3BIO: Journal of Biological Science, Technology and Management 2(2):61-72
e-ISSN: 2655-8777 https://journals.itb.ac.id/index.php/3bio

Production of Bioethanol and Crude Cellulase Enzyme Extract from Napier Grass (*Pennisetum purpureum* S.) through Simultaneous Saccharification and Fermentation

Taufikurahman*, Sherly

School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*) Corresponding author; e-mail: taufik@sith.itb.ac.id
Received 12 December, 2019
Accepted for publication 18 August, 2020

Abstract

Napier grass (*Pennisetum purpureum* S.) has been recognized for its high amounts of cellulose and hemicellulose which can be utilized for bioethanol production. Bioconversion of Napier grass to bioethanol can be performed by filamentous fungi. A fungi, *Neurospora sitophila*, can synthesize and secrete hydrolytic enzymes to breakdown the cellulose and hemicellulose into various monomeric sugars and simultaneously convert it to bioethanol. This study investigated the effect of Napier grass substrate concentrations and cultivation time on cellulase enzyme and bioethanol production by *N. sitophila*. The pretreatment of Napier grass was carried out using 1.5% (w/v) NaOH solution for 120 hours at 30 °C. After the pretreatment process, simultaneous saccharification and fermentation was conducted at 30 °C; pH 6.8-7.0 and agitation speed 130 Rpm with various of Napier grass substrate concentrations (10, 20 and 30 g l^{-1}) and cultivation period within 24; 48; 72; 96 and 120 hours. The results show that the highest enzyme activity is 0.28 FPU/ml which was achieved at 120 hours of cultivation and 20 g/L substrate concentration of Napier grass. The highest ethanol content was obtained by 96 hours of simultaneous saccharification and fermentation (1.25 g l^{-1}) using 20 g l^{-1} substrate concentration and the maximum ethanol yield is 0.30 g/g cellulose conducted at 30 g l^{-1} substrate concentration and 96 hours of fermentation.

Keywords: Bioethanol, Enzyme Activity, Neurospora sitophila, Napier grass, Simultaneous saccharification, Fermentation

DOI: 10.5614.3bio.2020.2.2.3

1. Introduction

Napier grass (*Pennisetum purpureum* Schumach) is a species of perennial tropical grass with fast-growing characteristics and low input requirements (e.g., low nutrient, fertilizer, and water requirements). The annual productivity can reach about 78 tons of dry weight/ha [1].The Napier grass also contains high sugars, comprising 30-37% cellulose, 20-31% hemicellulose, and 8-21% lignin [2]. The high percentage of cellulose and hemicellulose with low lignin made it as an attractive raw material for bioethanol production. Moreover, Napier grass does not compete with other food crops therefore it is reducing the food versus energy competition.

Napier grass can be converted to bioethanol by conducting several steps include pretreatment, hydrolysis, and fermentation. The pretreatment aims to breakdown the chemical bonds of lignin and cellulose and removes the lignin. The pretreatment mainly involves chemical processing (alkali or acid pretreatment) to reduce the crystallization of lignocellulose and to increase the specific areas of cellulose for enhance the process of hydrolysis [3]. Hydrolysis can be conducted using either acid or enzymatic hydrolysis. Hydrolysis using acid is reportedly not friendly because in general, the process involves an explosion to high temperatures and can generate furfural compounds that can inhibit the fermentation process [4]. Recently, enzymatic hydrolysis is known as an environmentally friendly and safe method. The enzymatic hydrolysis can be performed in separate hydrolysis and fermentation (SHF) or in the process of simultaneous saccharification and fermentation (SSF). SHF process has the main advantage to perform the saccharification and fermentation under optimal condition. However, the degrading enzyme could be affected by accumulation of high glucose obtaining from saccharification process. Correspondingly, SSF technology was developed in recent years because it enables a reduction in investment costs, saves energy and achieves higher ethanol productivity by reducing the inhibition of end-products compared to separate hydrolysis and co-fermentation [5].

Numerous of filamentous fungi are known to degrade complex substrates of lignocellulose into various monomeric sugars and simultaneously convert it to bioethanol. With this promising characteristic, one of the filamentous fungi, *Neurospora* sp. has been used for bioethanol production. The fungus has ability to secrete enzyme for cellulose and hemicellulose hydrolysis as well as enzymes to produce bioethanol [6]. However, the enzyme activity and bioethanol production is proportional with some parameters such as substrate concentration and fungi cultivation period. This work is aimed to study effects of parameter and selecting the best condition for bioethanol production from Napier grass using *Neurospora* sp.

2. Materials and Methods

2.1. Microorganism N. sitophila.

Stock cultures were cultivated on dextrose potato agar at room temperature for 3 days. The resulting conidia (spore) suspension were then calculated in a haemocytometer and achieving about 10^6 spores per milliliter. Afterward the spore suspension was transferred into liquid media (Potato Dextrose Broth or PDB) by scraping the sporulating colonies and suspended in sterile distilled water containing 0.85% (v/v) Tween 80 as wetting agent and was incubated at room temperature for 3 days on a benchtop orbital shaking at 120 Rpm. The culture was used as inoculum for bioethanol and cellulase production in the submerged fermentation.

2.2. Napier grass (Pennisetum purpureum S.)

Napier grass seedlings were obtained from CV Bintang Tani, Bogor, West Java, Indonesia. Then Napier grass was cultivated in screen house at ITB Jatinangor and harvested with the growing age of 3 months. The grasses was washed and dried by shade drying. Dried Napier leaves was milled using a blender and then sheaved to obtain the particle size of +35/-60 mesh.

2.3. Neurospora minimal medium

The growth medium for *N.sitophila* under SSF process using its minimal medium which is recommended by National Collection of Industrial Microorganisms (NCIM) as optimum medium for initial revival or subculture [7]. The medium consisted of the following mineral medium:(in g L^{-1}) KH_2PO_4 2,.0; $NH4NO_3$ 2.0; $MgSO_4.7H_2O$ 0.5; NaCl 0.1; $CuCl_2$ 0.0001; $ZnCl_2$ 0.0002; $MnCl_2$ 0.00002; $FeCl_3$ 0.00002; $Na_2MoO_4.2H_2O$ 0.000002; H_3BO_3 0.000001; and biotin 0.000001, supplemented with 0.2% wt glucose. The pH was adjusted to 6.8-7.0.

2.4. Alkaline pretreatment methods

Twenty grams of Napier grass powder was pretreated in 400 ml of 1.5% wt NaOH solution (ratio 1:20) at room temperature for 120 hours. Subsequently, the treated Napier grass were filtered, washed, sterilized, and dried at 105°C [8] was subjected to further SSF process.

DOI: 10.5614.3bio.2020.2.2.3

2.5. Simultaneous saccharification and fermentation (SSF) using N. sitophila.

Simultaneous saccharification and fermentation was carried out in a 250 ml flask with various substrate concentrations of Napier grass (10 g l^{-1}; 20 g l^{-1} and 30 g l^{-1}). For 10 g l^{-1} of substrate concentration, one gram of pretreated Napier grass was put into the flask. Then, the substrate was mixed with 90 ml of *N.sitophila* (90% total volume). The mixture was sterilized in a water bath at 80 °C for an hour. After sterilization, 10 ml of PDB broth containing fungus suspension was inoculated aseptically to the flask, resulting 100 ml in total. The pH of each culture was adjusted until 6.8-7.0. The flask were enclosed by cotton plugged to release the carbon dioxide produced. The culture was shaken (130 Rpm) and incubated at room temperature for 72 h in aerobic condition. After 72 hours of cultivation, the agitation was stopped. The culture was harvested periodically over 24 h and analyzed for the fungus growth, substrate availability, enzyme activity and fermentation products. Each flask was harvested everyday to represent an experimental point. All treatments were conducted in triplicate.

2.6. Quantification of fungal biomass

The mycelial biomass of the fungal in the culture was harvested by filtration through filter paper, washed with stipulated hot water to separate the mycelia and the substrate, then dried at 105 °C overnight. The dried residues were weighted and recorded. Every flask was harvested to represent an experimental point. Specific growth rate and doubling time of fungal were determined in log phase.

The specific growth rate (μ) and doubling time were calculated according to the Eq. (1) and Eq.(2), respectively.

$$\mu = \frac{\ln Xt - \ln X_0}{t} \quad (1)$$

$$dt = \frac{\ln 2}{\mu} \quad (2)$$

Where X_0 denotes the initial biomass concentration after the elapsed time t and Xt is the biomass concentration after the elapsed time [9].

2.7. Analytical method

Lignocellulosic contents of Napier grass was analyzed according to the Chesson-Datta method [10]. SSF products (bioethanol, acecate, glucose and xylose) were analyzed using high performance liquid chromatography (HPLC) equipped with an ion moderated partition chromatography column, Aminex HPX-87H (Bio-Rad, CA). The HPLC was used in combination with a Waters (2414) refractive index detector. The flow rate of the mobile phase (5 mM of H_2SO_4) was adjusted at 0.6 ml/min and the temperature was set at 60 °C. The amount of reducing sugar released by saccharification and remained in the supernatant was determined by Dinitrosalicylic Acid (DNS) method [11]. Protein concentration was measured by the method of Bradford [12] using bovine serum albumin as a standard. Filter paper activity (FPA) was determined as described by Ghose [13]. The enzyme activity was expressed in international units (IU), defined as the amount of enzyme required producing 1 mmol of glucose per minute under the assay condition [14].

3. Result and discussion

3.1. Alkaline pretreatment for Napier grass

In this study, the lignocellulosic component of Napier grass before and after pretreatment with 1.5% (w/v) NaOH solution were determined (Figure 1). After the pretreatment process, lignin and hemicellulose content in Napier grass were decreased, however cellulose content was increased. The percentage of lignin removal in this study was 54.9%. It is clear that alkaline pretreatment aims to eliminate lignin to increase porosity and specific surface area and lead to an increase in the enzymatic digestibility of biomass. The extractive content of Napier grass was about 32.5% and increased to 38.63% after the pretreatment process. It means lignin removal have made the extractive is more accessible to the enzyme or microorganism. Cellulose content was also increased from 30.7% to 37% after the pretreatment process. This percentage of levitation occurs based on solvation and saphonication by alkaline catalysts, which induce depolymerization and division of lignin-carbohydrate linkages, resulting the substance that are not biodegradable are more accessible by extracellular enzyme [15]. In addition, these chemicals are able to break the ester bonds between lignin, hemicellulose and cellulose so that lignin and hemicellulose are concurrently removed [16].

3.2. Growth kinetics and substrate consumption of Neurospora sitophila

The growth of fungi in submerged fermentation can be determined by estimating the biomass dry weight concentration. In general, four phases in the growth of filamentous fungi have shown, namely, the lag phase, the log phase, the stationary phase, and the death phase. Figure 2 depicts the growth curve of fungi during cultivation with Napier grass. However, the fungal growth has increased significantly within 24 h of incubation thus the lag phase of *N.sitophila* can not be distinguished. According to Dewi [17], the adaptation or lag phase of fungi should be found at 12-16 hours of cultivation. After that, fungal growth reached

DOI: 10.5614.3bio.2020.2.2.3

the log phase in 16-24 h of cultivation and leaded to increasing biomass exponentially. Table 2 summarises the specific growth rate and doubling time of *N.sitophila* on various Napier grass substrate concentrations. In this study, specific growth rate of *N.sitophila* in 10 g l^{-1} of substrate concentration was 0.136 h^{-1}. Similar result was reported by Oguntimein [18] that obtained the specific growth rate of *Neurospora* sp on corn stover 1% (10 g l^{-1}) was about 0.14 h^{-1}. Thereafter, the biomass of fungal was decreased on 48 h of cultivation onwards. This phenomenon occurs because the availability of lignocellulose as a carbon source is depleted with increasing cultivation time. In addition, Figure 5 also shows reducing sugars in the medium was also decreased. This result shows nutrient depletion was attributed to growth restriction. This result was similar to other study [19] that showed biomass of fungal *Neurospora* sp decreased in three days of cultivation.

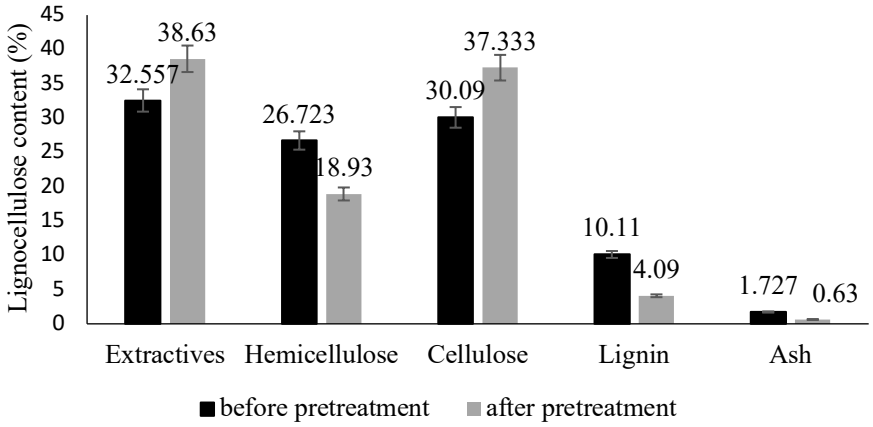


Figure 1 Composition of lignocellulose on Napier grass after pretreatment*

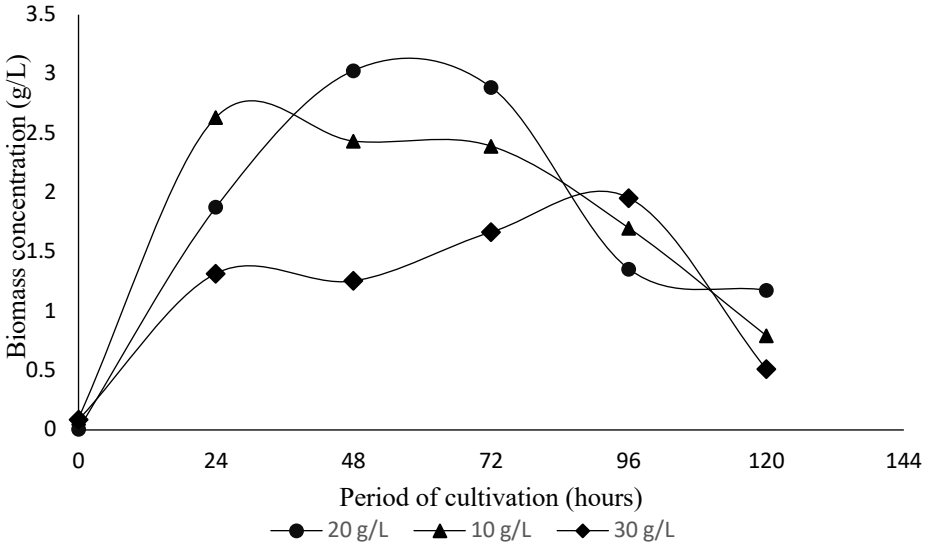


Figure 2 Growth curve of *N.sitophila* during simultaneous saccharification and fermentation in various substrate concentration of Napier grass

DOI: 10.5614.3bio.2020.2.2.3

N.sitophila grown best in 20 g l^{-1} of Napier grass as indicated by the highest rate of specific growth and the shortest hour of doubling time. Growth of *N.sitophila* on the substrate of 20 g l^{-1} was performed better than substrate concentration of 10 g l^{-1}. The increasing of initial substrate concentration induced cells to grow faster so the biomass concentration will also increase. However, *N.sitophila* on the substrate of 30 g l^{-1} having a slow growth rate compared to 20 g l^{-1} substrate concentration. This may be occured due to low oxygen transfer to fungi cells that are close contact with the substrate. As shown in Figure 3.a, the cell did not grow well since the presence of substrate restricted the oxygen transfer. Oxygen is the elementary components for substrate utilization. Hence, the limited supply of oxygen to a cell will lead to a slow growth rate [20].

Profile substrate component availability was reported in Figure 4. Based on the graph., it showed at 24 hours of cultivation, *N. sitophila* consumed simple sugars in extractives and at same time, it started to degrade the cellulose and hemicellulose by secreting hydrolytic enzyme [21]. As a consequence, after 24 hours, for the treatment using 10 g/L of Napier grass in the culture showed in a low content of sugar sources (extractives, hemicellulose and cellulose) hence the growth of cell also decreased. A different result obtained from the treatment using 20 g l^{-1} of Napier grass which was shown a high content of sugars and started to decreased gradually. For the treatment using 30 g l^{-1}, the substrate availability was gradually decreased and thereafter became stationer since the substrate components were depleted. However, there an error was detected at 72 hours of cultivation which shown an increase of cellulose content whereas the content of oteher components were decreased.

3.3. *Production of crude cellulase extract*

Profile of cellulase activity on various the Napier grass substrate concentrations were presented in Figure 5. Cellulase enzyme activity has been detected in 24 h of cultivation. Based on statistical analysis, there was not any significant difference ($p>0.05$) of enzyme activity recorded as varying the substrate concentrations. However, the enzyme activity at 20 g l-1 of Napier grass is greater than 10 g l-1 of substrate concentration. But, the enzyme activity was relatively declined on the culture with substrate concentration of 30 g l-1. Cheng and Anderson reported that substrate concentration is one of the main factors that affect the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results increases the yield and reaction rate of the hydrolysis [22]. However, this result showed that the enzyme activity at 30 g l-1 is lower than 20 g l-1

The fungal growth could be correlated with enzyme activity. This is might correlated with poor fungal growth which was observed on treatment with 30 g l-1 substrate concentration Napier grass in the culture. It seemed that the substrate was too compact lead to reduce surface contact area [21].

The highest enzyme activity on 10 g l-1 substrate concentration was 0.185 FPU/ml with 48 h of cultivation (Figure 6.a). Similarly, Oguntimein [18] also stated that the activity of cellulase enzyme produced by Neurospora on 1% corn stover and Chinese baggase was 0.15-0.18 FPU/ml. This activity also occurred in 40-60 hr of cultivation. The enzyme activity became stationer after 48-72 hours of cultivation. After 72 hours of cultivation, cellulose availability is low hence enzyme activity also was decreased. The growth of biomass also decreased and could cause factors in enzyme activity to decreased. A different result was obtained from 20 g l-1 substrate concentration. Figure 6.b shows the activity of cellulase has a stationary trend then peaked at the end of cultivation (120 hrs). When the sugar is present in high concentrations, it could inhibit cellulase performance. After the reducing sugars is started to depleted, the fungi will degrade hemicellulose and cellulose thus the availability of cellulose and hemicelluose in substrate was decreased (Figure 4). Generally, cellulase produced from substrate concentration of 20 g/L has greater stability than other treatments. At the end of cultivation, the cellulase activity remains high, approximately 0.28 FPU/ml. By increasing enzyme stability, the yield of products could be increased [23].

There was no salient increase of enzyme activity at 30 g l-1 substrate concentration even though the substrate concentration was the highest. This condition occurred because the fungi did not growth well on substrate concentration of 30 g l-1 compared to other treatments. When growth is not optimal, it will influence the performances of enzymes. Cellulase activity was reached its peak when 72 hours of cultivation thereafter decreased in the stationary phase of fungal growth. Enzyme activity was low also because the sugar concentration is high and may lead to repression of cellulase expression [24].

DOI: 10.5614.3bio.2020.2.2.3

(a) (b) (c)

Figure 3 Growth of *N.sitophila* in various Napier grass substrate concentrations (a) 10 g l^{-1} (b) 20 g l^{-1} (c) 30 g l^{-1}

Table 1 Specific growth rate and doubling time of *N. sitophila* in various Napier grass substrate concentrations

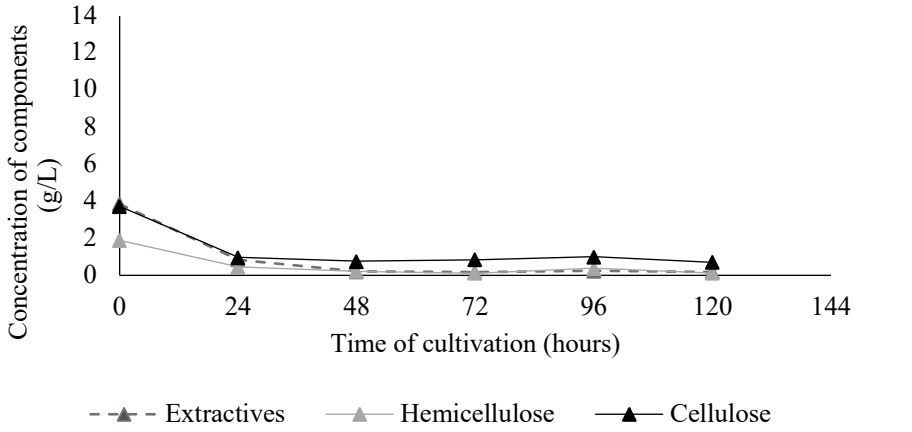
Treatments	Specific growth rate ($hours^{-1}$)	Doubling time (hours)
10 g l^{-1}	0.1363	5.0845
20 g l^{-1}	0.2274	3.0478
30 g l^{-1}	0.1107	6.2609

3.4. Ethanol Production

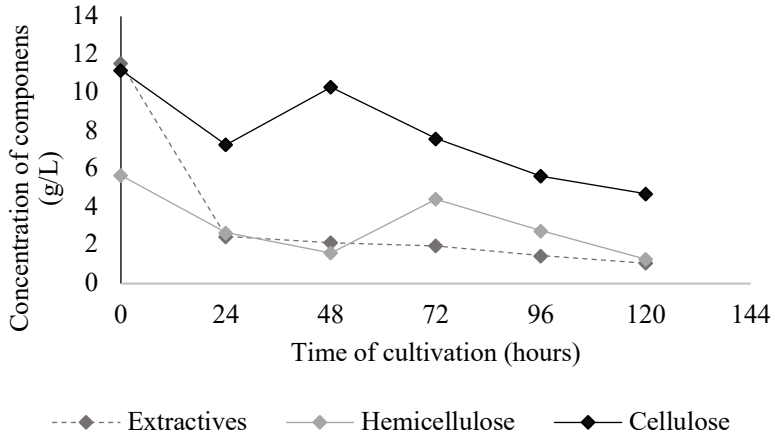
Neurospora sitophila is one of the few microorganisms that able to convert cellulose and hemicellulose into ethanol in two stages, by producing the required hydrolytic enzymes during aerobic growth stage and fermenting the produced simple sugars to ethanol in anaerobic or micro-aerobic conditions (fermentation stage). Ethanol was produced based on the Embden-Meyerhof glycolytic pathway with resulting 2 mol of ethanol being produced per a mol of glucose utilized [25]. Neurospora sp secreted alcohol dehydrogenase (ADH) which is an enzyme for ethanol production. Profile of bioethanol production on various Napier grass substrate concentrations was investigated in this study (Figure 7). The results of ethanol, reducing sugars, acetate concentration and ethanol yield from all treatments were presented in Table 2. These results indicate an increasing substrate concentrations will lead to an increasing concentration of ethanol that produced. Based on this study, it was found that ethanol was already produced in 24 hrs of cultivation in the fermentation of 10 g l-1 substrate concentration. It indicates that the ADH enzyme has performed to produce ethanol. However, also in this treatment, the ethanol concentration was decreased with increasing time cultivation. It seems the cells started to grow, the sugar availability was starting depleted to low concentration of 0.07 g l-1 (Table 2). The low concentration of sugars were not sufficient for fungi, and as a result, Neurospora sp utilized the ethanol produced as a carbon source for growth. Recent studies by Lin [26] and Dewi [17] also observed that under some conditions, Neurospora sp can utilize and metabolize the produced ethanol for growth. Alcohol dehydrogenase 1 gene (adh-1) is reported to be responsible for ethanol degradation while the adh-3 enzyme is responsible for ethanol synthesis [27].

Ethanol produced by Neurospora sp on substrate concentration of 20 g l-1 in 48 h of cultivation was 0.189 g l-1. There was an increase in ethanol concentration as time cultivation increased because reducing sugar concentrations were remained in high value. This happens during the process of lignocellulose degradation leads to sugar availability to produce metabolites such as ethanol. Whereas, the highest ethanol was achieved on substrate concentration of 30 g l-1. It reached about 1.25 g l-1 of ethanol at 96 hours of cultivation. However, there was a decline in ethanol concentration at 120 hrs of cultivation. This decrease might be caused by the formation of acetate, resulting in lower yield of ethanol. A similar result was obtained from the other study [17] which indicates that the formation of acetate was a simultaneous phenomena with ethanol reduction.

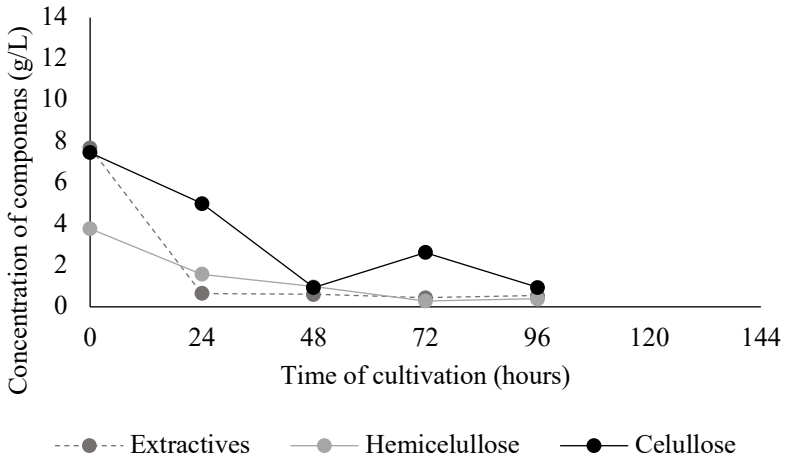
DOI: 10.5614.3bio.2020.2.2.3



(a)



(b)



(c)

Figure 4 Lignocellulosic components of Napier grass after simultaneous saccharification and fermentation a) 10 g l^{-1} b) 20 g l^{-1} c) 30 g l^{-1}

DOI: 10.5614.3bio.2020.2.2.3

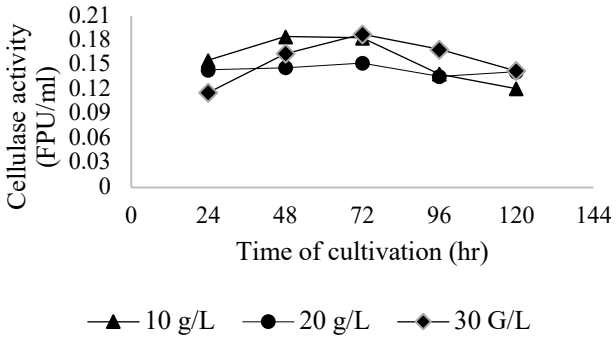
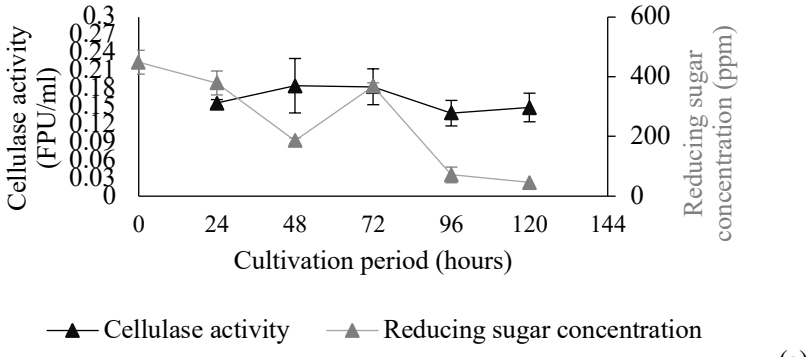
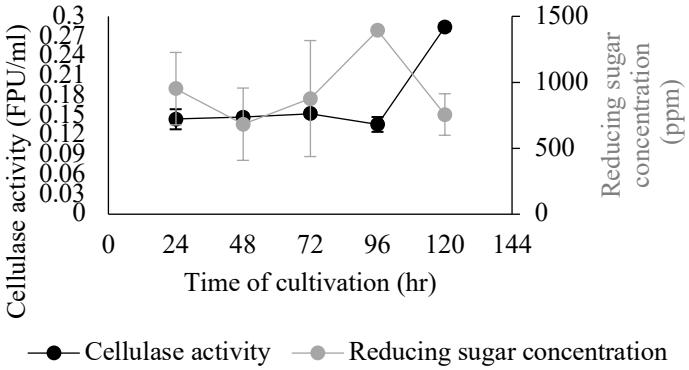


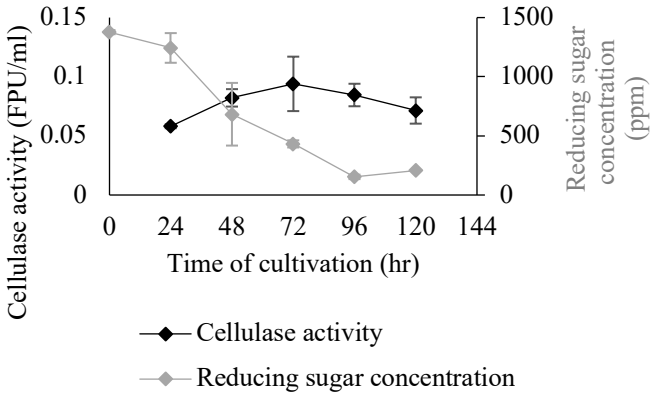
Figure 5 Profile cellulase activity in various Napier grass substrates concentration



(a)



(b)



(c)

Figure 6 Profile cellulase activity and reducing sugar concentration from simultaneous saccharificaton and fermentation using various Napier grass substrate concentration a) 10 g l^{-1} b) 20 g l^{-1} c)30 g l^{-1}

DOI: 10.5614.3bio.2020.2.2.3

Table 2 Reducing sugars concentration, ethanol concentration, ethanol yield and acetate concentration in various Napier grass substrate concentrations and time of cultivation

Substrate concentrations	Cultivation period (hrs)	Reducing sugars concentration (g l^{-1})	Ethanol concentrations (g l^{-1})	Ethanol yield (g ethanol/g cellulose)	Acetate concentrations (g l^{-1})
10 g l^{-1}	24	0.38	0.42	0.15	0
	48	0.17	0.17	0.06	0
	72	0.40	0.29	0.10	0
	96	0.07	0.19	0.07	0
	144	0.05	0.21	0.07	0
20 g l^{-1}	24	0.95	0	0	0.23
	48	0.68	0.19	0.02	0.01
	72	0.88	0.73	0.09	0.84
	96	1.39	0.53	0.08	0.06
	144	0.76	0.85	0.12	0.09
30 g l^{-1}	24	1.24	0	0	0.16
	48	0.68	0.99	0.23	0
	72	0.43	0	0	0
	96	0.15	1.25	0.30	0
	144	0.20	1.19	0.22	0.02

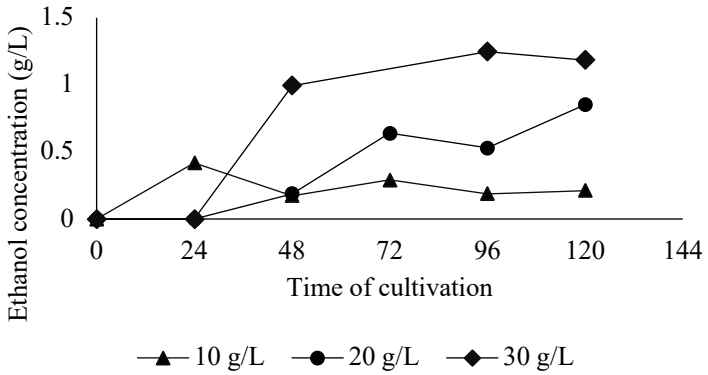


Figure 7 Profile of ethanol accumulation in various Napier grass substrate concentrations

This study showed that ethanol accumulation was maximum at the stationary phase. This result also was reported by Colvin [26]. Ethanol produced in this study still has a low value in comparison to other studies. Colvin reported that accumulation ethanol by Neurospora sp can reach 4.5 g/L. Dewi also observed that the highest ethanol produced using Neurospora sp on leaves of vetiver grass was 4.5 g/L within 48 h of cultivation. This is could be correlated that the culture was still containing oxygen. The headspace still containing 60% of void space since the working volume is 40% of total volume. This indicates the bioreactor headspace is occupied by air and could enhance enhancing oxygen mass transfer to a culture. Eakin, R. T., & Mitchell reported a wild type strain of Neurospora has consumed oxygen at rate of 66 mmol of oxygen/minutes [28]. Based on theoretical calculation, during the cultivation time, the oxygen in the culture remain high and have not reach microaerobic condition. As a result, the fermentaion process was inhbited. This phenomenon is probably related to Pasteur effect. The Pasteur effect, the inhibition of fermentation by respiration, may be operative in Neurospora sp [25].

The ethanol yield from all treatments are shown in Table 2. Based on the result, the highest ethanol yield was 0.30 g/g cellulose conducted by 96 hours of fermentation. This value was lower than the study of Ingram and Doran [29], that reported ethanol yield could reach 0.30 g/g cellulose based on lignocellulose material. Low ethanol yield indicates the

DOI: 10.5614.3bio.2020.2.2.3

substrate that consumed is not fully used for ethanol production. Substrate utilization for cell maintenance was not led to biomass formation [9]. Fermentation products and substrate analyzed in HPLC and the chromatograph had shown in Figure 8.

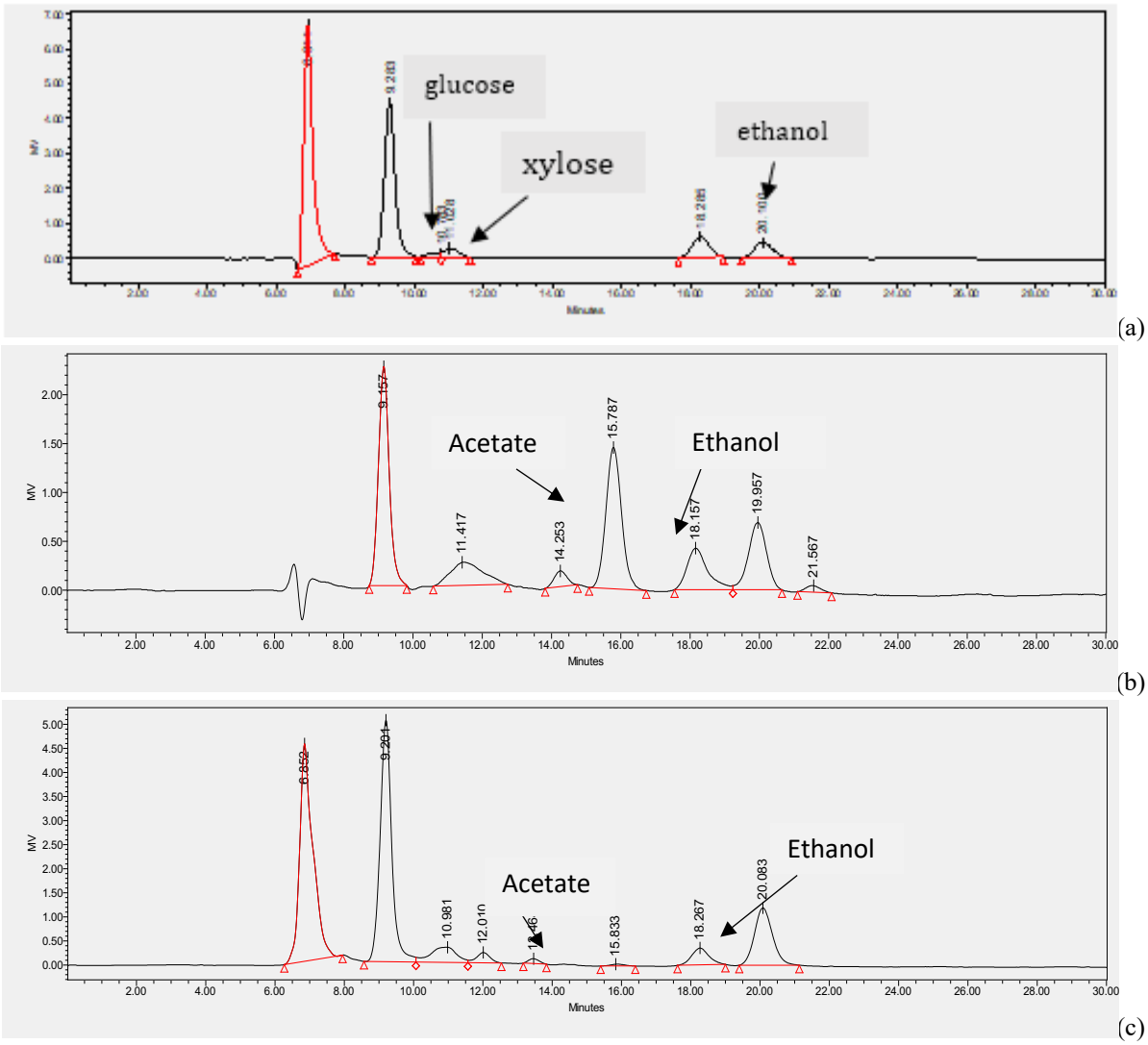


Figure 8 Chromatograph of fermentation samples using various substrate concentrations of Napier grass a) 10 g l^{-1} b) 20 g l^{-1} c) 30 g l^{-1}

However, there is a room for further improvement by optimizing the microaerobic condition for bioethanol production. In addition, it is also possible for using Neurospora sp and yeast simultaneously for enzyme production and obtaining bioethanol production.

At T_1 and T_2, the diversity index value in the treatment does not have a certain trend. Spraying herbicide may not have a significant effect on the species diversity index at the time of T_2. But on T_3 (before harvest), the diversity index value in all treatment groups experienced an increase (Figure 4).

4. Conclusion

The highest cellulase enzyme activity produced by *N. sitophila* is 0.28 FPU/ml occurred at 120 hours of cultivation and 20 g l^{-1} substrate concentration. However, the ethanol yield was 0.30 g/g cellulose, which achieved at 30 g l^{-1} substrate concentration and by 96 hours of fermentation. Napier grass could serve as a good substrate for production of value-added products such as crude extract cellulase enzyme and bioethanol through simultaneous saccharification and fermentation using *N. sitophila*.

DOI: 10.5614.3bio.2020.2.2.3

References

[1] Negawo, A. T., Teshome, A., Kumar, A., Hanson, J., & Jones, C. S.. Opportunities for Napier grass (Pennisetum purpureum) improvement using molecular genetics. *Agronomy*, 2017; 7(2): 28.

[2] Yasuda, M., Ishii, Y., & Ohta, K.. Napier grass (Pennisetum purpureum Schumach) as raw material for bioethanol production: pretreatment, saccharification, and fermentation. *Biotechnology and Bioprocess Engineering: BBE*, 2014; *19*(6): 943

[3] Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., & Ladisch, M.. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, 2005; *96*(6): 673-686.

[4] Pandey, A.; Soccol, C. R.; Nigam, P.; Soccol, VT.; Vandenberghe, L. P. S.; Mohan, R. Biotechnological potential of agro-industrial residues. II: cassava bagasse. *Bioresource Technology*, 2000; 74 (1): 81-87

[5] Luo, P., & Liu, Z. Bioethanol production based on simultaneous saccharification and fermentation of wheat straw. In *2010 International Conference on Challenges in Environmental Science and Computer Engineering* 2010, March; (pp. 48-51). IEEE.

[6] Dogaris, I., Mamma, D., & Kekos, D.. Biotechnological production of ethanol from renewable resources by Neurospora crassa: an alternative to conventional yeast fermentations?. *Applied microbiology and biotechnology*, 2013; *97*(4), 1457-1473.

[7] Medium V 4.0 *Media Formulations* 195–216

[8] Selig, M.J., Todd B. Vinzant, T.B., Himmel, M.E. and Decker, S.R... The effect of lignin removal by alkaline peroxide pretreatment on the susceptibility of corn stover to purified cellulolytic and xylanolytic enzymes, *Appl Biochem Biotechnol* 2009.

[9] Nielsen, J., Villadsen, J., & Keshavarz-Moore, E.. Bioreaction engineering principles. *Trends inBiotechnology*, 1995; *13*(4): 156.

[10] Chesson, A. & Datta, R.. Acidogenic Fermentation of Lignocellulose-Acid Yield and Conversion of Components. *J. Biotechnology and Bioengineering,* Vol. XXIII, 1981; Pp. 2167

[11] Miller, G.. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 1958.

[12] Kruger, N. J.. The Bradford method for protein quantitation. *The protein protocols handbook* 2002; (pp. 15-21). Humana Press.

[13] Mandels M, Andreotti R, Roche C Measurement of saccharifying cellulase. *Biotechnol Bioeng Symp* 1976; 6: 21-33.

[14] Ghose, T. K.. Measurement of cellulase activities. *Pure and Applied Chemistry*, 1987; *59*(2): 257-268.

[15] Rollin, J. A., Zhu, Z., Sathitsuksanoh, N., & Zhang, Y. H. P. . Increasing cellulose accessibility is more important than removing lignin: A comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. *Biotechnology and Bioengineering*, 2011; *108*(1): 22-30.

[16] Gáspár M, Kálmán G, Réczey K. Corn fiber as a raw material for hemicellulose and ethanol production. *Process Biochem* 2007; 42:1135–1139

[17] Dewi, Arinta.. *Pengaruh Temperatur terhadap Proses Biokonversi Lignoselulosa Daun Akar Wangi* (Vetiveria zizanioides L. Nash) *Menjadi Bioetanol Menggunakan* Neurospora sp. 2016; Skripsi Sarjana, Rekayasa Hayati, Institut Teknologi Bandung.

[18] Oguntimein, G., Vlach, D., & Moo-Young, M.. Production of cellulolytic enzymes by *Neurospora sitophila* grown on cellulosic materials. *Bioresource Technology*, 1992; *39*(3): 277-283.

[19] Li, Y., Peng, X., & Chen, H.. Comparative characterization of proteins secreted by *Neurospora sitophila* in solid-state and submerged fermentation. *Journal of Bioscience and Bioengineering*, 2013; *116*(4): 493-498.

[20] Augustine, A., Imelda, J., & Paulraj, R.. Biomass estimation of Aspergillus niger S, 4 a mangrove fungal isolate and A. oryzae NCIM 1212 in solid-state fermentation. *Journal of the Marine Biological Association of India*, 2006; *48*(2): 139-146.

[21] Dogaris, I., Vakontios, G., Kalogeris, E., Mamma, D., Kekos, D.. Induction of cellulases and hemicellulases from Neurospora crassa under solid-state cultivation for bioconversion of sorghum bagasse into ethanol. *Industrial crops and products* 2009; 29.

[22] Cheng, S.W. and Anderson, B.C.. Investigation of ethanol production from municipal primary waste water. *Bioresource Technology*, 1997; 59: 81-96.

[23] Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R.. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology*, 2007; *40*(6): 1451-1463.

[24] Zhang, Y., Tang, B., & Du, G.. Production of cellulases by *Rhizopus stolonifer* from glucose-containing media based on the regulation of transcriptional regulator cre. *Journal of Microbiology and Biotechnology*, 2017; *27*(3): 514-523.

DOI: 10.5614.3bio.2020.2.2.3

[25] Colvin, H. J., Sauer, B. L., & Munkres, K. D.. Glucose utilization and ethanolic fermentation by wild type and extrachromosomal mutants of *Neurospora crassa*. *Journal of Bacteriology*, 1973; *116*(3): 1322-1328.

[26] Lin H, Warmack RA, Han S , Kasuga T, Fan Z.. Alcohol consumption and tolerance of *Neurospora crassa*. *Ferment Technol* 2016; 5:136.

[27] Xie, X., Wilkinson, H. H., Correa, A., Lewis, Z. A., Bell-Pedersen, D., & Ebbole, D. J.. Transcriptional response to glucose starvation and functional analysis of a glucose transporter of *Neurospora crassa*. *Fungal genetics and biology*, 2004; *41*(12): 1104-1119.

[28] Eakin, R. T., & Mitchell, H. K.. Alterations of the respiratory system of *Neurospora crassa* by the mi-1 mutation. *Journal of bacteriology*, 1970; *104*(1): 74-78.

[29] Ingram, L. O., & Doran, J. B.. Conversion of cellulosic materials to ethanol. *FEMS Microbiology Reviews*, 1995; *16*(2-3): 235-241.

DOI: 10.5614.3bio.2020.2.2.3