Rapid Assessment of Diverse Trichodermal Isolates of Indonesian Origin for Cellulase Production

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Abstract

Trichoderma is a well-known candidate to be promoted as cellulase producer for the hidrolysis of lignocellulose that contain in woody biomass. The number of trichodermal isolates in our laboratory collected from diverse ecosystem types in Indonesia increases significantly during the last 5 years. It is our aim to assess the cultures for its ability in producing cellulase. Sixty-six trichodermal isolates used in this experiment are obtained from Biotechnology Culture Collection (BTCC), Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). The 31 isolates were isolated from District of Liwa (South Sumatra, Indonesia) and the 35 isolates from District of Maros (South Sulawesi, Indonesia). The isolates were screened qualitatively, 7 isolates from Liwa and 12 isolates from Maros showed cellulolytic activity. From the results of quantitative test, two strains (ID08-T004 and ID08-T63) showed the higher cellulolytic activity among the selected strains, 133.5 and 133.5 U/ml, respectively. These extracellular enzymes were characterized their temperature and pH optimum. The temperature optimum for both enzymes was the same, 50°C, with activity 213.6 U/ml for enzyme extracted from ID08-T004 and 197.3 U/ml for enzyme from ID08-T0063. The pH optimum was pH 5 of ID08-T004 with activity 137.7 U/ml and pH 6 for ID08-T063 with activity 75.0 U/ml. The enzymes from ID08-T004 and ID08-T063 were stable in their temperature and pH optimal condition even after 90 minutes incubation with activity 179.0 U/ml and 86.7 U/ml, respectively. The enzyme stability was approximately 150 minutes for both enzymes in the temperature and pH optimum.

Key Words: *Trichoderma*, cellulase, Culture Collection

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Introduction

The demand for fuel energy increases sharply, while the supply continuously decreases by time. The use of fossil fuel also is no longer attractive, as it is contributing to the increasing of gas emission and getting worse of climate change and eventually causes global warming. For these reasons, search for an alternative energy, a biomass based energy, attracted interest as an alternative solution to the crises. Production of bioethanol from biomass has now being practiced in various parts of the world. Most of biomass used e.g. sugarcane, corn and cassava, but it is conflicting to food security program. Wood biomass would probably be an excellent alternative raw material for bioethanol. Wood biomass, however, more difficult to be converted to monomeric sugars as feedstock in bioethanol industry compare to the sugary and starchy materials obtain from sugarcane, corn and cassava. For this reason, enzymes and enzymes system for conversion of wood biomass to monomeric sugars need to be developed. *Trichoderma* is a well known candidate to be promoted as enzymes producer for this purpose (Kumar *et al*, 2008).

Trichoderma was first proposed as a genus by Persoon in 1794 on the basis of material collected in Germany. In the 1969 publication of Rifai's thesis (Rifai, 1969), became a groundbreaking monograph of *Trichoderma*. After that, in about 1995, the use of DNA sequence analysis became the new paradigm in fungal systematics and *Trichoderma* workers quickly incorporated sequence data to the developing taxonomy of *Trichoderma* and *Hypocrea* (Samuel, 2006). Currently, there are

89 species of *Trichoderma* anamorph and that of the *Hypocrea* teleomorph.

Although the genus *Trichoderma* is represented in soils and other organic matter collected at all latitudes, some species are widely distributed while other species are geographically limited in their distribution. In the term of uses, *Trichoderma* have been known since at least the 1920s for their ability to act as biocontrol agents against plant pathogens. *Trichoderma* have been known to be able to attack other fungi, to produce antibiotics that affect other microbes, and to act as biocontrol microbes. Indeed, *Trichoderma* were demonstrated to be very efficient producers of extracellular enzymes, with cellulases as the first example. Later, these fungi were found to produce a wide range of other extracellular enzymes (Harman, 2006).

Cellulase of *Trichoderma resei* (syn *Hypocrea jecorina*) are commonly used for the hidrolysis of lignocellulose. *T. reesei* could produce extracellular cellulolytic enzymes in a significant amount. The cellulolytic enzymes produced by this fungi usually belong to endoglucanase, cellobiohydrolase (exoglucanase) and β-glucosidase and work very efficient and synergy on the degradation of organic cellulosic waste (Viikari *et al*, 2007). The genome of this organism was released by Martinez *et al* (2008). Sterenberg (1976) found that *Trichoderma* could also produce β-glucosidase but at low concentration. Low concentration of β-glucosidase production by *Trichoderma* due to the fermentation conditions particularly pH. The decrease of pH during the course of fermentation is the major handicap which causes inactivation of the enzymes synthesis. Recently, study on fermentation conditions related to the synthesis of hydrolytic enzymes on solid state fermentation was also studied (Kristensen *et al*, 2009). It is however believed that the ability of *Trichoderma* in producing enzymes complex related to the conversion of biomass varied significantly.

Study on the ability of large number of *Trichodermal* isolates of Indonesian origin to produce complex enzymes system for the conversion of wood biomass should be of great value in boosting bioethanol production from wood biomass. Therefore, by using trichodermal collections, we first, investigated the cellulase activity of our trichodermal collections.

Materials and Methods

Trichodermal isolates. Sixty-six trichodermal isolates used in this experiment are obtained from Biotechnology Culture Collection (BTCC), Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). The isolates were isolated from soils and litters collected from District of Liwa (South Sumatra, Indonesia) and District of Maros (South Sulawesi, Indonesia). The isolation method of trichodermal isolates from soils was as follows: 0.5 g of soil samples were diluted in 5 ml of sterile water, mixed for 5 minutes, and leaved at room temperature for 5 minutes to release their spores. Then, the trichodermal isolates were isolated by dilution and UV irradiation method as described by Davet & Rouxel (2000). The isolation method of trichodermal isolates from litters was as follows: 0.2 g of cutting leave-litters in size 3×1 cm was put in 5 ml sterile water and 2 g of glass beads. The tube was mixed by vortex for 3 minutes and the crashed litter was filtered by 0.3 mm stainless steel mesh. The crashed litter was resuspended with 5 ml sterile water, then, the trichodermal isolates were isolated by dilution and UV irradiation method as described by Davet & Rouxel (2000) same as in soil samples.

Cultivation medium. All cultures of trichodermal isolates obtain in the form of cultures as slope Potato Dextrose Agar (PDA, Oxoid), containing 4 g potato extract, 20 g dextrose, and 15 g agar in 1 L distilled water and incubated at 30°C for 5-7 days. Spore from the slope PDA culture was transferred to fresh slope PDA medium and incubated in the same temperature. The cultures rejuvenate once a month during the course of study. For the purpose of experiment, spores suspension was used by suspend the spores on a 0.1% Tween 80 (Sigma).

Screening of cellulolytic enzymes. Screening of cellulolytic enzymes used Cellulose Congo-red Agar (CCA) medium containing 0.2 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, 0.752 g carboxymetyl cellulose (CMC), 0.08 g congo red, 0.04 g cyclohexamide, 5.2 g agar, 40 ml

soil extract, and 360 ml distilled water in pH 7.0 as described by Hendricks *et al* (1995). The trichodermal spores was inoculated on CCA medium and incubated at 30°C for 5 days. Clear zone around the colony was measured.

Crude enzymes preparation. Crude cellulase was prepared using solid state fermentation on a medium consisted of 20 g of wheat bran and 20 ml of distilled water in an Erlenmeyer flask. In the sterile medium, the spores of *Trichoderma* was inoculating spread out, mixed thoroughly, and incubated at 27-30°C for 5 days. The cellulolytic enzymes were extracted with 5 volumes of sterile distilled water, mixed thoroughly, leaved for 2 hours, then the extract was filtered through filter cloth. The filtrate was centrifuged at 8,000 rpm for 20 minutes to remove some contaminating spores, and the supernatant was used as crude cellulase.

Cellulase (CMCase) activity. CMCase activity was determined by the method of Haggett *et al* (1979) with modification. The 0.5 ml of diluted crude enzyme was added with 0.5 ml of 1% CMC prepared in 20 mM phosphate buffer pH 7.0 as substrate, and mixed by vortex. The mixture was incubated at 30°C for 30 minutes, and the reaction was stopped by the addition of 1.5 ml DNS (3, 5-dinitrosalicylic acid) solution, boiled for 15 minutes, and cooled in water for color stabilization. A non-incubated solution of 0.5 ml crude enzyme, 0.5 ml of 1% CMC prepared in 20 mM phosphate buffer pH 7.0 and 1.5 ml DNS and an incubated solution of 0.5 ml sterile water, 0.5 ml of 1% CMC prepared in 20 mM phosphate buffer pH 7.0, and 1.5 ml DNS were used as a negative control. CMCase activity was determined by measuring of absorbance of reducing sugar from CMC solubilized in 20 mM phosphate buffer pH 7.0. The absorbance was measured at 540 nm. One unit (U) of CMCase activity was defined as the amount of CMCase that released 1 µmol of glucose per minute.

Optimization of temperature and pH. Effect of temperature to the enzyme activity was determined by reacting the crude enzyme solution, substrate prepared in 20 mM phosphate buffer pH 7.0 for 30 minutes on different temperatures, with interval ranges 10°C from 30-80°C. Effect of pH to the enzyme activity was determined by reacting the crude enzyme solution, substrate prepared in 20 mM phosphate buffer with different pH from 3-8 for 30 minutes on 30°C. Cellulase activity was measured using DNS method as described by Haggett *et al* (1979).

Enzyme stability on optimum condition. Enzyme stability was tested on optimum temperature and optimum pH with incubation of the crude enzyme with sterile distilled water and without substrate. The cellulase activity was measured every 30 minutes for 150 minutes by the method of Haggett *et al* (1979).

Results and Discussion

Trichodermal isolates were isolated from soils and litters collected from District of Liwa, Lampung Province, Sumatera and District of Maros, South Sulawesi Province in Indonesia. These two regions are separated by Wallace line where flora and fauna of these two regions sharply distinct. Some of the nature of sampling sites and code of sample is shown in Figure 1.

Sixty-six trichodermal isolates are preserved at Biotechnology Culture Collection (BTCC), Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). The 31 isolates were isolated from District of Liwa (South Sumatra, Indonesia) and the 35 isolates from District of Maros (South Sulawesi, Indonesia). The isolates were screened qualitatively for cellulolytic activity by using CMC as substrate in CCA medium. As results, 7 strains from District of Liwa and 12 strains from District of Maros showed cellulolytic activity by formation of clear zone around the colony measuring at 5 days incubation (Table 1 and Figure 2). The selected trichodermal isolates could utilize CMC in the medium for growth. The morphology of some trichodermal isolates are shown in Figure 3 as refer to Kubicek & Harman (1998).

From the results of quantitative test of cellulase enzyme activity, two trichodermal isolates (ID08-T004 and ID08-T063) showed the higher cellulolytic activity among the selected isolates, 133.5 and 113.5 U/ml, respectively (Figure 4). Cellulolytic enzyme system of filamentous fungi, especially *T.*

reesei, composed two cellobiohydrolase exoglucanase (CBH1 and CBH2), four endoglucanases (EG1, EG2, EG3, EG5), and β-glucosidase. These enzymes act synergistically to catalyze the hydrolysis of cellulose physical parameters such as pH, temperature, adsorption, chemical factors such as nitrogen, phosphorus, the presence of phenolyc compounds and other inhibitors that can improve lignocellulose bioconversion (Viikari *et al*, 2007).

Figure 1. Examples of isolation sources of trichodermal isolates

Table 1. Qualitative test of cellulose activity of *some trichoderma isolates* on CCA medium.

Culture No.	Diameter zone (cm)	Culture No.	Diameter zone (cm)
District of Liwa		**District of Maros**	
ID08-T004	4.5	ID08-T040	1.7
ID08-T010	2.0	ID08-T042	2.5
ID08-T011	2.5	ID08-T045	1.5
ID08-T012	3.75	ID08-T049	2.9
ID08-T013	1.4	ID08-T051	2.5
ID08-T026	2.5	ID08-T054	1.7
ID08-T029	1.9	ID08-T057	1.9
		ID08-T059	1.6
		ID08-T062	1.5
		ID08-T063	3.4
		ID08-T064	2.1
		ID08-T065	1.8

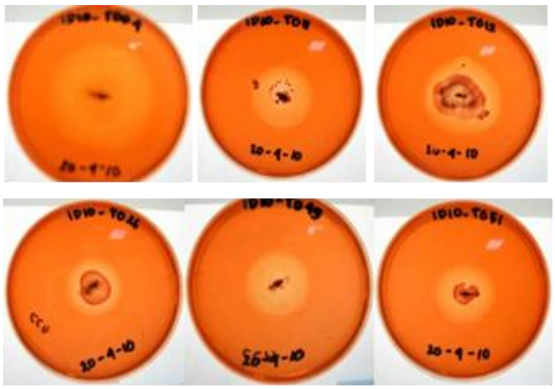

Figure 2. Examples of trichodermal potential cellulolytic strains in CCA medium.

Cellulosic bioconversion is a complex process and requires a synergistic action of three enzymatic components consisting of exoglucanase, endoglucanases, and β-glucosidase. *Trichoderma* has a component of endo and exoglucanase high but lower content of β-glucosidase, and therefore limited its efficiency in hydrolysis of cellulose (Soni & Soni, 2010).

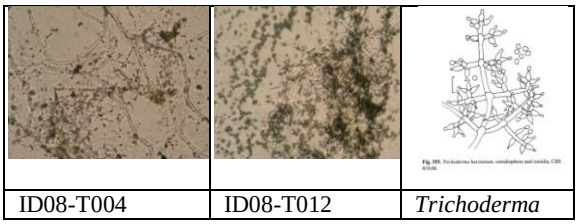

Figure 3. The morphology of trichodermal isolates

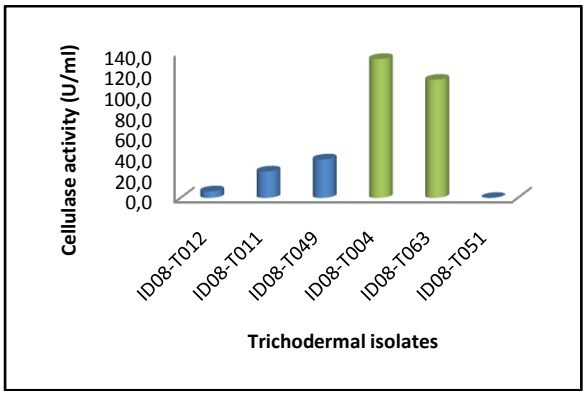

Figure 4. Cellulase enzyme activity of some trichodermal isolates

The optimum temperature enzyme activity was determined with range of the temperature from 30 to 80°C with an interval of 10°C. As shown in Figure 6, the optimum temperature of cellulase enzyme of *Trichoderma* sp. ID08-T004 and *Trichoderma* sp. ID08-T063 was the same, 50°C, with enzyme activity of 213.6

U/ml and 197.3 U/ml, respectively. Temperature has a profound effect on lignocellulose bioconversion. Temperature for cellulase enzyme activity test usually in the range 50-65°C to various strains of fungi such as *Aspergillus wentii*, *Penicillum rubrum*, *Aspergillus niger*, *Aspergillus crassa* and *Neurospora ornatus* (Dashbanth *et al*, 2009).

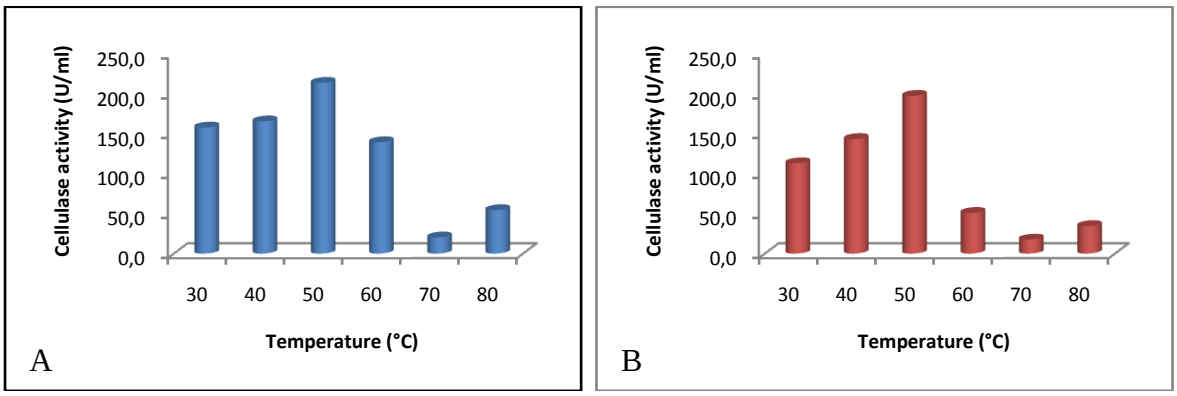

Figure 6. Cellulase enzyme activity of *Trichoderma* sp. (A) ID08-T004 and (B) ID08-T063 on various temperatures

The optimum pH enzyme activity was determined with range of the pH from 5 to 8 with an interval of 1.0. As shown in Figure 7, the optimum pH of cellulase enzyme of *Trichoderma* sp. ID08-T004 was pH 5 with the enzyme activity of 137.7 U/ml; while *Trichoderma* sp. ID08-T063 was pH 6 with the enzyme activity of 75.0 U/ml, compared with the control treatment without any phosphate buffer. Different physical parameters will affect the bioconversion of cellulose, and pH are important factors that influence the production of cellulase. Kim *et al* (1988) have reported that maximum adsorption of cellulase from *Aspergillus phoenicus* occurred at pH 4.8 to 5.5. The pH range of 4.6 to 5.0 is found optimum for CMCase, FPase and production of β-glucosidase from *Aspergillus ornatus* and *T. reesei* ATCC 26921 (Kumar *et al*, 2008).

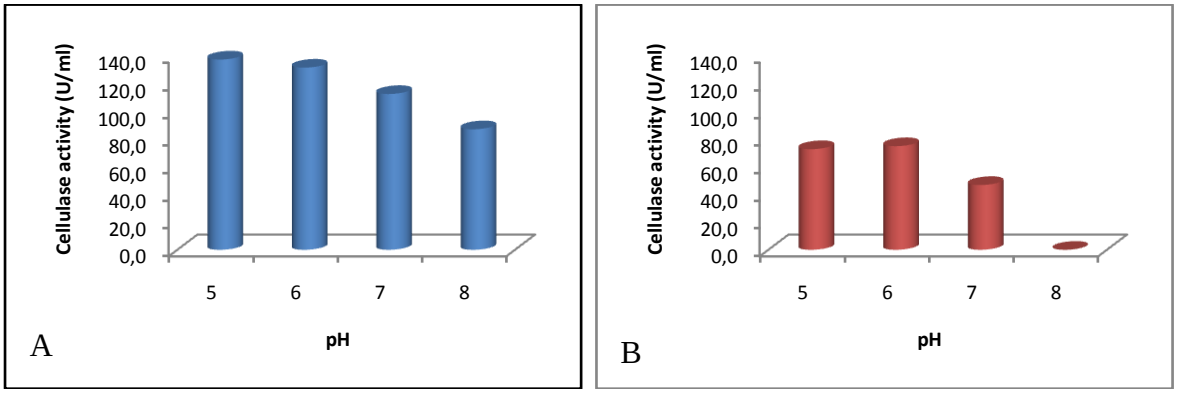

Figure 7. Cellulase enzyme activity of *Trichoderma* sp. (A) ID08-T004 and (B) ID08-T063 on various pHs.

Enzyme stability of cellulase of *Trichoderma* sp. ID08-T004 and ID08-T063 on its temperature and pH optimum was determined in 150 minutes heating. The cellulose enzyme stability of *Trichoderma* sp. ID08-T004 was high enough that is equal to 179.0 U/ml in incubation for 90 minutes of heating and *Trichoderma* sp. ID08-T063 is 86.7 U/ml in the heating for 90 minutes heating (Fgure 8). remained stable in temperature and pH optimum for heating 150 minutes, and likely still will increase, due to the minute-150 enzyme activity has not decreased to half of normal enzyme activity. Similarly, *Trichoderma reesei* strain showed higher growth at 28°C, but its maximum cellulase activity at 50°C and about 98, 59 and 76% of CMCase activity, F Pase and β-glucosidase, after incubated 48 hours in 40°C in a medium containing rice straw that has

been delignification as a substrate (Maheshwari *et al*, 2000).

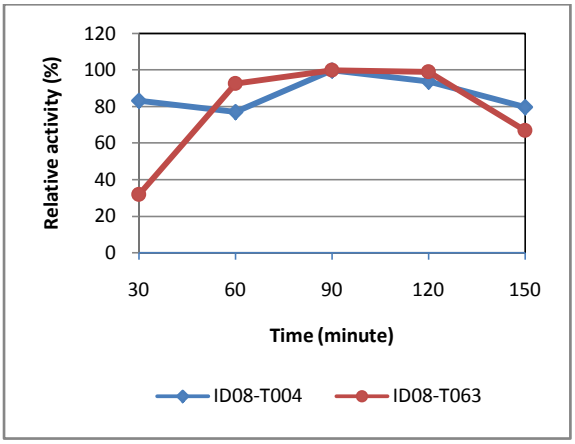

Figure 8. Cellulase enzyme activity of *Trichoderma* sp. ID08-T004(♦) and ID08-T063 (●) on 50°C and pH 5.0 for 150 minutes heating.

Conclusion

Seven isolates from Liwa and twelve isolates from Maros showed cellulolytic activity. From the results of quantitative test, two strains (ID08-T004 and ID08-T63) showed the higher cellulolytic activity among the selected strains, 133.5 and 133.5 U/ml, respectively. The temperature optimum for both enzymes was the same, 50°C, with activity 213.6 U/ml for enzyme extracted from ID08-T004 and 197.3 U/ml for enzyme from ID08-T0063. The pH optimum was pH 5 of ID08-T004 with activity 137.7 U/ml and pH 6 for ID08-T063 with activity 75.0 U/ml. The enzymes from ID08-T004 and ID08-T063 were stable in their temperature and pH optimal condition even after 90 minutes incubation with activity 179.0 U/ml and 86.7 U/ml, respectively.

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