Screening of Thermophilic Bacteria Produce Xylanase from Sapan Sungai Aro Hot Spring South Solok

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Abstract - Xylanase is one of the enzymes with great prospects as hemicellulose hydrolyzing enzyme. Global annual market demand for this enzyme reach US $ 200 million. This enzyme catalyzes the xylan (hemicellulose) reactions breaking into xiloooligosakarida and xylose. Xylanase can be applied to various industrial sectors such as bread, sugar xylose, biofuels, especially in bleaching paper (bleaching) pulp. Xylanase is able to replace conventional chemical bleaching using chlorine that is not friendly for the environment. Currently xylanase production is extracted from the thermophilic bacteria for enzyme stability at high temperatures that are suitable for industrial applications. Thermophilic bacteria can be isolated from a hot spring, one of the which is a source of Sapan Sungai Aro Hot Spring, located in the district South Solok. The aim of this study was to select and identification of thermophilic bacteria can produce xylanase. This roomates is a descriptive study, which was Carried out in the Laboratory of Microbiology, Mathematic and Science Faculty of Padang State University, and Laboratory of Bacteriology, Baso Veterinary Research Center. The research procedure consisted of the preparation and sterilization of materials and tools, medium manufacturing, regeneration, selection and identification. Selection is performed by using a semiquantitative screening plate that contains xylan substrate. Identification is based on microscopic and biochemical characteristics until the genus level. Selection results showed 12 out of 16 isolates had xilanolitik activity, with the highest activity is SSA2 with xilanolitik index of 0.74. The top five index producehigestxilanolitik isolates that are SSA2, SSA3 and SSA4 identified as Bacillus sp. 1., and SSAS6 and SSA7 is Bacillus sp. 2.

1. Introduction
Enzymes are bio-catalyst. Enzymes improve chemical reactions that occur in the cells of organisms without changing the structure itself[1]. Chemical reactions can walk 108 to 1020 times faster than normal in the presence of enzymes. Are specifically bound enzyme with its substrate in catalyzing the reaction and produce products[2].

In line with the advancement of applied biotechnology enzymes to catalyze chemical reactions outside the cells or their environment. Enzymes are used in various industrial sectors such as textiles, food, detergents, paper and cosmetics, as well as biofuels. Needs of the enzyme in the world market in 2012 was US $ 4.5 billion and nearly US $ 4.8 billion in 2013, it is estimated that the market needs in 2018 amounted to US $ 7.1 billion[3].

Xylanase enzyme is one of the enzymes that were instrumental in the industrial world. Market demand for the xylanase is also quite high, at US $ 200 million[4]. This enzyme catalyzes the cracking
reactions xylan (hemicellulose) into xilooligosakarida and xylose. Xylanase can be used in pulp bleaching, hemicellulose conversion into a source of raw material for production, biofuel and the production of sugar xylose. Very important xylanase used in the process. Pulp bleaching Xylanase replacing bleaching chemically conventional using chlorine unsustainable[5]. In addition, in the production of bread xylanase also used to be a softer dough structure[6].

Production of xylanase enzyme for industrial use is extracted from various species, such as bacteria, fungi, yeast and plants. When this has been known to almost 4000 types of enzymes, 200 of which came from the microorganisms and used commercially[7]. Microorganisms produce enzymes that can be utilized humans in the number and types vary widely. They are easy to be cultured and potential and in accordance to the industry[8]. Microorganism one right choice for use in industrial processes, because it is thermostable. For example, brown stain removal process or bleaching of paper material (pulp bleaching), for optimal results of this process should use high temperatures and alkaline conditions[9].

Or termozim thermostable enzymes suitable for use in high-temperature reactions. Some of the main advantages of using high-temperature reaction is to reduce the risk of microbial contamination, lower viscosity, increases the rate of movement of the molecules, and increases the solubility of the substrate[10]. Thermostable enzymes capable of increasing the speed of the reaction thus saving time, labor and operational costs, facilitate the separation of volatile compounds, and more stable at a longer storage period[11].

Thermophilic bacterium grows optimally at a temperature range of 60-108 0C. The bacteria can be found in various places in nature, either sea or land which is heated by geothermal energy, such as hot springs, volcanic sediments from the mainland, hydrothermal vent systems, and ventilation hydrothermal seafloor in[12]. Indonesia is one that is quite a lot of hot springs, so it has the potential to generate sources of microorganisms that can produce thermostable xylanase enzyme. The microorganisms capable of producing thermostable xylanase enzyme is a valuable biological assets for the benefit of industrial biotechnology. Thermophilic bacteria are known to produce thermostable xylanase is Bacillus subtilis[7] and Pseudomonas[13], while the fungus known to produce this enzyme is Streptomyces[14], Aspergillus and Trichoderma. From 5 hot springs in the Northern Himalayas, Himachal Pradesh. 101 isolates were obtained and tested enzymatic activity. Through analysis of the 16S ribosomal RNA gene sequences identified genus Bacillus and Paenibacillus has the potential to produce xylanase[15].

Indonesia is a region that is pretty much had the hot water sources. One of these hot springs are hot springs Mudiak Sapan South Solok. Telah dilaporkan ada 19 thermophilic bacteria, selected 13 of them showed xylanase activity. The hot springs have temperatures up to 93 0C with a pH of 8. The obtained 18 isolates the overall shape and relatively Gram-positive bacilli and 1 isolate shaped Gram-negative bacilli[16].

In addition to hot springs Mudiak Sapan, in South Solok district also has other hot springs. The hot springs are a source of hot water Sapan Aro River located in the South Solok District. The hot springs have a temperature of 750C and pH 8. It was reported 16 isolates (7 comes from water samples and 9 of the samples), sludge From these hot springs obtained 16 isolates belonging to the shaped bacilli and gram-positive bacteria and produce endospores, and also showed amylolytic activity[17]. Synthesis influenced by the presence of an enzyme substrate. So a bacterium that produces amylase in starch medium containing potentially produce xylanase on medium containing xylan [18].

Microscopic characteristics are very helpful in the identification phase. But the bacteria with the same shape as likely to have metabolic and physiological activity are different. Biochemical characteristics are also required to specify a type of bacteria. In fact, the most closely related bacteria that can be separated into a particular species with biochemical tests, one of which is determined by their ability to ferment the carbohydrate group selection[19].

Currently it has developed a wide range of biochemical tests, such as tests TSI(TripleSugarIron), gas production, the production of H2S, catalase, oxidase, motility, IMViC(Indole, Methyl-red, Voges Proskauer, citrate), urease, oxidative-fermentative (OF), and
fermentation of various kinds of carbohydrates (glucose, sucrose, lactose, mannitol, sorbitol, arabinose, and others), the hydrolysis of casein, gelatin and many other tests. The various tests can be used to determine the genus or even species of a bacterium[20].

2. Methods

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%, K2HPO40.1%, MgSO4.7H2O 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.

1) The Spread Plate
The Medium used for isolating the thermophilic bacterium is sterilized NA, poured in a sterile petri. After 0.1 mL of hot water samples was solid, then it was distributed with hockey sticks on the surface of agar, it was incubated for 24 hours at a temperature of 60 ° C, each of hot water samples was made in duplicate. While for the isolation of "sludge" sediment process is done by weighing samples of sediment as much as 1 g. Then it was put into a test tube containing 9 mL of distilled water. Then it was divortek until the sediments homogeneous, then took 0.1 mL suspension using a micropipette and inserted into a petri plate which filled with NA medium.

2) The Streak Plate
For the purification of bacteria, each bacterial colonies which were growing in culture were previously taken with one inoculating loop an it was scratched intopatri plate to obtain pure cultures of bacteria.

a. Xylanase Activity
Xylanase activity test carried out by inoculating all pure isolates obtained from the previous stage. Isolates thermophilic bacteria grown in Nutrient Agar medium 24 hours. Then the isolates which have grown were tested in a suspension by taking a 1-2 inoculating loop of isolate bacterial culture into a sterile test tube which already contain physiological saline solution 0.85%. The mixture was homogenized with vortex mixture turbidity, and then it was compared with turbidity Mac Farland scale of 1 equivalent to 3.108CFU / mL. A total 0.1mL bacterial suspension was pipetted on Nutrient Agar medium + 0.5% Beechwood Xylan. The cultures were incubated for 72 hours at a temperature of 60 ° C [10]. Bacterial isolates were grown drops by 1% congo red solution and allowed to stand for 30 minutes, then it was dropped by 2M NaCl to see the clear zone. After that, it was measured to see the diameter of clear zone. The Isolates which produce xylanase indicated by a clear zone around bacterial colonies. Clear zone diameter of bacteria which have formed, then it is measured by using a caliper [11].

b. Catalase Test
Two drops of hydrogen peroxide (H2O2) 3% are dropped on a glass slide, then placed one inoculating loop of thermophilic bacteria and flatted. The positive catalase test is characterized by the existence of air bubbles. The existence of air bubbles indicates the amount of oxygen is produced.

Observations

Observations are carried out by observing the characteristics of bacteria and enzyme activities of xylanase that is produced by termophilic bacteria in hot springs Mudiaek Sapan, South Solok District.

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Biochemical Characterization of thermoxyanololytic 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 H2S, oxidase, motility, IMViC (Indole, Methyl-red, Voges Proskauer, Citrate), urease, oxidative-ferментative (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
zigzag, 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 H2S, 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 H2S production.

**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 produces a red ring on the surface and show negative if it produces an orange ring on the surface.

**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 anaerobe 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 50°C for 18-24 hours. When the color of the medium turn yellow, it means the only farming acid from the fermentation of carbohydrates. When there are air bubbles in the Durham tube, means of fermentation gas are 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 temperature of 40°C, medium will remain liquid after cooling. If not hydrolyzed gelatin medium will remain solid.

**3. Results and Discussion**

**3.1 Screening of thermophylic bacteria**

Xylanase activity journal in this study was conducted using Congored, selected 12 of the 16 isolates showed xylanolytic activity. This activity is characterized by the formation of clear zone around bacterial colonies. The measurement results xylanolytic index (IX) can be seen in Table 1.

This clear zone arising from the hydrolysis of xylan by xylanase activity extracellular thermophylic bacteria which grow on the medium xylanolytik. Xylan is contained in the medium decomposed by xylanase into xylose and xilooligosakarida. Therefore, dyes Congo red that bind strongly to xylan hydrolysis is not found in areas[29].

Clear zone formed in this study ranged from 7.09 mm to 10.45 mm with xylanolytic index in the range of 0.18 to 0.74. Isolates SSA 2 clear zone most (8.77 mm) to 0.74 xylanolytic index than other isolates as shown in appendix 1 of the clear zone formed includes a small if compared with Winanda study[16] with the isolates from Sapan Mudia hot springs, South Solok regency, which has xylanolytic index range between 0.54 to 1.39. So also with the results obtained by Susilowati[13] from the hot springs Sonai, Southeast Sulawesi, with xylanolytic index range between 0.4 to 1.8. But xylanolytic Index thermophilic bacteria from hot springs Sapan Aro River, South Solok District is still greater than the results obtained Habibie[30] of mud Lapindo Sidoarjo, with xylanolytic index range from 0.3 to 0.66.

Of the 12 isolates, xylanase enzyme hydrolytic activity in xylan hydrolyze show different values. Firstly it relates to the variation of xylanase genes of each of these bacteria. Differences in the amino acid sequence making up a protein (enzyme xylanase) cause differences activity. For example Thoyib et al[31] reported the activity of isolates identified as Pseudomonas sp. xilanolitik have higher activity than the isolates were identified as Bacillus sp.

Second, the difference is certainly related activities with factors that affect the action of the enzyme. Some of the factors that affect the activity of enzymes include enzyme concentration, substrate concentration, temperature, pH, and inhibitors. The enzyme concentration is directly proportional to the speed of the reaction, but the concentration of the enzyme is the basic factor that is dependent on other factors. The higher the concentration of substrate which will increase reaction speed to maximum speed is achieved.
Table 1. Xylanolytic Index xylanase-producing thermophilic bacteria from hot springs Sapan Aro River, South Solok District, West Sumatra Indonesia

| No. | ISOLATE | Xylanolytic index |
|-----|---------|-------------------|
| 1   | SSA2    | 0.74              |
| 2   | SSA4    | 0.61              |
| 3   | SSA3    | 0.58              |
| 4   | SSA6    | 0.46              |
| 5   | SSA7    | 0.40              |
| 6   | SSA5    | 0.37              |
| 7   | SSA15   | 0.37              |
| 8   | SSA1    | 0.35              |
| 9   | SSA8    | 0.31              |
| 10  | SSA16   | 0.31              |
| 11  | SSA13   | 0.23              |
| 12  | SSA14   | 0.18              |
| 13  | SSA9    | 0                 |
| 14  | SSA10   | 0                 |
| 15  | SSA11   | 0                 |
| 16  | SSA12   | 0                 |

Temperature and pH are the two factors that work specifically, has a certain optimum value. For example, experimental results Susilowati et al. [13] states that the optimum temperature and pH for xylanase activity *Pseudomonas sp.* is 50 °C and pH 9.0. While [30] reported that thermophilic bacteria isolates that she gets having xylanase activity optimum at a temperature of 50 °C and pH 7.0. The presence of an enzyme inhibitor capable of inhibiting the action of the enzyme. Likewise xylanase enzyme, a compound in wheat (*Triticumaestivum*) called TLXI able to inhibit xylanase activity. TLXI is a non-competitive inhibitor [32].
B. Identification of Thermophilic bacteria

We have found five isolates have xilanolitik index tertinggiyaitu SSA2, SSA3, SSA4, SSAS6, and SSA7 been selected for identification. Identification of the five isolates based on microscopic and biochemical characters can be seen in Table 2. Characteristics of the five isolates of the same microscopic, that belonged to the Gram-positive, and have shaped bacillus endospores. It is also confirmed from the results of previous studies that have been done by Yenti [17]. These results are slightly different from those obtained Habibie[30], of 14 isolates obtained from Lumpur Lapindo Sidoarjo 11 shaped bacilli and the other 3 are cocci, but all isolates obtained also included a group of Gram-positive and has endospores. Endospores is a special structure that is created when the environmental conditions are not favorable, as a strategy to survive when extreme environmental changes occur such as changes in extreme heat, toxic chemicals, and water shortages. This structure is commonly encountered in Gram-positive bacteria, such as the genus Bacillus and Clostridium. Therefore, these bacteria are often found in the environment termofil[19].

| No. | Test          | SSA2, SSA3, SSA4 | SSAS6 and SSA7 |
|-----|---------------|------------------|-----------------|
| 1   | Aerobic / Anaerobic | A                | A               |
| 2   | GRAM; shape   | (+); basil       | (+); bacillus   |
| 3   | spores        | (+)              | (+); basil      |
| 4   | TSIA          | m | m | m     |
| 5   | Gas           | (-)              | (-)             |
| 6   | H2S           | (-)              | (-)             |
| 7   | catalase      | (+)              | (+)             |
| 8   | Oxidase       | (-)              | (-)             |
| 9   | Motility      | (+)              | (-)             |
| 10  | Indole        | (-)              | (-)             |
| 11  | Urease        | (+/-)            | (-)             |
| 12  | Citrate       | (+/-)            | (-)             |
| 13  | Lactose       | (-)              | (-)             |
| 14  | Glucose       | (-)              | (-)             |
| 15  | Sucrose       | (+/-)            | (-)             |
| 16  | Mannitol      | (+/-)            | (+)             |
| 17  | MR            | (-)              | (-)             |
| 18  | VP            | (+)              | (-)             |
| 19  | OF            | (-)              | (-)             |
| 20  | Gelatin       | (+)              | (-)             |

Genus: Bacillus sp.

| No. | Test | Isolates          |
|-----|------|-------------------|
| 1   |      | Bacillus sp.      |
| 2   |      | Bacillus sp.      |

Notes: A: Aerobic, m | m: red - red (negative test result), (-) negative test results, (+): positive test results, (+/-): results of the test can be positive or negative.

Based on the characteristics in Table 3, the identification referring to Bergey's Manual of Bacteriology Systemathics. The identification results show that all isolates are similarities with the
genus Bacillus. This is in accordance with the characteristics of this genus of gram-positive bacilli, forming endospore, and aerobic[33]. 3 isolates namely SSA2, SSA3 and SSA4 have similarities in terms of its ability to decompose H2O2, motile, asetoin production, and gelatin hydrolysis expressed as Bacillus sp.1. While SSAS6 similar to SSA7, only showed a positive test on catalase test is expressed as Bacillus sp. 2. Thoyib [31], reported that it has gained 5 alkalofilik bacterial genus that has xilanolitik activity of calcareous soils Krakitan limestone hill region of Klaten. It consists of 5 genus Pseudomonas, Bacillus, Flavobacterium, Micrococcus, Paracoccus, and Alcaligens. Sharma et al.,[22] reported that the genus Bacillus and Paenibacillus has the potential to produce xylanase of 5 hot springs in the Northern Himalayas, Himachal Pradesh.

Bacillus sp. 1 and Bacillus sp.2 have catalase or peroxidase enzymes to break down hydrogen peroxide (H2O2) to form oxygen and water. This capability is needed bacteria to protect themselves from product O2, which is toxic, because it is a strong oxidizing agent that can be destructive to the metabolism in cells. Bacteria that have this enzyme generally is that aerobic or facultative anaerobes[34]. Bacillus sp. 1 shows the positive results of the motility test, this leads to ownership structures which help the movement, such as flagella. This genus generally have flegel peritriks, the flagellum located around the edge of the cell as in B. subtilis, B. circlulans, B. brevis and B. polimyxa[35].

Gelatin hydrolysis capability was also demonstrated by Bacillus sp.1. This capability is based on the presence of the enzyme gelatinase. This enzyme is able to hydrolyze gelatin condensed into liquid (liquid). Sometimes these traits also indicate the pathogenicity of a particular bacterium. Gelatinase production relates to the ability of bacteria damages the collagen and into the host body [20].

The genus Bacillus have a high abundance in ecosystems hot springs[22] [30][15]. Abundance is related danganproperties Bacillus which make it capable of living in this ecosystem. One important property is the ability to form spores. When the environmental support to the necessities of life begin to disappear, or when changes in extreme environmental conditions, such as temperature changes, bacteria will soon form resistant structures called endospores. When the environment back ends meet bergerminaasi forming endospores will soon return vegetative cells[35]. Willey et al.[36] added that adaptation of thermophilic bacteria which makes it able to withstand the high temperature environment are as follows: a. Have a good protein stability against heat, especially enzymes. This protein has a hydrophobic amino acid organization that is very good in the interior and more hydrogen bonding, as well as bonding nonkovalen. The number of amino acid proline more so that the polypeptide chain is more rigid and stable to heat. This protein is also stabilized and assisted in pelipatannya by a chaperon protein; b. A kind of histone proteins also help in stabilizing the thermophilic bacteria DNA to heat; c. Thermophilic bacterial membrane lipids tend to saturate, more branches and larger molecular weight, it is able to increase its melting point.

4. Conclusion
From the results of research that has been done can be concluded as follows.
1. It has been selected 12 of 16 isolates having xylanolytic activity, with the highest activity being SSA2 with xylanolitic index 0.74.
2. The top five isolates producing the highest index xilanolitik were SSA2, SSA3, and SSA4 identified as Bacillus sp. 1., and SSAS6 and SSA7 are Bacillus sp. 2..

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