrifuge (Biofuge Primo USA), autoclave (EYELA Singapore), incubator (Sanyo Japan), hot plate (Advantec Japan), water bath (Sibata Japan), analyte balance (Fujitsu Japan), oven (Sanyo Japan). Others included a magnetic stirrer (ARE China), pH meter (METROHM 620 Indonesia), muffle (EYELA Singapore), high-performance liquid chromatography (Shimadzu Japan), spectrophotometer (Spectronic 200 Germany), and cabinet dryer (Indonesia).

Making Starter

T. reesei PK1J2 and *R. oryzae* AT3 were rejuvenated using Potato Dextrose Agar (PDA) media incubated at 30 °C for 7 days. In this study, *T. reesei* PK1J2 isolate was prepared into a starter by inoculating 1 mL of spore suspension ($10^6/\text{mL}$) in a mixture of 100 g of rice and 50 g of sterile rice husks (75% water content). Meanwhile, the *R. oryzae* AT3 starter was produced by inoculating 1 mL of spore suspension ($10^6/\text{mL}$) in 200 g of sterile rice (75% water content). Both were incubated at 30 °C for 7 days, then dried at 50 °C.

Pretreatment

Pretreatment was performed using the Maas method (2008) with slight modifications. Straw with a size of 5 mm as much as 60 g was mixed with Ca(OH)_2 with a content of 0.15 g/g dry biomass and added with distilled water with a ratio of solids to distilled water of 15% (w/w). Incubation was then carried out at 85 °C for 16 hours. Furthermore, it was washed with distilled water, neutralized with 20% (v/v) H_2SO_4 to pH 7, and dried at 50 °C until the water content was <10%. Before and after the pretreatment, cellulose, hemicellulose, and lignin content as well as sample weight were analyzed to determine the pretreatment yield.

Separate Hydrolysis and Fermentation - Hydrolysis by *T. reesei* PK1J2

Hydrolysis process was conducted using the Fatma et al. method (2010) with slight modifications. Straw without and with pretreatment as much as 3 g was put in a plastic box measuring 5x4x4 cm and mineral solution was added until the water content was 75%. The mineral solution used was KH_2PO_4 2 g/L, $(\text{NH}_4)_2\text{SO}_4$ 1.4 g/L, peptone 0.75 g/L, urea 0.3 g/L, CaCl_2 0.3 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3 g/L, yeast extract 0.25 g/L, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 20 mg/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5 mg/L, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.4 mg/L, and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.25 mg/L. Subsequently, sterilization was carried out at a temperature of 121 °C for 20 minutes. *T. reesei* PK1J2 starter was aseptically inoculated into straw, and the spore concentration at the beginning of hydrolysis process was approximately 10^6 per gram of material. Incubation was performed at room temperature with 91% humidity for 5 days. During hydrolysis process, reducing sugar, cellulase and xylanase enzyme activity, dry weight loss, as well as lignin, cellulose, and hemicellulose content were analyzed each day.

Separate Hydrolysis and Fermentation - Fermentation by *R. oryzae* AT3

Fermentation process was carried out by applying the Soccol method (1994) with slight modifications. The hydrolyzed straw was adjusted to pH 6 using 1 M HCl or

1 M NaOH solution. A total of 8 g of hydrolyzed straw was placed in a 5x4x4 cm plastic box and sterilized at 121 °C for 20 minutes. The *R. oryzae* AT3 starter was inoculated into a sterile substrate and the spore concentration at the beginning of fermentation process was 10^6 per gram of material. Incubation was performed at room temperature with 91% humidity for 6 days. During fermentation process, sugar reduction, pH, lactic acid content, and dry weight loss were analyzed each day.

Simultaneous Hydrolysis and Fermentation

Straw without and with pretreatment as much as 2 g in a 5x4x4 cm plastic box was added to the mineral solution until the substrate water content was 75% and sterilized at 121 °C for 20 minutes. The sterile straw was inoculated with *T. reesei* PK1J2 and *R. oryzae* AT3 starters and the concentration of each spore at the beginning of the process was around 10^5 per gram of material. Incubation was conducted at room temperature with 91% humidity for 6 days. During hydrolysis and fermentation process, reducing sugar was analyzed every day.

Chemical Analysis

Pectin, oligosaccharide (water-soluble substances), cellulose, hemicellulose, and lignin content were analyzed using the thermogravimetric method (Carrier, 2011). Reducing sugar concentration was measured using the DNS method (Wang), dry weight loss was determined using the thermogravimetric method (Christensen et al., 1989), and lactic acid content was calculated using the UV-HPLC method (Ahmed et al., 2014).

Cellulase Enzyme Analysis

A 0.5 mL enzyme solution was added into 1 mL of Na-citrate pH 4.8, heated at 50 °C, with Whatman paper 1, and incubated at 50 °C for 60 minutes. This continued with colorimetric analysis of reducing sugar using DNS. Furthermore, reducing sugar was analyzed on Spectro

Zero, enzyme blanks, as well as glucose standards, and enzyme activity was measured using Equation 1.

$$\text{FPU} \left(\frac{\text{units}}{\text{mL}} \right) = \frac{\text{Resulting reducing sugar } (\mu\text{mol per mL})}{60 \text{ minutes}}$$

$$1 \text{ unit} = 1 \mu\text{mol/minute} \quad (1)$$

Ghose (1987)

Xylanase Enzyme Analysis

The substrate was 1.0% birchwood 4-O-methyl glucuronoxylan in citrate buffer pH 4.8. Approximately 1.8 mL of the substrate solution was heated to 50 °C, added with 0.2 mL of the enzyme solution, incubated at 50 °C for 5 minutes, and measured for reducing sugar using DNS. Reducing sugar analysis was also performed on reagent blanks, enzyme blanks, and xylose standards. Xylanase activity was calculated using Equation 2.

$$\text{Xylanase activity} \left(\frac{\text{units}}{\text{mL}} \right) = \frac{\text{Resulting reducing sugar } (\mu\text{mol per mL})}{5 \text{ minutes}}$$

$$1 \text{ unit} = 1 \mu\text{mol/minute} \quad (2)$$

Bailey (1992)

RESULTS AND DISCUSSION

Pretreatment of Straw

Changes in the percentage of cellulose, hemicellulose, and lignin after pretreatment using $\text{Ca}(\text{OH})_2$ were presented in Table 1.

The hemicellulose content after pretreatment decreased significantly by 8.32% (5% significance level). This decrease gave open access for cellulase enzymes to cellulose. In this process, there was a possibility of cutting the bonds between hemicellulose and cellulose and lignin, as well as cross-linking between hemicellulose components to form several products

Table 1. Changes in straw components before and after pretreatment

Straw Components	Without pretreatment	With $\text{Ca}(\text{OH})_2$ pretreatment	Sig.
	Content (%)	Content (%)	
Water soluble substances	13.66 ± 1.33	13.6 ± 0.137	0.736
Cellulose	18.26 ± 1.89	35.91 ± 3.4	0.000
Hemicellulose	27.55 ± 1.76	19.32 ± 1.11	0.000
Lignin	24.22 ± 2.12	16.98 ± 2.69	0.005
Ash	16.31 ± 1.2	14.18 ± 0.516	0.006

with shorter chain lengths (Nguyen et al., 2010 and Sun et al., 2002). From the original difference test, lignin content decreased significantly by 7.24%. Lignin needed to be removed, as it could block enzyme access to its substrate (Gu et al., 2013). The percentage of cellulose increased with the degradation of lignin and hemicellulose. Furthermore, the effect of pretreatment with $\text{Ca}(\text{OH})_2$ differed from wheat straw, where there was no decrease in lignin content and only a slight change in the percentage of cellulose and hemicellulose (Maas, 2008). In this study, there was a significant loss of rice straw mass because the heat process resulted in the degradation of macromolecular components into smaller or water-soluble components. The yield of this pretreatment process was 61.11%.

Separate Hydrolysis and Fermentation Process - Hydrolysis by *T. reesei* PK1J2

Hydrolysis of straw using *T. reesei* PK1J2 showed that high reducing sugar resulted in straw with pretreatment compared to without pretreatment (Figure 1). This was because the lignin content in straw with pretreatment was reduced, as a result, enzymes accessed lignocellulose faster and broke it down to produce reducing sugar.

The cellulase (Figure 2) and xylanase (Figure 3) enzyme activity in straw with and without pretreatment showed that the activity of both enzymes was higher in straw without pretreatment. Although the activity was high, the reducing sugar content in the sample without pretreatment was low (Figure 1). This pretreatment left inhibitory compounds that could affect the work of the enzyme, such as the remaining $\text{Ca}(\text{OH})_2$ used in the pretreatment process, the remaining H_2SO_4 used to neutralize straw after pretreatment, or the presence of furfural, 5-hydroxymethyl furfural, and syringaldehyde compounds as by-products of hemicellulose and lignin

hydrolysis during the pretreatment process.

Although the produced reducing sugar varied (Figure 1), same as cellulase (Figure 2) and xylanase (Figure 3) enzyme activity, the treatment with and without $\text{Ca}(\text{OH})_2$ did not affect the high growth rate of *T. reesei* PK1J2, as showed by the dry weight loss of straw during hydrolysis (Figure 4).

With the growth of *T. reesei* PK1J2, there was a change in the percentage of cellulose, hemicellulose, and lignin (Figure 5). After 1 day of hydrolysis, the hemicellulose content in straw without pretreatment decreased by 17.02%. Meanwhile, in straw with pretreatment, the hemicellulose content decreased by 22.78% after 1 day of hydrolysis and decreased again by 11.34% after 2 days of hydrolysis. At the same time, when viewed from the xylanase enzyme activity in straw without pretreatment, it was higher than in those with pretreatment. This was due to the presence of other enzymes that hydrolyzed hemicellulose, such as β -xylosidase and α -L-arabinofuranosidase (Gyalai-

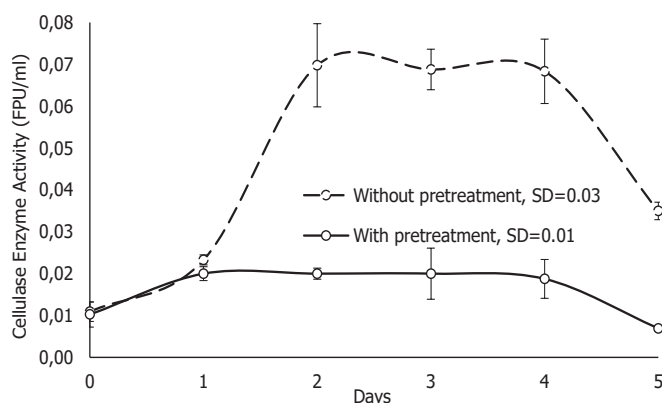


Figure 2. Graph of cellulase enzyme activity during hydrolysis process

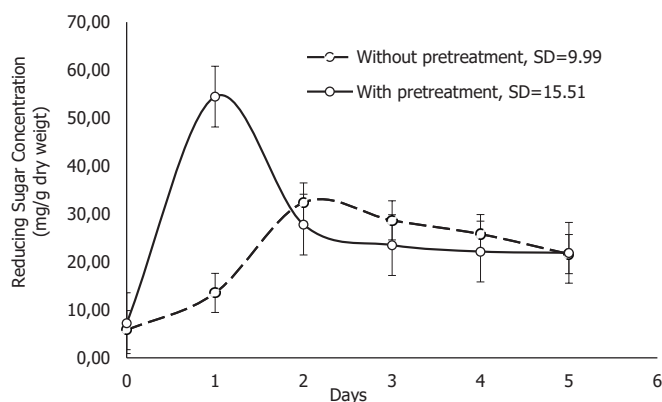


Figure 1. Graph of reducing sugar content during hydrolysis process

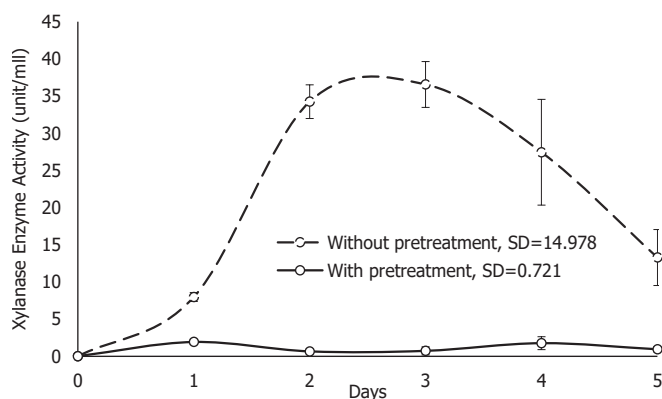


Figure 3. Graph of xylanase enzyme activity during hydrolysis process

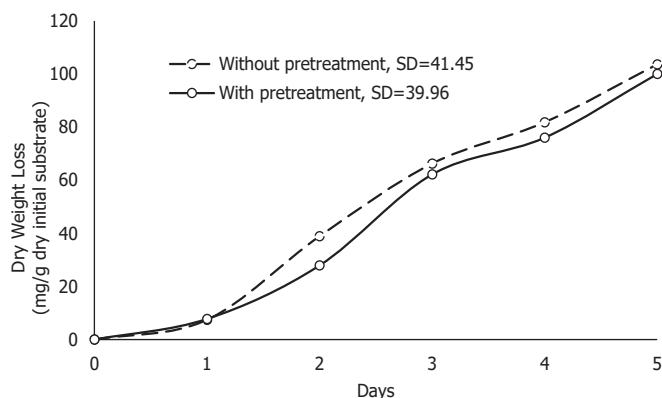


Figure 4. Dry weight loss of straw during hydrolysis process

Korpos, 2012). Without pretreatment, the percentage of lignin components also decreased by 45.32% and 5.59% after 1 and 2 days of hydrolysis respectively. The percentage of straw with pretreatment decreased by 2.45% after 1 day and decreased again by 8.69% after 2 days of hydrolysis. This decrease in percentage was relatively large, specifically in straw without pretreatment. When viewed from the high decrease in the percentage of lignin in straw without pretreatment, *T. reesei PK1J2* had an enzyme that could hydrolyze lignin.

The percentage of cellulose components in straw without pretreatment increased by 78.21% after 1

day and decreased slightly by 11.38% after 2 days of hydrolysis. This increase was due to the decrease in lignin and hemicellulose, which was supported by the low cellulase activity (Figure 2) causing it not to be converted into reducing sugar. After 2 days of hydrolysis, the percentage of cellulose decreased along with the increase in cellulase activity produced by *T. reesei* PK1J2 in straw without pretreatment (Figure 2). The percentage of cellulose components in straw with pretreatment increased after 1 and 2 days of hydrolysis, namely 2.61% and 6.90%. This increase was due to the decrease in the percentage of lignin and hemicellulose, which was quite high due to hydrolysis. To ensure the availability of reducing sugar to be used in fermentation process, hydrolysis process was stopped when the reducing sugar content was highest. This was on 2 days of hydrolysis process for straw without pretreatment and on 1 day of hydrolysis process for straw with pretreatment.

Separate Hydrolysis and Fermentation Process - Lactic acid fermentation using *R. oryzae* AT3

Before fermentation process, straw hydrolysis results were sterilized to stop the activity of *T. reesei*. However, sterilization could decrease reducing sugar (Figure 6). Before sterilization, the reducing sugar content from straw hydrolysis without and with pretreatment was 32.39 and 54.46 mg/g dry sample. After sterilization, the reducing sugar content decreased

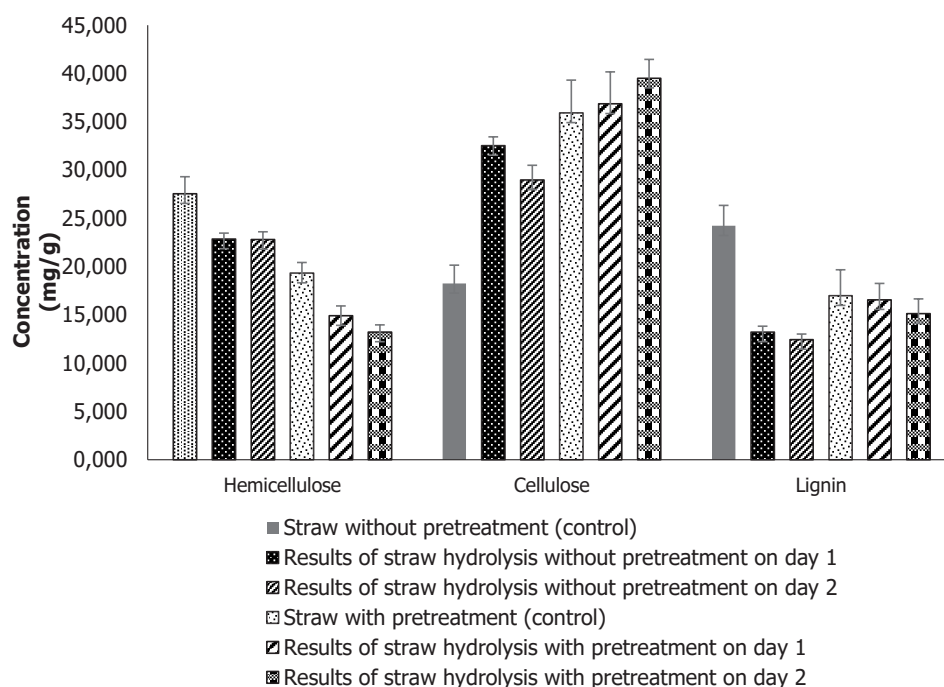


Figure 5. Diagram of changes in the percentage of cellulose, hemicellulose, and lignin after one and two days of hydrolysis

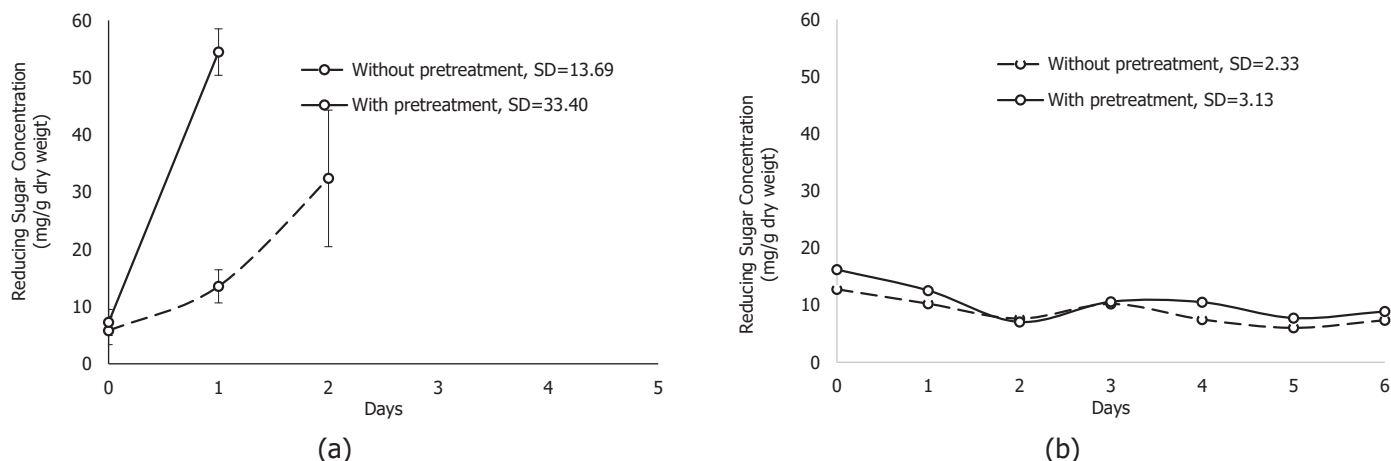


Figure 6. Reducing sugar content in (a) hydrolysis and (b) fermentation processes

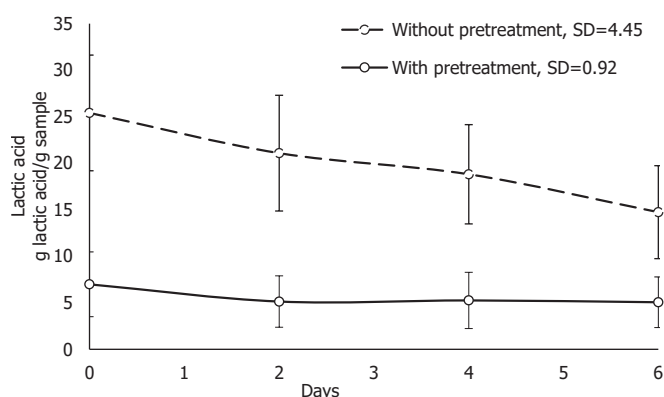


Figure 7. Lactic acid content during fermentation process

to 12.78 and 16.22 mg/g dry sample. This decrease was caused by the Maillard reaction during the sterilization process due to the influence of heat and the availability of amino acids from *T. reesei* PK1J2.

During fermentation process, reducing sugar tended to decrease. However, the rate of reducing sugar decrease was not significant and until the 6th day, the reducing sugar was not completely consumed. This showed that *R. oryzae* AT3 could not grow well because the process conditions did not support its growth. The relative humidity (RH) for *Rhizopus* to grow well was 95 to 97% (Han, 2003), although this RH could not be achieved.

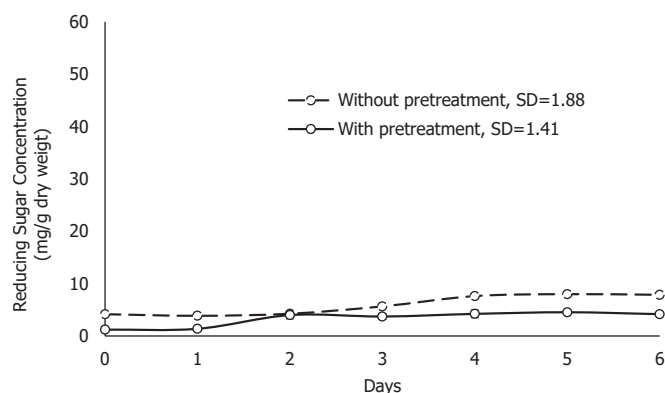
At the beginning of fermentation process, there was already lactic acid in rice straw (Figure 7) of 25.41 g lactic acid/g sample for straw without pretreatment and 6.98 g lactic acid/g sample for those with pretreatment. This was because fermentation in this study was solid substrate fermentation which was non-aseptic fermentation, as a result, other microflora potentially grew and produced lactic acid.

The result of this study showed that lactic acid decreased, and at the end of fermentation process, its content was 14.74 g lactic acid/g sample in straw without pretreatment and 5.06 g lactic acid/g sample in straw with pretreatment. This was likely due to conditions that did not support the metabolism of *R. oryzae* AT3 to produce lactic acid, partly because the RH in this study did not reach 95 to 97%.

Simultaneous Hydrolysis and Fermentation Process

Sugar reduction increased slightly in simultaneous hydrolysis and fermentation process, as shown in Figure 8 (0.62 and 0.49 mg/g dry sample/day in straw without and with pretreatment). The increase in reduced sugar was due to its production by *T. reesei* PK1J2 being directly consumed by *R. oryzae* AT3 for growth.

In simultaneous hydrolysis and fermentation process, the biomass growth of *T. reesei* PK1J2 and

Figure 8. Reducing sugar produced during hydrolysis by *T. reesei* PK1J2 and fermentation by *R. oryzae* AT3

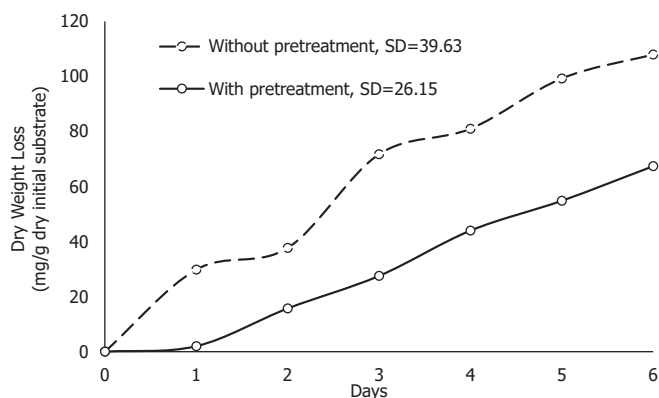


Figure 9. Dry weight loss during hydrolysis and fermentation by *T. reesei* PK1J2 and *R. oryzae* AT3

R. oryzae AT3 on straw without pretreatment was greater than with pretreatment (Figure 9). The cause was similar to what occurred during hydrolysis process in separate hydrolysis and fermentation process. In this study, the growth of *R. oryzae* AT3 was more dominant and higher than *T. reesei* PK1J2.

At the beginning of simultaneous hydrolysis and fermentation process, there was already lactic acid in the substrate with a content of 18.59 g lactic acid/g sample in straw without pretreatment and 5.05 g lactic acid/g sample in straw with pretreatment (Figure 10). This was because other microflora potentially grew and produced lactic acid in simultaneous hydrolysis and fermentation process.

In simultaneous hydrolysis and fermentation process of straw without pretreatment, lactic acid initially decreased. However, after 4 days, there was an increase in lactic acid content, which did not reach a peak until 6 days of hydrolysis and fermentation. An increase in lactic acid production could still be observed on the following day. At the end of the process, lactic acid content in straw without pretreatment was 16.76 g lactic acid/g sample. Meanwhile, in straw with pretreatment, lactic acid content immediately increased. This showed that at the beginning of the process, *T. reesei* PK1J2 and *R. oryzae* AT3 easily accessed the substrate in straw with pretreatment (already modified) and directly converted it into fermented products. After 4 days of hydrolysis and fermentation, there was no increase in lactic acid content and even tended to decrease showing a 6.28 g lactic acid/g sample.

CONCLUSION

In conclusion, pretreatment could reduce lignin, but this process was unnecessary because *T. reesei*

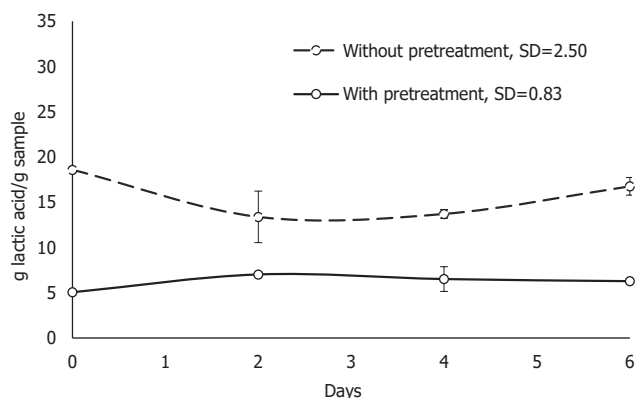


Figure 10. Lactic acid produced by *T. reesei* PK1J2 and *R. oryzae* AT3 during hydrolysis and fermentation

PK1J2 could degrade lignin in hydrolysis process. Fermentation process conditions in this study showed no support for *R. oryzae* AT3 in producing lactic acid. According to biomass formation, simultaneous hydrolysis and fermentation system (108 mg/g dry initial substrate) was superior to separate hydrolysis and fermentation system (104 mg/g dry initial substrate).

ACKNOWLEDGMENTS

The authors were grateful to the Ministry of Research, Technology, and Higher Education Indonesia for their funding through the Higher Education Excellence Study Program for Fiscal Year 2016 with the number 788/UN1-P.III/LT/DIT-LIT/2016 and to all parties who have assisted in the implementation of this study.

CONFLICT OF INTEREST

The authors declared no conflict of interest with any party in this study.

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