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Identification, characterization, and purification of xylanase PC-01 isolated from Pacet hot spring

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Abstract

This research aims to characterize, identify and purify the xylanolytic enzymes by hydrophobic interaction and anion exchange chromatography via the endo-β-xylanase activity test with the DNS method. The best results obtained from anion-exchange chromatography of third fraction pH 5 with the purity level of 78.25 times higher than crude extract. In conclusion, Characteristics of bacterial isolates PC-01 are rod-shaped bacterial cells, Gram-positive, and have subterminal oval endospores.

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Keywords: Endo-B-Xylanase, Hydrophobic, Interactions, Chromatography.

Identificación, caracterización y purificación de xilanasa PC-01 aislada de aguas termales de Pacet

Resumen

Esta investigación tiene como objetivo caracterizar, identificar y purificar las enzimas xilalanolíticas por interacción hidrofóbica y cromatografía de intercambio aniónico a través de la prueba de actividad endo-β-xilanasa con el método DNS. Los mejores resultados obtenidos de la cromatografía de intercambio aniónico de tercera fracción pH 5 con un nivel de pureza de 78.25 veces mayor que el extracto crudo. En conclusión, las características de los aislamientos bacterianos PC-01 son células bacterianas en forma de bastón, grampositivas y tienen endosporas ovales subterráneas.

Palabras clave: Endo-B-Xilanasa, Hidrofóbico, Interacciones, Cromatografía.

1. INTRODUCTION

Enzymes are highly important for a wide range of industrial processes including food and beverage, pharmaceutical, pulp and paper, agricultural, cosmetic, textiles, and leather industries. The use of enzymes is gaining interest owing to their stability, catalytic efficiency, non-toxic, and can be manipulated through molecular engineering process. The needs for finding safely environmental methods with reduction in cost and natural resource depletion are the

main factors for the increasing demand for enzyme usage. World demand for enzymes is expected to increase approximately USD 11.03 Billion by 2026, according to a new report by Reports and Data. Enzymes are biological molecules that highly specific; only accelerate the rate of particular reaction by lowering the activation energy without undergoing any permanent change in them, and therefore, are vital biomolecules that support life (ALDRIDGE, 2013; USAK, KUBIATKO, SHABBIR, DUDNIK, JERMSITTIPARSERT, & RAJABION, 2019).

One type of enzyme that is widely used in industrial activities is degradative enzymes. The enzyme included in this type is the hemicellulase enzyme which is part of the group of lignocellulose enzymes. The hemicellulase complex enzyme has a degradative activity against hemicellulose which is one of the raw materials in biomass industry activities. Lignocellulosic biomass contains lignin, hemicellulose, and cellulose. Hemicellulase is usually a mixture of various hydrolytic enzymes that work on xylan (KUMAR, CAMPBELL & TURNER, 2016). Xylan is the main component of hemicellulose which is polymer of β (1-4) D-xylopiranosa (xylose) with β-1,4-glycoside bonds and chains containing acetyl, glucoronosil, and arabinosil depending on the plant species. Complete degradation of xylan involves complex enzymes that are responsible for hydrolyzing the various linkages in hemicellulose: endo-β-D-1,4-xylanase (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), α-Larabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139),

acetyl xylan esterase (EC3.1.1.72), and ferulic acid esterase (EC3.1.1.73).

Endo-β-D-1,4-xylanase degrades internal glycosidic linkages of the xylan backbone-producing xylooligosaccharides, followed by β-D-xylosidase that cleaves the nonreducing termini of xylobiose and xylooligosaccharide fragments into xylose. Xylan abundance in the biosphere makes the role of xylanolytic enzymes highly important for xylan bioconversion. Xylan biodegradation is carried out by the xylanase produced by various organisms such as bacteria, algae, fungi, protozoa, crustaceans, insects, and plant seeds. The industrial process ran at high temperatures. Therefore, the discovery of bacteria capable of producing xylanolytic enzymes that work optimally at high temperatures is very beneficial in industrial processes, especially the pulp and paper industry. Xylanolytic enzymes that are resistant to high temperatures can be produced from thermophilic bacteria. Thermophilic bacteria can be obtained from hot springs, volcanic craters, deep-sea troughs, and submarine volcanoes (POLIZELI, RIZZATTI, MONTI, TERENZI, JORGE & AMORIM, 2005). Thermophile xylanolytic enzymes isolated from Indonesia hot spring have already characterized and successfully cloned and expressed in Escherichia coli system, such as β-xylosidase GBtXyl43A, β-xylosidase GBtXyl43B and α-L-arabinofuranosidase from Geobacillus thermoleovorans IT-08 (RATNADEWI, FANANI, KURNIASIH, SAKKA, WASITO, SAKKA, NURACHMAN & PUSPANINGSIH, 2013). The structure also already determined (ROHMAN,

OOSTERWIJK, KRALJ, DJIKHUIZEN, DIJKSTRA & PUSPANINGSIH, 2007).

In this research, PC-01 isolate was obtained from Pacet hot springs, Indonesia, which has positive xylanase activity. Exploration of xylanolytic bacteria from various different hot springs is expected to produce bacteria with different characteristics. Preliminary research has succeeded in isolating bacteria from Pacet hot springs and coded PC-01 bacterial isolate. PC-01 was able to produce xylanolytic, which has not been identified or the genus. The information obtained from this study will is important for further application of the enzyme in industrial field. In this study, identification of xylanase produces bacteria that will be done, followed by characterization and purification of the enzyme.

2. METHODOLOGY

The sample used was a bacterial isolate obtained from mud of Pacet, Hot Spring, East java. One ose of single colony of PC-01 isolate was inoculated into 20 mL of liquid media and incubated in a shaker incubator for 16 hours at 50^{o}C. The culture was then centrifuged at 5000 rpm at 4^{o}C for 10 minutes. Cell pellets were removed, while supernatants collected for further analysis. Staining of bacterial spores using Malachite Green solution of 5% based on the procedure of Schaeffer Fulton method, after gram coloring with safranin dye. A colony of bacteria was inoculated and smear on the object-glass and

burned carefully for fixation. Malachite green was then added over the heat fixed bacterial smear and subsequently heated over a steam bath for two minutes followed by cooling, during this stage both the spore and vegetative cells appear as green color. Water used as decolorizing agent for vegetative cells. The cells appear in red or pink color (PRESCOTT, HARLEY & KLEIN, 2005).

The endo-β-xylanase activity test with the DNS method was prepared as the following step. A mixture of 100 ul enzyme and substrate, respectively was incubated at 50 0C for 30 minutes Control was prepared the same way except that active enzyme replaced with inactive enzyme. Samples and controls were then added 600 µL DNS respectively and heated in boiling water for 15 minutes followed by incubation in an ice bath for 20 minutes. The absorbance is read at a wavelength of λ_{550} nm. One unit of enzyme activity (IU) is defined as the amount of enzyme needed to release 1 **µ**mol reducing sugars per min under assay conditions. The specific activity of the enzyme was expressed in units per milligram. Xylose was used as the standard reducing sugar.

$$\text{Unit activity} = \frac{[\text{product}]\text{x volume of test solution x dilution factor}}{\text{xylose molecular weight x time of incubation (min)x volume of enzyme}}$$

The activity test of xylan degrading enzymes, such as α-L-arabinofuranosidase, glucuronidase, β-xylosidase, and acetyl xylan esterase was carried out with p-nitrophenol (pNP) derivative substrates, such as pNP-α-L-arabinofuranosida, pNP-β-D-glucuronide,

pNP-β-D-xylopiranoside, and pNP-acetate. Each 1 mM substrate (0.9 mL) was incubated together with an enzyme (0.1 mL) at 50^0C for 30 minutes. The reaction is stopped by adding 0.1 mL of 0.4 M Na_2CO_3. The enzyme activity is determined by the release of p-nitrophenol. One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol p-nitrophenol within 1 minute under experimental conditions (MOTTA, ANDRADE & SANTANA, 2013; SHABBIR, ABBAS, AMAN, & ALI, 2019).

Determination of the optimum temperature of xylanase was carried out by determining the xylanase activity at various incubation temperatures from 30 to 80°C. For determination of optimum pH of xylanase was determined by incubated enzyme with substrate at pH 4 to 10 (phosphate citrate buffer for pH 4, 5, 6; phosphate buffer for pH 6, 7, 8; Tris-HCl buffer for pH 8, 9 and glycine buffer- NaOH for pH 9, 10) at the optimum temperature of the enzyme. While pH stability is determined by regulating enzymes at pH 4 to 10 and subsequently incubated in the water bath at the optimum temperature of the enzyme for 1 h, then each enzyme was set to its optimum pH and measured its activity (BRADFORD, 1976).

3. RESULTS

The bacterial isolate PC-01 isolated from Pacet hot springs, East Java has the following characteristics: white-cream bacterial colonies, rounded shapes with spread edges, and raised elevations shown in

Figure 1. Based on the results of Gram stain of PC-01 bacterial isolates having rod shape (Bacil) with a single arrangement and classified into Gram-positive bacteria shown in Figure 2. Based on the results of spore staining, PC-01 bacterial isolates have oval-shaped endospores located subterminal shown in Figure 3.

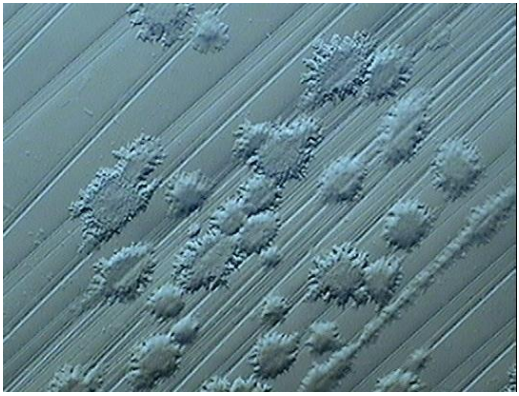

Figure 1: PC-01 bacterial isolate grown on LB media

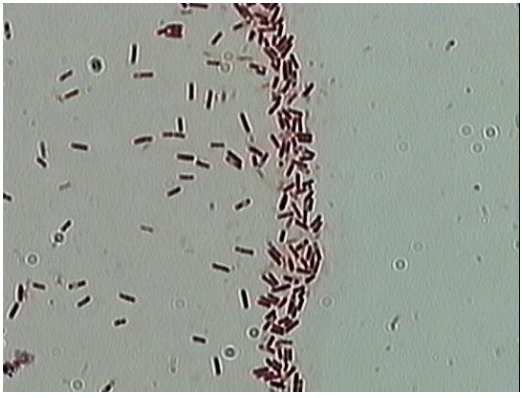

Figure 2: The results of Gram stain of bacterial isolates PC-01 with 1000x magnification

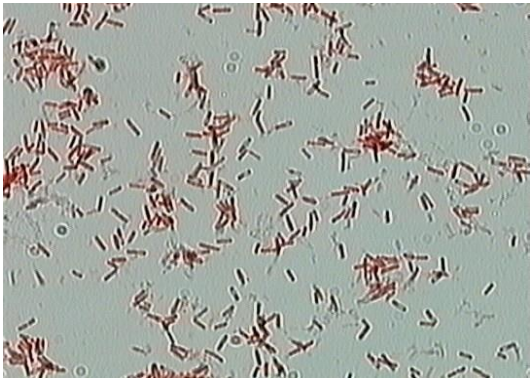

Figure 3: The results of staining of bacterial spores PC-01 with a magnification of 1000x

The optimum temperature of xylanase from PC-01 isolate was obtained at 60^{o}C with the activity of 10.70 x 10^{-2} U mL^{-1}. However, at a temperature of 40 ^{o}C and 50 ^{o}C, xylanase still has high activity reaching 66.73% and 80.47% of xylanase activity at optimum temperatures as shown in Fig 5. The optimum pH of xylanase was reached at pH 5 with activity of 8.97 x 10^{-2} U mL^{-1} as shown in Fig 5b. At pH 4 and 6 enzyme activity was still quite high, reaching around 97.77% and 80.49% of optimal pH activity. The stability of the xylanase produced by PC-01 isolates is quite good where the enzyme is stable at 60 ^{o}C for 6 hours. Until the 6 hours, the residual activity of the xylanase was still above 50% while above the 6 hours the residual activity was below 50% as shown in Fig 5c. The xylanase produced by PC-01 isolates has a pH range that varies greatly from 4 to 10. At pH 4-10 this xylanase has a residual activity above 50% as shown in Figure 5.

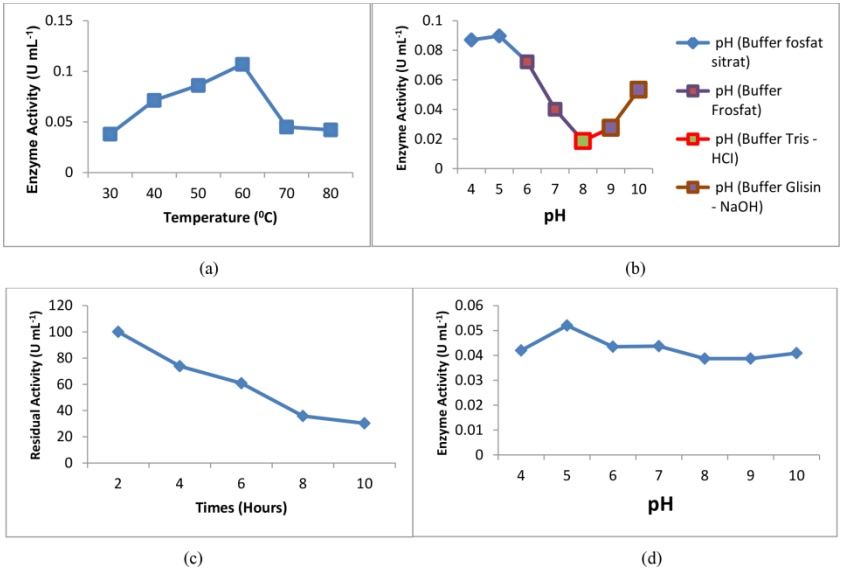

Figure 4: Effect of temperature (a) & pH (b); thermostability (c) & pH stability (d) on xylanase activity

Fractions from hydrophobic interaction chromatography with similar activity (2,3) as shown in figure 6 were collected as one fraction (fraction A). While fraction number 7 was named as fraction B. Further purification of fraction A by anion exchange chromatography after dialysis revealed that fraction 3 has the highest specific activity of 3.83 x 10^{-2} mg mL^{-1}.

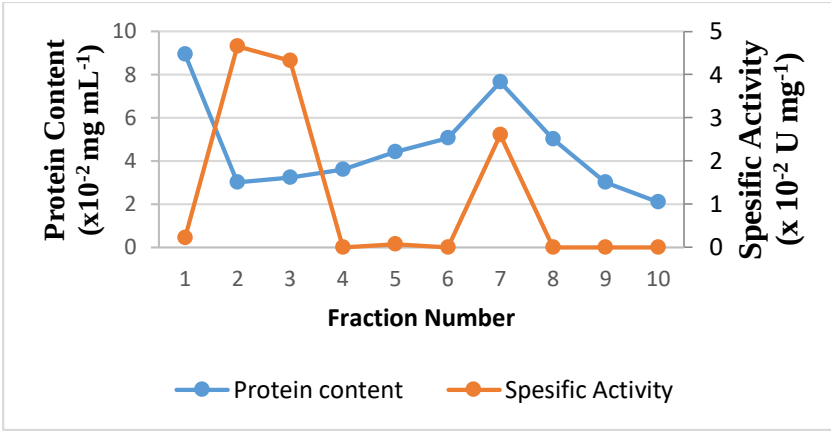

Figure 5: Hydrophobic interaction chromatography curve (ammonium sulfate eluent with 50% saturation in pH 7 Tris HCl buffer)

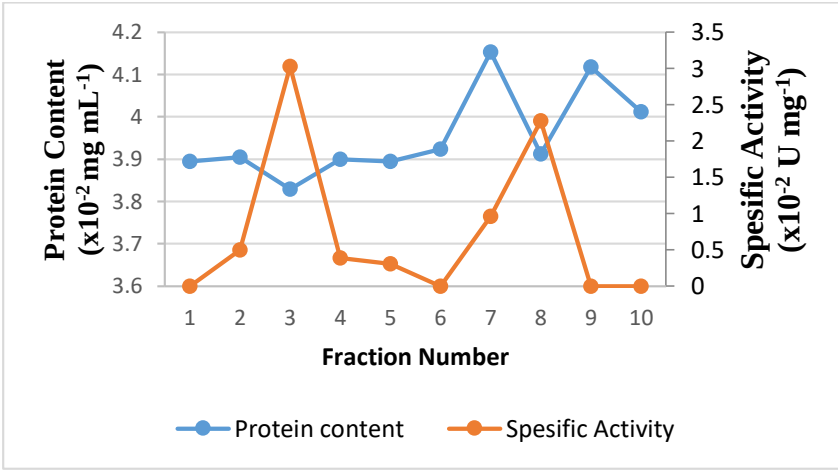

Figure 6: Chromatographic anion exchange of fraction A curve (pH 5, eluent NaCl [0-0,5] M in Tris HCl)

Table 1: Purification of xylanase

Purification step	Protein total (mg)	Activity total (U)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Ammonium sulfate precipitation	0.44	0.13	0.30	100	1
Chromatographic anion exchange	0.038	0.12	3.16	92,3	10.5

4. DISCUSSION

Many studies have been conducted to isolate xylanase from genus Bacillus, but still exploration of enzymes from these bacterial isolates is in demand PC-01 bacterial isolate shows general characteristics possessed by the genus Bacillus (SUNNA, GIBBS & BERQGUIST, 2000). In Bergey's manual of determinative bacteriology, bacteria with rod-shaped features, Gram positives are grouped into the genus Bacillus. The physiological test results using the Microbact identification kit, PC-01 isolate showed positive results on the test of lysine, xylose, and ONPG, and negatively tested the ornithine, H_2S, glucose, mannitol, indol, urease, VP, and citrate. From the results of physiological tests, and confirmation with the species characterization table of the genus Bacillus (SHALLOM, 2003), it is thought that PC-01 bacterial isolates were Bacillus subtilis species.

The estimation is because not all physiological tests in the characterization table are carried out. This data can be further analysis with 16S rRNA for a more accurate result. Some Bacillus sp. has known to produce xylanase, in this research, PC-01 Bacillus sp. produces two kinds of xylanase activity: endo-b-xylanase and arabinofuranosidase. To characterize xylanase produced by PC-01 isolate, temperature and pH optimum was conducted.

At the optimum temperature, the energy obtained by the enzyme is the same as the energy needed to start a reaction. Whereas at temperatures above the optimum temperature can cause enzymes to denaturation. At temperatures above 60^{o}C there is a decrease in activity, this occurs because atoms in enzyme molecules have enough energy to move caused by changes in the structure of enzymes due to increased thermal vibrations of the components of the atoms so that proteins form denatured enzymes. The occurrence of enzyme conformational changes due to changes in pH can cause a decrease in enzyme activity because the enzyme confirmation is no longer the same as the substrate conformation. At pH 4-10 this xylanase has a residual activity above 50%. The width of the pH range is beneficial in its application because it can work in wide pH conditions. Although having a wide pH range this enzyme turned out to show optimum activity at pH 5 even though the use of thermophilic enzymes in industrial processes generally required an alkaline pH. Alternatively protein engineering can be used to engineer the enzyme to obtain the desirable pH. Thermostability is very important since industrial processes mostly work at high temperatures, enzymes that have low-

temperature stability will cause costs to procure enzymes to be increased otherwise if it has high-temperature stability it can reduce costs for the procurement of enzymes. The stability of the xylanase produced by PC-01 isolates is quite good where the enzyme is stable at 60^{o}C for 6 hours. The previous study by MOTESHAFI, HASHEMI, MOUSAVI, MOUSIVAND (2016) reported that xylanase from Bacillus subtilis D3d kept activities after 3 hours at 50^{o}C, 72 hours at 40^{o}C. In contrast β-D-Xylosidase from Geobacillus thermoleovorans by RATNADEWI ET AL. (2013) produce the optimum working pH and temperature of GbtXyl43B were 6.0 and 60 °C, respectively. GbtXyl43B was thermally stable up to 60 °C and pH stable at 6–8 and α-L-arabinofuranosidase from Geobacillus thermoleovorans produce the optimum pH was determined by incubation at 70°C for 30 min in the pH range from 4 to 10 (YAMANI, JONET, BAKTIR, ILLIAS & PUSPANINGSIH, 2017).

Removal of the non-target protein from mixture of xylanase and arabinofuranosidase was initiated by precipitation using ammonium sulfate. Ammonium sulfate is the most common method to remove non-target protein through a series of concentrations. Different proteins will be precipitate at different degrees of ammonium sulfate. In this process a specific activity of xylanase of activity of xylanase was 0.30 U mg^{-1} shown in table 1 from 50 % degree of ammonium sulfate precipitation**.**

Ammonium sulfates residue in protein fractions was purified by dialysis as it worked by selecting molecules based on the size. Ammonium sulfate has a low molecular weight compared with protein

molecules and results will be moved out through the dialysis tube while protein molecules retained in the tube. The specific activity obtained from this step is 0,30 U mg^{-1}. This activity has been increased compared with fraction from ammonium sulfate precipitation indicating removal of some non-target protein.

Xylanase purification products using hydrophobic interaction chromatography as described in figure 6 showed that xylanase with saturation of ammonium sulfate 50% and pH 7 has the highest specific activity. Fractions obtained from this chromatography method were collected as one fraction as they have similar activity and further purified by anion exchange chromatography. Anion exchange chromatography principle of purification is separation of protein mixtures based on the net charged of the protein. Xylanase from PC-01 with positive charge will bound to the negative charge of the matrix used for purification. The protein target is eluted from the column using increasing concentration of NaCl solution.

Based on the results in figure 7, it can be informed that after optimization of the pH of anion exchange chromatography fractions, the third fraction of pH 5 is the best result of enzyme purification with 3.16 U mg^{-1} specific activity shown in table 1. Optimization of protein engineering can be done to obtained enzymes with desirable pH, for application in the industrial process.

5. CONCLUSIONS

Characteristics of bacterial isolates PC-01 are rod-shaped bacterial cells, Gram-positive, and have subterminal oval endospores. The bacterial isolate PC-01 belongs to the genus Bacillus. The xylanase from PC-01 isolates had an optimum pH of 5.0 and optimum temperature of 60°C, stable at 60°C for 6 hours and has a pH range of 4-10. The best results obtained from anion exchange chromatography in third fractions pH 5 fraction with a purity level 78.25 times higher than crude extract.

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