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Xylanase Enzyme Stability and Biochemical Characteristics Thermoxylanolytic Bacteria From Mudiak Sapan Hot Springs at Solok Selatan District

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ABSTRACT

Xylanase is an enzyme that has the highest prospects in the industry. Xylanase enzyme plays an important role, especially in the pulp industry, food and beverage as well as the production of xylose. Production of xylanase enzyme for industrial extraction of various types of living creatures including microorganisms. One of the microorganisms that produce xylanase enzyme is thermophilic bacteria. Enzyme generated from these bacteria are thermostable indispensable in the industrialized world. Mudiak Sapan Hot Springs of South Solok with temperatures at 93°C and pH 8 potentially contain a thermophilic bacterium that can produce xylanase enzyme. This study aims to determine the genus of thermophilic bacteria and determine the stability of the enzyme xylanase produced. Xylanase activity used Bailey modified methode and protein value used Lowry method. The result showed the genus Bacillus sp. on biochemical testing. Fifth isolates were classified into four isolates of Bacillus sp, including MS11 (Bacillus sp.1), MS12 and MS15 (Bacillus sp.2), MS16 (Bacillus sp.3) and MS18 (Bacillus sp.4), the five isolates xylanase enzyme that is stable, and isolates the most stable MS18 (Bacillus sp.4).

Keywords: *Xylanase Enzyme, thermoxylanolytic bacteria, Mudiak Sapan.*

INTRODUCTION

An enzyme is a biopolymer molecules composed of a series of amino acids in the composition and arrangement of chains constant and regular [1]. For catalytic, enzyme is a biocatalyst in cells in small amounts can speed up a chemical reaction without changing its structure [2].

One type of enzyme that is much needed in the world market is a xylanase enzyme. Xylanase an extracellular enzyme that can hydrolyze xylan [hemicellulose] into short chain xylooligosacharide and xylose. Xylanase can be used in pulp and paper manufacturing process as the manufacturing and paper bleaching [3]. Xylanase enzyme also plays a role in industrial applications such as bioconversion of lignocellulosic materials for fermentation products, juice purification, improved consistency of beer, increase the development of bread and animal feed industry and the field of pharmacology [4].

Xylanase can be produced by microorganisms. One is thermophilic microorganisms. The ability of thermophilic bacteria to produce thermostable enzyme that makes the researchers interested to use enzyme in the field of industrial. Stability is a trait that must be possessed of enzymes as biocatalysts.

Enzymes can be said to be stable if the enzyme can maintain its activity during storage and use, and can maintain stability against temperature and pH extremes and a variety of compounds that can damage, such as acid or alkaline solvent [5]. Xylanase enzyme stability can be seen from the enzyme residual activity, where the enzyme is said to be stable if the remaining enzyme activity of more than 50% of the original enzyme activity [6].

The Xylanase enzyme-producing bacteria found in hot springs. One of the hot springs is Sapan Aro River, located at Koto Parik Gadang di Ateh, South Solok District as showed in figure 1. The hot springs have a temperature of 75°C and pH 8. Santoso research (2015), has secured the 12 isolates showed xylanolytic activity. Highest isolates found in isolates SSA2 with xylanolityc index of 0.74 mm.

Unknown stability of enzymes produced by thermophilic bacteria and unknown genus of thermophilic bacteria such. Based on the above researchers have conducted research on the stability of the xylanase enzyme and bacteria thermoxylanolytic Biochemical Characteristics of Mudiak Sapan hot springs at South Solok District.

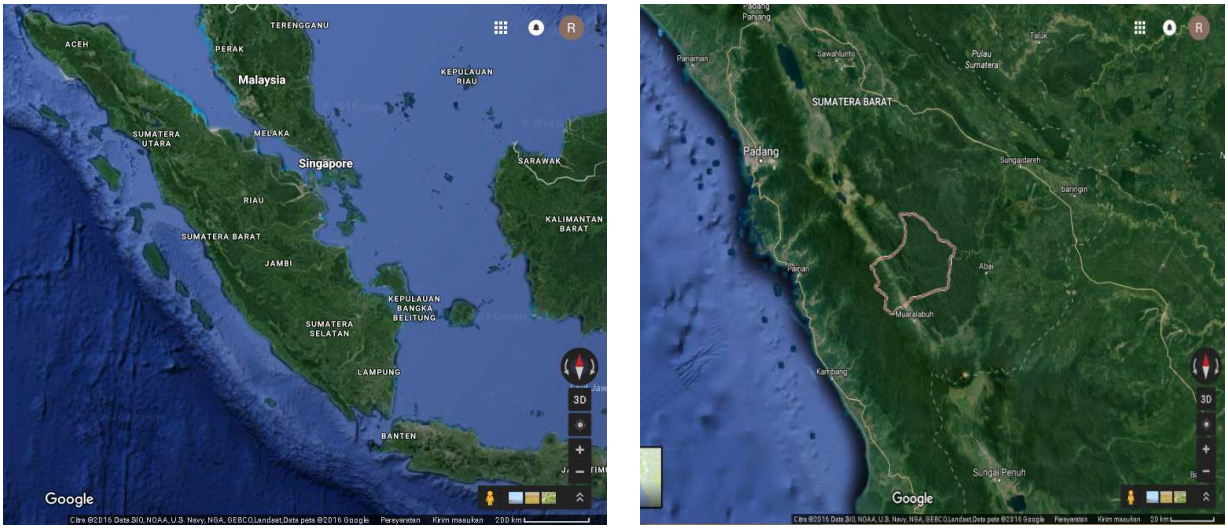

Figure 1. Map of Koto Parik Gadang di Ateh, South Solok District, in Sumatera Island, Indonesia

MATERIALS AND METHODS

TOOLS AND MATERIALS

Sterilization of Apparatus

All the tools that will be used first sterilized. Sterilization and medium performed by autoclaving at 121°C and a pressure of 15 psi for 15 minutes.

Medium Formation

Medium Nutrient Agar (NA)

Medium used that medium to regenerate NA thermophilic bacteria. A total of 10 g of NA was dissolved in 500 mL of distilled water and heated until homogeneous, then sterilized in an autoclave at 121°C at a pressure of 15 psi for 15 minutes.

Medium Production Beechwood Xylan

Bactopepton as much as 0.5%, yeast extract 0.1%, K_2HPO_4 0.1%, $MgSO_4.7H_2O$ 0.02%, and 0.1% beechwood xylan, dissolved in 1 L of distilled water. Then heated using an electric stove and stir until homogenous. After boiling, medium poured into the erlenmeyer and sealed with gauze and aluminum foil. Sterilization in an autoclave at a temperature of 121ºC and a pressure of 15 psi for 15 minutes (7).

Preparation of Inoculum
The inoculum was prepared by inoculating one loop of the isolates were selected from the screening results into 25 mL of liquid medium beechwood xylan in 100 mL Erlenmeyer and incubated at 60°C for 24 hours on a shaker with a speed of 150 rpm (8).

Implementation Research
Enzymes Stability Test
Getting a stable enzyme performed stability tests on the enzyme-producing bacteria thermophilic xylanase to the conduct of inoculum preparation, then taken 2 ml inoculum, then put in a 100 mL medium beechwood xylan in 250 mL Erlenmeyer. After that, be shaked at a temperature of 60^{o}C with a speed of 150 rpm for 24 hours. After 24 hours of each interval of 1 hour for 12 hours a sample is taken as much as 0.5 mL then isolated and tested xylanase enzyme activity (8).

Isolation Xylanase Enzyme
Isolation of xylanase enzyme was performed according to the Susanti method (2003), the enzyme xylanase in beechwood xylan medium separated from the cells by centrifugation at a speed of 5000 rpm for 15 minutes. Supernatant formed a crude extract xilanase. Measurement of enzyme xylanase activity carried out on the supernatant.

Test of Xylanase Enzyme Activity
Xylanase enzyme activity test was performed according to Bailey *et al*. (1992) are modified, using 1% (w/v) of xylan in buffer phosphate (pH 8.0) for reaction. Test mixture (0.5 ml substrate solution and 0.25 mL of coarse enzyme) was incubated at 60°C for 10 minutes and the reaction was stopped by addition of 0.5 ml dinitro-salicylic acid reagent (DNS), followed by keeping at 90°C for 15 minutes to stop the enzyme reaction and measured absorbance at 540 nm (9).

Xylose Standard Curve
Xylose standard curve was made in the range of 10, 20, 30, 40, 50, 60, 70 80, 90 and 100 mg/mL. A total of 0:25 mL of each standard solution is mixed with 0:25 mL of distilled water, then add 0.5 mL DNS reagent. The tube is inserted in a boiling water bath for 15 minutes, then cooled and absorbed at a wavelength of 540 nm.

Measurements of Protein Content
The protein content of the filtrate enzymes were measured by using the Lowry method (1976). The protein content in the sample can be determined with 0.1 ml enzyme sample was added 0.9 ml of reagent C and then shaken and allowed to stand for 15 minutes. add the coloring reagent Folin ciocalteau 3 ml ago in the shake and let stand for 30 minutes. Then define a spectrophotometer absorbance at a wavelength of 500 nm. The protein content of enzymes is determined by comparing the results with the standard curve absorbance Bovine Serum Albumin (BSA). Blank is determined by means of 0.1 ml of distilled water mixed with 0.9 ml of reagent C and 3 ml of Folin Ciocalteau reagent.

Measurement of specific activity
Xylanase enzyme specific activity (U/mg) is the ratio of xylanase enzyme activity (U/ml) to protein content (mg/ml).

Biochemical Characterization of Thermoxylanolityc Bacteria
To determine the type of bacteria producing thermophilic xylanase enzyme necessary to do some biochemical tests. Biochemical characterization performed on 5 isolates showed the highest xylanase activity. Biochemical characterization was conducted on the test: TSI (Triple Sugar Iron), gas production, the production of H_2S, oxidase, motility, IMViC (Indole, Methyl-red, Voges Proskauer, Citrate), urease, oxidative-fermenteative (OF) and fermentation of various kinds carbohydrates (glucose, sucrose, lactose and mannitol), and the hydrolysis of gelatin.

Triple Sugar Iron Test (TSI)
TSI testing using Slant Jelly Medium containing 1% lactose and sucrose, and 0.1% glucose. Then Phenol red was added to the medium that is used as a pH indicator which will detect the formation of acid from the fermentation of carbohydrates. Inoculation of bacteria on the surface of the medium in a zig-zag, then incubated for 18-24 hours. Some of the reactions that might occur in the medium are as follows.

(1) Base yellow (K) and red inclined surface (M), leading to fermentation of glucose (Phenol red indicator turns yellow due to the presence of acid in the base). The sloping surface remains red (alkaline) due to limitations of glucose in the medium.
(2) Base yellow (K) and an inclined surface of red (M), refers to the fermentation of lactose and sucrose (concentration of the glucose is high) which make acid formation occurred throughout the medium.
(3) Establishment of gas, occurs when the order was split by the presence of air.
(4) The formation of H_2S, visible through the black color in order.
(5) The base and red inclined surface (M|M), indicating none of fermentable sugars, and no gas or H_2S production (14).

Oxidase Test
Testing is done by adding reagents oxidase tetramethyl-p- phenylenediamine dihydrochloride 1% on filter paper. Then the bacterial cultures were taken with ose and smeared on the filter paper. The paper will be blue, black or purple in areas be oiled by culture if positive test results.

Test of IMViC (Indole, Methyl-red, Voges-Proskauer, and Citrate)
Test of Urea Indole Motile (MIU)
Colonies that will be identified retrieved using ose. Colonies were inoculated on the test medium motile indole urea in the form of semi-solid manner in order to be stabbed. Media incubated at room temperature for 18-24 hours. The bacteria movement indicated by the spread of bacteria colonies around the puncture. Urea positive reaction indicated media color change to pink. Indole positive reaction is indicated by the addition of Kovac reagent will then produce a red ring on the surface and show negative if it produces an orange ring on the surface (10).

Test of Methyl Red (MR)
Colonies that will be identified retrieved using ose. Colonies were inoculated on media MR-VP. The media were incubated at 37°C for 18-24 hours. Then add three to four drops of methyl red indicator. The red color indicates a positive reaction.

Test of Voges-Proskauer (VP)
Colonies that will be identified retrieved using ose. Colonies were inoculated on media MR-VP. The media were incubated at 37°C for 18-24 hours. Then added reagent Barrit. The suspension was shaken for 20-30 seconds. Positive VP reaction, in case of acid formation marked change of medium color to pink after addition of Barrit reagents.

Test of Citrate
Colonies that will be identified retrieved using ose. Colonies implanted scratch zigzag on the seeding media *simmons citrate* in form of slant jelly. Then the media were incubated at 37°C for 18-24 hours. Blue colonies showed positive results, while green colonies showed a negative reaction.

Urease Activity Test
Urease activity by inoculating bacteria on the medium Christensen's Urea Agar and the use of a pH indicator phenol-red. Incubation process in the tube for 24-48 hours at 50°C. Positive test results will increase the pH which causes the indicator changes from red/orange to pink or dark purplish red. While negative if it does not change color.

OF Test
Test OF preceded by inoculation of cultures in two tubes of OF, which containing media Hugh and Leifson. One tube was added oil for making the anaerob condition. Both of them were incubated at 50°C for 24-48 hours. The Positive fermentative result if there is a color change from green to yellow medium on both the tube and the positive oxidative if only a tube that is not covered in oil that changes color to yellow. Negative result if there is no change in the medium tube. The color could turn into a bluish due to the amine metabolism peptone by bacteria.

Carbohydrates Fermentation Test
Colonies that will be identified retrieved using ose. The colony was inoculated into tubes which each contain glucose, lactose, mannose, maltose, and sucrose. Into each of the sugar solution is added phenol red indicator (phenol red) and inserting to Durham tubes in upside down position. The microbial suspension was incubated at

50°C for 18-24 hours. When the color of the medium turned yellow, it means the colony forming acid from the fermentation of carbohydrates. When there are air bubbles in the Durham tube, means of fermentation gas is formed.

Test Hydrolysis Gelatin

This test is performed by inoculating cultured in a nutrient medium gelatin. Then the medium was incubated for 24 hours at 50°C. Before the test medium is incubated again in the refrigerator at a temperature of 40°C until the bottom freezes. If gelatin was hydrolyzed, medium will remain liquid after cooling. If not hydrolyzed gelatin medium will remain solid.

RESULTS AND DISCUSSION

Biochemical Activity of Thermophilic Bacteria

The results of biochemical tests were conducted on five isolates of the bacteria producing the highest xylanase enzyme from Hot Springs Mudiak Sapan South Solok District can be seen in Table 1.

Table 1. Results of Biochemical Activity of Xylanase Enzyme Thermophilic Bacteria

No	Test	Names of Isolates				
		MS11	MS12	MS15	MS16	MS18
1	A/An	A	A	A	A	A
2	TSIA	m | m	m | m	m | m	m | m	m | k
3	Gas	(-)	(-)	(-)	(-)	(-)
4	H_2S	(-)	(-)	(-)	(-)	(-)
5	Catalase	(-)	(+)	(+)	(-)	(+)
6	Oxidase	(-)	(-)	(-)	(-)	(-)
7	Motility	(+)	(+)	(+)	(+)	(+)
8	Indol	(-)	(-)	(-)	(-)	(-)
9	Urease	(+)	(-)	(-)	(-)	(+)
10	Citric	(-)	(-)	(-)	(-)	(-)
11	Lactose	(-)	(-)	(-)	(-)	(-)
12	Glucose	(-)	(-)	(-)	(-)	(+)
13	Sucrose	(-)	(-)	(-)	(-)	(+)
14	Mannitol	(-)	(-)	(-)	(-)	(+)
15	MR	(-)	(-)	(-)	(-)	(+)
16	VP	(-)	(-)	(-)	(-)	(+)
17	OF	(-)	(-)	(-)	(-)	(-)
18	Gelatin	(+)	(+)	(+)	(+)	(+)
Genus		*Bacillus* sp.1	*Bacillus* sp.2		*Bacillus* sp.3	*Bacillus* sp.4

Description: (+): positive test results, (-) test result is negative, A: Aerobic, m | m: red-red (negative test result), m | k: red-yellow (the formation of acid), Mudiak Sapan (MS).

The results of biochemical tests showed that isolates MS11, MS12, MS15, MS16 and MS18 on gelatin media testing showed positive results, characterized by melting gelatin medium at a temperature of 4ºC. Gelatin is a protein that is not perfect because it does not have the amino acid tryptophan. Gelatin may freeze when they are below 24ºC temperature and melt at temperatures above 25ºC. This indicates that all isolates were able to produce gelatinase enzyme that plays a role in hydrolyzing gelatin condensed into liquid (liquid) (10). In testing isolates TSIA MS11, MS12, MS15, MS16 symbolized by (m | m) which indicates a negative test result, the lack of the fermentation, the production of gas or H_2S (14). While the 18 isolates obtained positive test results are symbolized by (m | k) indicating the presence of glucose fermentation.

Results of testing positive motility characterized by the growth of bacteria that spread from the stab (inoculation) and followed by a color change medium into cloudy. It is due to the movement of bacteria. The Movement of bacteria aided by a long thread-like structure called the flagellum grow in the cell membrane (13).

At the catalase test isolates showed positive results, namely, MS12, MS15 and MS18, indicated the presence of bubbles of oxygen because of the breakdown of H_2O_2 (hydrogen peroxide) by the catalase enzyme produced by bacteria. While the urease testing positive results only indicated by two bacteria, namely, MS11 and MS18 which indicates that both isolates were able to attack the carbon and nitrogen to produce the enzyme urease to form ammonia compounds. The presence of ammonia causes the color change medium phenol-red color changed to pink (10).

Tests on the positive results of carbohydrate fermentation only shown in carbohydrates do MS18. Test of isolates consisting of sucrose, glucose and mannitol, positive results obtained marked by changes in color of the medium changed to a yellow color, which states the bacteria form acid from the fermentation of carbohydrates (10).

The results of the testing of the MR-VP in isolates 18 showed positive results indicating isolates can ferment the mixed acid, by changing the environmental pH medium becomes lower. Positive results marked the change in color to red on the medium (14). Meanwhile, the VP test result is positive color change to pink or red after the addition of reagent containing naphtol indicating that the presence of acetone (10).

The big difference in the results of some biochemical tests performed on five isolates, it can be concluded that there are four groups of *Bacillus sp*. of all five isolates. All of isolates, *Bacillus sp*. among them MS11 (*Bacillus sp.1*), MS12 and MS15 (*Bacillus sp.2*), because both these isolates have similar results on each test, then MS16 (*Bacillus sp.3*) and MS18 classified into groups (*Bacillus sp.4*).

Genus of Bacillus have physiological properties were attractive because each type has different capabilities, such as: (1) capable of degrading organic compounds such as proteins, starch, cellulose, hydrocarbon and so, (2) capable of producing antibiotics; (3) role in nitrification and detoxification Denitrification; (4) nitrogen-fixing; (7) are chemolitotrof, aerobic or facultative anaerobes, acidophilic, psycophilic, or thermophilic (11).

Stability The Xylanase Enzyme of Thermophilic Bacteria Isolates

From research conducted, showed that states that the stability of the xylanase enzyme activity produced by the five isolates of bacteria isolated from Hot Springs Mudiak Sapan South Solok stable until the second hour (Figure 2).

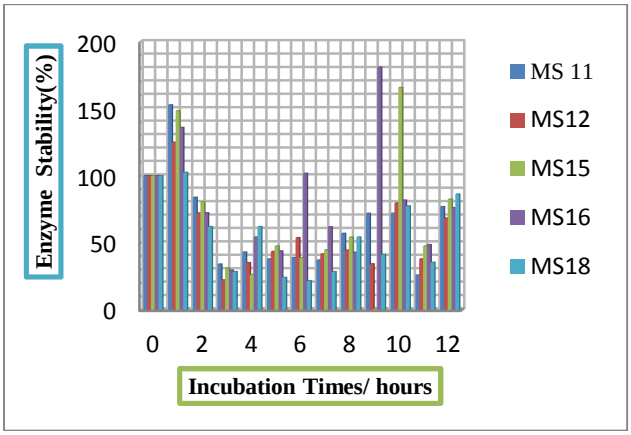

Figure 2. Xylanase Enzyme Stability

Isolates which had the highest activity and stable, up to 2 hours in produced by *Bacillus sp.1* (isolates MS11) with activities 83.9%, followed by *Bacillus sp.2* isolates belonging MS15 activity with 80.5%, 72.4% isolates MS16, MS12 isolates 72.2%, and very low activity experienced by 62.1% of the isolates MS18. Data of enzyme activity can be seen from (Figure 3).

As stated by (Chaplin and Bucke, 1990), the stability of a xylanase enzyme can be seen from the resulting residual enzyme activity, wherein the enzyme is said to be stable if the remaining enzyme activity of more than 50% of the initial activity of the enzyme (6). At the 3rd till the 12th of incubation times, enzyme activity decreased and the increase is very significant. The enzyme activity capable of maintaining stability above 50% until the second hour of incubation, further increasing and decreasing, until the 9th. The percentage of enzyme stability in all isolates increased again at the 10, 11 and 12. This may be due denatured enzyme protein structure resulting in decreased enzyme activity.

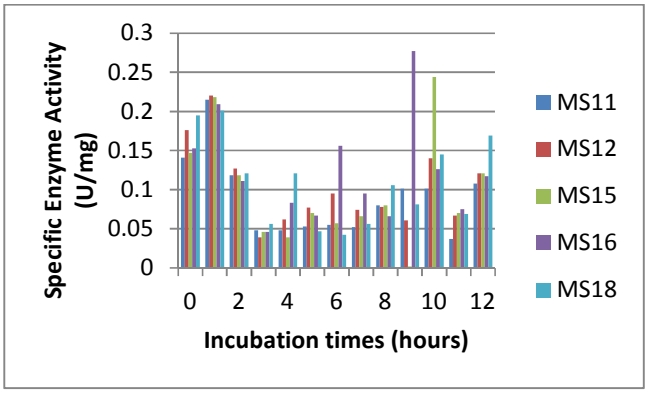

Figure 3. Xylanase Specific Enzyme Activity

The stability of the enzyme is an important trait that must be owned by an enzyme as biocatalyst. Enzyme can be said to be stable if the enzyme can maintain its activity during storage and use, and can maintain stability against temperature and pH extremes and a variety of compounds that can damage, such as acid or alkaline solvent (5). Activity and stability of enzymes is influenced by physical and chemical modification of the conditions that can cause structural changes in secondary, tertiary, and quaternary of the enzyme molecule, which is caused by the dissolution of covalent bonds and hydrophobic bonds (12).

From the data obtained at the 12th hour (after 12 hours of incubation), the stability of the enzyme increased the activity of a specific enzyme from hour to-2. The high activity of the enzyme that occurs in the harvest phase, indicating that the bacteria *Bacillus sp.* isolated from Hot Springs Mudiak Sapan South Solok has the ability to produce the enzyme xylanase on a long incubation at 60ºC temperature. As described by Prescott (2002), that the increase in enzyme activity due to increased production enzim.Dalam enzyme reaction, the enzyme reaction velocity is proportional to the concentration of the enzyme, when the concentration of the enzyme increases, an increase in enzyme activity (14).

The result showed that, of the five bacterial isolates were seen stability of enzyme activity, bacteria that have the most good enzyme stability is *Bacillus sp.4* (MS18). The stability of the enzyme on isolates can be seen from the specific enzyme activity resulting from hour-to-hour 0 to 2 is quite high and is above 50%, then increased activity at the time of harvest. Increased activity of enzymes that occur at harvest time also showed that the bacteria are able to produce an enzyme which is stable. Xylanolytic index produced MS18 is xylanolytic highest index among the five other isolates is 1:39 mm. The high value of the index xylanolytic on MS18, can also confirm that the bacteria have specific enzyme activity that most flattering and can be classified into bacteria capable of generating enzymes stable. While most low enzyme stability was found in isolates MS15, due to a significant decrease, on the hour all 9 isolates activity has decreased and has almost no activity is 0:08 U/ml (Appendix 3).

CONCLUSION

From the research that has been done about the stability of enzymes and biochemical tests of bacteria, it can be concluded as follows: The fifth genus is *Bacillus sp* isolates. *Bacillus sp.1* isolates as MS11, meanwhile MS12 and MS15 isolates for *Bacillus sp.2*. *Bacillus sp.3* isolates is MS16 and MS18 for *Bacillus sp 4* isolates. Then, the five isolates had a stable xylanase enzyme. The most stable enzyme found in *Bacillus sp.4* (MS18).

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