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An Active of Extracellular Cellulose Degrading Enzyme from Termite Bacterial Endosimbiont

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Abstract

Cellulase is an ezyme that specifically cleaves the 1,4-β-glycosidic bond of cellulose to produce the small fragments of simple carbohydrate. This work was aimed to characterize the extracellular cellulase from *Paenibacillus* spp., which was previously isolated from macro termites, *Odontotermes bhagwatii* in our laboratory. Two *Paenibacillus* isolates were used in this experiment, namely *Paenibacillus cellulositrophicus* SBT1 and *Paenibacillus*, sp. SBT8. Analysis of the total proteins in the supernatants showed that *P. cellulositrophicus* SBT1 and *Paenibacillus* sp. SBT8 roughly produced as much as 18.6 mg/l and 24.8 mg/l of extracellular cellulases, respectively. Enzymatic assay showed that SBT1 and SBT8 cellulase exhibited enzymatic acitivity of 0.17 U/mg and 0.12 U/mg, respectively. Temperature dependencies analysis indicated that both cellulases exhibited maximum activity at 35^{o}C. At the temperature higher than 55^{o}C, the enzymatic activities of both cellulases were roughly 20% reduced compared to the maximum activity. SBT1 and SBT8 cellulases were both active at acidic pH. At basic pH (pH 8) the enzymatic activities of both cellulases were reduced roughly 30% compared to that of acidic pH. Supplementing of Mg^{2+}, Zn^{2+}, and Ca^{2+} in range of 1-10 mM increased the enzymatic activity of both cellulases roughly 33 to 50%.

Keywords: *Cellulase, Paenibacillus cellulositrophicus SBT1, Paenibacillus sp. SBT8, divalent metal cation, 1,4-β-glycosidic bond*

Introduction

Cellulose is the most abundant plant material that can be used as renewable biomaterial and bioenergy. Production of bio-based material and energy resource from less costly renewable lignocellulosic materials would bring benefits to local economy, environment problem and national energy security. Cellulase has an important role for the production of bio-based materials and energy from less costly renewable lignocellulosic materials (Zhang *et al.*, 2013).

Cellulase (EC.3.2.1.x) is an enzyme that specifically cleaves the 1,4-β-glycosidic bond of cellulose polymer into smaller fragments of simple carbohydrate. Based on the catalytic mechanism, cellulases are classified into three classes of enzymes, namely endo 1,4-β-glucanase, exo 1,4-β-glucanase, and β-glycosidase. Endo 1,4-β-glucanase is cellulase that randomly cleaves internal glycosidic bonds of cellulose polymer. In contrast to the endo 1,4-β-glucanase, exo 1,4-β-glucanase attack the cellulose polymer from the end of polymer, either from reducing or non-reducing end. The processive exo 1,4-β-

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glucanse also referred as cellobiohydrolase. The later class of cellulase, β-glucanase convert cellobiose, the major product of endo- and exo-glucanase, are glucose (Li *et al*., 2006; Ogawa *et al*., 2007; Gao *et al*., 2008; Horn *et al*., 2012). Cellulase encompasses non-catalytic carbohydrate binding module (CBM) that may be located at the N- and C-terminus of the catalytic domain. CMB is amino acids sequence that functions as substrate binding module (Shoseyov *et al*., 2006). The catalytic domain contains two important catalytic residues, aspartate (D) and glutamate (E). Those two residues act as donor or acceptor proton during the enzyme catalysis. Therefore, the catalysis mechanism of cellulase obeys the general acid base catalysis (Mosier *et al*., 1999; Rye and Withers, 2000; Rabinovic *et al*., 2002).

Some plants, animals, protozoa, fungi, and bacteria produce cellulase. Because of the simplest preparation and manipulation, the bacterial cellulase is the only cellulase that has been intensively and profoundly studied. Cellulase producing bacteria can be isolated from soil, agricultural wastes, and also from animals (Beguin and Aubert, 1994).

Termite is a type of ecosocial insect that feeds wood materials, especially cellulose (Bignell *et al*., 2011). Higher termites would not be able to digest cellulose until the bacterial endosimbionts are present on their digestive tract (Ohkuma, 2003; Hongoh *et al*., 2005; Hongoh *et al*., 2008).

Previously, we have successfully isolated the cellulase-producing bacteria from *Odontotermes bhagwatii*, a species of higher termites collected from the soil of the yard of the Faculty of Agriculture, Universitas Gadjah Mada. Based on the molecular identification, all the cellulase-producing bacteria are belong to *Paenibacillus genera*. Therefore the objective of this work is to characterize the extracellular cellulase from *Paenibacilus* spp.

Materials and Methods

Isolates. Two isolates of *Peanebacillus* used in this work were *Paenibacillus cellulositrophicus* SBT1 and *Paenibacillus* sp. SBT8. Both *Paenibacillus* produce the extracellular cellulase and were isolated from *Odontotermes bhagwatii* in the Laboratory of Microbiology, Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada.

Chemicals and microbiological media. Carboxy methyl cellulose (CMC)(Sigma), ekstrak yeast (Oxoid), agar (Oxoid), KH_2PO_4 (Merck), $MgSO_4$ (Merck), Acetic acids buffer pH 4.8, Phosphate buffer, $MgCl_2$ (Merck), $ZnCl_2$ (Merck), $CaCl_2$ (Merck), BSA (*bovin serum albumin*)(Promega), BCA (*Bichinchoninic Acids*) *working reagent* (Promega), NaOH (Merck), HCl (Merck).

Isolation, selection and identification of cellulolytic bacteria. A termite was surface sterilized by using ethanol, and performed dissecting to take out the termite's gut. The termite's gut was disrupted and then inoculated into defined media supplemented with 1% CMC. After 2 to 3 days incubation, 100 µl culture was transferred into defined media agar supplemented with 1% CMC. The growing bacteria were selected randomly based on the morphological characteristic differences. The celulolytic activity was assayed by using 1% Congo red with 1M NaCl and the positive results was designated as the formation of clear zone surrounded the growing colonies (Kumar and Velayutham, 2014). Cellulolytic activity assay was measured by cellulolytic index, a ratio of clear zone diameter and the colony diameter. Bacterial identification was carried out by analyzing of 16SrRNA genes (Janda and Abbott, 2007).

Extracellular cellulase production. Bacterial strains were grown in defined medium supplemented with 1% CMC. Cultures were incubated at room temperature for 2 days. Aeration was provided by agitation of culture using shaker incubator that had been set at 150 rpm. Supernatants were harvested by centrifugation at 15000 g for 15 minutes and used as crude extract cellulase sources. The supernatants were kept at 4°C for further analyses.

Enzymatic activity assay. Cellulase activity was examined by using the DNS method. 250 ml of crude extract of cellulase (OD_{280}~0.1) was mixed with 10 mM acetic acids buffer (pH 4.8) supplemented with 0.5% CMC as substrate. The reaction mixtures were incubated at 35°C for 30 minutes. The reaction was stopped by addition 1 ml of 3,5-dinitrosalycilic acids. The reaction mixtures were then boiled for 15 minutes and solution mixture of *sodium potassium tartrate*, *phenol*, *sodium sulfite* was added and kept cool at room temperature. The mixtures were then centrifuge at 10000 rpm for 5 minutes and supernatants were measured by spectrophotometric at λ=540 nm (Miller *et al*., 1959). One unit is defined as amount of enzyme that cleave cellulose to produce 1 µmol of reducing sugars per minute at certain condition.

Effect temperature, pH and metal ions. To test the effect of temperature, pH, and metal ion on the enzymatic activity of cellulase, the reaction condition was similar as the enzymatic activity assay, with specific modification. For example when the pH was the point of assay then the reaction buffer was modified such that the reaction conditions reach the specific pH. To tests the effect of metal cation on the enzymatic activity, Mg^{2+}, Zn^{2+} and Ca^{2+} were added in the reaction mixture separately with the concentration from 0.5 to 50 mM.

Results and Discussion

Isolation, identification and cellulolytic activity. We have successfully isolated 4 isolates showed potential cellulolytic activity from *Odontotermes bhagwatii,* and those were designated as SBT1, SBT4, SBT6 and SBT8, respectively. Molecular identification based on the 16SrRNA gene sequence showed that SBT1 and SBT8 belong to the group of *Paenibacillus*. SBT1 was much closer to the *Paenibacillus cellulositrophicus*, while the SBT8 close to the *Paenibacillus* sp. SBT4 and SBT6 were close to the *Pseudomonas citronellolis* and *Pseudoxantobacter soli*, respectively.

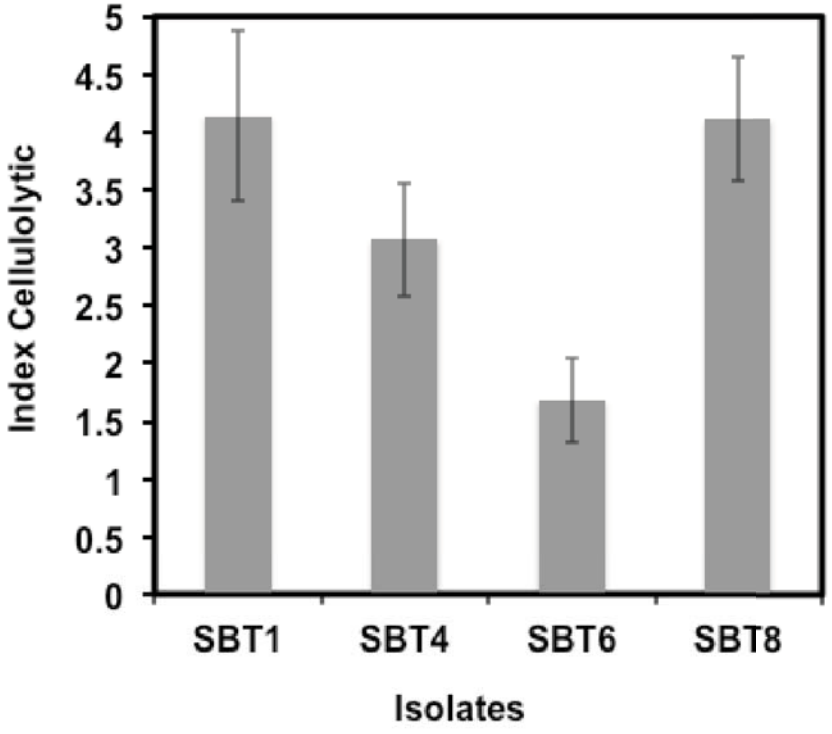

Fig. 1. Index cellulolytic of cellulolytic bacteria isolated from *Odontotermes bhagwatii.* Error bars represent the standard deviations of the corresponding values.

Cellulolytic activity assay showed that SBT1 and SBT8 exhibited the highest cellulolytic activity compared to SBT4 and SBT6 (Fig. 1). Therefore, we have chosen the SBT1 and SBT8 as a model for the extracellular cellulase production.

Extracellular cellulase production. To produce the extracellular cellulose, *Paenibacillus cellulsitrophicus* SBT1 and *Paenibacillus* sp. SBT8 were cultivated in defined or limited medium containing 1% CMC as a carbon sources. Cultivation was carried out for 2 days at room temperature. From this batch fermentation system, *Paenbacillus cellulositrophicus* SBT1 and *Paenibacillus* sp. SBT8 could produce extracellular cellulase roughly of 18.6 mg/l and 24.8 mg/l, respectively. This result indicated that those two isolates potentially could be used for large scaling production of the cellulase. For simplicity, hereafter, we designated the extracellular cellulase from *P. cellulositrophicus* SBT1 and *Paenibacillus* sp. SBT8 as SBT1 and SBT8 cellulase, respectively.

Effect of temperature on the cellulase activity. To test temperature dependencies of extracellular cellulase produced from SBT1 and SBT8, the SBT1 and SBT8 cellulase were test the their enzymatic activity on different temperature conditions. Both cellulases showed the temperature dependencies that were nearly identical (Fig. 2). Both celluases showed the highest activity at 35°C. At the temperature higher than 55°C, the enzymatic activity of both cellulases were roughly 20% reduced compared to the activity at the optimum temperatures. At the optimum temperature, the SBT1 and SBT8 cellulases exhibited the specific enzymatic activity of 0.17 and 0.12 U/mg, respectively. The result indicated that the SBT1 cellulase was more active than SBT1 cellulase. Based on the temperature dependencies assay, it clearly showed that both enzymes belong to the mesophilic enzymes at which optimum enzymatic activity were reached at moderate temperature.

Effect of pH on the cellulase activity. The enzymatic activities as function of pH were

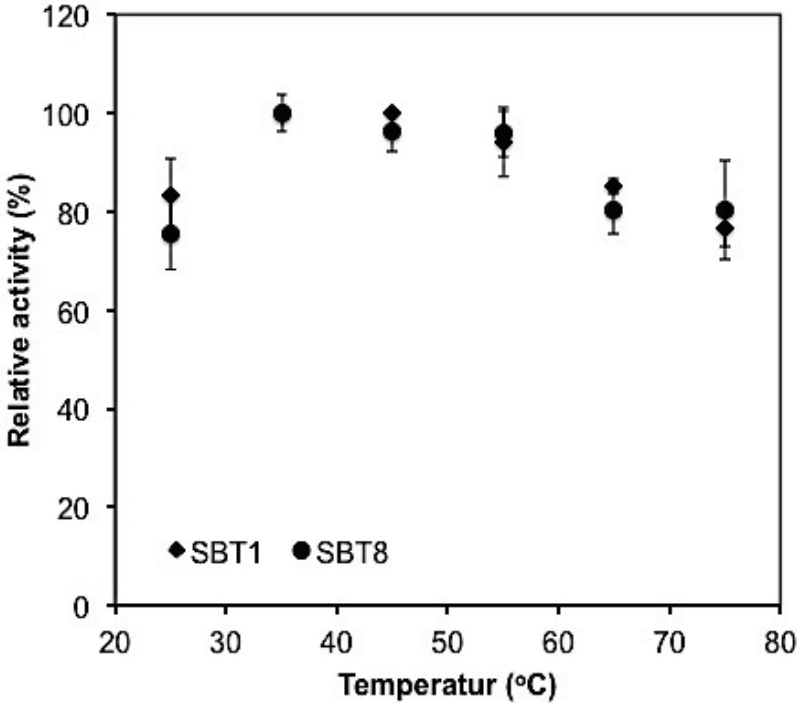

Fig. 2. Relative activity of SBT1 and SBT8 cellulase at various temperatures. Error bars represent the standard deviations of the corresponding values.

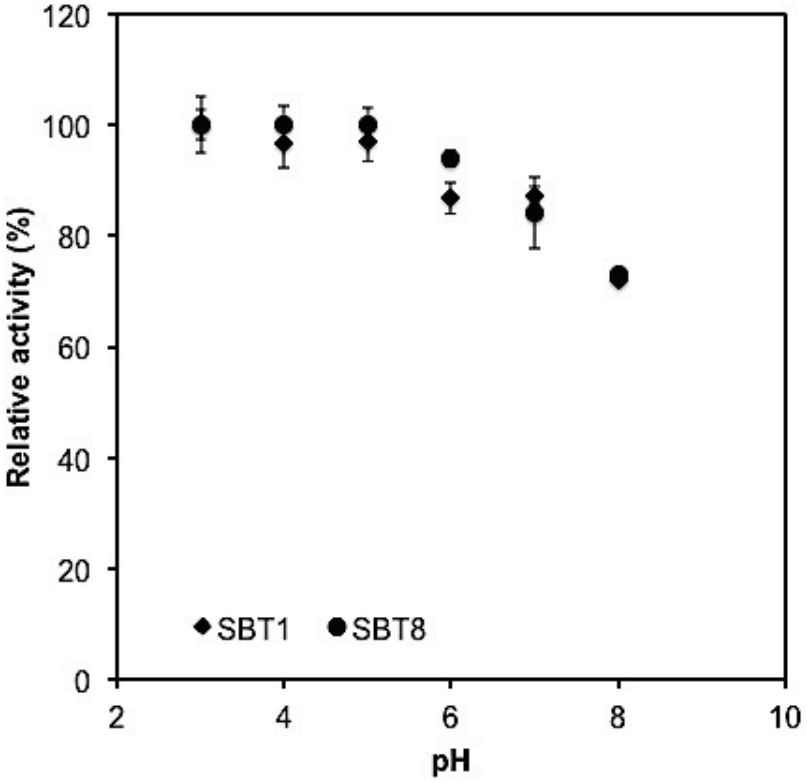

Fig. 3. Relative activity of SBT1 and SBT8 cellulase as a function of pH. Error bars represent the standard deviation of the corresponding values.

also examined in this work (Fig. 3). The results showed that both cellulase enzymes were exhibited the enzymatic activity at acidic pH, with the optimum pH was determined to be 3. The enzymatic activity of cellulose SBT1 and SBT8 were determined to be 0.19 and 0.13 U/mg, respectively. Lin *et al* (2012) reported that the *Bacillus thuringiensis* cellulase also exhibited enzymatic activity at the acidic pH (Fig.3).

Catalytic side of cellulase has two important catalytic residues, aspartate (D) and glutamate (E) (Davis and Henrissat, 1995). Aspartate and glutamate both have side chain confers with carboxyl group. The pK values of carboxyl group of asparatate (D) and glutamate (E) side chain are determined to be 3.65 and 4.25, respectively (Buxbaum, 2007). Those two residues function as acid and base during enzymatic catalysis. At the lower pH the carboxyl group of aspartate (D) residues undergoes deprotonated, therefore it could be proton donor for nucleophilic activation of water molecules. The nucleophile activated water molecules attack the glycosidic bond of cellulose (Mosier *et al*., 1999).

Effect of divalent metal cation on the cellulase activity. Several enzymes require the divalent metal cation for their enzymatic activity. Divalent metal cation plays important role for one third of the enzymes (Glusker *et al*., 1999). In order to know whether divalent metal cation affect the enzymatic activity of SBT1 and SBT8 cellulase, we have examined three divalent metal cations, Mg^{2+}, Zn^{2+} and Ca^{2+} on the enzymatic activity of both cellulases. The concentration of divalent metal cations was varied from 0.5 to 50 mM.

The results showed that addition of divalent metal cations at concentration of 0.5 to 10 mM generally increases the enzymatic activity of both cellulases (Fig. 4). The addition of Mg^{2+} and Zn^{2+} at the concentration

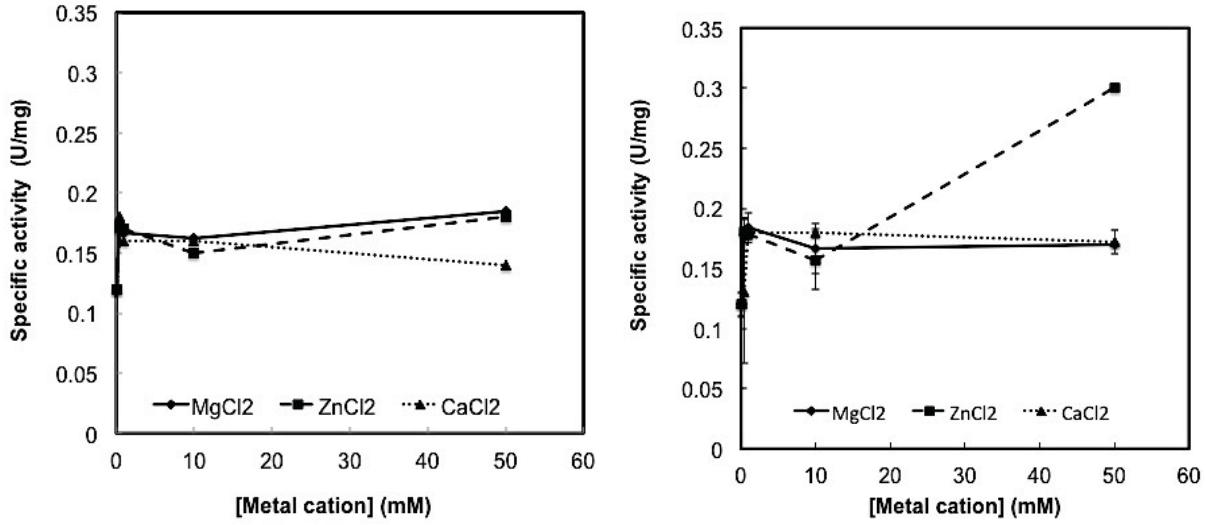

Fig. 4. Effect of divalent metal cations on the enzymatic acitivity of (a) SBT1 cellulase and (b) SBT8 cellulase. Error bars represent the standard deviation of the corresponding values.

above 10 mM, tend to increase further of SBT1 cellulase activity, while the Ca^{2+} tend to decrease enzymatic activity. However, in the case of SBT8, only Ca^{2+} at above 10 mM increases the enzymatic activity. It was surprisingly that both SBT1 and SBT8 showed similar dependencies on the present of Mg^{2+} and Zn^{2+} divalent metal cations. Both Mg^{2+} and Zn^{2+} have similar ionic radii, meanwhile Ca^{2+} has the largest ionic radii. The ionic radii of Mg^{2+} and Zn^{2+} are 0.65 and 0.72 Å, respectively, while ionic radius of Ca^{2+} is of 1.0 Å. In spite of the similar ionic radii, Mg^{2+} and Zn^{2+} both have dissimilar ligand binding. Mg^{2+} tend to form ligand with oxygen atom while Zn^{2+} readily to form ligand with nitrogen and sulfur. Although Ca^{2+} divalent cation has the largest ionic radii, this divalent cation has similar preference on the ligand binding to that of Mg^{2+} (Glusker *et al.*, 1999). To be note however, Zn^{2+} can bind to the oxygen atoms as ligand when it forms 6 coordination numbers. Therefore, it seems that the similarity or differences on the ionic radii cannot be ruled out for this discrepancies on the response of the enzymatic activity of SBT1 and SBT8 cellulases.

Conclusion

Extracellular cellulase from SBT1 and SBT8 both exhibited enzymatic activity. Both were active at acidic pH and broad range of temperature. The addition of divalent metal cations at 1 -10 mM significantly increased both cellulases activities.

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Authors Contribution

MSR performed data analysis, data interpretation and writing the manuscript; EP performed enzyme isolation and data acquisitions for enzymatic activity, respectively; YK performed bacterial isolation and identification; TY and EM contributed on critical reading the manuscript.

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