jurnal βeta kimia
e-ISSN: 2807-7938 (online) dan p-ISSN: 2807-7962 (print)
Volume 4, Nomor 1, Mei 2024
http://ejurnal.undana.ac.id/index.php/jbk

Bioethanol Production from Presto Pretreatment of Sorghum Stem by Simultaneous Saccharification and Cofermentation Technique and Purification Using *Ende* Natural Zeolites

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Info Artikel:
Dikirim:
15 April 2024
Revisi:
27 April 2024
Diterima:
28 April 2024
Keywords:
Bioethanol; presto retreatment; sweet sorghump; simultaneous saccharification and co-fermentation; Zeolite.

Abstract*-One form of new renewable energy source with promising prospects in dry climate areas is bioethanol produced from sweet sorghum. In this research, bioethanol was produced from sweet sorghum stem pulp, which had been treated by boiling in a pressure cooker (presto) for varying times. The fermentation technique used is Simultaneous Saccharification and Cofermentation (SSCF) using a mixed culture of Trichoderma reesei-Saccharomyces cerevisiae. Next, the bioethanol is purified using adsorbent made from Ende natural zeolite. The results showed that press-cooked sweet sorghum stalk pulp produced less ethanol than non-press-cooked sorghum pulp. The longer the pressure treatment time, the less ethanol produced. Throughout the three adsorption stages, the physically activated-Zeolite (only calcined at 600 °C for 3 h) increased the ethanol concentration from 94% to 99%. Zeolites activated with acid (HCl) can increase the ethanol concentration from 94% to 96%, while those activated with bases (NaOH 4M) can increase the ethanol concentration from 94% to 95.5%.*

INTRODUCTION

One type of new renewable energy source that can be exploited is biomass, namely organic material from plants, including special energy-producing plants, food and feed crops, agricultural plant residues and waste, wood residue and waste, aquatic plants, waste, animals, residential waste, and other waste materials. Bioethanol, a form of new and renewable energy produced from plants, is an attractive fuel because it has good combustion qualities and is environmentally friendly. Bioethanol has been widely used in the form of a mixture with gasoline (10% ethanol and 90% gasoline), even flex fuel technology has developed car engines that are capable of using ethanol and gasoline as well as a mixture of both [1]. Bioethanol is ethanol produced through the fermentation of sugar from plants or food waste containing sugar, starch, or cellulose. In recent years, bioethanol has been developed on a large scale as a result of increasing fuel prices. Governments in several countries provide subsidies and tax reductions to promote its use as fuel [2]. As a fuel, bioethanol has advantages compared to gasoline, because: 1) its combustion is more complete so that hydrocarbon and particulate emissions are lower; 2) Has a higher octane number so that it can provide more efficient and economical power [2]; 3) Does not contain methyl tertiary butyl ether (MTBE), a compound that is suspected of being a carcinogen which is commonly used in gasoline [3]; 4) Does not add greenhouse gases to the atmosphere because the CO_2 produced by burning ethanol is equivalent to the CO_2 absorbed by replanting biomass to produce bioethanol [4].

One plant that has very good prospects as a source of raw material for making bioethanol is sweet sorghum (*Sorghum bicolor L. Moench*). According to Barcelos et al. [5], sweet sorghum has several advantages compared to other plants that have been used as a new renewable energy source, namely: 1) Short growth period (3–5 months) makes this crop suitable as a complementary feedstock to sugarcane in marginal areas or in crop rotations; 2) Relatively high biomass productivity and low input requirements; 3) Sweet sorghum is a drought and heat tolerant, multi-purpose crop that can be cultivated on a wide range of soils; 4) Can be used for the production of sugar, alcohol, syrup, jaggery, fodder, fuel, bedding, roofing,fencing, paper and chewing; 5) Sweet sorghum is also known for its easy cultivation from seeds, possibility of multiple crops per season and its large breeding potential. The advantages of the sweet sorghum plant are that it is very suitable for the conditions of several regions in Indonesia such as Gunung Kidul Yogyakarta, West Nusa Teggara, and East Nusa Tenggara which have a lot of dry and less fertile land [6].

The composition of sweet sorghum stems is approximately 70% water by wet weight, soluble carbohydrates by approximately 58.2% by dry weight, and insoluble carbohydrates, namely cellulose and hemicellulose by 22.6% by dry weight [7]. Sweet sorghum stems consist of three main components, namely water, dissolved sugar, and fiber. According to the *Direktorat Jenderal Tanaman Pangan dan Hortikultura* [8], the composition of sweet sorghum stalks is 73% water, 12.98% dissolved sugar, 11.72% fiber (lignocellulose), and 2.3% others. Dissolved sugar consists of the main compounds in the form of sucrose, glucose and fructose. Lesser compounds are galactose, arabinose, silose, mannose, ribose, sorbose, and polyglucose [9]. The fiber component (insoluble carbohydrates = lignocellulose) consists of cellulose, hemicellulose, and lignin.

Based on the composition above, it appears that the fiber content (insoluble carbohydrates = lignocellulose) is quite large or does not differ much from the dissolved sugar content. This means that if this fiber component can be converted into bioethanol like dissolved sugar, the results will also be quite significant.

Ordinary fermentation technology will only convert dissolved sugars (sucrose, glucose, and fructose) into ethanol, while the insoluble carbohydrate components in the form of cellulose and hemicellulose are not converted. However, if fermentation is preceded by hydrolysis of the cellulose and/or hemicellulose components, these two components can also be converted into ethanol so that the bioethanol yield obtained will increase. The application of this technique has been carried out on sweet sorghum stem substrates by several researchers, for example Mamma et al. [10] used *Fusarium oxysporum-Saccharomyces cerevisiae* with an increased ethanol yield of 20.7% and Jasman et al. [11] used *Trichoderma reesei-Saccharomyces cerevisiae* with ethanol yield increasing by 38.5%. In this research, we will introduce initial treatment in the form of pressed-cooking using a pressed-cooker (*presto*) on sweet sorghum stem pulp before continuing with simultaneous co-fermentation. This is intended to further soften the crystalline texture of lignocellulose in the substrate so that it is easier to be hydrolyzed.

For the purposes of hydrolysis of cellulose or lignocellulosic components, several types of fungi such as *Tricoderma reesei* can be used. This type of fungus can produce cellulolytic enzymes which will catalyze the hydrolysis of lignocellulosic materials into glucose, a type of soluble sugar. Thus, to convert sweet sorghum stems into ethanol, it can be done by combining or coupling hydrolysis and fermentation through a co-fermentation technique (joint fermentation of two or more different substrates in one container) between dissolved sugar and cellulose with a mixed culture of *Tricoderma reesei-Saccharomyces cerevisiae*. In this case, *T. reesei* plays a role in hydrolyzing lignocellulose and *S. cerevisiae* converting dissolved sugars into ethanol. The combination of these two processes, hydrolysis and fermentation is known as the simultaneous saccharification and fermentation (SSF) technique. If in this case the fermentation is co-fermentation, it is called a co-fermentation technique and SSF is combined, it is called simultaneous saccharification and co-fermentation (SSCF).

The resulting bioethanol will be purified to a concentration of 99% through multilevel distillation followed by molecular filtration using natural Ende zeolite, a type of adsorbent

obtained from zeolite minerals which are widely found in the Ende region of East Nusa Tenggara. There has been a lot of research on making bioethanol from sorghum sap and purifying bioethanol using zeolite, but only this research has carried out pretreatment with a pressure cooker, fermentation using the simultaneous saccharification and co-fermentation (SSCF) technique and purification using natural Ende zeolite.

METHODS

Tools and Materials

Tools: Blender, knife, pressed-cooker (*presto*), flannel cloth, filter paper, freezer, laminar air flow, fermenter, distillator, alcoholmeter, and glassware.

Materials: Sweet sorghum stalks is obtain from air force farm in Penfui Kupang; yeast extract and peptone from Himedia (India); KH_2PO_4, $MgSO_4.7H_2O$, glucose pa., tween 80, Pb acetate, $NaHPO_4$, KI, $Na_2S_2O_3$, $CuSO_4.5H_2O$, HCl pa, arsenomolybdate, H_2SO_4 98%, Na_2CO_3, $NaHCO_3$, Na_2SO_4, and NaK tartrate are purchase from Merck.

Microorganisms: *Saccharomyces cerevisiae* is in the form of instant yeast (Fermipan, France) and *Trichoderma reesei* FNCC 6012 from Gadjah Mada University.

Bioethanol Production Scheme

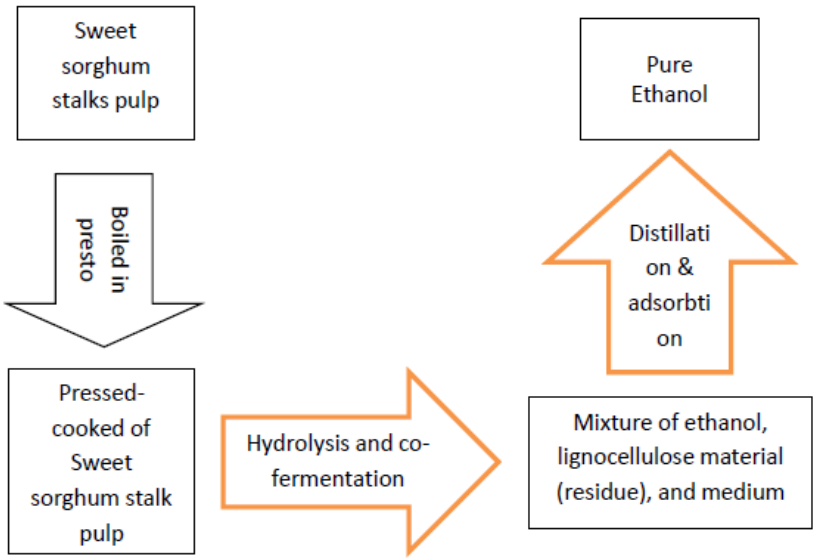

Figure 1. Scheme of Bioethanol Production

Experimental design

The first experiment carried out is in the Pretreatment section, namely boiling the sweet sorghum stem pulp using a pressure cooker (presto). The independent variable is boiling time: 0 (control), 15, 30, and 45 m, while the dependent variable is the concentration of fermented ethanol. The experiment was carried out in triplicate (3x for each treatment) so that there were a total of 12 units.

The second experiment that will be carried out is at the purification stage, namely purifying ethanol using ZAE. The independent variables are the mode of activation of the zeolite (Oven without reflux, refluxed with NaOH/oven, refluxed with HCl/oven) and the dependent variable is the concentration (%) of purified ethanol.

Working procedures

Inoculum preparation

The pure isolate of *T.reesei* was grown on potato dextrose agar (PDA) medium and stored in the refrigerator. The *T.reesei* inoculum that will be used in fermentation is made by suspending *T.reesei* seeds on agar slants in 250 ml of sweet sorghum stem pulp supplemented with 2 grams of urea, 2 grams of ammonium sulfate, 75 milligrams of K_2HPO_4 and $MgSO_47H_2O$ each. After incubation for 2 days and the spore suspension in the medium reaches a concentration of $1.8x10^8$ cells per ml, the inoculum is ready for use [12].

S. cerevisiae isolates were grown on malt extract agar (MEA) medium and stored in the refrigerator. The *S. cerevisiae* inoculum that will be used is prepared by culturing *S. cerevisiae* isolates in a suspension of sorghum stem pulp, which is added with 0.15% each of K_2HPO_4 and $MgSO_47H_2O$ as well as 5% each of yeast extract and peptone [13].

Pretreatment and Substrate Preparation

Sweet sorghum stem pulp was prepared by chopping sweet sorghum stems in to small pieces then 1000 grams of it was added in to 1000 ml of water and boiled in pressed-cooker for a certain time according to experimental design in Table 1 (exception for control substrate, it was not treated). After the cooling down, the material was grounded in blender machine until its form was turned to fine pulp.

Fermentation Process

An amount of 250 g of substrate was added with 250 mL distilled water then transferred into a 1000 mLfermentation bottle. Furthermore, this mixture was supplemented with 5 grams of urea, 5 g of ammonium sulfate, 150 mg of K_2HPO_4 and $MgSO_47H_2O$ each. After that, the mixture was inoculated with 50 mL of *S. cerevisiae* culture and 50 mL of *T.reesei* culture. Next, the acidity was adjusted to pH 5 using HCl and NaOH solutions and phosphate buffer solution. The fermentation bottle is closed tightly with a rubber stopper that can be tightened. All the above work is carried out aseptically. The incubator is set at 35 °C, and when the temperature is reached, the fermentation bottle is inserted into it, and the incubation is carried out for 24 h. After that, the fermented alcohol mixture and the remaining lignocellulosic fibers were separated using a filtration technique. As much as 70 mL of of this clear filtrate was added by 70 mL distilled water then distilled again at 100 °C until it got 70 mL distillate. The alcohol content in the distillate was determined using an alcoholmeter.

Yield Calculation

Ethanol yield will be calculated using the Eq.(1):

$$Yp/s = \frac{P-Po}{So-S} \quad (1)$$

Note: Yp/s = ethanol yield; P = product concentration (ethanol) at the end of fermentation; P_o = ethanol concentration at the start of fermentation; s = substrate (sugar) concentration at the end of fermentation; and s_o = substrate concentration at the start of fermentation [13].

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Purification of Bioethanol

a. Distillation

The fermentation results are put into a distillation flask and then heated to a temperature of 78.5 °C until the ethanol evaporates and condenses in the cooling column. Ethanol was collected in an Erlenmeyer flask which was cooled with ice water. The ethanol content of the distillation results is measured with an alcohol meter. The ethanol solution obtained was distilled repeatedly until it reached ethanol content ≥ 94%.

b. Natural Zeolite Activation

Ende Flores-NTT natural zeolite (ZA) is sorted, bright green and clean is selected, crushed to a size of 80-100 mesh. Next, 1000 grams of the powder were weighed and then soaked in ion-free water while stirring with a Wise Stirrer for 1 hour at room temperature, then left for 30 minutes and after that the filtrate and sediment were separated. The clean natural zeolite (ZA) precipitate was given hydrothermal treatment by putting the ZA into pressure cooking and mixed with 2500 mL of ion-free water. The zeolite was then refluxed for 3 h at a temperature of 100 °C, then filtered and washed then dried at a temperature of 120 °C in an oven for 6 hours. The obtained solid was calcined at 600 °C for 3 h, active natural zeolite (ZAA) was obtained.

c. Activation of Natural Zeolite Using HCl

The ZA product is the result of a hydrothermal process and dried at 120 °C in an oven for 6 hours, activated using HCl. A total of 500 g of ZA was soaked in 1 L of 3 M HCl solution, then refluxed in a closed container in a fume hood at a temperature of 90 °C for 6 h. Filtered and washed repeatedly until the HCl is gone, dried at 120 °C in the oven for 6 hours. The obtained solid was calcined at 600 °C for 3 h, H-ZAA was obtained.

d. Activation of Natural Zeolite Using NaOH

The ZA product is the result of a hydrothermal process and dried at 120 °C in an oven for 6 hours, activated using NaOH. A total of 500 g of ZA was soaked in 1 L of 3 M NaOH solution, then refluxed in a closed container in a fume hood at a temperature of 90 °C for 6 h. Filtered and washed repeatedly until neutral, dried at 120 °C in the oven for 6 h. The obtained solid was calcined at 600 °C for 3 h, OH-ZAA was obtained.

e. Test the water adsorption capacity of Zeolite

Performance tests of ZAA, H-ZAA and OH-ZAA in ethanol-water adsorptive dehydration were carried out in batches. 20 g of each and 100 mL of distilled bioethanol were stirred in an Erlenmeyer for 3 h, then filtered, then measured the ethanol content using an alcohol meter, followed by calculating the amount of water adsorbed.

RESULT AND DISCUSSION

Bioethanol Fermentation

The results of bioethanol fermentation are as seen as in Table 1

Table 1 Ethanol Concentration in Fermented Liquid

Pretreatment time (Presto treatment) (minutes)	Ethanol concentration (% m/v)		
	1	2	3
0	6.00	6.25	5.75
15	5.50	5.00	5.25
30	4.00	4.25	4.50
45	3.00	3.00	4.00

The data above are the results of alcohol meter readings on fermentation products that have been clarified by distillation [14]. Regarding to the addition of water from the pressure-cooking process until the fermentation process, thus the dilution factor (df) is 850/250) = 3.4. Thus, the actual ethanol concentration from the fermentation of an amount of sorghum stem pulp is as follows (Table 2):

Table 2 Ethanol Concentration Regarding to Dilution Factor

Pretreatment time with presto (minutes)	Ethanol concentration (% m/v)			
	1	2	3	Average
0	20.40	21.25	19.55	20.40±0.85
15	18.70	17.00	17.85	18.27±0.60
30	13.60	14.45	15.30	14.45±0.85
45	10.20	10.20	13.60	11.33±1.96

In graphic form, as seen in Figure 2

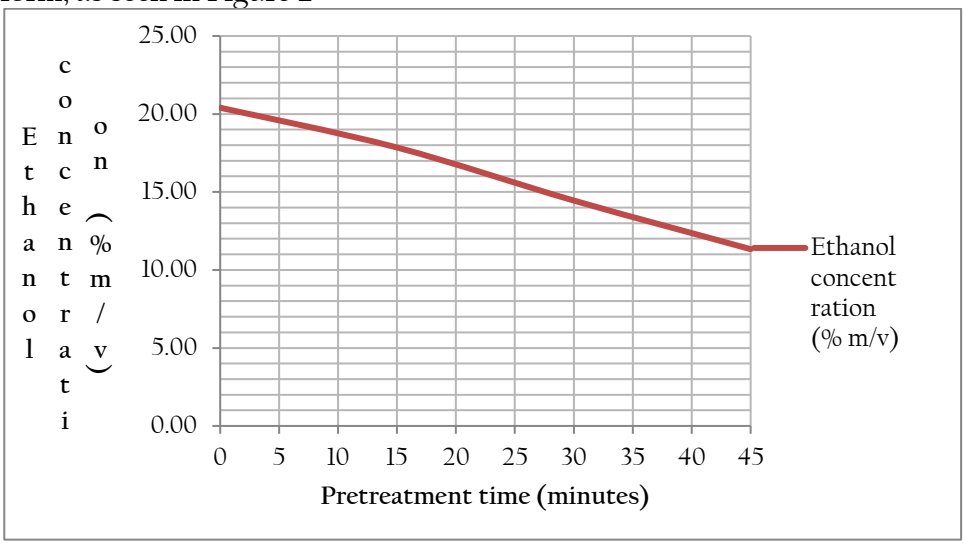

Figure 2. The relationship between the duration of substrate pretreatment using pressed-cooker and the ethanol concentration resulting from fermentation.

The ethanol yield obtained is calculated based on equation (1) and the results are shown in Table 3.

Table 3. Ethanol yield

Pretreatment time with presto (minutes)	Ethanol yield (p/s)			
	1	2	3	Average
0	0.49	0.51	0.47	0.49±0.02
15	0.45	0.41	0.43	0.43±0.02
30	0.33	0.35	0.37	0.35±0.02
45	0.24	0.24	0.33	0.27±0.05

From Table 2 and Figure 2, it appears that increasing the pretreatment time (pressure treatment) causes a decrease in the concentration of ethanol produced. As the concentration of ethanol produced from the same amount of material decreases, the ethanol yield also decreases (Table 5.3). Based on statistical analysis (one way ANOVA), the ethanol concentration and yield as well as the efficiency of the sugar to ethanol conversion process obtained were different for each treatment length. The significant difference (p>0) is shown in Table 5.4.

Table 5.4. Tukey test results for ethanol concentration for each treatment period

Results			
Pretreatment Time	N	Subset for alpha = 0.05	
		1	2

Tukey HSD[a]	45 minute	3	11.3333	
	30 minute	3	14.4500	
	15 minute	3		17.8500
	0 minute	3		20.4000
	Sig.		.057	.126

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

The data in column 1 is significantly different from that in column 2, while the data in the same column is not significantly different.

The results above were unexpected, because it was originally thought that the longer the pressed-cooking time, the more ethanol was produced, with the reason that the longer it was heated at high pressure the more the cellulose crystalline structure was damaged and the easier be accessed by enzyme during hydrolysis, glucose concentration increases and fermented ethanol also increases. Several research results show that pretreatment by boiling lignocellulose at high temperatures, known as liquid hot water (LWH), releases a lot of hemicellulose, produces little monomer sugar [15], cellulose undergoes depolymerization [16]. This is expected to produce a lot of glucose and then produce a lot of ethanol when fermented.

However, the data obtained in this study contradicts to the assumptions above. This forces researchers to look for different scientific explanations for this result. We thought that this difference appears to be caused by the degradation of glucose and fructose during heating at high temperatures [17]. At high temperatures, glucose and fructose undergo *Maillard* and caramelization reactions thus their concentration decreases [18]. The *Maillard* reaction produces a brown pigment product called melanoidin, which contains varying amounts of nitrogen. In addition, it also produces volatile compounds, aroma compounds and several other types of compounds [18]. Caramelization reactions can also occur in glucose and fructose due to heating which produces organic acid compounds, aldehydes and ketones [19].

The decrease in ethanol produced in fermentation is thought to not only because of reduced concentrations of glucose and fructose in the material, but also by what is more influential is the presence of inhibitor compounds against *S. cerevisiae* in the form of acetic acid, aldehydes and phenols [20] produced from the glucose caramelization. Apart from that, 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde compounds can also be formed due to hexose and pentose dehydration. Various kinds of phenolic compounds produced from the breakdown of lignin and carbohydrate degradation during pretreatment [21] can also inhibit the ethanol fermentation by *S. cerevisiae*.

Bioethanol Purification

Distillation

The result of distillation process are shown in Table 5

Table 5. Bioethanol concentration of distillation series

Initial concentration (%)	Ethanol concentration of distillation series (%)				
	I	II	III	IV	V
5.75	30.5	55	70	91	94.0

The results in Table 5 show that up to the 4th distillation, the increase in ethanol concentration was quite drastic, but after that the increase in ethanol concentration was only slight. This is possibly because the water-ethanol mixture at that time was approaching the *azeotrope* point, namely at an ethanol concentration of 94.5-95% [22]. The *azeotrope* condition is the condition where the composition of the liquid fraction is the same as the composition of the

vapour fraction [23]. Under azeotropic conditions, the water-ethanol mixture can no longer be separated by distillation. Therefore, the separation must be carried out by adsorption.

Adsorption

Ethanol concentration after a series of adsorption process are as seen in Table 6

Table 6. ethanol concentration as the results of adsorption process

Adsorbent	Adsorption stage (3 replications)	Initial concentration (%)	Final concentration
ZAA	I	94	96
	II	96	98
	III	98	99
H-ZAA	I	94	95
	II	95	95.5
	III	95.5	96
OH-ZAA	I	94	95
	II	95	95.5
	III	95.5	95.5

From the data in table 5.6, it can be seen that ZAA in three adsorption stages is able to increase the ethanol concentration from average of 96.00% to 97.67%, H-ZAA is able to increase the ethanol concentration from 94.83% to 95.52%, while HO-ZAA is able to increase the ethanol concentration from 94.83% to 95.33%. These results indicate that among the three forms of active zeolite, ZAA has a greatest adsorption capacity, while the adsorption capacity of H-ZAA is greater than that of HO-ZAA.

The results above indicate that for the conditions (calcination time, calcination temperature, adsorbent/ethanol ratio) used in this research, it seems that ZAA is more suitable for use in bioethanol purification when compared to H-ZAA and HO-ZAA. To achieve truly pure ethanol (close to 100%) or fuel grade, it is possible to achieve this by modifying the parameters used, for example by increasing the calcination temperature and reducing the zeolite particle size. This is because based on research by Susilawati et al. (2018) the optimum calcination temperature for *Pahae Natural Zeolite* is 700 °C and the particle size is 200 mesh [24], while the one used in this research is 600 °C and the particle size is 80-100 mesh.

CONCLUSIONS

Based on the results obtained in this research, it can be concluded that retreatment using boiling techniques using a pressure cooker is not appropriate to apply to sweet sorghum stalks for bioethanol production. The use of the SSCF technique with a mixed culture of *T.reesei - S.cerevisiae* on sweet sorghum stalk pulp without pressed-boiling pretreatment can convert almost all carbohydrates (soluble and insoluble) into ethanol. Bioethanol can be purified using the active Ende Natural Zeolite adsorbent. The zeolite activation method that produces adsorbents with the highest capacity to adsorp water molecules under the conditions applied in this research is physical activation with a calcination time of 3 h at a temperature of 600 °C.

ACKNOWLEDGEMENT

Thank you very much are expressed to the Leaders of FKIP Undana who have allocated funds for this research. We also appreciate the students involved in this research for the energy and time spent on field and laboratory work for data collection.

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