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Enzymatic Hydrolysis of Soluble Starch from Yam (*Dioscorea hispida* Dennts.) Using Enzyme from Termite (*Nasutitermes Sp.*) For Bioethanol Production.

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ABSTRACT

Termites have enzymes diversity in their gut, that can be used to hydrolyzed feedstock into bioethanol, but at present its use is limited to a substrate lignocellulose materials. Yam (*Dioscorea hispida* Dennts) is one of the most potential raw materials for bioethanol production, but it gets less attention. The aim of this work was to optimized crude enzyme from *Nasutitermes sp*. for hydrolysis yam as the substrate for bioethanol production. The crude enzymes that were extracted from worker termites was used to hydrolyze yam starch at different pH from 3.0 to 6.0, incubation time for 10 to 100 minutes, temperature at 30 to 75ºC, and substrate concentration between 1 to 5% w/v. The reduced sugar was fermented with peptone and urea as nitrogen sources for 24, 48, 72 and 96 h. The optimum condition for enzymatic hydrolysis of yam starch using crude enzyme from *Nasutitermes sp*. was at pH 5.4 for 20 min of incubation at 60ºC. The highest fermentation efficiency was 49.22% for 96 h with peptone as the nitrogen source. As the conclusion, the crude enzyme from *Nasutitermes sp.* was able to hydrolyze starch of yam (*D. hispida* Dennts) for bioethanol production.

Keywords: Termite, Yam (*D. hispida* Dennts), *Nasutitermes sp.,* Bioethanol

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INTRODUCTION

In the recent years, bioethanol has received much attention due to its renewability. Bioethanol is a liquid biofuel, which has higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline and ethanol contains oxygen (35% of oxygen content) in the fuel, it can effectively reduce particular matter emission in the diesel engine. Bioethanol can be produced from several feedstocks and through several conversion methods. The starchy material is one of the raw materials for bioethanol production, such as potato, cassava, rice and yam [1-3].

Yam (*D. hispida* Dennts) is a member of the family widely grown in Indonesia. The yam (*D. hispida* Dennts) is materials carbohydrate, but they are not been explore yet, because of it has high content of toxic substances, i.e. alkaloids and hydrogen cyanide in both free and bound forms. The yam (*D. hispida* Dennts) contain about 20 g carbohydrates in every 100 g of the tuber (wet basis) [4-5].

One of the green processes for bioethanol production is enzymatic hydrolysis [6]. However, the price of pure enzymes are expensive due to production cost, which contributes 40–60 % of the total cost [7]. One way to reduce the cost is by using an alternative source of hydrolytic enzymes like the termites. It has been used the gut extract of subterranean termite, *Odontotermes obesus* Rambur for conversion of lignocellulosic biomass [8]. Moreover, Lima et al reported enzymes diversity in *Nasutitermes corniger* termite gut: cellubiose, hemicellulase, proteinase, amylase, cellulase [9]. The use of enzymes from termites is still limited to raw materials containing lignocellulose, whereas the yam (*D. hispida* Dennts) has a potential for use as a feedstock for bioethanol production.

The aim of this research was to investigate the optimum condition of enzymatic hydrolysis of *Nasutitermes sp.* crude extract, and study the effect of nitrogen source and time of fermentation on bioethanol yield using yam (*D. hispida* Dennts) as a feedstock.

MATERIAL AND METHODS

Collection of termites

Worker termites were collected from decaying tree trunk in the forest nearby Andalas University, Padang, West Sumatera, Indonesia. The termites were transferred to laboratory packaged into a plastic box. Identification of the termites was done at insect ecology laboratory, Faculty of Agriculture, Andalas University. The termite was confirmed as *Nasutitermes sp*.

Preparation of crude enzyme

An adaptation of the method described by Kumar et al [8] and Lima et al [9]; was used to the preparation of crude extract. 40 g of worker termites *Nasutitermes sp.* was added 500 mL NaCl 1% w/v bit by bit into the blender. The homogenates were centrifuged at 1200 g at 4 °C for 30 min. The supernatant was stored at refrigerator (10°C) in 0.05 M sodium acetate pH 5.6 containing NaCl 0.17 M. The protein content was determined by the Lowry's method using bovine serum albumin as a standard [10].

Amylase activity assay

The Amylase enzyme activity was determined according to Lima et al [9] and Liu et al [11] with slight modifying ; 500 µL of enzyme solution was added to 4500 µL soluble starch from yam (*D. hispida* Dennst) 1% w/v in 0.05 M sodium acetate buffer (pH 5.4) containing 0.17 M NaCl, at 50ºC for 10 min. The reaction was stopped by the addition of 1.0 mL Nelson-Somogyi reagents and the mixture was boiled for 20 min. After cooling to room temperature, the mixture was added 1.0 mL phosphomolybdate reagent and diluted with 7.0 mL distilled water then measured at 540 nm [12]. The amount of reducing sugar was determined using glucose as standard ($Y = 0.0022x - 0.0297$); Y is the absorbance at 540 nm; X is the concentration of glucose in mg/L), and amylase activity determines based on Akansha et al. Formula [13]. One unit (U) of enzyme activity is the amount of one µmol of reducing sugar per minute under the defined reaction conditions [14].

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Substrate collection and preparation

In this study, Yam (*D. hispida* Dennts) was used as a substrate. Yam (*D. hispida* Dennts) was procured from a district in Bengkulu, namely Kepahiang. The substrate was gelatinization followed Suryani et al method [15].

Determination of optimum condition

The hydrolysis optimum conditions were determined by varying pH, incubation time and temperature. The pH variation was obtained using 0.05 M sodium acetate buffer with pH range between 3 to 6. 500 µL of crude extract from termites *Nasutitermes sp.* was added to the mixture containing 1% of soluble starch from yam (*D.hispida* Dennst) and sodium acetate buffer then incubated for 10 min at 50°C. After it is taken 1 mL, and added 1 mL Nelson-Somogyi reagent and placed into boiling water. After cooling to room temperature, the mixture is placed added 1 mL phosphomolybdate reagent, and 7 mL of distilled water and measured at the wavelength of 540 nm [12]. Effect of incubation time on the glucose levels was determined by incubating the mixture (pH optimum) for 10 to 100 minutes at 50°C. The influence of temperature on the enzyme activity of crude extract of termites *Nasutitermes sp.* by incubating the mixture (at pH, and incubation time Optimum) at different temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, and 75°C).

Determination of enzyme kinetic

The K_M and V_{max} were determined to use yam (*D. hispida* Dennts) as the substrate. The enzyme was determined at varying substrate concentrations from 1% to 6%. The K_M was obtained by constructing a Lineweaver-Burk plot.

Bioethanol production

Bioethanol production method was adopted by Moshi et al [16] and Kumar et al [17] method with slight modifying, 62 g soluble starch of yam (*D. hispida* Dennts) was added 200 mL 0.05 M sodium acetate buffer (pH 5.4), enzyme (9.605 mU) and incubated for 20 min at 60°C. About 1 mL of a mixture was taken for determination initial sugar Nelson-Somogyi method. The supernatant was added with 10% of inoculum of *Saccharomyces cerevisiae* which was allowed to grown on YEPD medium with different nitrogen sources (urea and peptone) and the fermentation was carried out at 30ºC for 24, 48, 72, and 96 h. After fermentation, the samples were analyzed for ethanol yield (GC-MS) and leftover sugar by Nelson-Somogyi method [12].

RESULTS AND DISCUSSION

Effect of pH

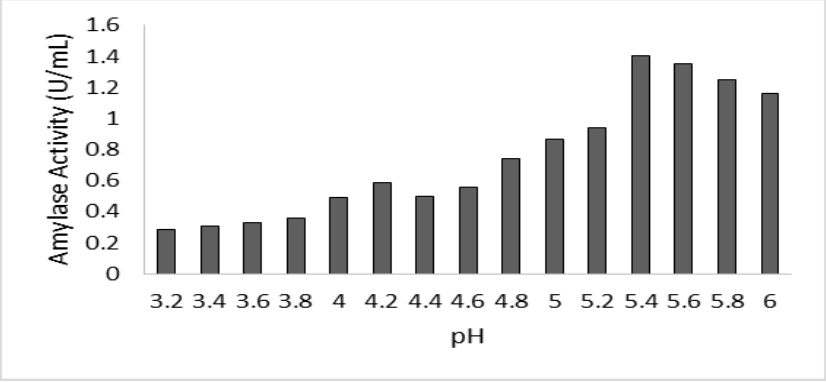

Figure 1. Effect of pH on amylase activity

A narrow pH range (3−6) was chosen for this study because it has been reported that amylase has the optimum condition at acidic environment [9]. Enzymes usually have their activity affected by pH, since the ionization of side chains of amino acids in both the active site and whole enzymes influences protein shape and

charge, as well the positioning of the substrate [18]. The effect of pH on the amylase activity of the crude extract from *Nasutitermes sp.* was presented in Figure 1 above.

The result showed that the amylase activity started increased up to 1.4055 U/mL but dropped gradually of about pH 5.4. The detection of activity at different pH values has been reported for amylase producer *Bacillus subtilis* BS5 was obtained from the hindgut of wood eating *Amitermis evuncifer* Silvestri termites was active optimally at pH 6 [19]. The optimum pH of amylase activity of the isolate LBPs and LOS strain from *Macrotermes Subhyalinus* and *Macrotermes Bellicosus* is optimal at 5 and 5.6 respectively [20].

Effect of Incubation time

Enzyme activity is also depending on incubation time [11]. Amylase activity in the crude extract was determined at different incubation time varied from 10−100 min (Figure 2). The optimum incubation time for amylase in the crude extract was 20 minutes. Subsequently, the glucose was decreased at longer period incubation. Optimal time of enzyme and substrate to interact are necessary to produce higher amount of products, afterwards all the proteins undergo denaturation and lost a large amount of activity [14].

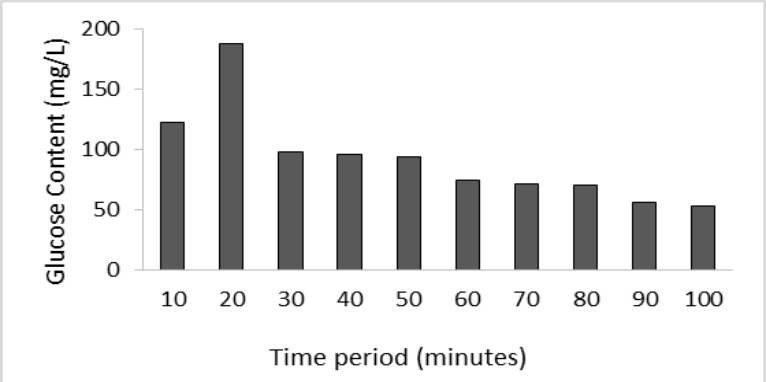

Figure 2. Effect of Incubation time on glucose content

Effect of temperature

Most chemical reaction proceeds at a faster velocity as the temperature is raised. An increase in temperature gives more kinetic energy to the reactant molecules resulting in more collisions per unit time. Enzyme catalyzed reaction behave similarly, up to a point. The true optimum temperature depends on the assay time chosen. It is the maximum temperature at which enzyme exhibits a constant activity over a time period at least during the assay. This can easily be established by pre-incubating the enzyme at different temperatures for one or two times at the prefered assay time and then measuring the activity at the possible lowest temperature for the protein not to denatured [14]. The effect of temperature on amylase activity of the crude extract from *Nasutitermes sp.* is shown in Figure 3.

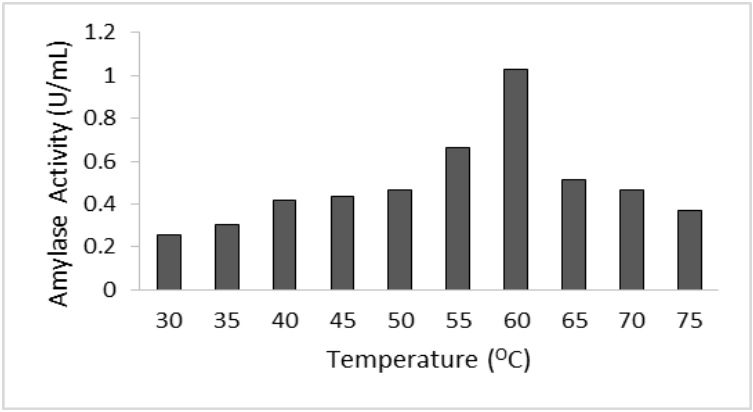

Figure 3. Effect of Temperature on Amylase activity

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The results showed that the enzyme activity increases with rising temperatures and achieved optimum at 60°C (1.0283 U/mL). Similar results have been reported for amylase from *Bacillus subtilis* by Kim et al [21].

Enzyme kinetic

One of the most fundamental factors affecting the activity of the enzyme is the substrate concentration. The rate of enzyme activity will increase by increasing levels of substrates. While the substrate reaches saturated concentration, the addition of more substrate will have no effect on the rate of enzyme activity [22]. Effect of substrate concentration on amylase activity in the crude extract was shown in Figure 4.

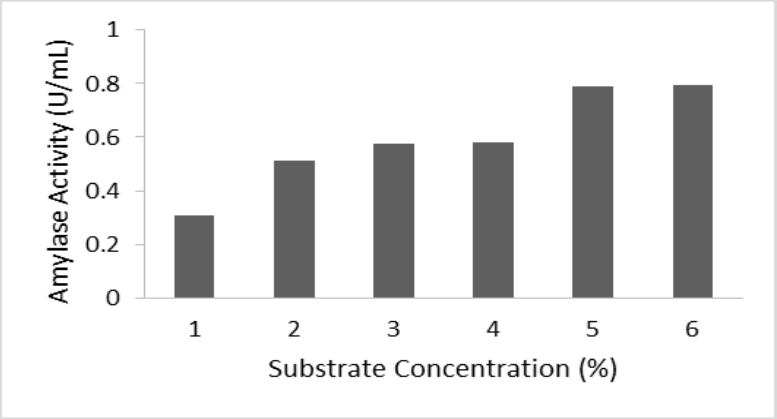

Figure 4. Effect of Substrate concentration on Amylase activity

In this study, the optimum substrate concentration was at 5% and then remained plateau. This is due to all the enzymes has established an enzyme-substrate complex, so there is no longer the enzyme active site that is free (the enzyme has been saturated with the substrate) [23]. Effect of substrate concentration was used to determine of enzyme kinetic. In any enzyme-catalyzed reaction that obeys Michaelis-Menten kinetics [23].

Bioethanol Production

Table 1. Bioethanol production efficiency by different nitrogen source and varying fermentation time

No.	Parameters	Nitrogen sources							
		peptone				urea			
		Fermentation time (h)				Fermentation time (h)			
		24	48	72	96	24	48	72	96
1.	Initial glucose (After hydrolysis) (mg/L)	275.6	520.3	434.8	458.5	312.4	658.4	298.3	268.8
2.	Total leftover glucose (After fermentation) (mg/L)	210.1	496.6	264.8	232.8	256.0	517.5	216.9	235.6
3.	Total glucose consumed (mg/L)	47.4	23.6	169.9	225.7	56.4	140.9	81.4	33.2
4.	Bioethanol fermentation efficiency (%)	17.2	4.5	39.1	49.2	18.0	21.4	27.3	12.3

Based on Table 1 with peptone as nitrogen source, the highest glucose resulted in fermentation for 96 h with fermentation efficiency of 49.22%. It was similar to that reported by Maurice [24], which reported the optimum fermentation conditions for 96 h at a temperature of 30°C with a concentration bioethanol fermentation 88.67%. Whereas in Table 1 with urea as nitrogen source, the highest fermentation efficiency was 27.27 % at fermentation time 72 h. Hashem [25] also reported the optimum fermentation conditions at 72 h with the resulting ethanol content of 11.62% (w/v) by using the yeast *Kluyveromyces sp.* GU133329 at 35°C and optimum pH 5.0-5.5. Low quantity of ethanol production from yam starch required further optimization of

fermentation conditions. According to Lin, *Saccharomyces cerevisiae* work optimally at pH 4.5 and a temperature of 25-26ºC [26].

CONCLUSION

The crude enzyme from *Nasutitermes sp.* was able to hydrolyze starch of yam (*D. hispida* Dennts) for bioethanol production.

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REFERENCES

[1] Taylor G. Energy Policy 2008; 36: 4406-9.
[2] Balat M. Explor exploit. 2007; 25: 195-218.
[3] Kim H, Choi B, Park S, Kim YK. Researchgate. 2005.
[4] Harijono, Estiasih T, Sunarharum WB, Hartono MD. International Food Research Journal. 2013; 20(5): 2279-2285
[5] Coursey DG. 1967. Yams. Longmans-Green, London.
[6] Carlo NH, Geertje VH, Andre PCF. Biomass and Bioenergy. 2004; *28*: 384-410.
[7] Dhillon GS, Brar SK, Kaur S, Metahni S, M'hamdi N. Biomass and Bioenergy. 2012; 41: 65-174
[8] Kumar NS, Sourimuthu M, Devaraj SB. African J. Biotech. 2015; 14, 1753-1756.
[9] Lima.TdA, Pontual EV, Dornelles LP, Amorim PK, Sá RA, Coelho LCBB, Napoleão TH, Paiva PMG. Comparative Biochemistry and Physiology.2014; *176*: 1-8.
[10] Lowry OH, Roserbrough NJ, Farr AL, Randall R. J. Bio. Chem. 1951; 193: 265–275.
[11] Liu XD, Xu Y. Bio. Technol. 2008; 99: 4315-4320.
[12] Nelson S. J.Biol.Chem. 1944; *153*: 375-380.
[13] Akansha K, Varsha N. Int. J. Phar. Biol. Sci. 2013; 3: 354-360.
[14] Segel IH, 1975. Biochemical calculations 2nd edition. California
[15] Suryani R, Nisa FC. Jurnal Pangan dan Agroindustri. 2015; *3*: 723-733.
[16] Moshi AP, Crespo CF, Badshah M, Hosea KM, Mshandete AM, Mattiasson B. Bio.Tech. 2014; 156: 348-356.
[17] Kumar MR, Kumaran MDB, Balashanmugam PA, Rebecca IN, Kumar DJM, Kalaichelvan PT. Pakistan.J.Bio.Scie. 2014; 17: 735-739.
[18] Purich DL. Elsevier. 2010; Amsterdam.
[19] Femi-Ola TO, Olowe BM. Research Journal of Microbiology. 2011; 6(2): 140-146.
[20] Dabonné K, Yahaya KB, Pamphile Z, Philomène, Simplie GT. Scientific Journal of Biological Sciences, 2013; 2(12): 244-255
[21] Kim TU, Gum GB, Jeong JY, Young SC, 1995. Appl. Environ. Microbiol, 61: 3105–12.
[22] Lehninger AL, Nelson DL, Cox MM. 2005. Lehninger principles of biochemistry, WH Freeman and Co, New York, US.
[23] Prasad NK. 2011. New Delhi
[24] Maurice ML. Shanghai Jiao Tong University. 2011; 1-42.
[25] Hashem M, Abdel NA, Zohri, Maysa MAA. African Journal of Microbiology Research. 2013; 7(37): 4550- 4561.
[26] Lin Y. Microbiol. Biotechnol. 2006; 627-642.