XYLANASE ENZYME FROM A LOCAL STRAIN OF *Pseudomonas stutzeri*

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ABSTRACT

Xylanase is an important enzyme, required in many industries to degrade xylan. A local xylanolytic bacteria, producer of xylanase enzyme has been screened and isolated from a liquid fermentation broth of organic waste. The bacteria was identified as *Pseudomonas stutzeri* species and showed high xylanolytic activity. In the medium with 2% (w/v) birchwood xylan and temperature of 37°C, the bacteria produced optimally xylanase enzyme for time of 27 hours, by adding of 0.5% (w/v) molasses. The yielded xylanase could work optimally at pH 5 and temperature of 50°C. This finding underlies the development of xylanase production on a large scale, using the bacteria in the future.

Keywords: xylanolytic bacteria, organic waste, xylanase, *Pseudomonas stutzeri*.

INTRODUCTION

Xylan is found abundantly on earth as the main component of hemicellulose, which is the second biggest biomass source in nature, after cellulose [1 - 4]. On molecular basis xylan is composed by β-1,4-linked D-xylosyl residues in a backbone, and many branched heteropolysaccharides substituents, such as L-arabinose, 4-O-methyl-D-glucuronic acid and acetyl groups that link to the backbone [4 - 6]. Xylan can be used as a potential raw material in the production of xylose and xylooligasaccharides. Subsequently, the products can serve as renewable resources to produce biofuel, prebiotics, solvents and other chemical preparations [7 - 8]. The complete degradation of xylan to useful products requires xylanolytic enzymes, i.e. endoxylanase, α-arabinofuranosidase, α-xylosidase, α-glucuronidase and acetyl-xylan esterase [1 - 2, 7]. Among the enzymes, endo-β-1,4-xylanase (EC.3.2.1.8) and β-1,4-xylosidase (EC.3.2.1.37) play a primary role for degradation of xylan’s backbone, where endo-β-1,4-xylanase digests the β-1,4-linked glycosidic between xylose residues to release xylooligosaccharides, and then β-xylosidase continues to hydrolyze the short-oligosaccharides to xylose [4].

The availability of multifunctional xylanolytic enzymes could be largely presented by a microbe that is quite widespread in the world [9 - 11]. Concerning the enzyme production, the microbe is usually used as a primary source, due to the ease of the microbe to grow in a medium rather than plant and animal cells. By using of the microbe, it is possible to develop production of enzymes on a large scale. Moreover, the microbe can be modified relatively easy by genetic engineering to get an enzyme with high activity [2, 9]. Various microorganisms including of bacteria, fungi, actinomycetes have been reported as xylanolytic enzyme producers. Some of the identified microorganisms are the Aspergilli, Trichodermi, Streptomycetes, Phanerochaetes, Chytridiomycetes, Ruminococci, Fibrobacteres, Clostridia and Bacilli [9, 12 - 16].

Indonesia has been known as one of the countries with the highest biodiversity in the world, taking the second rank after Brazil [7]. However, the potential
biodiversities have not been explored yet optimally to get a microbial source for production of xylanolytic enzymes. This paper presents an effort to screen and explore the local bacterial xylanolytic from a consortium microbe that can be obtained from liquid fermentation of organic waste. In addition, the paper also reports the properties of xylanase from the selected bacteria.

EXPERIMENTAL

Materials and methods
The object of the research was a consortium microbe, taken from a liquid fermentation of organic waste, after composting for 3 months. The chemicals for the research like birchwood xylan, MgSO₄.7H₂O, KH₂PO₄, Na₂HPO₄.7H₂O, citric acid, CaCl₂, NaCl, glucose (Aldrich), NaOH (Aldrich), Rocelle salt (Aldrich), 3,5-dinitrosalycilic acid (Aldrich), Na₂SO₃ (Aldrich), and xylose (Aldrich) were purchased from Sigma Aldrich; while bactoagar, yeast extract and peptone were purchased from Merck; the molasses were obtained from the sugar industry. The instruments used consisted of an analytical balance Mettler Toledo AL204, a pH meter Mettler Toledo, a Bachmen refrigerate centrifuge, a Memmert incubator, a Sanstat Water Bath type SYK-382-M and a Gerhardt, Laminar Air Flow Kottermann® 8580 electric autoclave, model No. 25X, vortex meter VM-300, and an UV-Vis spectrophotometer Shimadzu UV-1800.

Screening of microbial xylanolytic
A 100 mL sample of liquid fermentation was diluted with sterile water up to 10³ dilution. 100 µL of the diluted sample were spread onto a solid medium, composed of 0.02 % (w/v) yeast extract, 0.1 % (w/v) KH₂PO₄, 0.24 % (w/v) MgSO₄, 0.1 % (w/v)NaCl, 0.01 % (w/v) CaCl₂, 0.5 % (w/v) peptone, and 2.5 % (w/v)bacto agar. Next, it was incubated at 37ºC for 16 hours [8]. The cell colonies grown were selected in the same solid medium containing 1 % (w/v) birchwood xylan under incubated conditions of 37ºC for 16 hours. The cell colonies with a transparent halo were moved into a sterile medium. The width of the halo area of each colony was measured using calipers to calculate the xylanolytic index. The microbial isolates with the largest halo index were identified by a Microbact Identification System at the Laboratory of Microbiology, Faculty of Science and Technology, Airlangga University.

The growth curve
A microbial isolate with the biggest halo index was grown in 100 mL of liquid medium that was composed of 0.02 % (w/v) yeast extract, 0.1 % (w/v) KH₂PO₄, 0.24 % (w/v) MgSO₄, 0.1 % (w/v)NaCl, 0.01 % (w/v) CaCl₂, and 0.5 % (w/v) peptone. The cell culture was incubated at 150 rpm with an orbital shaker TS-330A, at temperature of 37ºC. The optical density of the cells in the culture was monitored every two hours with an UV-Vis spectrophotometer Shimadzu UV-1800, at λ 600 nm [18].

Optimization of the xylanase production
200 µL of microbial starter was added to 100 mL of liquid medium, then incubated at 150 rpm with the orbital shaker TS-330A at temperature of 37ºC for 16 hours. Every 3 hours, the culture was sampled and separated by centrifugation at 5000 x g at temperature of 4ºC for 20 minutes. The supernatant from this treatment contained crude xylanase enzyme. The optimization for the xylanase production was also done with a various molasses as a carbon source.

Assay of the xylanase activity
The assay was carried out by incubating a 100 µL sample, containing 0.2 mg/mL crude enzyme with 900 µL of 1 % (w/v) birchwood xylan in 0.2 M buffer of citrate phosphate pH 7 at 50°C for 5 min. To the mixture were added 1.5 mL DNS reagent, incubated in a boiling water bath for 15 min, and cooled immediately in ice water for 20 min. The absorbance of the product was measured at λ 540 nm. The reducing sugar for this assay was calculated by using xylose as standard. One unit of xylanase activity was expressed as the amount of enzyme (mL) to form 1 micro mole of reducing sugars equivalent to xylose per unit time under assay condition [2, 18, 19]. The enzyme activity was calculated by the formula given below:

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xylanase\ activity\ (Unit/mL) = \frac{P \times D}{T_x \times W \times V}
\]
where:
- \( P \) - amount of xylose produced, mg/mL
- \( T \) - time of reaction, min
- \( W \) - molecular mass of xylose
- \( V \) - enzyme volume, mL
- \( D \) - dilution factor.

**Characterization of the xylanase**

The optimum pH and temperature of xylanase were determined by an enzyme activity test at various values of pH and temperature. The pH was set in the interval of 3.0 - 8.0. A 0.2 M citrate phosphate buffer was used to adjust pH between 3.0 and 7.0, while the 0.2 M phosphate buffer was used to adjust pH between 6.0 and 8.0. The suitable pH value, obtained in this work, was then used for conditioning the assay of the enzyme activity at various temperature.

**RESULTS AND DISCUSSION**

**Identification of microbial xylanolytic**

For a long time, the present of xylanase activity in a consortium of microbe has been used as a biomarker that marks the presence of microbial xylanolytic [12]. In a previous study, xylanase activity in the liquid fermentation of organic wastes, composed by vegetable waste and fruit peels has been found.

By using a spread plate method, the microbial xylanolytic in the liquid fermentation was screened by planting on a solid medium containing 2 % (w/v) birchword xylan. Some dilutions for the planted liquid sample were developed to optimize the yields of microbial colonies which grew in the medium. After incubation for 24 hours at 37ºC, some microbial colonies grew on the medium. Of the colonies, six colonies with transparent halos around of their cells were obtained. This indicated that they exposed xylanolytic activity by release of xylanase enzyme to break down xylan in the solid medium. The greatest xylanolytic activities were presented by the X1 and X2 isolates, which showed xylanolytic indices of 4.57 and 4.00, respectively (Fig. 1). Identification of both isolates with the MicrobactTM Identification System kits showed that the X1 and X2 isolates had score similar to 99.39% with *Pseudomonas stutzeri*. Both isolates exhibited the same properties in a series of tests for oxidase, motility, nitrate reduction, lysine decarboxylase, ONPG, urea hydrolysis, and dihydrolase arginine assays. On the opposite, they showed negative properties in a series of tests for decarboxylic ornithine, \( H_2 S \) production, acid production from glucose, mannitol, inositol, sorbitol, ramnose, sucrose, lactose, arabinose, adonitol, raffinose, salicin and xylose, indole, citrate, malonate, gelatin hydrolysis, and the tryptophan deaminase test. Based on this result, it was decided that X1 and X2 were the same species. Furthermore, one of the isolates was used as a source to produce xylanase enzyme.

**The growth of *Pseudomonas stutzeri***

The growth of *Pseudomonas stutzeri* showed a sigmoid curve in the plot of optical density of cells versus time (Fig. 2). In the initial time up to 2 hours, the *Pseudomonas stutzeri* had no crucial growth. The bacteria had not used the nutrient well to divide its cell, but seemed to adapt to the new environment. This event is usually called “a lag phase” [16, 17], defined as an adaptative phase for bacteria when firstly introduced to a new medium. Thereafter, *Pseudomonas stutzeri* grew exponentially up to the time reaching of 20 hours. The bacteria had a significant growth, due to having an ability to use the nutrients optimally in the medium to divide its cells. This period is known as “the logarithmic phase” of
bacterial cell growth [17]. Because of the culture having a good growth, the cells in the condition were used to make a starter for production of xylanase in the following stage. The manufacture of the microbial starter was chosen in this condition, because at this time the bacteria had the needed quality and quantity of cells to act in the production of enzyme. In the period of 20 - 36 hours, the \textit{Pseudomonas stutzeri} showed a stationary growth, representing a balance between a number of cell life and death [16, 17]. Up to 36 hours, the growth of \textit{Pseudomonas stutzeri} was still a stagnant. The length of the stationary time might be triggered by the presence of molasses in the medium, so the nutrients are not quickly exhausted. This phenomenon was similar to that of \textit{Pseudomonas sp} XBP-6, which had a long death phase, around of 40 hours [6].

**Optimum conditions for xylanase production**

**The time of fermentation**

To determine the optimum conditions for \textit{Pseudomonas stutzeri} in the production of xylanase, the xylanase production curve, linking the xylanase activity with the time of \textit{Pseudomonas stutzeri} growth was developed. In this work, the \textit{Pseudomonas stutzeri} was cultured in a liquid medium containing 2 % (w/v) birchwood xylan, checking the xylanase activity in the culture for each 3 hours intervals. In the time interval of 0 - 12 hours, the \textit{Pseudomonas stutzeri} produced a low xylanase activity (Fig. 3). The high xylanase activity increased gradiently between 15\textsuperscript{th} - 18\textsuperscript{th} hour in the \textit{Pseudomonas stutzeri} growth. The highest activity in

![Fig. 2. The growth curve of \textit{Pseudomonas stutzeri}. The growth of \textit{Pseudomonas stutzeri} showed 3 phases. Between 0 and 2 hours, which was classified as an adaptative phase, the \textit{Pseudomonas stutzeri} started to adapt to the new environment, so there was no growth. In the 2 to 20 hours interval, there was a logarithmic phase, which meant that the \textit{Pseudomonas stutzeri} made a good growth exponentially, and in the last 20 to 36 hours, there was a stationary phase, in which the \textit{Pseudomonas stutzeri} showed a balance between cell growth and cell death.](image1)

![Fig. 3. Activity of xylanase in each fermentation time of \textit{Pseudomonas stutzeri}. The highest activity of xylanase was produced by the bacteria on the 27th hour of the fermentation time.](image2)
the growth of *Pseudomonas stutzeri* (0.7733 U/mL) was obtained for 27 hours. After that, the xylanase activity decreased, because *Pseudomonas stutzeri* might also produce protease and so the xylanase could be degraded.

The amount of molasses that was used as a carbon source to support *Pseudomonas stutzeri* in xylanase production was also optimized. The xylanase activity increased gradually when *Pseudomonas stutzeri* was grown in a medium with molasses at concentration of 0 - 0.4 % (w/v) (Fig. 4). The highest xylanase activity was produced by *Pseudomonas stutzeri* in a xylan medium by adding of 0.5 % (w/v) molasses. Up to 0.5 % (w/v) molasses in the medium, *Pseudomonas stutzeri* produced a low of xylanase activity. This might be because at the higher levels of molasses, the bacteria has enough nutrient to support its growth. The bacteria action might not involve degradation of xylan to get the nutrient, so the it did not produce xylanase in these conditions. If there are two or more sources of carbon in the medium, the microbes generally prefer to use a simpler molecule [9, 16 - 17].

The xylanase activity was tested by the DNS method, using birch wood xylan as a substrate. The principle of the assay detects the amount of reducing sugars which are obtained by the enzyme reacting with xylose. Xylose reacts with the DNS reagent to produce 3-amino-5-nitrosalycilic acid (Fig. 5), the brown colour molecule which gives a maximal absorbance at 540 nm [18]. In this reaction, the reducing sugar is oxidazed to xylonic acid, while the 3,5-dinitrosalycilic acid is reduced to 3-amino-5-nitrosalycilic acid. The intensity of the colour of 3-amino-5-nitrosalycilic acid, which is formed in this reaction, depends on the concentration of reducing sugars [18, 19].

**Physical properties of xylanase**

**The optimum pH and temperature**

The enzyme function is closely linked to its structure, which further depends on the pH of its environment. The change of pH affects the conformation of the enzyme via ionization in the side groups of the amino acids of the protein. It triggers changes of the interactions between the functional groups of the amino acids, thus affecting to the structure and function of enzyme [2-3]. The optimum pH of an enzyme is not always same with the normal environmental pH from its source [6]. The

![Fig. 5. Reaction of xylose with 3,5-dinitrosalycilic acid (Toledo, 2012). The dye molecule, 3-amino-5-nitrosalycilic acid could give an optimum absorbance at λ 540 nm.](image-url)
The highest activity of xylanase from *Pseudomonas stutzeri* took place at pH 5, which was equal to 0.0791 U/mL (Fig. 6). At pH 4 and 6, the xylanase activity was still quite high - 0.0511 and 0.0609 U/mL, respectively.

In addition to pH, the temperature also affects the characteristics of the enzyme. It is related to the energy, required by the enzyme to initiate the reaction with a substrate. If the temperature is low, the energy for supporting the reaction between the enzyme and the substrate is insufficient, so the reaction does not go well. At the optimum temperature, the energy received by the enzyme is equal to the energy needed for starting the reaction between the enzyme and the substrate, so the reaction can take place properly. Above the optimal temperature, the enzyme might be denatured, so the reaction again does not go well.

Xylanase showed a quite low activity at temperatures of 30°C and of 40°C (Fig. 7). At these temperatures, the enzyme might not work properly. The optimum was obtained at a temperature of 50°C with the activity equal to 0.0947 U/mL. The activity decreased dramatically above the optimum temperature. At 60°C and 70°C, the activity of the xylanase remained 67.02 % and 25 %, respectively, from that at the optimal conditions. At the highest temperatures, the structure of the enzyme might have changed and it might have been denatured.

**CONCLUSIONS**

A selected bacteria, *Pseudomonas stutzeri*, that exhibited a high xylanolytic activity, could be isolated from the fermentation liquid of organic waste. The bacteria could produce optimally with extracellular xylanolytic activity at 27 hours of fermentation time of its cells, using molasses with concentration 0.5 % (w/v), as a carbon source. The xylanolytic enzyme of the bacteria had an optimum activity at pH 5 and temperature of 50°C. This finding underlies the development of xylanase production using the bacteria on a large scale in the future.

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