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Isolation and Characterization of A3 and S3 Isolate Thermophilic Bacteria from Lapindo Sidoarjo Mud, East Java

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Abstract: The purpose of this study is to identify thermophilic bacteria isolated from Lapindo mud water and solid sediment with morphological and biochemical characteristics based on biochemical tests (microbact identification kits) compared to 16S rDNA test in Kagoshima University laboratory. The method used is exploratory method. Research was conducted by isolating dominant bacteria from water samples and sediment solid of Lapindo mud as isolates A3 and S3, then the characterization of morphological and biochemical properties of bacteria using morphological test colonies and bacterial cells. Biochemical tests were compared with test results of 16D rDNA then by testing the physical and chemical environmental parameters in Lapindo Sidoarjo mud. Results show that isolates A3 and S3 are included in the group of thermophilic bacteria that grow in an environment with temperature over 45^{o}C. Thermophilic bacteria are known to have a thermostable enzyme that can be used in fish processing industries such as the manufacture of chitin and chitosan. From A3 and S3 isolates identification refer to the bacterial identification guidelines (Bergey's Manual of Determinative Bacteriology), the possibility of species *Pseudomonas pseudomallei* with accuracy percentage of 99.48%. From these results compared with the results of identification using 16S-rDNA test of previous study, it was found that A3 isolate contains *Pseudomonas stutzeri* species and S3 isolatecontains *Bacillus niabensis* species.
Keywords: thermophilic bacteria, microbact identification kits, thermostable enzyme, Lapindo mud.

Introduction

Bacteria that live in areas of geothermal, which is known as the thermophilic bacterium has many inviting appeal to scientists because of the enzymes produced are resistant to heat and are able to catalyze a variety of reactions quickly at high temperatures [1].

In Indonesia, research on thermophilic bacteria began to gain attention. Some local isolates of thermophilic bacteria have been isolated from a number of places. Some thermophilic bacteria were isolated from various hot springs in Indonesia, among others *Geobacillus thermoleovorans* growing at a temperature of 43°-75° C from the hot water source of Wayang crater, Pangelangan [2]. Besides, *Geobacillus thermoleovorans* bacteria is also found in the hot springs of Mount Pancar, Bogor with growing temperature between 42-70° C [3]. The purpose of the isolation of thermophilic bacteria from several habitats is to find the use of the bacteria and the resulting thermostable enzyme which can be applied in the intensified industrial world [1].

Thermostable enzymes makes possible occurrence of process at high temperatures, which is clearly beneficial because it has reactivity, stability, and higher yields, while they have viscosity and lower contamination problems. Isolation thermostable enzyme from a thermophilic organism has a number of commercial advantages for its stability. The advantages of bioprocess taking place at high temperature are higher diffusion rate, lower the viscosity of the substrate, higher reaction rate, increasing solubility of the reactants, and reducing risk of contamination with microbial pathogens [4].

One of the locations that can be used as a sample to obtain isolates of thermophilic bacteria is Lapindo mudflow in Sidoarjo. Lapindo mud that is considered detrimental to the surrounding community turned out to have benefits by containing thermophilic bacteria that live there to be exploited. Thermophilic bacteria are capable of producing thermostable enzyme known widely used in fish processing industry. Besides, the environment of thermophilic bacteria is reported to contain quite high levels of heavy metals, so that only heavy metal resistant bacteria are able to live in it. Therefore, its ability to absorb heavy metals known as bioremediation can be examined. It can be developed on quality control of industrial waste containing heavy metals. The purpose of this study is to identify thermophilic bacteria isolated from Lapindo mud water and solid sediment with morphological and biochemical characteristics based on biochemical tests (microbact identification kits) compared to 16S rDNA test in Kagoshima University laboratory.

Materials and Methods

Study Site

Mud and water samples were taken from the solid sediment of Lapindo mud in Sidoarjo. This research was conducted at the Laboratory of Microbiology LSIH (Central Laboratory of Biological Sciences) Brawijaya University, Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya and Chemistry Laboratory, Faculty of Science, University of Brawijaya from October 2013-August 2014.

Research Materials

This research was initiated by culture, isolation, characterization and test morphological identification of bacteria that includes biochemical tests, test microbact identification kits and comparison to the results of 16S rDNA tests conducted by Sukoso.

The materials used in this study were the water and sediment obtained from Lapindo mud in Sidoarjo area within 200 meters from the center of explosion, the growing media include Nutrient Agar (NA) and Nutrient Broth (NB) which is obtained from the microbiology laboratory of the Faculty of Fisheries and Marine Science, University of Brawijaya (UB); Biochemical test media include simon citrate agar, MR-VP broth, sulphites Indol Motility Agar, tryptone sugar iron agar, urea agar, nitrate broth, biochemical test reagent that includes 3% KOH, methyl Red, Kovac, a-naphthol, mineral oil, TDA, which is obtained from the microbiology laboratory medicine UB. Gram staining reagent taken from Central Laboratory of Biological Sciences, University of Brawijaya, alcohol 70%. The additional materials consisting of a blue tip rubbing alcohol, 42 whattman paper, gloves, masks and filter paper, as well as additional materials that are used to test heavy metals consisting of HCl, HNO_3, Ammonium persulfate ((NH_4) 2S2O8), H3¬PO4, NaIO4, diphenylcarbazide, and dimethylglioxime obtained from the chemical laboratory of Faculty of Mathematics and Natural Sciences, Brawijaya University.

Methods

This study was an exploratory study, a study aimed to investigate a problem or situation to gain a good knowledge and understanding and depth of the problem or situation that made the object of research.

Explorative method in this study was to collect data by conducting research. The study began with a test of Lapindo mud environment which included temperature, pH, salinity and heavy metals to determine the environmental conditions of the bacteria, and then performed the isolation and estimation through morphological characterization of bacteria that included the colony morphology test and Gram stain and bacterial isolates physiology and biochemical tests includes MR, VP, SIM, nitrate, urea, simon citrate, and TSIA tests. The following process was to determine the estimation of species isolates with microbact identification kits that refer to bacterial identification guidelines (Bergey's Manual of Determinative Bacteriology) and to know the role of thermophilic bacteria in the fish processing industry.

Physiological Test

Testing the physiological properties of bacteria is used to determine the biochemical properties of bacteria isolated from Lapindo mud water and sediment. Physiological test used in this study is biochemical and microbact identification kit tests. Biochemical tests performed in this study include the H_2S sugar fermentation test, methyl red test, Voges-Proskauer test, citrate test, urea test, and Sulfites Indol Motility nitrate test. Moreover, for the comparison, microbact identification kit tests is performed in this study including the oxidation test, motility test, nitrate test, test lysine, ornithine test, H_2S test, glucose test, mannitol, xylose test, ONPG test, indole test, urea test, VP test, citrate test, TDA test, gelatin test, malonate test, inoxytol test, xorbytol test, rhamnosse test, sucrose test, lactose test, test arabinose, adonitol test, raffinose test, salicin test, and arginyne test.

Results and Discussion

Environmental Conditions Sampling

From the environmental test parameters, the following data were obtained (Table 1).

Table 1. Environmental Parameter of Lapindo Mud

Sample	Environmental Parameter		
	Temperature	pH	Salinity
Lapindo water	45°C ± 0.29	7.8 ± 0.13	30 ppt
Lapindo sediment	48°C ± 0.20	7.5 ± 0.10	-

Lapindo mudflow has a high temperature for Lapindo mudflow is one of geological disasters due to an error at the time of drilling that resulted in the opening of the volcanic flow pressured by natural gas below drilling locations.

The results of measurements of salinity on Lapindo mud water is 30 ppt (30,000 ppm). SIEJ [5] stated that brackish water with high salinity has 10,000 ppm up to 35,000 ppm salinity. While the salinity of sea water above is 35,000 ppm. Therefore, Lapindo mud is classified as brackish water with high salinity levels.

For the parameters of pH, Lapindo water and sediment can be categorized as base because it has a pH greater than 7 due to the high salinity levels. The sea water pH ranged from 7.6 to 8.3 [6]. There is a contribution of ground water or sea water to water that comes out of the center of the Lapindo mudflow [7]. However, the salinity of sediment samples is not detected due to the solid form sample. Otherwise, the results of heavy metal showed in Table 2.

Table 2. Heavy Metal Levels in Lapindo Water and Sediment

Heavy Metals	Sample (ppm)		TUT* (ppm)
	Water	Sediment	
Pb	0.69	2.69	0.04
Hg	0.23	0.59	-
Cu	0.07	0.27	0.00
Fe	0.76	6.48	0.52
Zn	0.03	0.49	0.36
Cr	0.02	0.06	0.03
Cd	-	0.03	0.01
Ni	1.02	3.39	0.02
Mn	39.16	528	0.56
Au	0.42	2.12	not found

***Based on research at Toyohashi University of Technology (2012)**

Characterization of Bacteria

In characterizing the bacteria it needs to know its main features that include morphological characteristics, chemical composition of the cell, the genetic properties, metabolism and pathogen state. To determine these characteristics, it may take few morphological and physiological tests [8].

The Nature of Morphology

Morphological characteristics of bacteria observed in the study included colony morphology and cell morphology. Bacterial cell morphology observed included Gram stain, colony morphology, cell shape and motility of bacteria. Bacterial colony morphology can be seen in Table 3. As for the bacterial cell morphology can be seen in Table 4.

Figure 1. Isolated Colonies of Water in Lapindo Mud

Based on the table 3, the morphology of bacteria isolated from water and sediment over the same shape that is round. Colonies of bacteria isolated from water has a smooth periphery shape and colonies of bacteria isolated from sediments have a wavy shape of the fringes. The shape of the elevation of colonies of bacteria isolated from water is convex shape, while those from the sediment have a flat shape. Bacterial colonies visible to yellowish white water while white sediment colonies. Colonies of bacteria that will be in isolated from the water can be seen in Figure 1 and the bacterial colonies that will be isolated from the sediment can be seen in Figure 2.

Figure 2. Colonies of Sediment that has been Insulated

Table 3. Morphology of Bacterial Colonies

Isolate	Form	Periphery Form	Elevation Form
A3	Round	Flat	Convex
S3	Round	flat	Flat

To obtain pure bacterial isolates, the scratch cup method (zig-zag scratch), and gram staining were carried out, and then observations of cell morphology was done. Observation of cell morphology include cell shape and color grams

Table 4. Results of Cell Morphology Observation

Isolate	Cell Form	Gram Straining
Water	Basil	Negative
Sediment	Basil	Negative

In Table 4, as observed by using a microscope with a magnification of 1000x it can be seen that both isolates are gram-negative, gram-negative. The red gram-negative indicates that the bacteria contain lipids, or fatty substances, such as fat in a higher percentage than that contained in Gram-positive bacteria, in addition to the gram-negative bacterial peptidoglycan is also thinner than the peptidoglycan of gram-positive bacteria [9]. The results of isolate water gram staining can be seen in Figure 3. The result of sediment isolates gram staining can be seen in Figure 4.

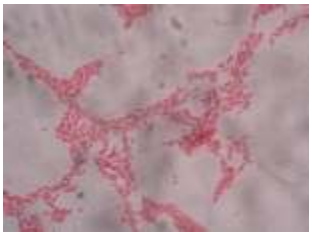

Figure 3. Results of Gram Isolates A3

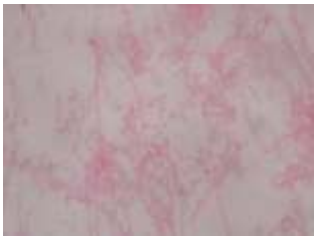

Figure 4. Results of Gram Isolates S3

Nature of Physiology

Methyl Red Test

Based on Table 5, the observation of methyl red test showed that all A3 and S3 isolated bacteria are not able to oxidize glucose which is characterized by the absence of color changes in the media after a few drops of the methyl red.

Table 5. Results of Biochemistry Bacteria Test

Biochemical Test	Isolat A3	Isolat S3
TSIA	Alkali/Alkali, G+, H_2S-	Alkali/Alkali, G+, H_2S-
SIM	Neg/Neg/Pos	Neg/Neg/Pos
Urease	Negative	Negative
Citrate	Positive	Positive
MR	Negative	Positive
VP	Negative	Negative
Nitrat	Positive	Positive

Description: Positive (+): has activity; Negative (-): not having activity; G +: capable of producing gas

Methyl red test (methyl red test) aims to determine the ability of microorganisms to oxidize glucose to produce acid at high concentrations as the end result. Hexose monosaccharide glucose is the main substrate oxidized by all enteric organisms as energy source. The end result of this process will vary greatly depending on the specific enzymes that exist in bacteria [10].

Voges-Proskueur Test

Based on Table 5, from the results of the VP test, A3 and S3 isolates showed negative results marked by the absence of color change in the media. This shows that the bacteria do not have the ability to do fermentation with 2.3-butanediol final result for growth.

Voges-Proskueur test is used to identify microorganisms that do fermentation with the end result of 2.3 butanediol. When bacteria ferment carbohydrates to 2.3-butanediol as the main products, there will be a buildup of such materials in the growth medium [11].

Sugar Fermentation Test (TSI) and H_2S

Based on the results in Table 5, A3 and S3 isolates show the same results that are at the top of the media in order to form a pink means shows alkaline reaction (alkali) and at the bottom to form a pink which means shows alkaline reaction (alkaline) of above reaction means isolates A3 does not ferment sugars. In the media TSIA looks a bit broken, it indicates that the positive isolates forming gas A3 and A3 isolates do not produce H_2S; it is characterized by the formation of black on TSIA media.

Glucose fermentation test is used to determine the ability of bacteria to ferment glucose to produce acid and gas. In TSIA media can be known occurrence fe ~ tion of glucose, lactose, or sucrose and gas production from glucose that is characterized by the formation of cavities at the bottom of the order. The red color on the surface and yellow at the bottom of the tube shows the fermentation of glucose but not lactose and sucrose. Yellow color on the surface and the bottom of the tube shows the fermentation of glucose, lactose, and sucrose [12]. The ability of bacteria to produce H2S at TSIA media (triple sugar iron agar) containing compounds FeS04. In this medium H2S will react with metals Fe 2+ contained in the medium, into FeS (Ferro Sulfide) are colored black [11].

Sulfites Indol Motility Test

Based on Table 5, from the results of SIM test, isolates A3 and S3 showed negative results for the production of sulfur which is characterized by the formation of a black color on the SIM medium, the two isolates also showed negative results for the formation of indole because it does not form a red ring on the SIM medium, and both isolates showed positive results for motility (movement). It is characterized by the presence of fog in the SIM media as a sign of the movement of these bacteria.

According Raihana [13], motility test is used to look at the movement of bacteria, if after incubation for 24 hours arising turbidity like fog means signifies the bacteria move. Indol test used to view the indole formation by bacteria. If the red ring is formed it mean positive and negative forms yellow ring. Red ring formation by bacteria is to form indole from tryptophan as a carbon source.

Urease Test

Based on Table 5, the results of the urea test showed that isolates A3 and S3 do not produce the enzyme urea which is characterized by the absence of color changes in the media so that the bacteria cannot degrade urea and utilize urea in cell metabolism.

Some bacteria are able to produce the enzyme which decomposes urea into ammonium and CO_2. The urea enzyme activity can be observed by the growing of the bacteria in a culture medium containing urea and a pH indicator (usually phenol red). When urea hydrolyzes, $NH4^+$ accumulates in the culture medium and causes the pH to alkaline media. Changes color from red-orange to red purple is an indication of occurrence of hydrolysis of urea [11].

Nitrate Test

Based on Table 4, from these observations on the nitrate test isolates A3 and S3 showed positive results marked by the growth in nitrate broth media. This shows that these bacteria have the ability to reduce nitrate.

Citrate Test

Based on Table 5, from the results of the citric test isolates A3 and S3 showed positive results are indicated by a color change to blue media. This shows that these bacteria have the ability to use citrate as a carbon source for energy. The medium used in this test is Simmons citrate which is a synthetic medium with Na citrate as the only carbon source, NH4 + se a source of N and brom thymol blue as a pH indicator. When microorganisms capable of using citric acid will then be removed from the culture medium, causing an increase in the pH of the medium and change the color to greenish blue [11].

Microbact Identification Test Kits

Based on the results of biochemical tests using identification kits microbact NAM 12A / B / E, 24E of the isolates, it was obtained from water and sediment A3 code with code S3, obtained genus obtained from the calculation of octal numbers. From the results obtained based on the properties of bacteria matched with reference to Bergey's Manual of Determinative Bacteriology for Microbact Testing Results Identification Kits can be seen in Table 6.

Table 6. Results of Testing Microbact Identification Kits

Test	Isolate A3	Isolate S3
Oxidase	+	+
Motility	+	+

Nitrate	+	+
Lysine	+	+
Ornithine	+	+
H_2S	+	+
Glucose	+	+
Mannitol	+	+
Xylose	-	-
ONPG	-	-
Indole	+	+
Urease	+	+
VP	-	-
Citrate	+	+
TDA	+	+
Gelatin	-	-
Malonate	+	+
Inositol	+	+
Sorbitol	-	-
Rhamnose	+	+
Sucrose	+	+
Lactose	-	-
Arabinose	-	-
Adonitol	-	-
Raffinose	-	-
Salicin	-	-
arginine	-	-

Prediction of Bacteria Type

Bacteria which have properties of gram-negative, rod-shaped cells, can use nitrate as an electron acceptor and approached the genus *Pseudomonas sp* motile [14]. To be sure in the estimation of bacteria was examined by microbact identification kits, obtained from a series of test estimation results for isolates of water and sediment are the type of *Pseudomonas pseudomallei* with a percentage of 99.48% accuracy. *Pseudomonas pseudomallei* has physiological characteristics are gram-negative, rod-shaped cells [15]. As for the biochemical characteristics that nitrate positive, positive glucose, arabinose negative, positive mannitol, citrate positive and oxidase positive. *Pseudomonas pseudomallei* are able to live in environments that have high levels of heavy metals and pH high enough. The above results was compared with the results of 16S-rDNA DNA test conducted by Prof. Ir. Sukoso M.SC Ph.D whose obtained results for isolates A3 is *Pseudomonas stutzeri* and the *Bacillus niabensis* bacterium isolate S3. The bacterial estimation results are presented in Table 7.

Table 7. Bacteria Estimated Type Results

Isolate Code	Biochemical Test	Microbact Identification Kits Test	DNA 16S-rDNA Test
A3	Pseudomonas sp.	Pseudomonas pseudomallei	Pseudomonas stutzeri
S3	Pseudomonas sp.	Pseudomonas pseudomallei	Bacillus niabensis

Bacteria isolated from water and sediment Lapindo Sidoarjo mud is a thermophilic bacteria, this is because these bacteria grow in an environment that has a temperature of + 45°C to + 48°C water mud and silt sediments. At that stage until the isolation of bacterial culture using temperature 47°C, this is done to adjust the maintenance condition with the original environmental conditions.

Role of Thermophilic Bacteria in Fishery Products Technology

Thermophilic bacteria are commonly used for the fishing industry because these bacteria are capable of producing thermostable enzyme or enzymes are stable at high temperatures one of which is the enzyme chitinase. Based on research Donderski [16], one of the chitinase enzyme-producing bacteria of the genus *Pseudomonas* sp is. Chitinase is an enzyme that degrades chitin into N-acetylglucosamine. Compounds derived from chitin and chitosan were made with the help of chitinase through deacetylation process. In the process of

deacetylation, N-acetyl groups in chitin will be lost and replaced with the amine group which, when dissolved in acid will be positively charged, so it is polycationic chitosan [17]. Applications use of chitosan can be widely used in various food industries, waste management, health, biotechnology and agriculture.

The results of the identification of bacteria isolated from water and sediment Lapindo mud show that from biochemical test results the *Pseudomonas* sp is found. for both samples. Meanwhile, according to test results obtained microbact identification kits estimation results for both samples are species of *Pseudomonas pseudomallei* with a percentage of 99.48% accuracy. From the above results compared with the results of identification using 16S-rDNA test conducted by Prof.Ir. Sukoso M.Sc Ph.D obtained results for isolates of *Pseudomonas stutzeri* A3 is a species and to isolate S3 is *Bacillus niabensis* species. The bacteria are able to live in an environment that levels of salinity and heavy metals is quite high and can produce thermostable enzyme chitinase enzymes one of them is involved in the processing of shells of shrimp chitosan can be used in the food industry.

It is suggested that further research on the enzyme activity of chitinase and other thermostable enzymes produced by *Pseudomonas pseudomallei* which can be useful in the fish processing industry needs to be done.

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