CELLULASE ENZYME PRODUCTION BY Trichoderma reesei THROUGH A SOLID SUBSTRATE FERMENTATION PROCESS

by Sri Wahyu Murni

Proceeding of the 1st ACIKITA International Conference of Science and Technology (AICST), 2011
ISBN No. 978 979 16415 9 3

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Sri Wahyu Murni dan Siti Diyar Kholisoh

Chemical Engineering Departement, FTI, UPN "Veteran" Yogyakarta, SWK 104 Street (North Ring Road), Condong Catur Yogyakarta-55283, Indonesia

E-mail: wahyuswm@yahoo.com, diyar.kholisoh@upnyk.ac.id

ABSTRACT

Shortage of energy is one of the serious problems facing the world today. Ethanol produced from vegetable materials is one of the most prospective alternative energy to reduce the dependence on fuel oil. Lignocellulosic biomass has enormous potential to be processed into ethanol. The main inhibitor in the enzymatic saccharification of biomass for ethanol production is costly cellulase enzymes. Cellulase production costs can be reduced by various approaches, for instance, using cheaper lignocellulosic biomass substrate for fermentation and applying more efficient fermentation strategies, such as solid state fermentation. (SSF). In this research, cellulase was produces by using a solid culture of Trichoderma reesei FNCC 6012. Enzyme production was conducted in Erlenmeyer flask and rotary drum fermenter (RDF). The results showed that the cellulase activity on rice straw substrate was higher than the mahogany sawdust. Moisture content influenced on the activity of enzymes produced. The best results obtained on rice straw substrate at 60% moisture content and 6 days fermentation, with the activity of CMC-ase and FP-ase respectively 0.00584 and 0.00318 U / ml respectivelly. The best result on mahogany sawdust was achieved at 70% moisture content and 7 days fermentation, with CMC-ase and FP-ase activity of 0.00434 and 0.00125 U/ml respectivelly. Enzymes productivity of in RDF was observed better than the flasks. It was obtained in RDF that CMC-ase and FP-ase activity was 0.00645 and 0.00334 U/ml, for rice straw substrate, and 0.00579 and 0.00302 U/ml for mahogany sawdust.

***Keywords*: Enzyme activity, fermentation, solid subtrate, cellulase, cellulose**

INTRODUCTION

Ethanol production from lignocellulosic biomass is one of the most important technologies for the sustainability of fuels production for transportation. Ethanol has higher octane number and produces less emissions than gasoline. Therefore, it is recommended as a substitute ingredient or additive on gasoline. (Sukumuran (2009)). According to these advantages, nowadays there is an increase on commercialization of technology for ethanol production from cheap biomass, including its role as a renewable alternative fuel. Ethanol production from lignocellulosic biomass includes the following steps: pretreatment, hydrolysis (saccharification), and ethanol fermentation. Hydrolysis of biomass is an important step to produce sugar which therefore it can be converted into ethanol by microbes. Two

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methods of hydrolysis (namely: acid and enzyme hydrolysis) was developed. Efficiency of this process strongly depends on the conditions of pretreatment, type of biomass, and the hydrolyzing agent. Acid hydrolysis has the disadvantage such as the acidic waste produced and the difficulty in separating the sugars.. Enzymatic method has some advantages as follows: it can be performed at room conditions and there is no toxic waste (acid waste) produced. However, commercialization of ethanol production from lignocellulosic materials is hampered by the costly manufacture of cellulase used for saccharification.

Cellulase production cost can reduced by optimizing several aspects that influence on enzyme production, from raw materials selection and development of microbial strains. The use of cheaper raw materials and effective fermentation strategy, such as fermentation solid culture, may increase the economic aspects of cellulase production. This research was aims to produce cellulase enzymes that have high activity through a batch fermentation of solid culture medium using *Trichoderma reesei* and some cellulose substrates, namely rice straw and sawdust mahogany. The moisture content of substrate was varied in the range 50-70%.

Lignocellulosic materials

Lignocellulosic materials have three main different kinds of polymers as follows: lignin, hemicellulose, and cellulose, which bind each other to form one unified structure. The ability of materials to be degraded is strongly influenced by chemical composition of them. Composition of each component depends on the type and age of biomass, and environmental conditions where biomass is grown, as well. Rice straw has a cellulose content of 32-47%, hemicelluloses (especially polymers of xylos) of 19-27%, and lignin of 5-24% (Binod et. Al, 2009). According to Bailey and Ollis (1986), hard wood consists of 40-55% cellulose, 24-40% hemicellulose, and 18-25% lignin. Cellulose $(C_6H_{10}O_5)_n$ is a major component of lignocellulose in the form of microfibril-macrofibril of homopolysaccharides and consists of 1,4-β-D-glucopyranose units. Cellulosa consists of crystalline the amorphous structure. The cristalinity determines its ability on both chemical and enzymatic hydrolysis.

Cellulase Enzyme

Cellulase can be categorized in a class of hydrolase enzymes. There are 3 (three) main kinds of cellulase produced by fungus, namely: (1) endoglucanase, hydrolyzes cellulose randomly into oligosaccharides with various chain lengths, (2) cellobiohydrolase, break down cellulose from its non-reducing ends and releases cellobiose, (3) β-glucosidase (BGL), breaks down cellobiose to produce glucose. (Ward, 1985)

Studies on cellulase are widely performed throughout the world. Cellulase has a lot of benefits, particularly converting lignocellulosic materials or hydrolyzing cellulose into glucose. Nowadays cellulase is widely used in cereal processing, brewing, alcohol production, extraction plants, fruit processing, beer industry, and waste processing. In alcohol fermentation, cellulose used in degradation of starch can increase the yield of alcohol. Production of sugar from cellulosic materials also requires cellulase. Several types of cellulase-producing microbes such as: *Aspergillus niger, Trichoderma reesei, Penicillium funiculosum, and Rhizopus sp*. In addition, cellulase is also produced by *Clostridium thermocellum, Sporotrichum celluphilum, Thielavia*

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terrestris, Thermoascus aurantiacus, Scytaladium lignicola, Alternaria alternata, Cellumonas uda, Aspergillus ustus, A. fumigates, A. aculeatus, and Dhicomitus squalens (Frost and Moss, 1987). According to ward (1985), cellulase produced by *Trichoderma reesei* work most effectively on the crystalline cellulose. Such cellulase is resistant to chemicals inhibitors and stable when performed in a stirred reactor at pH 4.8 and temperature of 50°C for 48 hours.

MATERIAL AND METHOD

Mikroorganism and Inoculums Preparation.

A laboratory strain of *Trichoderma reesei FNCC 6012* obtained from Pusat Antar Universitas (PAU) *Pangan dan Gizi* University of Gadjah Mada (UGM), Yogyakarta was used. The stock culture of fungus was grown on potato dextrose slants agar and sub-cultured for 5 days. For preparation of fungal inoculation, about 2 ml of sterile distilled water containing 0,1% Tween 80 was introduced into spore by using a pipette. The spore-suspensions of *T. reesei* containing ~10^7 spores/ml was used as inoculum.

Mineral Salt Medium for Fermentation

The mineral salt medium used for cellulase productions consisted of (in g/L): urea, 0.3; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; peptone, 0.75; yeast extract, 0.25;$CaCl_2.2H_2O$, 0.4; and trace elements: $FeSO_4.7H_2O$, 0.005; $MnSO_4.7H_2O$, 0.0016, $ZnSO_4.7H_2O$, 0.0014; and $CoCl_2$, 0.002.

Pretreatment of Raw Materials

Rice straw and mahogany sawdust as substrates were dried at 70°C in an oven to remove their moisture. Feed stock was therefore milled into 20 mesh size. Sample were treated by 0,1 N NaOH solution for 1 hour at 120°C in a pressure cooker. After cooling, the samples were washed several times by distilled water to neutralize the pH and therefore dried at 80°C. This pretreated was aimed to remove lignin in the material (delignification), hence increasing the ability of the surface area (porosity) of cellulose in the substrate. (Silvi et. al., 2011)

Enzyme Production in Flasks

In the Erlenmeyer flask, 5 g substrate (rice straw and mahagony sawdust, respectively) was moistened by mineral salt medium until moisture content of 50%, 60%, and 70% respectively. Therefore 1 ml spores suspension of *T. reesei* was inoculated into flask. The mixture was stirred uniformly and incubated at room temperature. After incubation within 7 days, enzyme was extracted using 50 ml of 0.1 N citrate buffer (pH 4.8). The extract was therefore centrifuged at 10,000 rpm for 10 min at 4°C and used as crude enzyme samples.

Fermentation in Rotary Drum Fermenter (RDF)

Cellulase production in RDF was conducted by using the same substrate and mineral salt medium as previous stage (in flasks), but at its best condition. Experimental conditions at each run are

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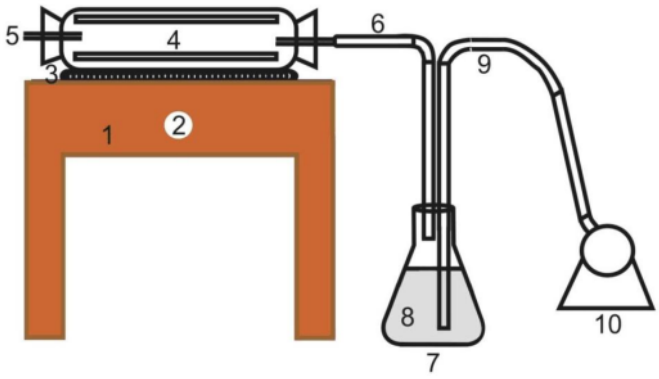
presented in Table 1. Fermentation was performed aerobically with aeration rate 384 ml /min of sterile air/min and rotor rotation s[eed of 10.5 rpm.

Table 1. Condition for fermentation in the RDF

Substrate	Rice straw	awdust from mahogany wood
Substrate weight (g)	20	40
Moisture (%)	60	70
Volume of inokulum (ml)	4	8
Concentration of spora (spora/ml)	4×10^7	$4{,}8 \times 10^7$
Fermentation time (day)	6	7
Volume of buffer added (ml)	200	400
Volume of enzyme extract (ml)	132	315

Equipment

The main equipment used to produce cellulase in this study was a rotary drum fermenter (RDF) illustrated in Figure 1. The RDF has specification as follows: length = 24 cm, inside diameter = 9 cm, outside diameter = 11.5 cm, the number of baffles = 4 , baffles length = 19 cm and baffle width = 1.5 cm.



Remark:

1&2	:	Desk & rotation electric switch	6&9	:	Pipe for introducing air into RDF
3	:	rotor	7&8	:	Flask containing H_2SO_4 solution
4	:	Fermenter (RDF)	10	:	Pump
5	:	Ole for releasing air			

Figure 1. Rotary drum fermenter (RDF)

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Analitycal Methods

Cellulase activities were evaluated through the Filter Paper Activity (filter paper assay, FP-ase) and Carboxy Methyl Cellulose assay (CMC-ase) tests, using the method of Ghose (1987). One unit of cellulase activity is proportional to 1 µmol substrate hydrolyzed per minute. Measurement of reducing sugar content was conducted by using DNS (dinitro salicylic acid) methods (Miller, 1959).

RESULT AND DISCUSSION

Effect of Moisture Content on Cellulase Activity

This research investigated that the type of substrate and moisture content affected enzyme activity. Cellulase is a sort of extracellular inductive enzyme. In this study cellulose was synthetized by *T. reesei* FNCC 6021 and induced by two different substrates. Rrice straw and mahogany sawdust were used as a substrate and inducer as well. Effect of substrate moisture content (50%, 60% and 70%) on cellulose activity produced by *Trichoderma reesei* FNCC 6012 is presented in Figure 2 and 3. The highest cellulose activity for rice straw and mahagony sawdust was obtained atb60% and 70% moisture content, respectively. They were achieved at 6 and 7 days fermentation, respectively. In addition, it was observed that cellulose activity measured by CMC was higher than filter paper (FP).

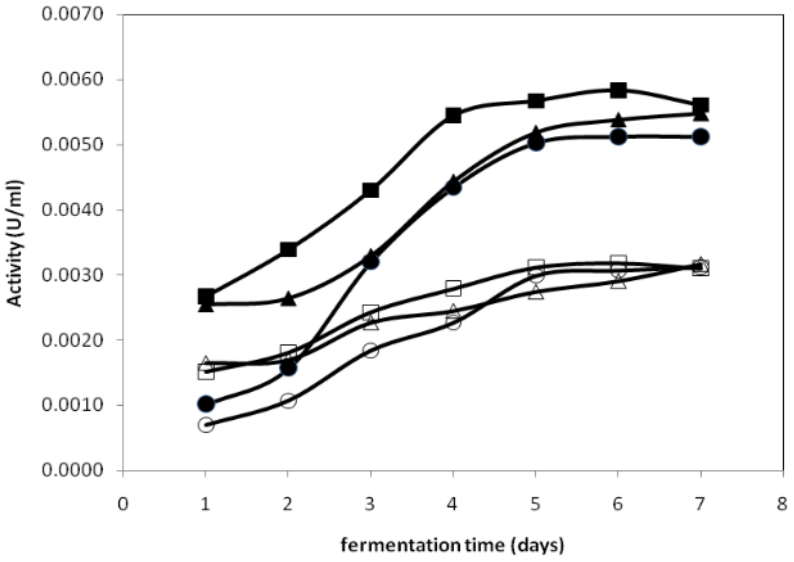

Figure. 2. Effect of moisture content on cellulase activity, for rice straw; ● CMC-ase (50%); ■ CMC-ase (60%); ▲ CMC-ase (70%); ○ FP-ase (50%); o FP-ase (60%); Δ FP-ase (70%)

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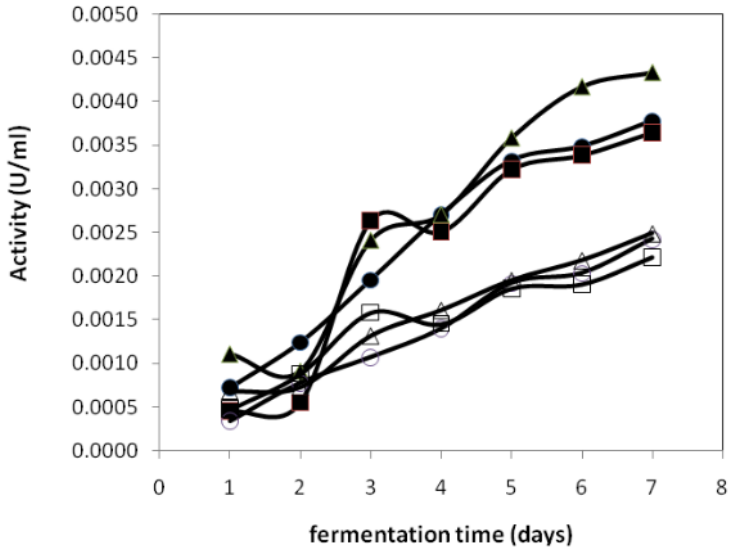

Figure 3. Effect of moisture content on cellulase, activity, for mahogany sawdust; ● CMC-ase (50%); ■ CMC ase (60%); ▲ CMC-ase (70%); ○ FP-ase (50%); o FP-ase (60%); Δ FP-ase (70%)

Rice staw has alower density and ahigher porosity than mahagony sawdust. It could be observed from the wight of substrate introduced to RDF. More mahagony sawdust was needed to ferment in the RDF than rice straw. Therefore, rice straw was relativlely easier to be moistened by mineral salt solution. This fact caused mahagony sawdust needed higher moisture content and longer fermentation than rice straw to produce cellulose at its highest activity. Besides, this moist substrate condition is actually a pre-requiement for substrate utilization tobe used in solid-state fermentation. Generally fungus can grow very well on the moist solid-substrate.

Effect of Type of Substrate on Cellulase Activity

Figure 4. shows the profile cellulase activity produced by *T. reesei* FNCC 6012 at different substrates. Cellulase activity produced on rice straw was higher than mahogany sawdust, both for CMC-ase and FP-ase. This was supported by a fact that the enzyme extract from rice straw contained higher reducing sugar. Higher content of reducing sugar indicated that more substrates converted into reducing sugar by T. reesei FNCC 6012.

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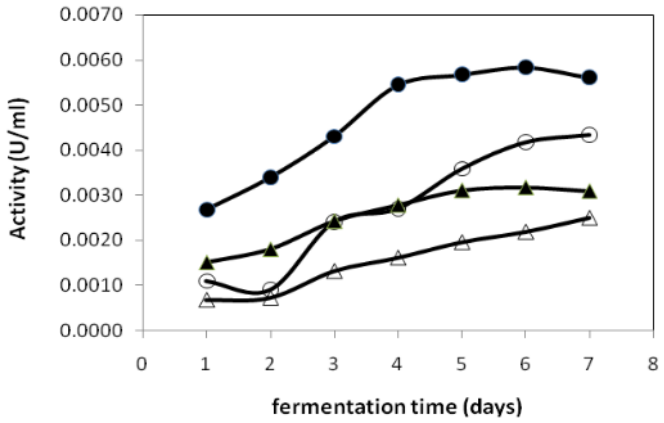

Figure 4. CMC-ase and FP-ase activity on rice straw: ● CMC-ase (60%); ▲ FP-ase (60%), and mahogany sawdust: ○ CMC-ase (70%); Δ FP-ase (70%)

Reducing Sugar Content in the Fermentation Medium

Profile of reducing sugar concentration during is presented in Figure 5 and 6. Reducing sugar produced by *T. reesei* simultaneously these microbes broke down cellulose from rice straw and mahogany sawdust. In this study, there was afact that the concentration of reducing sugar increased during fermentation. More microbe cells grew up at longer fermentation, therefore more enzymes was produced,and more cellulose was consequently hydrolyzed into reducing sugars.

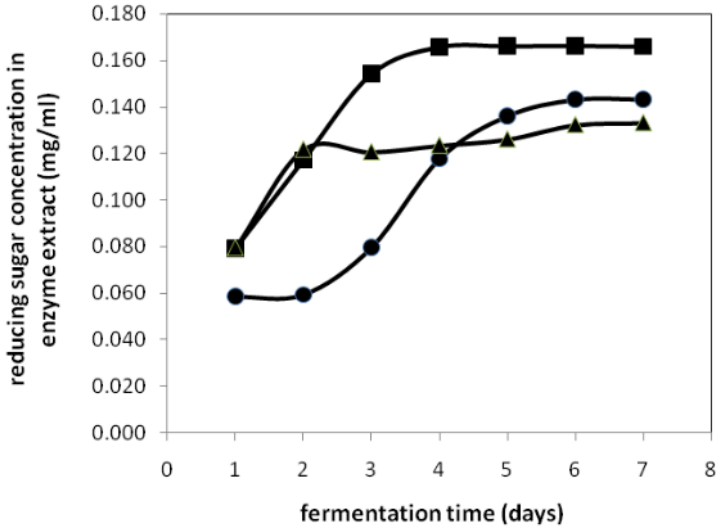

Figure 5. Profile of reducing sugar concentration in rice straw at different water content (%): ● (50); ■ (60); ▲ C (70)

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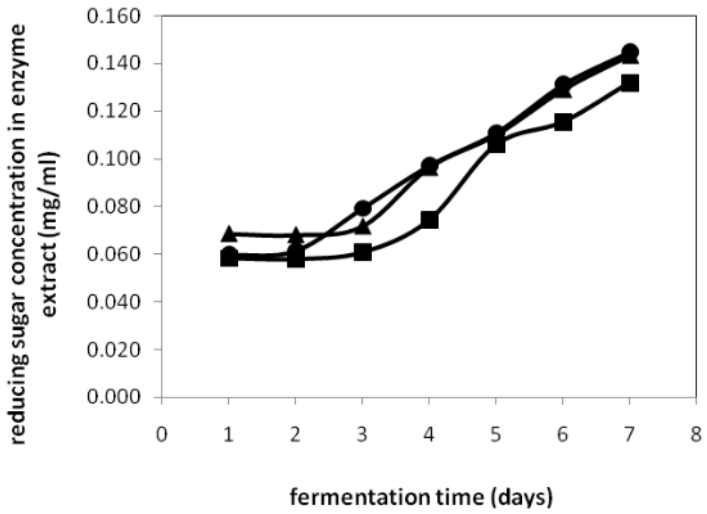

Figure 6. Profile of reducing sugar concentration Ion mahagony sawdust at different water content (%): ● (50); ■ (60); ▲ C (70)

As illustrated in Figure 5, sugar concentration yielded for the rice straw indicated a rapid increase until about 3 days fermentation time at the moisture content of 60 and 70%. The sugar content was therefore relatively constant after 3 days. However, at moisture content of 50% sugar concentration increased slightly during the first 2 days fermentation. Furthrmore, it increased sharply at 2-5 days fermentation. Thus, it might take longer time to break down cellulose in the substrate at lower moisture content. For mahagony sawdust (Figure 6), sugar concentration increased very slightly during the first 2 or 3 days fermentation and therefore increased gradually after it. Generally,it could be observed that mahagony sawdust took longer time to degrade compared to rice straw. This is enabled by mahagony wood is a type of biomass that has higher lignin content compared to rice straw (Silvi, 2011).

Cellulase Enzyme Activity in Rotary Drum fermenter (RDF)

Table 2. presents the comparison between cellulase activity produced through fermentation in flasks and RDF. Cellulase produced in RDF showed higher activity compared to the flasks, for both CMC-ase and FP-ase. It was caused by the RDF was well-aerated therefore the supply of oxygen or air for the growth of *T. reesei* was sufficient.

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Tabel 2. Comparison of cellulase activities produced in the flask and RDF

Substrate	Erlenmeyerf lask		RDF	
	CMC-ase (U/ml)	FP-ase (U/ml)	CMC-ase (U/ml)	FP-ase (U/ml)
Rice straw	0,00584	0,00318	0,00645	0,00334
mahogany wood	0,00434	0,00125	0,00579	0,00302

Additionally, the rotation of drum fermenter with a relatively low speed resulted in better growing of *T. reesei* on most of the substrate surface, notmerely grew on the top surface of the substrate. This rotating of drum had a role to increase the surface area or effective contacts between fungus and substrate. At the same time, it also served to increase the effectiveness of aeration.

RDF was operated at room temperature, atmospheric pressure, aeration rate 384 ml/min, and rotor rotation speed of 10.5 rpm. As presented in Table 2, cellulase activities obtained at these conditions were still relatively very low (i.e. in the range from 0.00125 to 0.00334 U/ml for FP-ase, and from 0.00434 to 0.00645 U/ml for CMC-ase). For subsequent studies, it is strongly recommended to increase the cellulose activity produced, through optimization of process conditions (such as aeration rate and drum rotation speed), improvement of the delignification process of the substrate used, optimization in the design of RDF , searching for superior fungal strains to produce cellulase, and isolation and concentration of crude enzyme, as well

CONCLUTIONS

Cellulase could be produced by *T. reesei* FNCC 6012 using rice straw and mahagony sawdust substrate that initially treated by using natrium hydroxide solution.. Enzyme activity measured as CMC-ase was higher than FP-ase. Enzyme activity observed on rice straw was higher than mahogany sawdust. Moisture-content of substrate influenced on the productivity of cellulose. For rice straw substrate, the highest productivity was achieved at 60% moisture content and 6 days fermentation with CMC-ase and FP-ase activity of 0.00584 and 0.00318 U/ml, respectivelly. The highest cellulase activity on mahogany sawdust (CMC-ase and FP-ase 0.00434 and 0.00125 U/ml, respectively) was obtained at 7% moisture content and 7 days fermentation.

Acknowledgement
We thank *Lembaga Penelitian dan Pengabdian kepada Masyarakat* (LPPM) UPN "Veteran" Yogyakarta for financial support (with the agreement number: B/65A/V/2010/LPPM. We also thank Handrian and and Yusvita Primadani Putri for performing the laboratory work and collecting data.

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