



The Potential of Microbial Symbionts *Macrotermes gilvus* Hagen Termite Gut as Degrading Agents of Cellulose in Bioethanol Production

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Abstract

Water hyacinth is a potential feedstock for bioethanol production because of their high cellulose. The microbial symbionts of the *Macrotermes gilvus* termite's gut have a high endoglucanase enzyme activity. This research was aimed to analyze the pH, temperature and agitation effects towards cell density, endoglucanase enzyme activity and reducing sugar, and to determine the effective optimum condition that can produce maximum reducing sugar. This research used *central composite design* (CCD) with the total number of run was . The independent variables were including pH (5.9, 6.4, 7.0, 7.6, 8.0), temperature (30 °C, 33 °C, 37 °C, 41 °C, 44 °C) and agitation (90 rpm, 114 rpm, 150 rpm, 185 rpm, 210 rpm), with six replications at central points. Parameters measured were cell density, endoglucanase enzyme activity and reducing sugar, thus analyzed by the statistical software package MINITAB 18.0. The Student's t-test result showed the primary sequence influencing cell density as pH > agitation > temperature and towards endoglucanase enzyme activity and reducing sugar as pH > temperature > agitation, $P < 0.05$. The maximum reducing sugar ($60.13 \pm 3.16 \text{ mmolL}^{-1}$) was obtained at pH 6.95, temperature 37 °C and agitation 150 rpm. The results of this research can be used to explore the more potential microbial symbionts of the *Macrotermes gilvus* Hagen termite's gut.

How to Cite

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INTRODUCTION

Bioethanol is one of potential energy sources that can be developed as renewable fuel and eco-friendly. Current bioethanol development has switched using lignocellulosic biomass, such as water hyacinth (*Eichhornia carssipes*). It is because the abundant availability of water hyacinth and does not compete with edible and also it has a high cellulose yield (Das *et al.*, 2016).

As a bioethanol feedstock, water hyacinth should be de-lignified to degrade and remove lignin and hemicellulose from cellulose. The next step is saccharification to depolymerate complex sugar into simple sugar. Delignification can dissolve more hemicellulose and increase the amount of cellulose that in the sample of delignification can produce cellulose (35,4%) and hemicellulose (19,6%) while in the sample of non-delignification can only produce cellulose (24,7%) and hemicellulose (32,2%) (Das *et al.*, 2016). It shows that delignification is important, that is why the water hyacinth in this research was delignified with H₂SO₄ (2%, v/v) under steam at a constant temperature (121 °C) for 20 min as same as the research of Reales-Alfaro *et al.* (2013).

The de-polymerization of cellulose into glucose and hemicellulose into glucose, manose, and xilose need complex cellulolytic enzyme (Sánchez *et al.*, 2011). It becomes a consideration in developing bioethanol because 40-60% from the total etanol production cost for commercial enzyme purchases (Dhillon *et al.*, 2012). Thus, many researchers tried to reduce the production cost, as a substituting commercial enzyme by microbial symbionts of the *Macrotermes gilvus* Hagen termite's gut. *Macrotermes gilvus* Hagen is higher termite which identified have two cellulolytic microbial of termite gut symbionts that have high cellulolytic activities, *Bacillus megaterium* and *Paracoccus yeii* (Ferbiyanto *et al.*, 2016)

On other hand, one of the effort to reduce the production cost by combining sacarification and fermentation steps into single step (simultaneous saccharification and fermentation (SSF)). SSF is more economical comparing with separate hydrolysis and fermentation (SHF) because the time needed to produce reducing sugar is shorter yet still can increase hidrolisis rates thus requiring lower enzyme loadings and also having low rate of contaminating risk (Sudiyani *et al.*, 2014; Scully & Orlygsson 2015). Thus, many factors of SSF, such as pH, temperature and agitation, are urgently necessary to be optimized by central composite design (CCD) to increase enzyme production (Deka *et al.*, 2013).

This aims of this research were to analyze the pH, temperature, and agitation effects towards cell density value, endoglucanase enzyme activity, and reducing sugar and also to determine the effective optimum condition that can produce maximum reducing sugar.

METHODS

Preparation and delignification of water hyacinth biomass

Fresh water hyacinth was collected from a Rawa Pening lake, Ambarawa, Semarang. It is washed to remove impurities. The leaves and stems (7 kg each) were dried separately. Drying is done for ± 7 d. The water hyacinth is chopped and ground using a grinding machine with a 0.6 mm sieve. After that, the applying of delignification as the method of Reales-Alfaro *et al.* (2013). Then, there was a Chesson (Datta 1981) assay step towards the delignification result filtrate and the last cellulose with amount of 42.24 ± 0.24% was obtained as bioethanol feedstock.

Preparation of the microbial symbionts of the *Macrotermes gilvus* Hagen termite's gut

Live termites were taken from Semarang State University mini forest. It were collected in a clean jar bottles (Subekti & Febriana 2016). After that one hundred *Macrotermes gilvus* caste worker termites was sterilized with 70% ethanol (v/v) (Tay *et al.*, 2010). Termite guts were taken aseptically using micro-tweezers and suspended at 1 mL NaCl (0.85%, w/v) (Sharma *et al.*, 2015). The suspension was further centrifuged at 95 g in 1 min (Tay *et al.*, 2010). Then, 0.5 mL aliquots were cultured with 4.5 mL of liquid medium I (Sharma *et al.*, 2015), followed by incubation at 30 °C for 7 d. Then 1 mL result of spread plate culture on sterile solid medium (Dickerman & Starr 1951), followed by incubation at 30 °C for 24 h. While microbial screening was performed by culturing the inoculum obtained from spread plate on 50 mL of the second liquid medium (Sharma *et al.*, 2015 modified without CMC) and incubated at 30 °C for 72 h.

Preparation of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae was obtained by culturing Fermipan (10%, w/v) in the second liquid medium (Sharma *et al.*, 2015 modified without CMC) and incubated at 30 °C for 72 h (Jumiyati *et al.*, 2012; Kurniawan *et al.*, 2014).

Optimization SSF factors

Filtrates of 1.5 g were added in the er-

lenmeyer and added 80 mL 0.05 M buffer pH $\text{KH}_2\text{PO}_4\text{-NaOH}$ (5.9, 6.4, 7.0, 7.6, 8.0) and 20 mL microbes (30%, v/v) (the microbial symbionts of the *Macrotermes gilvus* Hagen termite's gut-*Saccharomyces cerevisiae*, ratio 1:1), those were shaken with shaker for 24 h at temperature (30 °C, 33 °C, 37 °C, 41 °C, 44 °C) and agitation (90 rpm, 114 rpm, 150 rpm, 185 rpm, 210 rpm). Then, the suspension (the cell density measurement) was centrifuged at 2400 g for 20 min to separate filtrate and supernatant (to endoglucanase enzyme activity and reducing sugar assay).

Cell density, endoglucanase enzyme activity and reducing sugar assay

Cell density of the microbial symbionts of the *Macrotermes gilvus* Hagen termite's gut-*Saccharomyces cerevisiae* was done by using spectrophotometer UV-Vis (Parkin Elmer, Model lambda-45) at $\text{OD}_{600\text{nm}}$ (Sakolvaree & Deevong 2016). Endoglucanase enzyme activity was done with 0.5 mL cell-free supernatant added with 0.5 mL CMC (1%, w/v) which was dissolved in buffer pH 7.0, and then was incubated at 37 °C for 30 min. Reducing sugar assay was done based on standard method by using benedict quantitative reagent, and the absorbance measurement was done by using spectrophotometer UV-Vis (Parkin Elmer, Model lambda-45) at $\text{OD}_{477\text{nm}}$ (Sambo *et al.*, 2015). The sample of reducing sugar was obtained by calculating χ value in a formula that was obtained from the measuring of standard solvent (glucose 0 mmolL^{-1} , 2.5 mmolL^{-1} , 5 mmolL^{-1} , 7.5 mmolL^{-1} and 10 mmolL^{-1}).

RESULTS AND DISCUSSION

Water hyacinth biomass as the result of the delignification that was used in this research obtained cellulose $42.24 \pm 0.24\%$. Similar research that was conducted by Reales-Alfaro *et al.*, (2013) was producing less cellulose with amount of 31.67%. The different percentage of that lignocellulose component was reported by Reales-Alfaro *et al.*, (2013) that because the condition of nutrition habitate affect the metabolism process of the plant that generates different chemical characteristic. On the other hand, these differences might caused by the geographic location and growing condition of water hyacinth (Das *et al.*, 2016).

After that, the cellulose was converted into glucose by cellulase enzyme through simultaneous saccharification and fermentation (SSF). In this research, cellulase enzyme was obtained from the microbial symbionts of the *Macrotermes*

gilvus Hagen termite's gut which was optimized on some factors such as pH, temperature and agitation by central composite design (CCD). The advantage of CCD is representatif in showing optimum condition with less run amount (Montgomery 2013).

Actual and prediction values (from the result of data CCD analyzing) for cell density value, endoglucanase enzyme activity and reducing sugar from the result of SSF sample testing, were presented in Table 1.

Based on the Table 1, it showed that the mean of actual value from those three responses had considered small difference towards the prediction value, thus can be indicated that those three responses has valid value. On the other hand, the testing result of Student's t-test showed that the influence of pH and agitation towards cell density value was significantly different while the temperature had influence that was not too different from the value of $P \leq 0.05$. Those results mean that the influence of pH \neq agitation \neq temperature towards cell density value. Yet the result of Student's t-test for endoglucanase enzyme activity and reducing sugar shows that only pH wich has significant different, while the temperature and agiation indicated insignificance. It means that pH \neq temperature \neq agitation, towards endoglucanase enzyme activity and reducing sugar.

The result of the optimization confirmed that pH has most effect towards cell density value, endoglucanase enzyme activity and reducing sugar. It is because pH influences many chemical reaction by affecting chemical product transport and enzyme that are passing through cell membrane (Liang *et al.*, 2010). Similar research about the influence of pH towards cell density and endoglucanase enzyme activity was reported by Deka *et al.* (2013) and Sakolvaree & Deevong (2016).

The influence of pH towards cell density value could be seen from the maximum respond that reach $3.50 \pm 0.13 \text{ selmL}^{-1}$ (prediction value). It showed that microbial symbionts of *Macrotermes gilvus* termite's gut was optimum on considered neutral pH. That microbes included in neutrophil microbes with the rate of pH around 5.5-8.0. Sakolvaree & Deevong (2016) explained that microbial symbionts of termite gut can live far below its optimum pH and some of bacteria symbionts reported forming endospore which help the bacteria in it to survive and improve their ability to live in high temperature and extreme condition. That is related with the range of termite gut pH where the microbial symbionts

Table 1. Actual and prediction value for cell density (selmL⁻¹), endoglucanase enzyme activity (UmL⁻¹) and reducing sugar (mmolL⁻¹)

Codes	Cell density (selmL ⁻¹)		Endoglucanase enzyme activity (UmL ⁻¹)		Reducing sugar (mmolL ⁻¹)	
	Actual	Prediction	Actual	Prediction	Actual	Prediction
A ₋₁ B ₋₁ C ₋₁	3.22±0.0	3.53±0.26	0.003±0.0	0.004±0.00	16.07±0.0	22.28±4.12
A ₁ B ₋₁ C ₋₁	2.65±0.0	2.76±0.26	0.006±0.0	0.006±0.00	35.00±0.0	30.22±4.19
A ₋₁ B ₁ C ₋₁	4.08±0.0	3.53±0.26	0.005±0.0	0.005±0.00	25.42±0.0	25.04±4.12
A ₁ B ₁ C ₋₁	3.85±0.0	2.76±0.26	0.004±0.0	0.006±0.00	22.62±0.0	32.98±4.19
A ₋₁ B ₋₁ C ₁	3.87±0.0	4.23±0.26	0.003±0.0	0.004±0.00	15.18±0.0	19.98±4.10
A ₁ B ₋₁ C ₁	3.67±0.0	3.46±0.26	0.005±0.0	0.005±0.00	28.04±0.0	27.92±4.17
A ₋₁ B ₁ C ₁	4.56±0.0	4.23±0.26	0.004±0.0	0.004±0.00	24.23±0.0	22.74±4.10
A ₁ B ₁ C ₁	3.13±0.0	3.46±0.26	0.004±0.0	0.006±0.00	24.11±0.0	30.68±4.17
A _{-1,68} B ₀ C ₀	3.75±0.0	4.21±0.32	0.005±0.0	0.005±0.00	27.44±0.0	26.50±6.35
A _{1,68} B ₀ C ₀	1.90±0.0	2.90±0.30	0.009±0.0	0.008±0.00	48.81±0.0	44.97±5.88
A ₀ B _{-1,68} C ₀	3.85±0.0	3.50±0.13	0.004±0.0	0.004±0.00	22.62±0.0	20.81±6.13
A ₀ B _{1,68} C ₀	3.73±0.0	3.50±0.13	0.005±0.0	0.005±0.00	28.33±0.0	25.64±6.13
A ₀ B ₀ C _{-1,68}	2.53±0.0	2.91±0.30	0.004±0.0	0.005±0.00	24.11±0.0	23.36±5.97
A ₀ B ₀ C _{1,68}	4.75±0.0	4.09±0.30	0.004±0.0	0.003±0.00	22.20±0.0	18.17±6.07
A ₀ B ₀ C ₀	2.87±0.0	3.50±0.13	0.011±0.0	0.011±0.00	56.85±0.0	60.13±3.16
A ₀ B ₀ C ₀	3.60±0.0	3.50±0.13	0.009±0.0	0.011±0.00	48.75±0.0	60.13±3.16
A ₀ B ₀ C ₀	3.04±0.0	3.50±0.13	0.012±0.0	0.011±0.00	62.80±0.0	60.13±3.16
A ₀ B ₀ C ₀	4.22±0.0	3.50±0.13	0.011±0.0	0.011±0.00	61.43±0.0	60.13±3.16
A ₀ B ₀ C ₀	3.05±0.0	3.50±0.13	0.014±0.0	0.011±0.00	78.04±0.0	60.13±3.16
A ₀ B ₀ C ₀	3.31±0.0	3.50±0.13	0.010±0.0	0.011±0.00	52.68±0.0	60.13±3.16

Description: *) the values were obtained from the OD cell density mean multiplied by dissolving factors (mean ± SD, n=3); **) the values were obtained from the formula standard curva; , while Y is the value of OD reducing sugar and X is the value of reducing sugar; ***) the values were obtained from the formula: , while Y is enzyme activity, C is reducing sugar concentration, Fp is the serial dilution, T is the time of incubation and MW is the molecule weight

breed. Brune & Ohkuma (2011) reported that the pH of *Cubitermes* termite's gut are crop (pH 4.0), midgut (pH 4.0-7.0), mixed segment (pH 7.0-12.0), pouch (P1) (pH 12.0), P3 (pH 8.0-12.0), P4 (pH 6.0-8.0) and P5 (pH 4.0-6.0).

Cell density value showed the amount of microbes cells that can produce endoglucanase enzyme. That enzyme has a function to hidrolise CMC that has crystalline structure and hard to dissolve in the water and become the source of carbon needed as the energy and ability to growth (Sakolvaree & Deevong 2016).

Microbial symbionts of *Macrotermes gilvus* termite's gut are the consortium microbes including *Bacillus megaterium*, *Bacillus flexus*, *Bacillus corensis*, *Bacillus abyssalis*, *Bhargavaea cecembensis*, *Paenisporosarcina indica*, *Planococcus fifiotoensis*, *Paracoccus huijuniae*, *Paracoccus aminovorans*, *Paracoccus denitrificans*, *Paracoccus thiocyanatus*, *Paracoc-*

cus yeei, *Oceanicola marinus*, *Sulfitobacter donghicola*, *Donghicola eburneus* (Ferbiyanto *et al.*, 2016). *Bacillus* reported by König *et al.*, (2013) has cellulase activity to degrade cellulose, 1,4-β-xylanase and 1,3-β-galactanase activities to degrade hemicellulose, β-glucosidase activity to oxidize C6 sugar, β-D-galactosidase, α-L-arabinosidase, β-L-xylosidase activities to oxidize C6 and C5 sugar, and also has an ability to oxidize aromatic components as same as role of *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Serratia*. *Bacillus* has higher endoglucanase enzyme activity with amount of 138.77 Ug⁻¹ (Kamsani *et al.*, 2015). As same as Ferbiyanto *et al.*, (2016) explained that *Bacillus megaterium* and *Paracoccus yeei* have highest cellulytic activity.

Besides the influence of pH, agitation is also confirmed more affecting in obvious towards cell density value than the temperature, otherwise

temperature factor is more affecting the endoglucanase enzyme activity value and reducing sugar compare with agitation factor. It is because microbial symbionts of *Macrotermes* termite's gut included in obligate aerob and facultative anaerob that needs oxygen in their growing rate (Ferbiyanto *et al.*, 2016; Muwawa *et al.*, 2016). On the other hand, temperature is affecting cell division (Black & Black 2015), and also affecting the secretion extracellular enzyme by transforming cell membrane physically (Sharma *et al.*, 2015). Lai *et al.*, (2015) also reported that reducing sugar concentration is increasing at temperature 35-45 °C because catalyst enzyme is reacting faster when the temperature is increasing. It corresponds with surface plot curve (Figure 1) which shows that the maximum value of reducing sugar is at temperature 37 °C.

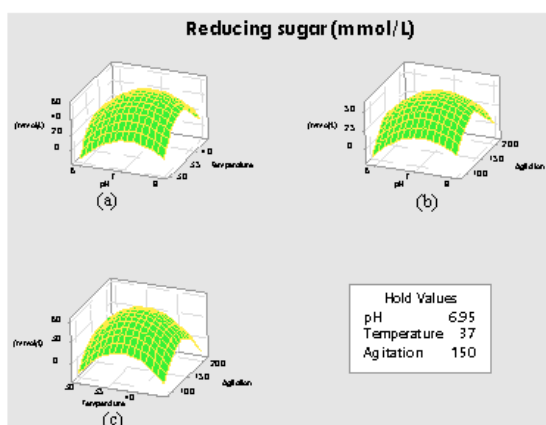


Figure 1. Interaction of (a) pH and temperature, (b) pH and agitation, (c) temperature and agitation towards the value of reducing sugar (mmolL^{-1}) on a maximum point

Figure 1 shows that the maximum value of reducing sugar is obtained in pH 6.95, temperature (37 °C) and agitation (150 rpm), with amount of $60.13 \pm 3.16 \text{ mmolL}^{-1}$. Reducing sugar was obtained by converting cellulose by cellulase enzyme. It means that reducing sugar is associated with cellulase enzyme activity especially β -glucosidase enzyme which have a role in converting cello-oligosaccharide or gluco-oligosaccharide into reducing sugar. On the other hand, cello-oligosaccharide is obtained from the random excision of internal bound of cellulose chain by endoglucanase enzyme (Manavalan *et al.*, 2015).

Saccharomyces cerevisiae in this research was used as fermentative yeast to degraded glucose into ethanol. Fermentative yeast, such as *Saccharomyces* sp., has growing characteristic on the base of the medium and forming sediment in the

growing assay by Sabouraud Broth (SB) medium (Jumiyati *et al.*, 2012). The limitation of *Saccharomyces* as fermentative yeast is only be able to degrade reducing sugar such as, glucose, fructose, and sucrose into ethanol, acetate, lactate acid and many others (Scully & Orlygsson 2015). While Chansoliya *et al.* (2016) explained that *Saccharomyces cerevisiae* was reported is more potential to be used as fermentation microbes than *Zymomonas mobilis*.

CONCLUSION

The result of the research shows that pH is really influencing towards the amount of cell density which is associated with endoglucanase enzyme activity and reducing sugar. Agitation is more affecting after pH towards the amount of cell density than temperature, and so the otherwise for endoglucanase enzyme activity and reducing sugar. It is corresponding with the optimizing result of optimum condition that showed the maximum amount of reducing sugar which was 60.13 mmolL^{-1} in pH 6.95 at temperature (37 °C) and agitation (150 rpm).

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