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PURIFICATION AND CHARACTERIZATION OF CELLULASE OF MOLD ISOLATED FROM VERMICOMPOSTING PROCESS OF PALM OIL EMPTY FRUIT BUNCHES

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Abstrak. In previous investigation of vermicomposting process of palm oil empty fruit bunches, five cellulase-producing microorganisms had been isolated. An isolated VTMI identified as *Aspergillus* sp. as a cellulase producing mold, and proven produced extracellular cellulase in solid state fermentation of palm oil empty fruit bunches. The obtaining crude cellulase was purified to homogeneity by ammonium sulfate precipitation, gel filtration on Sephadex G-100 chromatography, and finally purified on ion exchange chromatography on DEAE Cellulofine A-500. The yield and purification fold were 12.89% and 107.50, respectively. The purified cellulase was easily released glucose when 5% CMC applied as substrate, as shown by TLC analysis. The purified cellulase had an optimal pH and temperature at 4.0 and 45°C, and was stable at pH 3-7 and 30-50°C, respectively.

Key words: extracellular enzyme, cellulase, purification, VTM 1 (*Aspergillus* sp.).

Cite

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INTRODUCTION

Cellulase is an essential enzyme for industry which categorized as 1,4- β -D-glucan selobiohidrolase (exoglucanase), 1,4- β -D-glucan 4-glukanohidrolase (endoglucanase), and β -glucoside-glukohidrolase or β -D-glucosidase (cellobiase). β -D-glucosidase itself synthesize by specific gene in microorganism (Fitri et al., 2017), thus the characteristic would be unique for each species. Cellulase hydrolyzes cellulose complex polysaccharides to simple polysaccharide form, which consist of oligo and sugar as monomers, called glu-

cose (Kim & Kim, 1995; Muzakhar et al., 2015; Muzakhar et al., 2017). Cellulose is composed of D-glucose which are bound by ties of $\beta(1\rightarrow4)$. Cellulase is produced by cellulolytic microorganisms, such as fungi, bacteria and actinomycetes (Ariffinet. al, 2006; Kanti, 2005). The application of cellulase in the industry is more desirable than using synthetic chemicals because high purity level of products that produced, do not cause corrosion, resulting in higher yields and do not require a lot of energy in the process of hydrolysis (Bakare et al., 2005; Masfufatun, 2009).

A huge amount of palm oil empty fruit bunches (POEFB) produced during oil palm production and about 40% of POEFB material is consist of cellulose fiber (Hayati, 2010). Decomposition of palm oil empty fruit bunches are naturally very slow, thus it is necessary to develop alternative method to degrade the waste faster and effectivelly. One way to degrade the waste of POEFB is using enzymatic reaction, with the help of cellulolytic microorganisms. Based on previous results, it is clear that five of cellulolytic molds (VTM1, VTM5, VTM6, VM9, and VT12) have significant responsible in vermicomposting process of palm oil empty fruit bunches (Yuniar, 2013), however no further study to elucidate these enzymes. In this study, an isolate VTM1 had been selected as a source for cellulase production, characterisation, and purification.

MATERIALS AND METHODS

Microorganism Used

Five isolates of mold namely VTM1, VTM5, VTM6, VM9, and VT12 from previous investigation has been used in this research.

Growth Analysis

This step was done by counting the isolates spores which inoculated to PDA, incubated for 1-7 days in 30°C. The spore were counted everyday with Haemacytometer.

Preparation of Crude Enzyme

All isolates were cultured in POEFB medium, containing 0.1% pepton, and incubated for 1-7 days on incubator at 37°C. Enzyme harvested daily (1-7 days) with 20 ml distilled water containing 1% NaCl and 0.01% sodium azide. Each culture was centrifuged at 4,000 rpm for 10 min. The clear supernatants were

collected as extracellular cellulase preparations.

Cellulase Assay

Cellulase activity was quantified ($\mu\text{g/ml/min.}$) from reducing-end of sugars produced in hydrolysates when of CMC or POEFB substrate used. To quantify of sugars produced, the Somogyi-Nelson method was employed. The cellulase activity was determined by incubating the enzyme (0.5 ml) in 0.5% CMC (0.5 ml) and 0.5% POEFB substrate in 0.02 M acetate buffer, pH 5.0 at 37°C for 2 h. The reaction was terminated by the addition of 0.5 ml Somogyi reagent and heated for 15 min in boiling water. The solution was cooled down to room temperature and 0.5 ml Nelson reagent was added. The resulting solution then diluted with distilled water (2.5 ml) and measured at optical density (OD) 500 nm against the blank. To calculate of sugar content in hydrolysate, the standard curve with known glucose concentration was used.

Enzyme Purification

Ammonium Sulfate Precipitation

The proteins in the crude preparation were precipitated by the addition of solid ammonium sulfate to 70% saturation. The precipitate was allowed to form at 4°C and was collected by centrifugation at 12,000 at 4°C for 20 min. The precipitate was re-dissolved in 10 ml of 0.02 M acetate buffer, pH 5.0.

Gel Filtration on Sephadex G-100

The enzyme concentrate obtained from ammonium sulfate precipitation was re-dissolved in minimal amount of buffer and dialyzed for 24 h against 0.02 M sodium phosphate buffer, pH 5.0 containing with changes of the buffer per 4 hour. Remaining debris was removed by centrifugation and the supernatant was slurry loaded on the prepared Sephadex

G-100 column (2.5 x 25.0 cm) and equilibrated with 0.02 M acetate buffer, pH 5.0. The elution was done at a flow rate of 5 ml/23 minutes. The protein concentration in every fraction was monitored at 280 nm and the cellulase activity was assayed using the procedure above. Fractions with cellulase activity were pooled and was stored at 4°C for the next purification step.

Purification by Ion Exchange Chromatography on DEAE Cellulofine A-500

DEAE Cellulofine A-500 column was packed into a column (2.5 x 25 cm). The peaks with highest cellulase activity from the gel filtration step were applied to the column. The column was equilibrated with 3 bed volumes of 0.02 M acetate buffer, pH 5.0. The column was washed to remove all unbound proteins and a stepwise concentration of 0, 0.1, 0.2, 0.3, and 0.5 M NaCl in 0.02 M acetate buffer pH 5.0 was used to elute any bound proteins. Fractions (5 ml) were collected at a flow rate of 5 ml/5 minutes. The elution was monitored for protein concentration at 280 nm and was also assayed for enzyme activity. Fractions containing cellulase activities were pooled together and stored at 4°C

Thin Layer Chromatography (TLC)

The enzymatic hydrolysis product was analyzed by TLC on a silica gel plate (silica gel 60 F254). Butanol : ethanol : chloroform:ammonia (4:7,5:4:8) was employed as a solvent system. Sulphuric acid was used for detection. The spots were dried by heating at 170°C. All experiments were carried out at room temperature.

Effect of pH and temperature on cellulase activity

In order to analyze the stability of the cellulase at different pH values, the purified

enzyme was incubated at 37°C in 20 mM acetate buffer (pH 3 to 5.5) and phosphate buffer (pH 5.5 to 8.5). The remaining activity of each sample was measured after 4 hours of incubation. For measuring its thermal stability, the purified cellulase was incubated in 20 mM acetate buffer pH 4 at 30°C to 60°C. Remaining activity was measured after for 4 hours of incubation. The effects of pH and temperature on the optimum activity of enzyme were also measured in series pH and temperature range as mentioned above, using 1% CMC with 2 hours incubation at 37°C.

RESULT AND DISCUSSION

Exponential Phase Analysis

Based on the pattern of growth over 7 days, the adaptation phase occurs on day 0 to day 1. It was characterized by the absence of an increase in the number of spores during that time. Adaptation phase is a phase adjustment of microorganisms to growth media. Logarithmic phase for mold isolates VTM1, VTM5, VM9 and VT12 occurred on the third day which was marked by an increase in the number of spores in each isolate mold, while the VTM6 occurred on the fourth day.

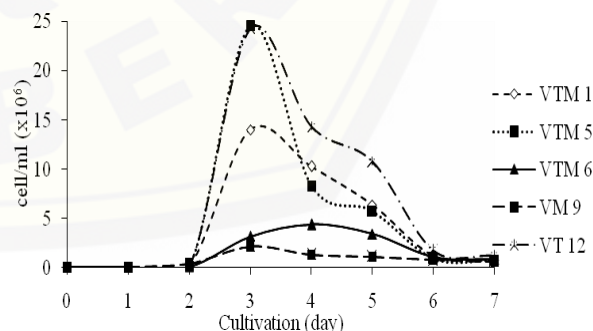


Figure 1. Growth curve isolates

Isolate Selection

Isolate selection used to choose one of mold isolate that has highest activity.

Table 1. Measurement of Enzyme Activity with CMC and POEFB Substrates

Isolate	Cellulase Activity Index		Enzyme Activity			
	*)		CMC (5%)		POEFB (5%)	
	CMC	TKKS	µg/ml	U/ml	µg/ml	U/ml
VTM1	3.12	3.65	71.33	4.13	34.28	3.97
VTM 5	8.7	5.4	14.42	0.83	22.57	2.61
VTM 6	2.7	5	18.33	1.06	25.03	2.90
VM 9	1.17	1.66	27.50	1.59	19.51	2.26
VT 12	3.5	6	15.25	0.88	24.66	2.85

*)Adapted from Yuniar (2013)

VTM1 readily hydrolyzed 5% CMC and 5% POEFB substrate and released reducing sugar 71.33 and 34.28 µg/ml. Based on this results, the cellulase produced by VTM1 was selected as a source cellulase for purification. As shown at Table 1, the highest activity of cellulase produced by VTM1 when 5% CMC either 5% POEFB used.

Enzyme Purification

Crude enzyme obtained from optimum cultivation was collected and purified. For the first step, the crude enzyme was brought to 70% of saturated ammonium sulfate, precipitated by centrifugation at 12000 rpm for 20 minutes, followed by dialysis on cellulose tube (10KDa pores) using 20 mM acetate buffer at pH 5 to remove remaining ammonium sulfate. After dialysis, the concentrated enzyme was loaded onto open column containing Sephadex G-100 pre-equilibrated with the same buffer. The elution profile is showed in figure 2.

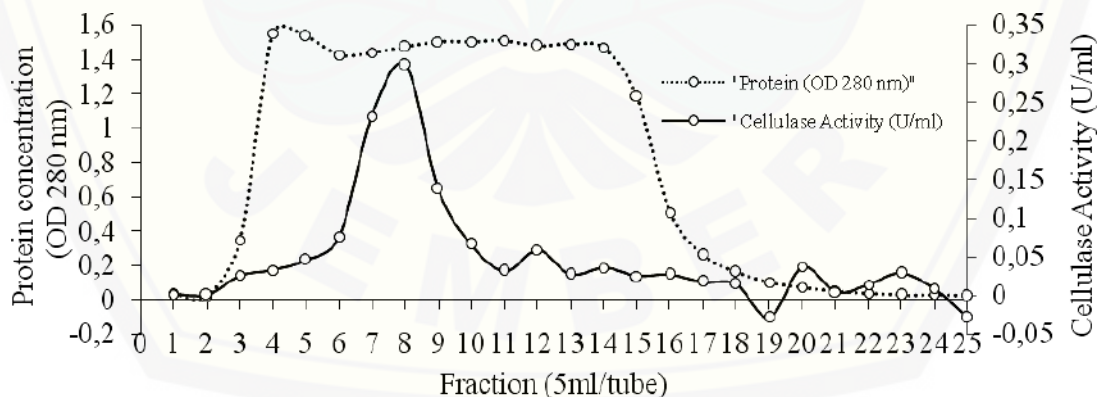


Figure 2. Elution profile of partially purified cellulase from VTM1 (*Aspergillus sp.*) on Sephadex G-100 column. The cellulase activity was determined in each fraction by the appearance of reducing end group in solution of CMC.

Finally, an ion exchange chromatography on DEAE Cellulofine A-500 was used to obtain purified cellulase. DEAE cellulose pre-equilibrated with the same buffer. For the frac-

tionation, the column was eluted using acetate buffer with the gradient 0-0.5 M NaCl. The active fractions were pooled and stored at 4°C. The elution profile is showed in figure 3.

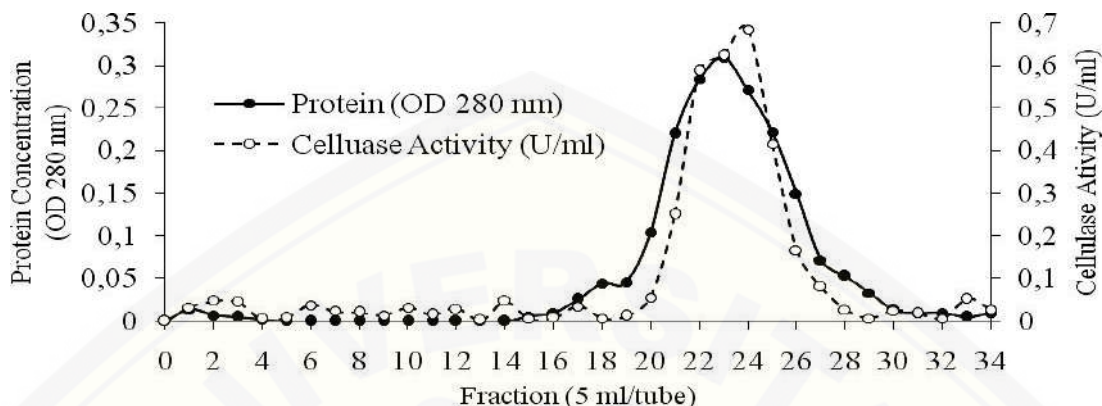


Figure 3. Elution profile of partially purified cellulase from VTM 1 (*Aspergillus sp.*) on DEAE-Cellulofine A-500 column. The cellulase activity was determined in each fraction by the appearance of reducing end group in solution of CMC.

The results of the purification of the cellulase from VTM 1 (*Aspergillus sp.*) are

summarized in Table 2.

Table 2. Summary of Purification of Cellulase from VTM 1 (*Aspergillus sp.*)

Purification Step	Enzyme Activity (U/ml)	Total Activity (U)	Total Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude enzyme	14.70	1470.43	5723.40	0.26	100	1
Ammonium sulfate	16.17	404.32	3006.25	0.13	27.50	0.52
Sephadex G-100	5.04	151.17	19.32	7.82	10.28	30.45
DEAE Cellulofine	4.74	189.47	6.86	27.62	12.89	107.50

As shown in Table 2, after loaded into ion exchange chromatography on DEAE Cellulofine A-500, the purified cellulase was obtained. The yield and purification fold were 12.89% and 107.50, respectively. The culture filtrates of VTM 1 (*Aspergillus sp.*) expressed cellulase which was subsequently purified to homogeneity. The gel filtration yielded only one peak with specific activities of 27.62 U/mg protein respectively. These values were greater than the values obtained for cellulase from *Aspergillus niger* (4.2×10^{-3} U/mg) (Hurstet et al.,

1977) and lower than *Aspergillus oryzae* (43.9 U/mg) (Yamane et al., 2002).

Cellulase Characterization

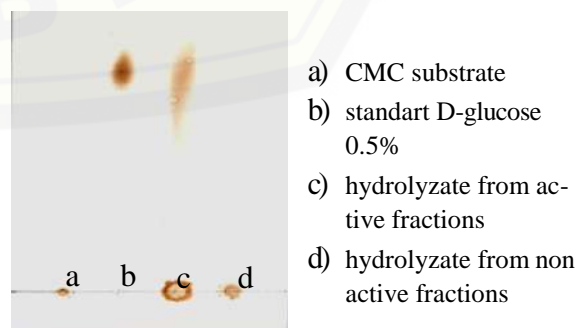


Figure 4. TLC Analysis of hydrolyzates

The spot on TLC show the presence of monosaccharides from CMC hydrolysis by cellulase enzymes. When compared with 0.5% glucose standard can be seen that the monosaccharide is glucose. When compared with

the results of the inactive fraction, showed that purified enzyme can produce monomers glucose. This evidence proved that the purified cellulase easily released glucose when 5% CMC as substrate as shown by TLC analysis.

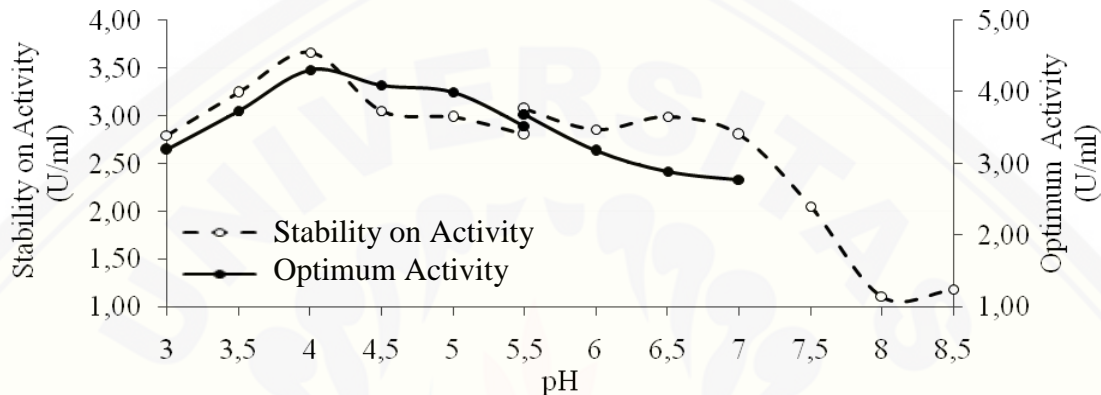


Figure 5. Effect of pH on optimum activity and stability of purified cellulase

As shown on Figure 5, the cellulase had optimum activity at pH 4 and the stability at pH 3 to 5. Furthermore, as shown on Figure 6, the

cellulase had optimum activity at 45°C and stability at 30 to 50°C, correspondingly.

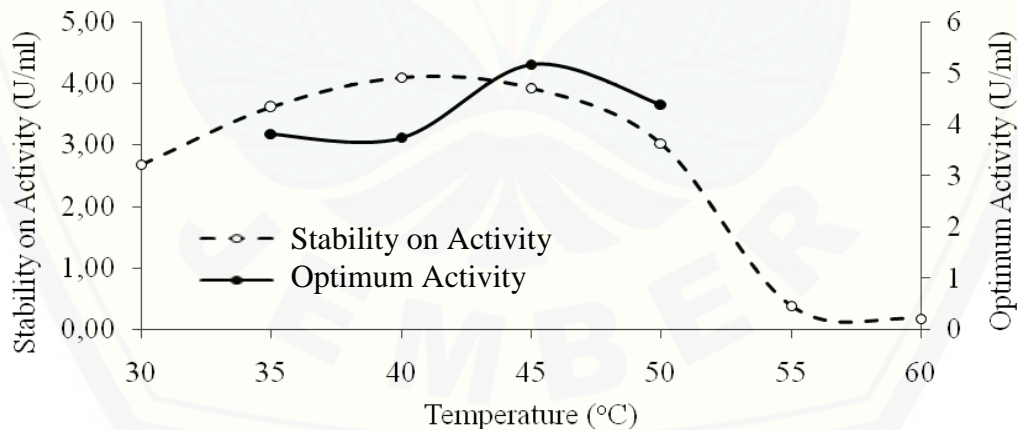


Figure 6. Effect of temperature on optimum activity and stability of purified cellulase

This result is similar to researches of Begum & Absar (2009) and Yamane et al., (2002) with *Aspergillus oryzae*; Devi & Kumar (2012) and Hurst et al., (1977) with *Aspergillus niger*; and Andrade et. al, (2011) with *Trichoderma*, sp., which the range of activity of cellulase enzymes from fungi at pH 3-

7,5 and at a temperature of 40-60°C, cellulase enzymes undergo denaturation at pH below 2.5 and above 7.5, and the temperature is below 30°C and above 80°C. At higher or lower pH and temperature than the stabil, enzyme activity decreases because of denaturation.

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