

## Research Article

# The Effects of Microwave-Assisted Pretreatment and Cofermentation on Bioethanol Production from Elephant Grass

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The process of acid hydrolysis using conventional methods at high concentrations results in products having lower yields, and it needs a longer time of process; therefore, it becomes less effective. In this study, we analyzed the effects of microwave-assisted pretreatment and cofermentation on bioethanol production from elephant grass (*Pennisetum purpureum*). We used a combination of delignification techniques and acid hydrolysis by employing a microwave-assisted pretreatment method on elephant grass (*Pennisetum purpureum*) as a lignocellulosic material. This was followed by cofermentation with *Saccharomyces cerevisiae* ITB-R89 and *Pichia stipitis* ITB-R58 to produce bioethanol. The optimal sugar mixtures (fructose and xylose) of the hydrolysis product were subsequently converted into bioethanol by cofermentation with *S. cerevisiae* ITB-R89 and *P. stipitis* ITB-R58, carried out with varying concentrations of inoculum for 5 days (48 h) at 30°C and pH 4.5. The high-power liquid chromatographic analysis revealed that the optimal inoculum concentration capable of converting 76.15% of the sugar mixture substrate (glucose and xylose) to 10.79 g/L (34.74% yield) of bioethanol was 10% (v/v). The optimal rate of ethanol production was 0.45 g/L/d, corresponding to a fermentation efficiency of 69.48%.

## 1. Introduction

In Indonesia, the national consumption of fossil fuels has been increasing annually. This increase is attributed to population growth, transportation use increase, and industrial activity upsurge. In addition, high fuel requirements are exacerbated by a decline in oil production capacity. Therefore, there is an immediate need to switch from using fossil fuels to biofuels [1].

Importantly, waste or nonfood materials with high biomass content, such as straw, grass, wood, and empty fruit bunches of palm trees, can be converted to bioethanol without affecting food supplies. One such nonfood material with a high biomass content is elephant grass (*Pennisetum purpureum*), which is abundantly found in tropical countries

such as Indonesia. Biomass of elephant grass is mostly in the form of cellulose (31.5%), hemicellulose (34.3%), and lignin (34.2%) [2]. The high content of cellulose and hemicellulose in elephant grass makes it an excellent source as feedstock for bioethanol production.

Typically, cellulose and hemicellulose from elephant grass can be obtained within 1–2 h by delignification process involving incubation in an alkaline solution at high temperatures (above 120°C) [3]. Next, the obtained cellulose and hemicellulose are converted to simple carbohydrates (D-xylose, D-glucose, D-mannose, D-galactose, and L-mannose) by acid hydrolysis, in which the  $\beta$ -(1,4)-glycosidic bond is broken by a strong acid, such as H<sub>2</sub>SO<sub>4</sub> or HCl. However, this process of delignification and hydrolysis is not without limitations. When the alkaline delignification process occurs

over a long duration, it may cause conversion of other lignocellulose components into secondary compounds such as xylitol, succinic acid, lactic acid, furfural, hydroxymethylfurfural, acetic acid, and glycerol, all of which can act as inhibitors in the next phase of hydrolysis [4]. In addition, the process of acid hydrolysis using conventional methods at high concentrations results in products having lower yields within a long period of time; therefore, the process becomes less effective. Furthermore, the residual acid waste can have a negative impact on the environment.

To overcome the aforementioned inefficiencies and environmental concerns of using conventional methods, a novel alternative method is to use a microwave oven to delignify and hydrolyze lignocellulose. In this process, the concentration of the base solution used is lower than that used in the conventional (reflux) method, with lower temperature conditions and shorter reaction times. Wang et al. demonstrated that the use of the microwave-assisted delignification process (base-catalyzed process) was more effective in increasing the release of lignin from biomass than conventional methods. They were also able to decompose up to 90% lignin in biomass samples by using an ionic liquid (1-ethyl-3-methylimidazolium acetate) as a solvent, which was more effective than the conventional delignification process, which released only 70%–80% lignin from lignocellulose components following refluxing the biomass for 16 h [4].

The microwave-assisted method has also been applied in the acid hydrolysis process of cellulose and hemicellulose, in which the rate of hydrolysis of starch to glucose increased 50–100 times as compared to the conventional heating method [5]. Because the concentration of acid used is also lower, this process requires shorter production time and is more environmental friendly and economical.

Fermentation processes by *Saccharomyces cerevisiae* and *Zymomonas mobilis* to produce ethanol typically use only glucose, not pentose sugars, as raw material, because either yeast cannot metabolize pentose sugars. However, some yeasts such as *Pichia stipitis* metabolize not only glucose but also other pentose sugars, such as xylose, to produce ethanol. A limitation of *P. stipitis*, however, is that its level of tolerance to ethanol is low, making cofermentation methods crucial. Cofermentation is a process of coculture, usually, *S. cerevisiae* with *P. stipitis*, wherein hexose and pentose sugars are gradually metabolized to produce ethanol [6]. In this study, we analyzed the effects of microwave-assisted pretreatment and cofermentation on bioethanol production from elephant grass.

## 2. Methods

**2.1. Chemicals.** DNS reagents ( $\text{Na}_{211}\text{S}_2\text{O}_3$ , NaOH, 3,5-dinitrosalicylic acid, and sodium-potassium tartrate) and glucose, xylose,  $\text{H}_2\text{SO}_4$ , NaOH, and HCl were purchased from

Merck. All the chemicals used were of analytic grade and were used without further purification. All aqueous solutions were prepared and dissolved in distilled water.

**2.2. Strains and Culture Media.** Bioethanol was produced from elephant grass by delignification and dilute acid hydrolysis using a microwave-assisted pretreatment method, followed by cofermentation using two strains of yeasts. *P. stipitis* ITB-R58 and *S. cerevisiae* ITB-R89 strains were obtained from the Microbiology Laboratory, Chemical Engineering Department, Institut Teknologi Bandung. Agar plates (10.0 g/L yeast extract, 20 g/L peptone, 20.0 g/L glucose, and 25.0 g/L bacterial agar) and an inoculum medium (5.0 g/L yeast extract, 5.0 g/L peptone, with 20.0 g/L mixtures of xylose and glucose as a carbon source) were used for maintaining the cultures. The fermentation medium (5.0 g/L yeast extract, 5.0 g/L peptone, 5.0 g/L  $\text{KH}_2\text{PO}_4$ , 0.4 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g/L  $\text{NH}_4\text{SO}_4$ , 18.71 g/L glucose hydrolysate, and 13.61 g/L xylose hydrolysate) was used to produce bioethanol.

**2.3. Cultivation Conditions.** The growth curves of *P. stipitis* ITB-R58 and *S. cerevisiae* ITB-R89 strains were determined according to the method of Arnata [7]. A single colony of each species was grown on a starter medium (0.5% (w/v) peptone, 1.0% (w/v) yeast extract, and 2% (w/v) glucose and xylose), followed by incubation at 37°C with vigorous shaking (150 rpm) overnight. Before the growth curve experiment, 0.1% of the culture was transferred to a production medium and incubated under the same conditions. The growth of both yeast cultures was monitored every day by measuring their optical density (OD) at 600 nm.

**2.4. Sample Preparation, Delignification, and Dilute Acid Hydrolysis of Elephant Grass Using a Microwave-Assisted Pretreatment Method.** Initially, the lignocellulose content of elephant grass was determined according to Surajit's method [8]. An amount of 1 gram of sample in 150 mL  $\text{H}_2\text{O}$  was refluxed for 2 hours at 100°C. The residual sample that had been dried was refluxed for 2 hours with 150 mL 0.5 M  $\text{H}_2\text{SO}_4$  at 100°C. The residue of the sample that has been dried was immersed in 10 mL 72% (v/v)  $\text{H}_2\text{SO}_4$  solution at room temperature for 4 hours, then diluted to 0.5 M  $\text{H}_2\text{SO}_4$ , and subsequently refluxed at 100°C for 2 hours, then dried. The residue was filtered and washed with demineralized water to neutral. The residue was then dried in an oven at a temperature of 105°C until the weight was constant and subsequently weighed (weight *d*). Afterward, the residue was fumed to ash and weighed (weight *e*). The composition of the lignocellulosic

components of the elephant grass sample was determined using the following calculation [8]:

$$\begin{aligned} \text{hemicellulose}(\%): & \frac{b-c}{a} \times 100\%, \\ \text{cellulose}(\%): & \frac{c-d}{a} \times 100\%, \\ \text{lignin}(\%): & \frac{d-e}{a} \times 100\%, \\ \text{ash content}: & \frac{e}{a} \times 100\%, \end{aligned} \quad (1)$$

with  $a$  is the initial weight of the dried powder of elephant grass samples;  $b$  is the weight of the dried sample residue after refluxed with hot water;  $c$  is the weight of sample residues after refluxed with 0.5 M H<sub>2</sub>SO<sub>4</sub>;  $d$  is the weight of sample residues after being treated with 72% (v/v) H<sub>2</sub>SO<sub>4</sub> solution; and  $e$  is the weight of ash from sample residues.

Elephant grass was washed and dried overnight before cutting them into 1 cm long pieces. Subsequently, the grass was blended and dried in an oven for 4 h at 60°C. The grass powder was then sieved to obtain a 100-mesh powder. The powder was characterized using a scanning electron microscope (SEM) (JSM-6510LA).

The delignification method was performed according to that proposed by Chen et al., with slight modifications [5], particularly in this study using the microwave-assisted delignification process. A total of 20 g of the sieved powder was placed in a 500 mL glass beaker and suspended in 300 mL of a 2% (w/v) NaOH solution. The suspension was placed in a microwave reactor SINEO MAS II, followed by heating under a 400 W microwave irradiation power for 30 min at 90°C. The heated mixtures were subsequently filtered and washed with hot water and neutralized with an acid. The resulting powder was dried, and the surface morphology of the powder was analyzed using an SEM.

Subsequently, the elephant grass powder from the delignification process ( $\pm 10$  g) was hydrolyzed by H<sub>2</sub>SO<sub>4</sub> solution (250 mL) and irradiated in the SINEO MAS II microwave reactor at an irradiation power of 400 W at 95°C [9]. The optimization conditions of the microwave-assisted hydrolysis of delignified elephant grass powder in this study were performed as shown in Table 1.

The hydrolyzed product was filtered and subsequently neutralized. The solid product was further characterized by scanning electron microscopy for surface topology. The reducing sugar content of the solution was measured with a DNS method using a Biochrom Libra S70 UV-Vis Spectrophotometer, and the xylose content was determined by high-performance liquid chromatography (HPLC) using an HP Aminex 87H column (at a flow rate of 0.6–0.8 mL/min, with a column temperature of 75°C).

**2.5. Cofermentation Process for Bioethanol Production.** Cofermentation of the sugar mixtures was performed according to the procedure of Yadav et al., with slight

modifications, using *P. stipitis* ITB-R58 and *S. cerevisiae* ITB-R89 cultures [10]. The fermentation medium was added to the hydrolysate solution, followed by autoclaving for 15 min at 120°C. The yeast cultures were transferred to the sterile fermentation medium and incubated anaerobically at 30°C. The pH of the medium was adjusted to 4.5 by adding NaOH or 2.0 M HCl solution. To evaluate the inoculum concentration on bioethanol production, cell concentrations were varied (i.e., 5.0%; 10.0%, and 20.0% (v/v)) during the cofermentation process. Residual glucose, xylose, and acetic acid were determined by HPLC [6]. The data were recorded every 5 days.

### 3. Results and Discussion

**3.1. Morphology of Elephant Grass before and after Treatment.** Elephant grass, as the lignocellulosic biomass used in this study, contains cellulose, hemicellulose, and lignin as its primary components [11]. However, its composition varies substantially, depending on the variety, climate, and soil fertility. Understanding its chemical composition is important for developing effective pretreatment methods to deconstruct its rigid structure and select the appropriate microorganisms to convert sugars into bioethanol. Hence, the structural carbohydrate contents of the grass were determined before and after the treatments both by monitoring the surface morphology of the grass and by measuring the chemical contents of the grass.

The surface morphology of the grass is shown in Figure 1. The results revealed that the surface morphology of the grass powder was compact and rigid before delignification and acid hydrolysis (Figure 1(a)). The treatment also removes acetyl and other acidic substitutions on hemicelluloses that protect cellulose. The scanning electron microscopy results revealed that the grass powder was broken after delignification (Figure 1(b)). The surface morphology of the grass powder after delignification and acid hydrolysis treatment underwent more significant damage, indicating that both treatments were successful in degrading the lignocellulosic parts of the grass powder (Figure 1(c)).

A delignification process using NaOH combined with microwave-assisted pretreatment technique helped degrade and release lignin from cellulose or hemicellulose materials. This is because lignin is not a sugar-based polymer and cannot be used as feedstock for bioethanol production via microbial fermentation as it inhibits microbial growth and fermentation process [4]. In addition, the treatment removes acetyl and other acidic substitutions on hemicelluloses that protect cellulose from attack by cellulases. The scanning electron microscopy results revealed that the grass powder was broken after delignification, as is shown in Figure 1(b). Furthermore, acid hydrolysis was required after delignification to degrade the  $\beta$ -1,4-glycosidic bond of linear glucan chains of cellulose or  $\beta$ -1,4-D-pyranosyl linkage of heterogeneous polysaccharides of hemicellulose to monomer sugars, such as glucose, xylose, galactose, arabinose, and mannose.

TABLE 1: The optimization conditions of the microwave-assisted hydrolysis of delignified elephant grass powder with acid catalyst using the SINEO MAS II microwave reactor.

Treatment	The concentration of H <sub>2</sub> SO <sub>4</sub> in % (v/v)	Irradiation power (watt)	Reaction time (minutes)	Temperature (°C)
1	0.5; 1; 2; 5; and 7	400	30	90
2	2	300; 400; 500; 600; and 700	30	90
3	2	400	5; 10; 20; 30; and 40	90
4	2	400	30	80; 90; 95; 100 and 110

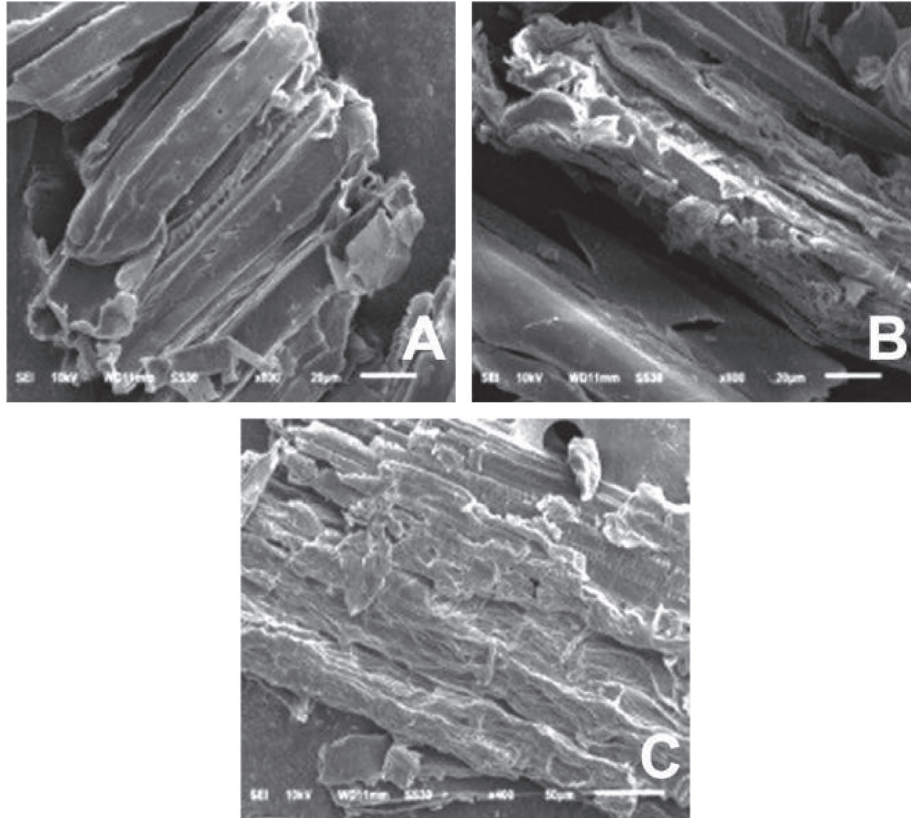


FIGURE 1: Morphology of the elephant grass powder: (a) before treatment; (b) after delignification process; and (c) after the delignification process followed by acid hydrolysis.

3.2. *Chemical Content of Grass Powder.* The results demonstrated that the cellulose content after alkaline delignification using the microwave-assisted pretreatment technique was higher (43.6%) than the usual alkaline delignification technique (37.1%) (Table 2).

Our findings demonstrated that the cellulose content after alkaline delignification using microwave-assisted pretreatment technique was higher (43.6%) than that obtained using the usual alkaline delignification technique (37.1%), as well as alkaline delignification combined with heat (31.2% as studied by Eliana et al. [3]). The high levels of cellulose content obtained in this study indicated that the alkaline delignification process with microwave-assisted pretreatment technique successfully damaged the structural linkages between lignin and carbohydrates and released the free cellulose into solution. However, the cellulose and hemicellulose contents were decreased after acid hydrolysis, as this process will also break down the glycosidic linkage of

cellulose or  $\delta$ -1,4-D-pyranosyl linkage of hemicellulose, producing its monosaccharides. Yadaf et al. reported that conventional acid hydrolysis of 100.0 g of elephant grass yielded 48.06 g of cellulose [10]. Meanwhile, in our study, 100.0 g of elephant grass resulted in 52.6 g of cellulose. Björling and Lindman used the combination of electron beam irradiation and alkaline delignification for pretreatment of corn stalks [12]. They reported that the glucose yield was 20% in untreated samples compared to 43% in samples treated with electron beam irradiation and NaOH delignification. Jeffries and Jin also reported that the hydrolysis rate of starch to glucose increased 100 times by using microwave irradiation [13].

All of the above results suggest that the microwave-assisted pretreatment techniques combined with alkaline delignification and acid hydrolysis methods are capable of degrading cellulose effectively and efficiently with a shorter reaction time, as well as substantially reducing the lignin

TABLE 2: The lignocellulose content of the elephant grass powder<sup>a</sup>.

Process	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
No treatment	29.9	20.2	21.1	7.2
After alkaline delignification	37.1	19.5	24.3	17.4
After alkaline delignification + microwave-assisted pretreatment techniques	43.6	21.1	10.8	10.7
After alkaline delignification and acid hydrolysis (both combined with microwave-assisted pretreatment techniques)	12.8	12.4	5.5	24.1

<sup>a</sup>Analyzed according to Surajit's method [8].

content and the side products, compared to conventional methods [5].

Fermentation of lignocellulosic hydrolysates was more difficult than the well-established process of bioethanol production (e.g., from sugar-cane juice), because the hydrolysates contain a broader range of inhibitory compounds. Inhibitor composition and concentration depend on not only the type of lignocellulosic materials but also the chemistry and nature of the pretreatment and hydrolysis processes [14]. Moreover, the hydrolysates of hemicelluloses contain both hexoses and pentoses, whereas xylose usually is the dominant sugar in the hydrolysates from hardwood hemicelluloses [15]. Therefore, the fermenting microorganisms that were used in our experiment should be able to produce bioethanol from the hydrolysates (including pentoses) with a high yield and productivity as well as be safe for humans.

### 3.3. The Concentration of the Reducing Sugar after Delignification Process

**3.3.1 Hydrolysis of Elephant Grass Powder without Microwave Assistance.** The effect of time-period on alkaline delignification and the optimal sulfuric acid concentration for acid hydrolysis was evaluated using the DNS method to determine the concentration of reducing sugars produced from the aforementioned treatments. All the experiments were conducted without microwave-assisted pretreatment. Four conditions for each treatment were tested: 1.5, 2.0, 2.5, and 3.0 h for delignification time, as well as 0.5%, 2.0%, 5.0%, and 7.0% (v/v) for sulfuric acid concentration. The results are presented in Figure 2, which are based on two time experiments represented as the mean concentration of reducing sugar (in g/L) produced (Table 3).

It was observed that 2.5 h for the delignification process and 7.0% (v/v) of sulfuric acid concentration were the best conditions for producing 9.41 g/L of the reducing sugar from elephant grass powder. However, this result is still lower than the previous result by Eliana et al., who conducted the usual delignification method and enzymatic hydrolysis of the cellulose fraction of the elephant grass and produced 34.4 g/L of the reducing sugar [3]. Therefore, in this study, the delignification process of the elephant grass powder sample was modified by following the acid hydrolysis using microwave irradiation heating.

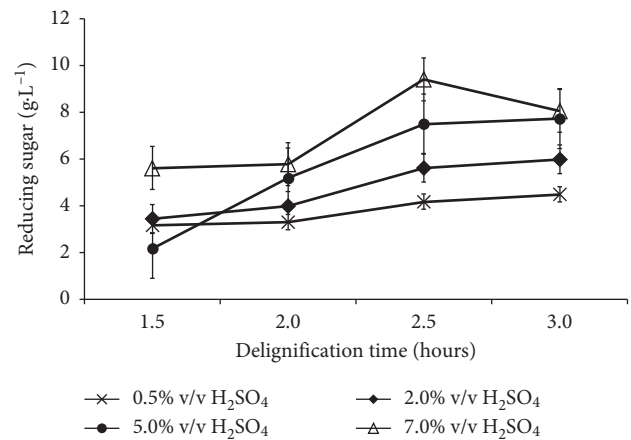


FIGURE 2: The concentration of the reducing sugar (g/L) produced from alkaline delignification of elephant grass powder followed by acid hydrolysis at various concentrations of sulfuric acid.

**3.3.2 Hydrolysis of Elephant Grass Powder through Microwave Assistance.** In this study, to obtain higher yields of glucose/reducing sugar, we modified the pretreatment process by including the microwave-assisted pretreatment technique, which used microwave irradiation to increase the reaction efficiency. Results of the delignification and hydrolysis of the elephant grass powder by using microwave irradiation heating are presented in Table 4. The power of microwave irradiation seemed to affect the amount of reducing sugars significantly. The results revealed that the concentration of reducing sugars was decreased with the increase of temperature and the microwave irradiation power (Table 4).

Based on the results of hydrolysis in Table 2 shows the increased concentration of sulfuric acid used in the hydrolysis process, the resulting reducing sugar concentration increased. However, it decreased at 5% (v/v) sulfuric acid concentration, 40 minutes of hydrolysis time, the temperature of 95°C, and irradiation power of 500 W. This condition was due to some products being converted into secondary compounds such as furfural and hydroxymethylfurfural (HMF) [16]. The most optimal condition for the acid hydrolysis of elephant grass combined with microwave-assisted pretreatment technique was at 2% (v/v) sulfuric acid concentration with 400 W irradiation power for 30 min at 95°C, which produced 26.63 g/L of the reducing sugar, corresponding to a hydrolytic efficiency of 66.57%. This result is higher than the delignification treatment

TABLE 3: The effect of delignification time and H<sub>2</sub>SO<sub>4</sub> concentration on the average glucose concentration produced without microwave-assisted pretreatment.

Concentration of H <sub>2</sub> SO <sub>4</sub> (in % (v/v))	Delignification time (hours)				The average reducing sugar (g·L <sup>-1</sup> )	Standard deviation
	1.5	2.0	2.5	3.0		
0.5	3.15	3.31	4.17	4.49	3.78	0.65
2.0	3.45	3.99	5.65	5.99	4.77	1.24
5.0	2.15	5.17	7.49	7.73	5.63	2.59
7.0	5.61	5.79	9.41	8.05	7.21	1.84

TABLE 4: The reducing sugar data produced from the delignification process of elephant grass powder followed by acid hydrolysis using microwave irradiation heating.

H <sub>2</sub> SO <sub>4</sub> (% v/v)	RS (g/L)	IP (W)	RS (g/L)	RT (min)	RS (g/L)	Temp (°C)	RS (g/L)
0.5	16.33	300	14.11	5	5.61	80	8.39
1	18.15	400	25.10	10	13.99	90	25.11
2	25.11	500	19.07	20	18.65	95	26.63
5	26.05	600	16.32	30	25.11	100	18.61
7	23.07	700	14.50	40	19.63	110	16.03

Abbreviations: H<sub>2</sub>SO<sub>4</sub>, sulfuric acid; RS, reducing sugar; IP, irradiation power; RT, reaction time; Temp, temperature. Variation in H<sub>2</sub>SO<sub>4</sub> concentrations of each parameter was carried out without repetition.

without a microwave, which is only 9.41 g/L with hydrolysis efficiency of 23.54% [3]. The reducing sugar concentration was increased by 45.05% compared to the one without microwave-assisted pretreatment. This optimal condition was also more effective than that in the previous work of Chen et al., who used 0.5% (v/v) sulfuric acid concentration with 900 W microwave irradiation power for 60 min at 121°C, producing 11.9 g/L of the reducing sugar [5].

Our findings demonstrated that the cellulose content after alkaline delignification using microwave-assisted pretreatment technique was higher (43.6%) than that obtained using the usual alkaline delignification technique (37.1%), as well as alkaline delignification combined with heat (31.2% as studied by Eliana et al. [3]). The high levels of cellulose content obtained in this study indicated that the alkaline delignification process with microwave-assisted pretreatment technique successfully damaged the structural linkages between lignin and carbohydrates and released the free cellulose into solution. However, the cellulose and hemicellulose contents were decreased after acid hydrolysis, as this process will also break down the glycosidic linkage of cellulose or  $\delta$ -1,4-D-pyranosyl linkage of hemicellulose, producing its monosaccharides. Yadav et al. reported that conventional acid hydrolysis of 100.0 g of elephant grass yielded 48.06 g of cellulose [10]. Meanwhile, in our study, 100.0 g of elephant grass resulted in 52.6 g of cellulose. Björling and Lindman used the combination of electron beam irradiation and alkaline delignification for pretreatment of corn stalks [12]. They reported that the glucose yield was 20% in untreated samples compared to 43% in samples treated with electron beam irradiation and NaOH delignification. Jeffries and Jin also reported that the hydrolysis rate of starch to glucose increased 100 times by using microwave irradiation [13]. All of the above results suggest that the microwave-assisted pretreatment techniques combined with alkaline delignification and acid hydrolysis methods are capable of degrading cellulose effectively and efficiently with

shorter reaction time, as well as substantially reducing the lignin content and the side products, compared to conventional methods [5].

**3.4. Growth of Cultures.** To determine the inoculation time of yeast, the cultivation of yeast cells was evaluated every day (Figure 3). The cell cultures were monitored, and the data were recorded daily for changes in cell numbers by determining the OD of each inoculum. *S. cerevisiae* required 12 h to adapt to the medium, to achieve an optimum OD of 0.706 at 48 h. After hour 60, *S. cerevisiae* entered the dead phase. Meanwhile, the cultivation of *P. stipitis* culture resulted in a slower increase in cell density. The exponential growth of *P. stipitis* started at hour 24 and lasted until hour 66, with a maximum OD of 0.682 at hour 60. The cells then entered into the dead phase at hour 84. These results suggest that the *P. stipitis* was inserted 12 h faster than *S. cerevisiae* into the elephant grass hydrolysates. Growth curve measurements were performed once without repeating every hour for 4 days.

**3.4.1 Effect of Inoculum Concentration.** To determine the effect of inoculum concentration on bioethanol production, serial experiments by varying the inoculum concentration of each yeast (i.e., 5 %, 10%, and 20% (v/v)) were conducted during the cofermentation process. The results of HPLC analysis of ethanol and acetic acid contents in products after fermentation were presented in the following figures.

The presence of ethanol and acetic acid in products after fermentation was confirmed by comparing the retention time of ethanol and acetic acid to the standards, as shown in Figures 4 and 5, and ethanol and acetic acid concentrations were calculated using the chromatogram data of HPLC analysis. Subsequent peak area data of each ethanol and acetic acid were inputted into each ethanol and acetic acid standard regression equation derived from the linear plot

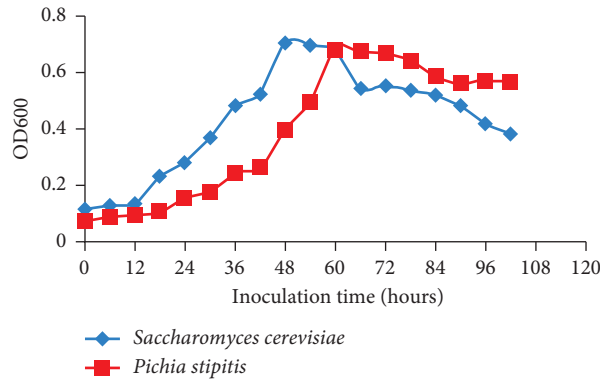


FIGURE 3: The growth curves of *Saccharomyces cerevisiae* and *Pichia stipitis*.

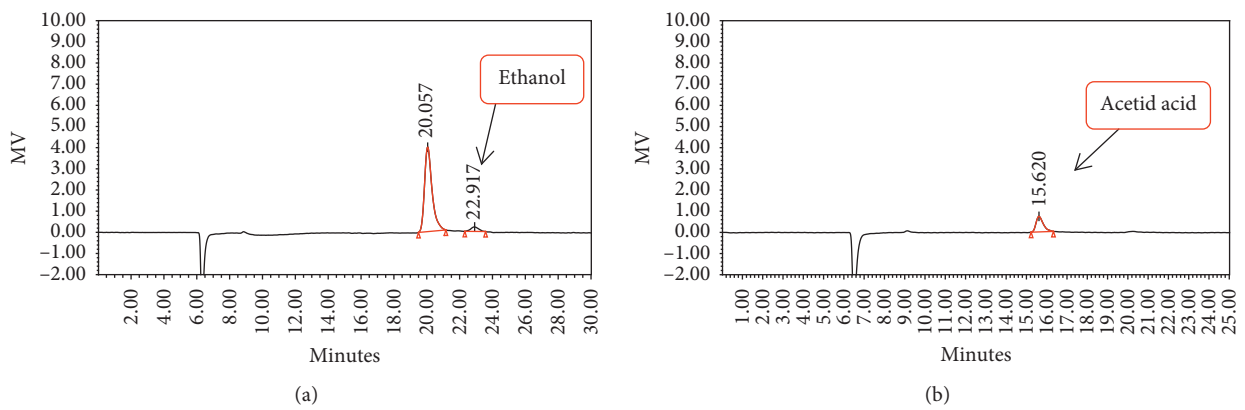


FIGURE 4: Chromatograms of ethanol and acetic acid standards.

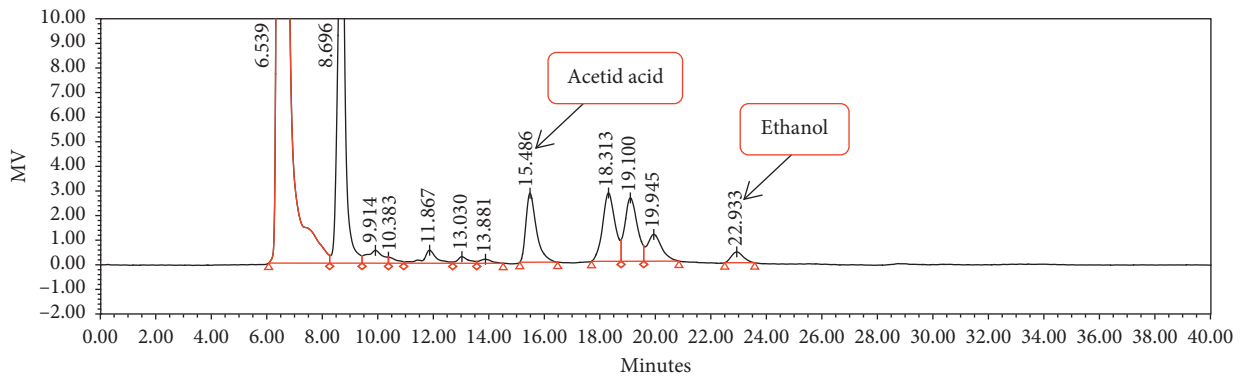


FIGURE 5: Chromatograms of ethanol and acetic acid contents in products after fermentation.

between peak area in chromatogram and concentration of ethanol and acetic acid, as presented in Figures 6 and 7, respectively, to get the ethanol and acetic concentration. The peak area of ethanol on the HPLC analysis

chromatogram of the product after fermentation was subsequently inputted into the ethanol standard regression equation as the variable (y) to get the concentration (x), as follows:

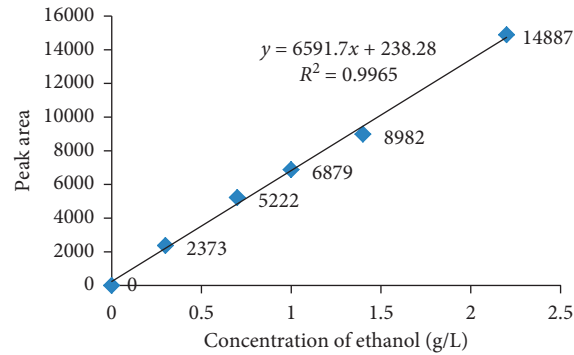


FIGURE 6: The linear plot between peak area in chromatogram and concentration of ethanol.

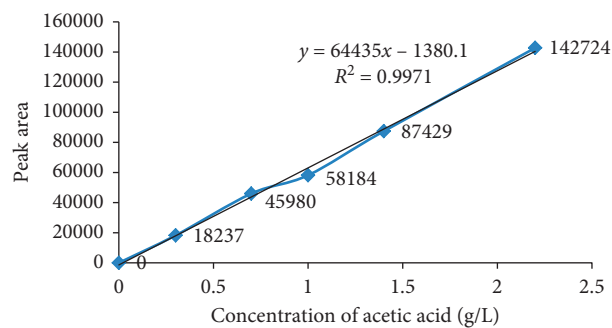


FIGURE 7: The linear plot between peak area in chromatogram and concentration of acetic acid.

$$\text{ethanol concentration (x)} = \frac{y - b}{a}, \quad (2)$$

$$\text{ethanol concentration (g/L)} = \text{ethanol concentration (\%)} \times \rho \times 10 \text{ (correction factor).}$$

The similar calculation was performed to get the concentration of acetic acid.

The initial concentration of glucose and xylose for 5%, 10%, and 20% (v/v) of inoculum treatments was 16.67 and 6.04 g/L; 17.43 and 16.31 g/L; and 18.45 and 8.45 g/L, respectively. The initial concentrations of glucose and xylose used were directly obtained from the pretreatment process of elephant grass. The OD, residual glucose and xylose concentration, and bioethanol and acetic acid accumulation were observed every day for 5 days. Both substrate concentrations decreased for all inoculum concentrations during cofermentation, as is shown in Figure 8. Measurement of the optimal concentrations of bioethanol and acetic acid revealed that the 5% inoculum concentration (Figure 6(a)) produced 5.26 g/L bioethanol and 2.24 g/L acetic acid at 48 h of cofermentation, and the concentration of both products decreased after the 72 h of cofermentation. Under these conditions, the bioethanol yield was 23%, with the highest productivity rate of 0.2 g/L per day. The fermentation efficiency reached a rate of 34.41% and substrate conversion efficiency by 56.38%. Moreover, the 10% inoculum concentration experiment resulted in an optimal bioethanol concentration at 48 h of cofermentation

(10.79 g/L, Figure 8(b)), with fermentation and conversion substrate efficiencies of 69.48% and 76.15%, respectively. The bioethanol concentration started decreasing after 72 h of cofermentation, indicating that the number of substrates was reduced and the yeasts entered into the dead phase after hour 72. Meanwhile, the 20% (v/v) inoculum concentration experiment (Figure 8(c)) revealed slightly different results for the optimal bioethanol concentration, which was 13.31 g/L at hour 96 of cofermentation. In addition, the efficiency of fermentation was 66.97%, and the substrate conversion rate reached up to 92.88%. The fermentation process is carried out once for each inoculum for 5 days.

**3.4.2 Substrates and Product Parameters Observed during Cofermentation.** In this study, the highest bioethanol concentration was 13.31 g/L, obtained on day 96 with 20% (v/v) inoculum concentration. However, in terms of the effectiveness and efficiency of the material used, the 10% (v/v) inoculum concentration demonstrated better results than other inoculum concentrations (Table 5). The ethanol yield ( $Y_{p/s}$ ) can be calculated using the following equation:



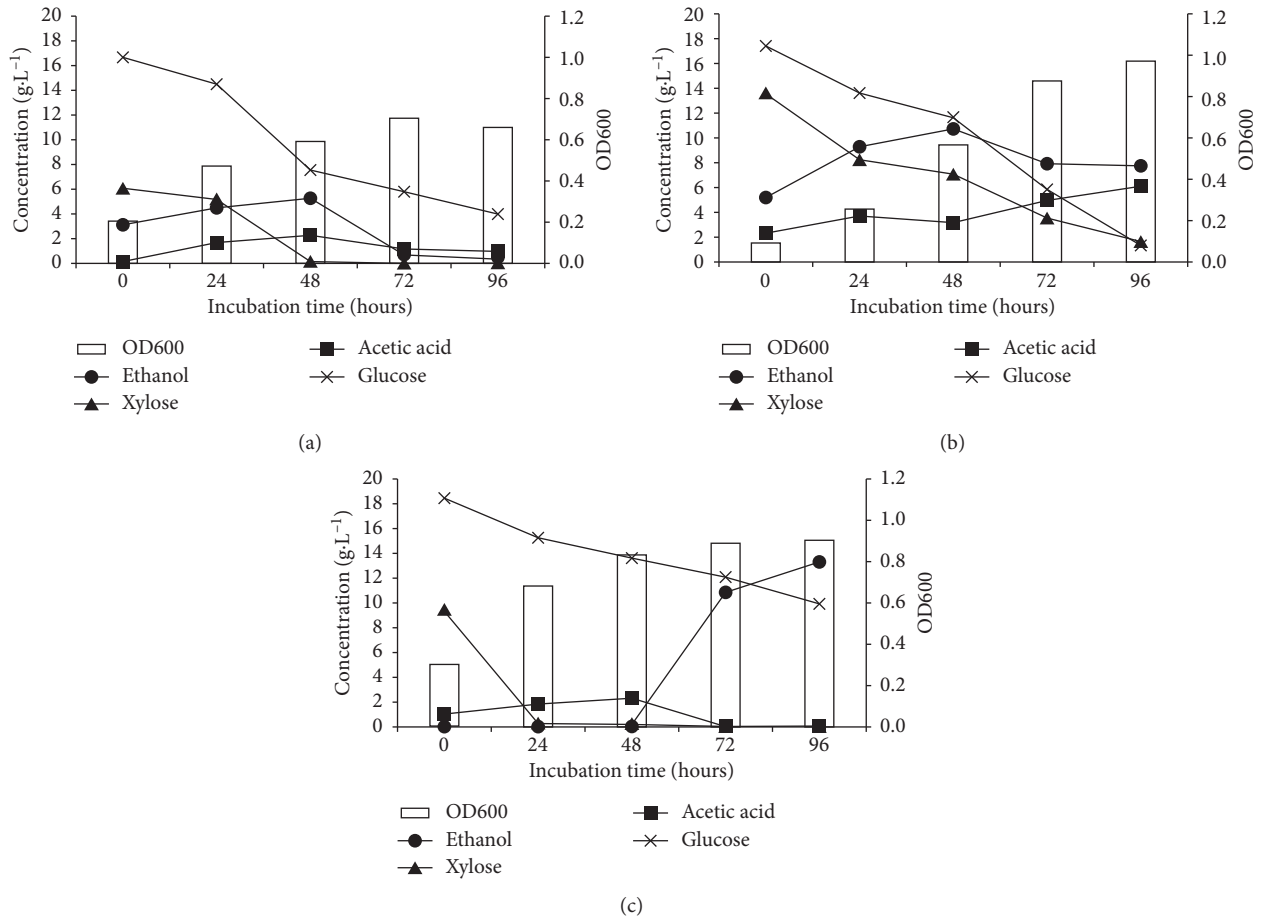


FIGURE 8: The effect of inoculum concentration: (a) 5%; (b) 10%; and (c) 20% (v/v) on bioethanol production.

TABLE 5: Substrates and product parameters observed during cofermentation.

Time (in h)	$Y_{p/s}$ (%)	$L_p$ (g/L, h)	EF (%)	Ks (%)
<i>Inoculum concentration 5% (v/v)</i>				
0	13.55	0.13	6.77	0.00
24	19.63	0.19	11.37	9.35
48	23.15	0.22	34.41	45.32
72	2.80	0.03	5.48	50.86
96	1.33	0.01	3.83	56.38
<i>Inoculum concentration 10% (v/v)</i>				
0	16.76	0.22	33.52	0.00
24	29.85	0.39	59.71	26.36
48	34.74	0.45	69.48	35.33
72	25.46	0.33	50.91	62.27
96	24.92	0.32	49.83	76.15
<i>Inoculum concentration 20% (v/v)</i>				
0	0.00	0.00	0.00	0.00
24	0.00	0.00	0.00	59.04
48	0.00	0.00	0.00	66.58
72	38.82	0.45	44.70	76.17
96	47.69	0.55	66.97	92.88

Abbreviations: Ks, total substrate conversion;  $Y_{p/s}$ , ethanol yields (theoretically = 51%);  $L_p$ , productivity rate of bioethanol per day.

$$\text{ethanol yield (\%)} = \frac{\text{ethanol concentration (g/L)}}{\text{reduction sugar concentration (g/L)}} \times 100. \quad (3)$$

Baker's yeast (*S. cerevisiae*) is the most commonly used microorganism for bioethanol production [13–17]. However, it cannot ferment xylose, as it lacks the genes for xylose reductase (XR) and xylitol dehydrogenase (XDH) [13, 18]. It was reported that the yeast can tolerate up to 12%–18% of ethanol concentration and about 5 g/L undissociated concentration of acetic acid, the most common carbocyclic acid found in the hydrolysates. For a range of temperatures from 4°C to 32°C, *S. cerevisiae* was able to ferment glucose, sucrose, and raffinose, exhibiting an optimal activity at 30–34°C, and not being active at temperatures above 40°C at pH of about 4.5. Ethanol production followed the Embden–Meyerhof–Parnas pathway (EMP) in which glucose was converted to pyruvate, and the pyruvate was converted to acetaldehyde and CO<sub>2</sub>, catalyzed by pyruvate decarboxylase. The acetaldehyde is then reduced to ethanol by the action of alcohol dehydrogenase [19]. The ethanol yields from hexose fermentation could reach up to 90%. Hence, this yeast was chosen for fermenting of the elephant grass hydrolysates.

As the OD value increased and the amount of substrate decreased, the ethanol concentration produced was also enhanced. It can be seen in Figure 8 that the optimum ethanol concentration obtained at the 48th hour is 5.26 g/L for the 5% inoculum concentration and 10.79 g/L for the 10% inoculum concentration. However, the concentration was decreased after the first hour. However, the concentration was decreased after 72 and 96 hours of fermentation process. This can be caused by the amount of substrate that has been significantly reduced and microorganisms that have begun to change into the phase of death that supported by ethanol to renew or oxidize into another product, namely, acetic acid. In contrast to the inoculum concentration of 20%, which at the beginning of the fermentation, the product also produced acetic acid, which reached up to 2.28 g/L, while ethanol was not detected. However, in the 48th hour, the concentration of acetic acid decreased, but the ethanol concentration increased. When viewed from the OD value, it can be said that the microbes are still in the stationary phase or the number of dead and living microbes were balanced; therefore, the fermentation could still be continued longer, because at the beginning of fermentation, only xylose was optimally converted while the remaining glucose levels are still high, i.e., 9.93 g/L. It was found that the optimum ethanol concentration obtained at the 96th hour was 13.31 g/L, while the concentration of acetic acid decreased. Therefore, the fermentation process can be continued with a longer time, so that more ethanol products were formed.

One of the major challenges in bioethanol production is the fermentation of xylose. Intensive efforts have been conducted to introduce organisms that can use xylose to produce ethanol. Hence, in this study, we used *P. stipitis* to convert pentose sugars such as xylose and produced ethanol by the pentose phosphate pathway. Yadav et al. reported that

the optimal temperature was 27–30°C and the pH was about 3.0–5.5 [11]. Furthermore, *P. stipitis* was able to convert xylose to ethanol anaerobically or moderately anaerobically with high yields, for example, 0.3–0.44 g of ethanol/g of xylose at 30°C [20]. Therefore, this yeast combined with *S. cerevisiae* was used as the microorganisms to ferment the hydrolysates of elephant grass to produce bioethanol.

Ethanol yield ( $Y_{p/s}$ ) in the elephant grass hydrolysate fermentation process, as shown in Table 5, is directly proportional to the rate of productivity ( $L_p$ ) and fermentation efficiency (EF). The optimum results were achieved when fermentation occurred at 48 hours, which reached up to 23.15%; however, there was a decrease at 72 hours and 96 hours. This decrease is due to the presence of other secondary products, and one of them is acetic acid, as evidenced in Figure 8, which results in a decrease in ethanol production as the concentration of acetic acid increased at 72 and 96 hours.

A previous study on bioethanol production from rice straw hydrolysates using coculture of *S. cerevisiae* and *P. stipitis* achieved bioethanol concentration of 12 g/L, with a productivity value of 0.33 g/L/d and fermentation efficiency of up to 95% [11]. Kosasih also reported that bioethanol concentration obtained from empty palm bunch hydrolysates by using *S. cerevisiae*–*P. stipitis* and *S. cerevisiae*–*Kluyveromyces marxianus* was 9.67%, with a productivity rate of 0.08 g/L/d [21]. In the present study, the highest bioethanol concentration achieved was 13.31 g/L, on day 96, by using the 20% (v/v) inoculum concentration.

#### 4. Conclusion

In this study, the biomass content after alkaline delignification using the microwave-assisted acid hydrolysis technique was 47.4% of cellulose, 24.3% of hemicellulose, 10.8% of lignin, and 10.7% of ash, at the following optimal conditions: concentration of H<sub>2</sub>SO<sub>4</sub>, 2%; temperature, 95°C; microwave irradiation power, 400 W; hydrolysis time, 30 min. The reducing sugar content was 26.63 g/L, indicating the hydrolysis efficiency of 66.57%. Sugar levels elevated by 45.05% compared to the unassisted microwave irradiation process. The optimal time for the growth of *S. cerevisiae* ITB-R89 and *P. stipitis* ITB-R58 was at hours 48 and 60, respectively. To investigate the optimal sugar mixtures of hydrolysis products (fructose and xylose), which are subsequently converted into bioethanol by cofermentation, the concentration of the inoculum was varied for 5 days (48 h) at a temperature of 30°C and pH of 4.5. The HPLC analysis revealed that 10% (v/v) inoculum concentration was the optimal concentration for converting 76.15% of sugar mixture substrate (glucose and xylose) into 10.79 g/L (34.74% yield) of bioethanol, and the optimal rate of ethanol productivity of 0.45 g/L/d corresponded to a 69.48% fermentation efficiency.

#### Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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