J.Kim.Terap.Indones. 20(1),
pp. 42–48, August 2018

e-ISSN:2527-7669

The Evaluation of Substrates and *Trichoderma sp.* Isolates for Cellulase Production

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ARTICLE INFO

Article history:

Received date :25 June 2018

Revised date : 25 July 2018

Accepted date: 8 August 2018

Available online at :
http://inajac.lipi.go.id

Keywords:

cellulase, Trichoderma sp., wheat bran, rice bran, EFBs.

Abstract

This research was more focused on the production of cellulase, especially on using the domestic microbes. *Trichoderma* sp. is considered as one of the most efficient cellulase producer. This study was investigated the performance of *Trichoderma* sp. on a variety of substrates to produce cellulase. Three types of substrate variations and three types of *Trichoderma* sp. were used in this experiment. Substrates were wheat bran, rice bran and oil palm empty fruit bunches (EFBs), whereas *Trichoderma* sp. isolates were encoded as T004, T051 and T063. Production of cellulase was conducted by solid fermentation for 7 days. The analysis of cellulase activity was based on National Renewable Energy Laboratory (NREL) method for filter paper assay. The results showed that the type of substrate affected the performance of *Trichoderma* sp. All types of fungus produced cellulase on wheat bran substrate with activity of 0.52 FPU /ml for T004, 0.23 FPU/ml for T051 and 0.27 FPU /ml for T063. With the rice bran substrate and EFBs, only T004 could produce cellulase with the enzyme activity analyzed were 0.08 FPU /ml and 0.008 FPU/ml respectively. Optimization of the buffer addition on enzyme extraction process produces the highest activity 0.85 FPU/mL for T004 with wheat bran substrate.

1. INTRODUCTION

World energy demand increases every year but production of fossil fuel continues to decrease. It encourages developing alternative energy such as biofuels. Global biofuels production, including bioethanol and biodiesel, remain static in 2012 in absolute volumes at roughly 110 billion liters, caused by high feedstock prices. The cost of biofuel production was 50% to 80% of total production costs [1]. Due to the high price of feedstock for first generation biofuels, lignocellulosic biomass was developed for feedstock of biofuels, particularly ethanol. Cellulose and hemicellulose, which represent the largest component of lignocellulose biomass such as straw from corn, cotton, soy and wheat, sugarcane bagasse, bond with lignin in the cell wall matrix that needs to be broken [2]. The potential of cellulose as an alternative raw material has stimulated research of bioconversion processes which hydrolyze cellulose into soluble sugars as feedstock for alcohol fermentations [3].

The hydrolysis process can be performed in different ways, either chemical or enzymatic [4]. Enzymatic hydrolysis gives two advantages of the process: low corrosion problems and low toxicity of the hydrolysates. Lignocellulosic hydrolysis used several enzymes, which the most popular are cellulases [5]. Cellulase is a generic name for the enzymes group which catalyzes the hydrolysis of cellulose and related cellu-oligosaccharide derivatives [6]. Cellulases are produced by a number of microorganisms. Cellulolytic microbes are primarily carbohydrate degraders and generally unable to use proteins or lipids as energy sources for growth [7]. Most commonly studied

e-ISSN:2527-7669

cellulolytic organisms include fungal species such as *Aspergillus, Trichoderma, Humicola, Penicillium*, bacteria species such as *Pseudomonas, Cellulomonas* and actinomycetes species such as *Actinomucor* and *Streptomyces* [5].

Three major types of cellulase activities are recognized in the cellulase systems of such fungi: 1) endoglucanases (EG), 2) exoglucanases including glucohydrolases and cellobiohydrolases (CBH) and 3) β-glucosidases or cellobiases [8]. The function of each component can be seen in Table 1. For growth and product formation, cellulolysis need appropriate conditions. Commercial cellulase preparations from most often used species *Trichoderma reesei* and *T. viride* are popular as they contains high activities of both exo-glucanase and endo-glucanase but low levels of β-glucosidase. *T. reesei* produces at least two CBHs, five EGs, and two β-glucosidases [9]. Assays for determining cellulase activity have been classified differently over years of cellulase research. Different cellulase assays that are classified within two groups: (1) total cellulase activity and (2) individual cellulase activity including endoglucanases, exoglucanases, and β-glucosidases. Total cellulase activity was measured by filter paper assay (FPA). Endoglucanases, activity was measured by carboxymethyl cellulase, exoglucanases was measured by Avicel, and β-glucosidases activity was measured by *p*NPG, cellobiose [10].

The important factors that affecting yield of enzyme production are the type of strain, culture conditions, nature of the substrate and availability of nutrients [11]. Cellulolytic fungi used cellulose as a primary carbon source. Pure, crystalline cellulose, such as Solka Floc, Avicel, and cotton are good cellulose inducers, but they are expensive. It is important to use a less expensive substrate for cost reduction [12]. It has been reported that low cost substrates like wheat flour, wheat bran, rice straws and molasses are suitably effective for growth and enzyme production [13, 14].

Table 1. Components and mode of action of aerobic fungal cellulases.

Enzyme	Mode of action
Endoglucanase (EG) (Endo-1,4-β-D-glucan cellobiohydrolase) EC 3.2.1.4	-G-G-G-G-G-G-G-G-G-G-G- ↑ ↑ Cleaves linkages at random
Cellobiohydrolase (CBH) (Exo-1,4-β-D-glucan cellobiohydrolase) EC 3.2.1.91	G-G-G-G-G-G-G-G-G-G-G ↑ ↑ Releases cellobiose either from the reducing or the non-reducing end
Glucohydrolase (Exo-1,4-β-D-glucan glucohydrolase) EC 3.2.1.74	G-G-G-G-G-G-G-G-G-G-G- ↑ Releases glucose from the non-reducing end
β –glucosidase or cellobiase (β-D-glucoside glucohydrolase) EC 3.2.1.21	G-G G-G-G G-G-G-G ↑ ↑ ↑ ↑ Releases glucose from cellobiose and short-chain glucose oligomers

Source: Bhat and Hazlewood, 2001.

In this study, the substrates used were wheat bran, rice bran and oil palm empty fruit bunches (EFBs). Wheat bran is produced in large quantities as residue of the milling process which is 14–25% of the grain [15, 16]. Rice bran is residue of the rice milling process. The milling of 1-ton rice will produce 60-80 kg rice bran [17]. The EFBs is produced as residue of Crude Palm Oil (CPO) production. The dry weight of EFBs is about 8% of the dry weight of Fresh Fruit Bunches (FFB) [18] or 39% of the weight of CPO produced [19]. Palm Oil plantations can produce 32 tons of FFB per hectare per year, and 6-7 tons of CPO per hectare per year [20]. Agro-industrial residues which were abundantly available have been thought as potential substrate in cellulase production.

The aim of this work was to investigate the performance of *Trichoderma* sp. Indonesian isolates in various type of substrates. These substrates were chosen from low cost raw material for the production of cellulase. After the combination of substrates and isolates which produce the highest cellulase activity were obtained, optimization of the buffer addition on enzyme extraction process will be conducted to get a higher cellulase activity.

e-ISSN:2527-7669

2. EXPERIMENTAL SECTION

2.1. Materials and methods

2.1.1 Substrates

Three different agro-industrial residues, wheat bran, rice bran and EFBs were used as substrates for cellulase production. Wheat bran was purchased from PT. Bogasari, rice bran was from local market, and EFBs was taken from an Palm Oil Plantation belongs to PT. Perkebunan Nusantara VIII, in Pandeglang, Banten, Indonesia.

2.1.2 Microorganisms and Culture Conditions

Three Indonesian isolates of *Trichoderma* sp. (T004, T051 and T063) were used for this study. T004 was isolated from District of Liwa in Lampung Province whereas T051 and T063 were isolated from District of Maros in South Sulawesi Province [21]. These isolates were obtained from Biotechnology Culture Collection (BTCC), Research Center for Biotechnology-Indonesian Institute of Sciences (LIPI) and maintained on potato dextrose agar (PDA) slants at 27°C. Fully sporulated cultures obtained after 7 days were preserved at 4°C. The cultures on PDA slants were used as inoculum, where cultures were transferred first onto PDA plates and grown for 7 days.

2.1.3 Preparation of Nutrient Solution

Nutrient solutions for three kinds of substrates consisted of 2g/L KH_2PO_4, 0.4g/L $CaCl_2.2H_2O$, 0.3 g/L $MgSO_4.7H_2O$, 1.4 g/L $(NH_4)_2SO_4$ and 0.3 g/L urea. For EFBs, 1g/L glucose was added. These components were diluted in 0.05M citrate buffer pH 4.8 and stirred [12] with modification

2.1.4 Preparation of Crude Enzyme

The crude enzyme was produced by solid fermentation method. Three plugs of each isolate was inoculated on each medium consisted of 10 g of substrate and 15 mL of nutrient solution in Erlenmeyer flask and then incubated at 27°C for 7 days. The solid fermentation process performed in two replicates for each variation of substrates and *Trichoderma* isolates. The cellulolytic enzymes were extracted with 25 mL of 0.5M citrate buffer pH 4.8, then mixed and preserved at 4°C. The extract was filtered by using filter cloth. The filtrate was used as a crude enzyme.

2.1.5 Optimization of enzyme activity by the addition of various buffer concentration on extraction process

Preparation of crude enzyme was performed by using substrate conditions and isolate which produced the highest cellulase activity. The cellulolytic enzymes were extracted by using of 0.5M citrate buffer pH 4.8 with 15, 20, 25 and 30 mL repectively, then mixed and preserved at 4°C for approximately 2 h. The extract was filtered by using filter cloth. The filtrate was considered as crude enzyme. The solid fermentation process performed in two replicates for each variation of buffer.

2.1.6 Measurement of enzyme activities

Filter paper activity (FPase) for total cellulase activity in the filtrate was determined according to a measurement of cellulase activities method from National Renewable Energy laboratory (NREL) [22]. The detection of glycosidic bond cleavage by this method involves the parallel and identical treatment of three categories of experimental tubes (assay mixtures, blanks and the appropriately diluted cultured filtrate as enzyme source was added to Whatman no. 1 filter paper strip (1 x 6 cm; 50 mg) immersed in one mL of 0.05M citrate buffer of pH 4.8. After incubation at 50°C for 60 min, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method [22]. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 2.0 mg of reducing sugar from filter paper per ml in 60 min. The calculation of FPU activity can be seen in equation 1.

$$FP\ activity = \frac{0.37}{[\text{enzyme}]\ \text{releasing 2.0 mg glucose}}\ {}^{\text{units}}/_{\text{mL}} \quad (1)$$

3. RESULTS AND DISCUSSION

The *Trichoderma* initially produces a dense pure white mycelium but sometimes mycelial mat on the casing layer gradually turns to a

e-ISSN:2527-7669

green color [23]. The isolated of *Trichoderma* sp. can be seen in Figure 1. Three types of *Trichoderma* sp. used have a different colony color. The colony color of T004, T051 and T063 were white, greenish white and dark green, respectively. The growth of three isolates of *Trichoderma* sp. (T004, T051 and T063) after incubation for 7 days at the three types of substrates showed that mycelium growth increased with pure white color for T004 and T051 and green color for T063. Figure 2. showed an example of the growth of *Trichoderma* sp. (T004, T051 and T063) on wheat bran substrate.

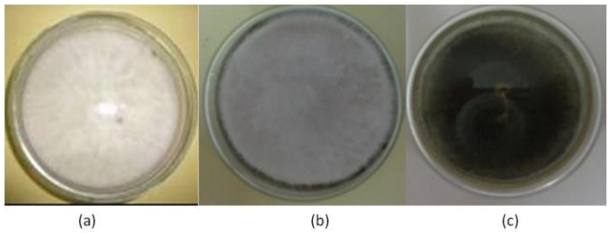

Figure 1. *Trichoderma* sp. in PDA medium (a) T004, (b) T051, (c) T063.

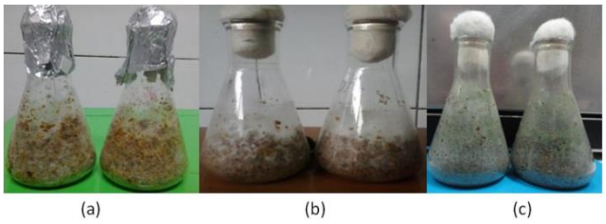

Figure 2. *Trichoderma* growth on wheat bran after 7 days' incubation time: (a) T004, (b) T051, (c) T063.

The variation of *Trichoderma* sp. with different substrates for cellulase production resulted a different activity of enzyme. Analysis of cellulase activity used NREL method for Filter paper assay (FPA). The FPA is the key method for analysis of total cellulase activity. To compare the efficacy of cellulase activity between microorganisms or their secreted enzymes, techniques for measuring total cellulase activity are required [10].

In this study, *Trichoderma* sp. T004 can work and produce cellulase with different activities on three types of substrates wheat bran, rice bran and EFBs which can be seen in Table 2. The Table shown that the highest cellulase activity was obtained on wheat bran substrate at 0.52 FPU mL, whereas the rice bran was 0.08 FPU/mL and EFBs was 0.008 FPU/mL. On two isolates of *Trichoderma* sp. (T051 and T063) only produce cellulase on wheat bran substrate, whereas on rice bran and EFBs the activity of cellulase were not detected. The cellulase activity data on T051 and T063 can also be seen in Table 2. The values of cellulase activity are the average data of two replications process of solid fermentation.

Table 2 showed wheat bran give higher activity of enzyme in every isolate of

Table 2. The cellulase activity of *Trichoderma* sp. T051 and T063 grown on different substrates

Media	*Trichoderma* sp.	Cellulase activity (FPU/mL)	Protein Concentration (mg/ml)	Specific activity (U/ mg protein)
Wheat bran	T004	0.52	113.1	0.005
	T051	0.23	85.8	0.003
	T063	0.27	141.9	0.000
Rice bran	T004	0.08	30.3	0.003
	T051	ND	46.2	-
	T063	ND	129.9	-
EFBs	T004	0.008	159.6	0.000
	T051	ND	163.2	-
	T063	ND	168.9	-

*) *ND = not detected*
*) *Above data showed enzyme activity value with validation measurement method. Precision method is 8.4% RSD (n=9)*

e-ISSN:2527-7669

Trichoderma sp. than the other substrates. It can be observed that the substrate as carbon source in the medium affects considerably in the production of the cellulolytic enzymes by *Trichoderma* sp. Wheat bran, rice bran and EFBs invariably affected the synthesis of cellulase in the medium. The carbon sources difference on the three type of substrates characteristics which the sum of cellulose, hemicellulose and starch between these substrates are approximately on same level concentration, about range 70%. EFBs has less carbon source with content of carbons about 50%, only cellulose and hemicelluloses. The significant difference was in lignin content, which is highest on EFBs. The presence of lignin causes difficulties for fungus to access and consume carbon sources. Lignin is the most difficult component of biomass to be degraded due to its complex structure, high molecular weight and high insolubility. Lignin was linked by carbon-carbon and other bonds to form tri-dimensional network associated with the hemicelluloses polysaccharides inside the cell wall [24]. The production of cellulase was induced only in presence of the substrate, and repressed when easily utilizable sugar are available [5]. The more lignin in substrate, the less sugar will be available for production of cellulase. However, wheat bran contained easier utilizable sugar and less lignin than rice bran. The easier access to sugar was suggested the cellulase from wheat bran has highest cellulase activity in all used three types of fungus.

The evaluation of substrates and *Trichoderma* isolates for cellulase production indicated that the use of wheat bran and T004 isolate produces the highest of enzyme activity 0.52 FPU/mL. Similar result was reported in previous studies. Cellulase production by using wheat bran and *Trichoderma koninggii* D-64 produce enzyme with activity 0.5±0.2 FPU/mL [25], sugar beet pulp and *T. reesei* achieved 0.46 IU/mL of filter paper activity was obtained [12]. All reported results were based on cellulase activity in crude form of enzyme. Crude enzyme usually has lower activity because there are so many impurities in the liquid. Although this number of activity is low even for crude form of enzyme, this result denoted that the whole process, especially wheat bran and T004, is good enough as a platform for cellulase production feasibility studies. Developments to get the higher activity of cellulase were obviously attractive.

The next step to get the higher enzyme activity from wheat bran substrate and T004 was by improving the extraction optimum condition such as the amount of buffer addition on extraction process. In the cellulase production by solid state fermentation, extra buffer for enzyme extraction was needed. In the first method, the buffer addition was 25 mL, then a variation of buffer used i.e. 15, 20 and 30 mL. The cellulase activities on various buffer addition can be seen in Table 3.

Table 3. The cellulase activity of *Trichoderma sp*. T004.

Substrate	Buffer addition (mL)	Cellulase activity (FPU/mL)
10g of wheat bran, 15 mL of nutrient solution	15	0.85
	20	0.31
	25	0.52
	30	0.33

Table 3. shows that the addition of buffer affected cellulase activity. Several previous studies using a variety of solution for enzyme extraction. Cellulase production from 5g of corn and 10 mL of distilled water using 100 mL distilled water or 100 mL pH solutions for extraction [26]. Cellulase from 20g of wheat bran and 20 mL of distilled water using 5 mL of distilled water for extraction [21]. Extraction cellulase from *Aspergillus oryzae* using 100 mL 0.2M sodium acetate buffer, pH 5.2 to extract 1 g of solid (myceliamat) [27]. Based on the differences in the solutions addition on the enzyme extraction, in this study conducted an additional variation of the buffer volume on enzyme extraction to determine its effect on cellulose activity. The highest activity was obtained at 15 mL of buffer addition that is 0.85 FPU/mL. The main step for the next study was purification system.

e-ISSN:2527-7669

Purification by using salt saturation, dialysis, ion exchange and gel filtration chromatography was relied to obtain high activity of cellulase. In average of this process increased total cellulase activity at about 50 times crude enzyme activity.

4. CONCLUSION

The present work showed that low cost substrate can be used as carbon source to produce cellulase. Wheat bran seems more suitable than rice bran and EFBs as substrate for cellulase production by three strains of Indonesian *Trichoderma* sp. indicating the highest activity in T004 isolate. The highest cellulase activity was 0.85 FPU/mL by using T004 and wheat bran at 15 mL buffer addition on enzyme extraction. This matter can be view based on the composition of substrate where wheat bran has many carbon sources and less blockage from lignin.

ACKNOWLEDGEMENT

This work was funded by National Priority Project of fiscal year 2013.

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