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Short Communication: The isolation of *Klebsiella variicola*'s cellulase from *Macrotermes gilvus* gut in Indralaya Peatlands, Indonesia

DWITA OKTIARNI[1,2,♥], HERMANSYAH[3,♥♥], EDDY IBRAHIM[4], MARSI[5], HASANUDIN[3], MIKSUSANTI[3], DEDE HERI YULI YANTO[6], NANIK RAHMANI[6], GETARI KASMIARTI[7]

[1]Doctoral Program of Mathematics and Natural Sciences, Graduate Program, Universitas Sriwijaya. Jl. Palembang-Prabumulih Km. 32, Indralaya, Ogan Ilir 30662, South Sumatra, Indonesia. Tel.: +62-711-580269 ext. 580056, Fax.: +62-711-580056, ♥email: dwita.oktiarni@unib.ac.id

[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Bengkulu. Jl. WR. Supratman Kandang Limun, Muara Bangka Hulu, Bengkulu 38371, Bengkulu, Indonesia

[3]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya. Jl. Palembang-Prabumulih Km. 32, Indralaya, Ogan Ilir 30662, South Sumatra, Indonesia. Tel.: +62-711-580269 ext. 580056, Fax.: +62-711-580056, ♥♥email: hermansyah@unsri.ac.id

[4]Department of Mine, Faculty of Engineering, Universitas Sriwijaya. Jl. Raya Palembang-Prabumulih Km. 32, Indralaya, Ogan Ilir 30662, South Sumatra, Indonesia

[5]Department of Soil, Faculty of Agriculture, Universitas Sriwijaya. Jl. Raya Palembang-Prabumulih Km. 32, Indralaya, Ogan Ilir 30662, South Sumatra, Indonesia

[6]Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Cibinong Science Center. Jl. Raya Bogor Km. 46 Cibinong 16911, West Java, Indonesia

[7]Doctoral Program of Environmental Science, Graduate Program, Universitas Sriwijaya. Jl. Padang Selasa No.524, Bukit Besar, Palembang 30139, South Sumatra, Indonesia

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Abstract. *Oktiarni D, Hermansyah, Ibrahim E, Marsi, Hasanudin, Miksusanti, Yanto DHY, Rahmani N, Kasmiarti G. 2023. Short Communication: The isolation of* Klebsiella variicola*'s cellulase from* Macrotermes gilvus *gut in Indralaya Peatlands, Indonesia. Biodiversitas 24: 4218-4222.* Termites are organisms that can inflict damage on agricultural, forest, and furniture materials. Furthermore, they can positively affect the ecosystem due to their ability to break down lignocellulose-containing materials. According to previous findings, the cellulolytic bacteria from the termite *Macrotermes gilvus* gut obtained in Indralaya Peatlands showed activity and hydrolyzed cellulose in a CMC agar medium. In this study, the cellulase enzymes of *Klebsiella variicola* isolated from *Macrotermes gilvus* gut showed higher cellulolytic indexes after staining with Congo red. Furthermore, the enzyme was purified and characterized using ammonium sulfate precipitation and dialysis. SDS PAGE and zymogram analysis reported a molecular mass of 20 kDa, with optimal activity at pH of 6 and temperature of 50°C.

Keywords: Cellulase, cellulose, termite

INTRODUCTION

In bioethanol production, 40-60% of the total bioethanol produced uses commercial enzymes (Susilowati et al. 2018). In the second generation of biofuel production using lignocellulosic biomass, this renewable and environmentally energy source has become a fundamental interest (Hermansyah et al. 2016, 2018). Bioethanol production with higher costs and hazardous chemicals needs to be avoided and reduced. This can damage the environment through physical, chemical, or physicochemical treatment (Hermansyah et al. 2018; Varghese et al. 2017; Batool et al. 2021). The enzymatic process is one of the green technologies in producing bioethanol from biomass and cellulase enzymes (Robak and Balcerek 2018). Several studies were conducted to reduce costs by replacing commercial enzymes with microbial symbionts (Ferbiyanto et al. 2015).

Termites are organisms with the capability to degrade cellulose into glucose by the bacteria and microbial symbionts within their digestive tract. The diet is highly varied as dead grass and other plant matter without wood sources. The chewed-up plant matter is used to construct elaborate fungus gardens in their nests (Ahmad et al. 2021). Termites that live in colonies are pests for oil palm and rubber plantations because they can damage these plants by making nests in these plants, in the ground, and mounds of the earth. The nests are usually located around palm and rubber trees. However, their primary source of food is obtained from cellulose, including wood, stems, roots, and leaves (Brune 2014; Hongoh 2011). Termites play a significant role in tropical peat swamp forests, food network, and maintain natural stability (Neoh et al. 2017).

The demand for cellulase in the industrial sector is rapidly increasing, and Asia-Pacific is the region with the most significant amount at about 32.84%. Cellulase enzymes are widely used as biocatalysts in food, beer and wine, textile, and agricultural products (Bhardwaj et al. 2021). The cellulolytic enzyme was isolated from bacteria in the termite gut and purified to obtain cellulase. Furthermore, this enzyme was characterized to determine the optimal pH and temperature conditions.

MATERIALS AND METHODS

Study area

Klebsiella variicola was isolated from the termite *Macrotermes gilvus* gut obtained from Tanjung Senai, Indralaya peatland area, Indonesia (Oktiarni et al. 2021, 2022). The isolated bacteria had the potential to produce cellulase enzymes.

Enzyme purification

Single colonies of *Klebsiella variicola* were inoculated in a flask containing 5 mL of 1% CMC media and incubated using a shaker incubator at 32°C and 150 rpm for 24 hours. The inoculum was transferred into a flask containing 100 mL of 1% CMC media and incubated using a shaker incubator at 45°C and 150 rpm for 24 hours. Subsequently, the inoculum was centrifuged at 4°C and 10,000 rpm for 10 minutes to obtain a supernatant. The protein content of the supernatant was precipitated using ammonium sulfate with a saturation level of 0%-20%, 20-40%, 40-60%, 60-80%, and 80-100% (w/v) and left overnight at 4°C. The residue was taken and centrifuged at 4°C and 10,000 rpm for 30 minutes, then dissolved in a small amount of 10 mM sodium phosphate buffer pH 6. Each enzyme fraction (1000 µL) was taken, and the activity was measured at 540 nm.

Gel electrophoresis

The enzyme fraction obtained was determined by molecular weight using SDS-PAGE. Meanwhile, 10 µL of protein sample was added to 10 µL of loading buffer at 70°C for 20 minutes in a PCR machine before loading into the gel (7% stacking gel and 12% separating gel). The initial electric current in the electrophoresis instrument was 200 W, 120 V, and 50 mA for 120 minutes. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 at room temperature and 40 rpm for 24 hours. Subsequently, it was washed with distilled water and decolorized with a destaining solution for 1 hour until clear and visible bands were obtained. The gel was photographed and documented, scanned and displayed, and the molecular weight was determined using the Vivantis protein ladder. The data obtained were analyzed by comparing the bands on the gel with the protein ladder.

The zymogram analysis was carried out by adding the enzyme solution with a buffer sample without β-mercaptoethanol containing: 15.5 mL 1 M Tris-HCl pH 6.8, 2.5 mL of 1% bromophenol blue solution, 7 mL of deionized water and 25 mL of 50% glycerol (v/ v). The sample was loaded on 5% stacking gel and 12% separating gel containing CMC. After electrophoresis, the gel was rinsed with Triton X-100 to remove SDS from the gel. Then stored in deep well maximizer in 50 mM sodium phosphate (pH 6) at 50°C for 1 hour. After rinsing with buffer, the gel was washed with aquadest for 2 minutes. Then the gel was stained with 0.1% Congo red for 30 minutes and decolorized with 1 M NaCl until a clean gel was obtained.

Cellulase assay

CMC (1% w/v) was dissolved in 50 mM sodium phosphate buffer pH 6, and 250 µL was added with the same quantity of enzyme and incubated at 30°C for 30 minutes. The reducing sugar was formed after adding 500 µL of dinitrosalicylic acid (DNS) reagent and boiled in a water bath for 10 minutes. After cooling, the absorbance of the samples was measured at 540 nm. Furthermore, 1 unit of cellulase activity is the amount of enzyme used to produce 1 mol of reducing sugar per minute.

Determine of protein

Determination of enzyme protein content can be conducted by measuring absorbance toward enzyme activity. The Bicinchoninic acid (BCA) reagent was used as a standard solution with various concentrations of 0, 20, 40, 60, 80, and 100 ppm. The enzyme protein content was determined with 10 µL of enzyme solution reacted with 20 µL of Bicinchoninic acid (BCA) reagent and incubated at 37°C and 90 rpm for 30 minutes. Meanwhile, the samples were incubated at room temperature for 10 minutes, and the absorbance was measured at 562 nm. The absorbance was substituted in the standard BCA linear regression equation as the protein concentration. The value of protein content was used in determining the specific activity of the enzyme.

Characterization of cellulase enzyme

Determination of pH

The optimum pH was determined by measuring the absorbance of the enzyme at a wavelength of 540 nm. The buffer solutions used were 50 mM citrate (pH 4-6), 50 mM sodium phosphate (pH 6-8), 50 mM Tris-HCl (pH 8-10), and 50 mM glycine-NaOH (pH 9-10). Furthermore, the cellulase enzyme was incubated with 100 mM sodium phosphate buffer solution pH 6 containing 1% CMC substrate, and the activity was measured at the optimum temperature.

Determination of temperature

Enzyme activity was measured at temperatures 30, 40, 50, 60, 70, 80, 90, and 100°C. The cellulase was incubated with 100 mM sodium phosphate buffer solution pH 6 containing 1% CMC as a substrate. Meanwhile, the activity was measured with a DNS reagent at 540 nm, and the sample solution without enzymes was used as a control.

RESULTS AND DISCUSSION

Enzyme purification

The cellulase enzyme was isolated in culture media obtained at optimum conditions. Fresh bacterial isolates were produced in 1 L of culture media and centrifuged to obtain the supernatant. The supernatant was then precipitated with ammonium sulfate. The addition was made slowly while stirring for the reaction between the enzyme and the ammonium sulfate to proceed effectively. The crude enzyme extract was stored in the refrigerator at 4°C and stirred at low speed for approximately 12 hours,

keeping the interaction of protein molecules with the ammonium sulfate at maximum for proper precipitation.

Crude enzyme in ammonium sulfate fraction 20-40% (Figure 1) showed the highest value of cellulase activity compared to other fractions at 0.195 U/mL. Protein fractionation aims to separate crude protein extract from other molecules to increase cellulase activity. The 0-40% enzyme fraction was centrifuged to obtain a precipitate/pellet, a crude extract of the cellulase. The extract was dialyzed using a dialysis bag to remove the remaining salts in the solution.

The activity of the crude enzyme was 0.19502 U/mL with a specific activity of 1.83925 units/mg and 0.10603 mg/mL of protein. After fractionation with ammonium sulfate, the specific activity slightly increased by 1.92828 units/mg. Furthermore, the enzyme was 1.92255 units/mg after dialysis, with a slight increase of 1.04529 folds (Table 1).

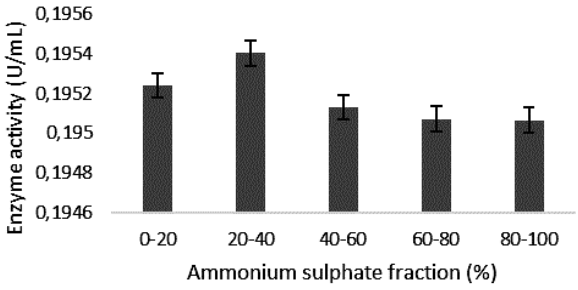

Figure 1. Activity cellulase enzyme with the ammonium sulfate precipitation

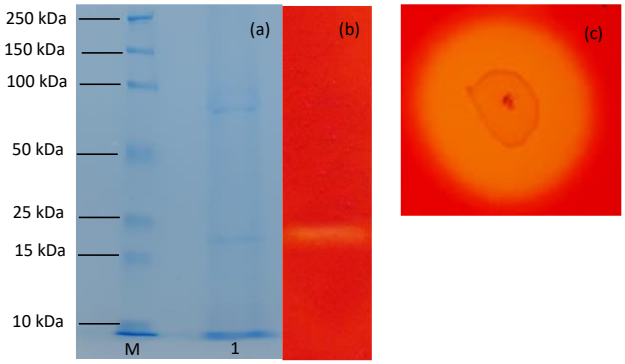

Figure 2. Gel SDS PAGE (a) M: marker protein; 1: crude enzyme, dan (b) zymogram analysis, (c) halo zone of hydrolyzing enzyme after staining with Congo red

Gel electrophoresis

The crude extract of the isolated enzyme determined the molecular weight by SDS PAGE. The protein bands that appear on the electrophoresis gel can be seen in Figure 2 (a). The crude extract had a molecular weight of about 20 kDa. The cellulase activity was indicated by the presence of a clear zone on staining with Congo red (Figure 2.C).

Characterization of enzyme

The enzyme's crude extract activity has an optimum value of 0.39066 U/mL at pH 6 with sodium phosphate buffer solution (Figure 3). The optimum pH conditions are required to activate all enzymes that bind to the substrate and convert them into products.

The optimum temperature of the crude extract of the enzyme was 50°C with an activity of 0.39097 U/mL (Figure 4). The temperature increases with the extract activity due to the higher kinetic energy.

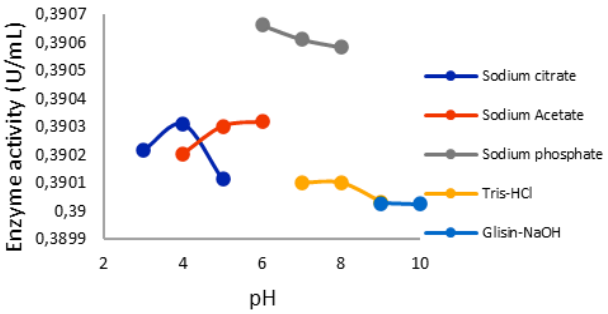

Figure 3. Effect of pH on enzyme activity

Discussion

In the previous study, *Klebsiella variicola* was isolated from *Macrotermes gilvus* gut in Tanjung Senai, Indralaya peatland area, Indonesia (Oktiarni et al. 2021, 2022). These bacteria showed a clear zone in the CMC agar medium and had the potential to degrade cellulose after staining in Congo red solution. The cellulolytic enzyme was isolated and purified using ammonium sulfate and dialysis.

The activity enzyme from *Klebsiella variicola* was 1.92255 units/mg, with a purification level of 1.04529 folds and a percentage yield of 5.0494. Furthermore, the purification fold for the enzyme from *Bacillus sphaericus* was 7.8, with 66.4 µ/mg specific activity protein and a yield of 35.8 (Ekwealor et al. 2017). Cellulase enzyme isolated from termite soldiers (*Ametermes eveuncifer*) had a specific activity of 5.04 U/mg with a percentage yield of 11.7% (Fagbohunka et al. 2017). The strain *Brucella* sp. and *Bacillus licheniformis* showed an enzyme activity of 96.37 U/ml and 98.25 U/ml, respectively (Behera et al. 2016).

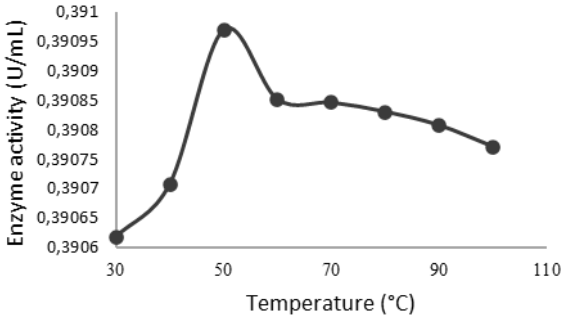

Figure 4. Effect of temperature on enzyme activity

Table 1. Summary of purification steps of an enzyme from *Klebsiella variicola*

Purification steps	Enzyme activity (U/mL)	Protein (mg/mL)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude enzyme	0.19502	0.10603	183.925	1	100
Ammonium sulfate	0.19540	0.10133	192.828	104.840	50.125
Dialysis	0.19509	0.10147	192.255	104.529	

SDS-PAGE showed this enzyme had a molecular weight of about 20 kDa, whereas relative molecular mass range of the enzyme from *Bacillus sphaericus* was estimated between 22.3 kDa-26.3 kDa (Ekwealor et al. 2017). However, the analysis of the partially purified cellulase enzyme from *Brucella* sp. and *Bacillus licheniformis* exhibited bands of approximately 55 and 72 kDa, respectively (Behera et al. 2016).

The optimum pH of the studied enzyme, *Bacillus sphaericus*, and *Bacillus cereus* sp.10 was 6.0, 9.0 (Ekwealor et al. 2017), and 5.0 (Titilayo et al. 2022). Moreover, the cellulase enzyme isolated from termite soldiers (*Ametermes eveuncifer*) had optimum pH of 8 (Fagbohunka et al. 2017).

The cellulolytic enzyme of *Klebsiella variicola* had an optimum temperature of 50°C, and similar of our research, the optimum temperature of cellulase enzyme from *Ametermes eveuncifer* had also of 50°C (Fagbohunka et al. 2017). Meanwhile, the cellulase enzyme isolated from *Bacillus sphaericus* was optimally active at 40°C (Ekwealor et al. 2017), and partially purified cellulase produced by *Bacillus cereus* sp.10 was active at a temperature of 60°C (Titilayo et al. 2022).

Termite are a threat to the world's agricultural community, but termites are organisms that are capable to maintain environmental cycle (Liang et al. 2020). Termites have cellulolytic bacteria in their gut, which have the ability to hydrolyze cellulose into glucose by their cellulase enzymes. The associated symbionts, the distribution, and enzyme production throughout the gut tunnels play a very important role in assisting in the degradation of lignocellulosic biomass (Bhujbal et al. 2021).

In industry field, the production cellulase enzyme is dominated by fungi *Aspergillus nidulans*, *A. niger*, *Penicillium* spp., and *Trichoderma reesei* (Battaglia et al. 2011). Enzymes derived from microorganisms can be used more widely in the industrial applications (Bušić et al. 2018). Enzymatic hydrolysis is influenced by specific conditions with pH and thermotolerance (e.g. pH of 6 and temperature below 50°C), so that it requires less energy and does not have a negative impact on the environment, eco-friendly technology (Mussatto and Teixeira 2010).

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