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Selection of Polymerase-Producing Thermophilic Bacteria from Ijen Crater, East Java

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ABSTRACT

Thermophilic bacteria are a group of bacteria that adapt to environmental conditions with high temperatures, ranging from 45° - 90° C. As the ring of fire, Indonesia has many volcanic areas as the source of thermophilic bacteria, once is in Ijen Crater, East Java. Thermophilic bacteria have the potential to produce heat-resistant / thermostable enzymes such as polymerase. DNA polymerase is a key enzyme in the amplification process of DNA fragment. The objective of this study was to find the novel's thermophilic bacteria isolated from Ijen Crater which has potential to be the source of DNA polymerase production. This study was carried out by growing the bacterial strains on suitable media, followed by thermal screening (70-90°C), isolating and identifying the morphological and molecular characteristics of the selected isolates. A total of 12 isolates has selected after thermal screening in 70°C, which were identified as positive and negative bacteria in gram staining assay. All the selected isolates were found to have the same colony color in white, but various types in shape, border, elevation, and border with. There were 4 isolates that selected after incubation in 90°C for 30 minutes, which were isolate 1, 5, 9, and 11. The genomic DNA of four selected isolates was then amplified using a universal primer (16S rDNA) and *pol1* to detect the presence of gene encoding polymerase. The result indicated that isolates 1, 5, 9, 11 were able to be the candidate thermophilic bacteria to produce DNA polymerase.

Keywords: *Thermophilic bacteria, 16srRNA, Ijen crater, Polymerase, Screening*

INTRODUCTION

Thermophilic bacteria are bacteria that can produce thermostable enzymes and used in various industries (1). The natural habitat of thermophilic bacteria is widespread throughout the earth's surface, including in hot springs, volcanic craters or volcanic areas (2). In Indonesia, there are many volcanic areas that can be the potential sources of thermophilic bacteria, one of them is the Ijen Crater which is located in East Java. There are many studies relating to the use of thermophilic bacteria in any fields. At this time thermophilic bacteria are studied and researched intensively for reasons of basic research development and biotechnological applications (3).

Thermophilic bacteria have the potential to produce heat-resistant / thermostable enzymes and can be used in industry, waste treatment processes and mineral weathering. The advantage of thermophilic bacteria is that it can produce enzymes that are resistant to high temperatures / thermostable enzymes. The use of enzymes that can withstand high temperatures in biotechnology can reduce operating costs and increase reaction speed, such as the use of enzymes from Thermus aquaticus bacteria in the Polymerase Chain Reaction (PCR) process (4). Among the reagents that are very important in the

PCR technique are the thermophile DNA polymerase. DNA polymerase is a key enzyme in the amplification process of DNA fragments. In addition, DNA polymerase is also able to provide the advanced usage of particular reagents, including in PCR properties (5).

The PCR technique is beneficial in molecular biology that is very commonly applied in various laboratories both in academic circles, government and private research agencies, as well as companies in the fields of biomedicine and biotechnology. Up to now, the provision of PCR reaction support reagents is still very dependent on the products produced by foreign companies. Indonesia is rich of natural sources that are potential to be developed in any field, including volcanic areas which are many thermophilic bacteria found. Until now, there has been little findings on the use of thermophilic bacteria (*Bacillus* sp) as a source of DNA polymerase production isolated from local natural resources, especially in East Java which has volcano craters.

Recently, observing the peculiar DNA polymerase source from various thermophilic bacteria has been one of the primary spotlights in molecular study. The need for screening is to find the best candidate bacteria producing the best polymerase. The objective of this study was to screen the novels thermophilic bacteria isolated from Ijen Crater which have potential to use as the source of DNA polymerase production. This approach is the first step to find and develop a polymerase-producing thermophilic bacterial isolate from Ijen Crater in Indonesia. Research on thermophilic bacteria was carried out by exploring the presence of these bacteria in the Ijen crater, East Java, Indonesia, namely by growing the bacteria on suitable media, then screening, isolating, and identifying the characteristics of thermophilic bacteria by investigating their morphological and biochemical properties.

METODE

Samples Collection

Samples in the form of surface water, hot spring, sand, pebbles, sulfuric pebbles and rock from different points of the thermal springs of Ijen Crater (Figure 1) respectively were collected in sterilized falcon laboratory and thermos which were kept in an icebox immediately during sampling and were brought to the laboratory and kept at 4 ^{0}C till further processing. The pH from water samples was recorded at the time of sampling.

Isolation and Growth Condition of Thermophilic Bacteria

Solid samples were incubated at 70 ^{0}C for 3 days before treatment. Bacterial strains were isolated from solid and liquid in ijen crater of east java by serial dilution method (6). A sample of 0.5 g (sand, pebbles, sulfuric pebbles and rock) and 0.5 ml liquid sample (surface water and hot spring) was suspended in 5 ml steril distilled water. 0.5 ml of sample suspension was serially diluted in 5 ml steril distilled water. A volume of 100 ul of sample suspension was spread onto nutrient agar plates and incubated at 37 ^{0}C for 24 h. The different of single colonies observed were screened and characterized.

Morphological characterization and Gram staining

Thermophilic bacterial isolates were identified for various morphological characteristics (elevation, color, shape, border) and gram staining (7). Morphological characteristics were carried out by growing isolates onto nutrient agar medium by streak quadrant. The growing single colony was observed using a microscope. Gram staining was done by dropping a solution of crystal violet, lugol, alcohol and safranin on the sample. If the results is red indicates Gram-negative, while if the bacteria are purple indicate Gram-positive.

Thermal screening

Thermal screening was carried out by growing isolates on nutrient agar media and incubating at 70 ^{0}C for 72 hours. The screened isolates were then grown in 5 ml of Nutrient broth media at 37 ^{0}C for 10 hours with shaker incubator. Temperature treatment was carried out by inserting 0.5 ml of each isolate in a microtube and heating with a temperature level of 80 and 90 ^{0}C for 30 min sing a waterbath. After that isolates were grown on nutrient agar by quadrant streak and incubated at 37^{0}C for 24 hours.

Genomic DNA Extraction

Genomic DNA from selected isolates after thermal screening were extracted by using Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd.). The samples were taken from bacterial cell grown in 3 ml nutrient broth. The genomic DNA extraction was done in four steps, sample preparation, lysis, DNA

binding, washing, and Elution. On the final step, sample was eluted in 100µl elution buffer then stored in -20^{0}C.

16S rDNA Amplification

The 16S rDNA of the isolates was amplified by using universal bacterial primer (F:5'AGAGTTTGAATCMTGGTTGCTCAG 3' and R: 5' TACGGYTACCTTGTTACGACTT 3'). The PCR conditions to performed DNA amplification was done in 30 cycles with following program: pre-denaturation at 95^{0}C for 5 min followed at 95^{0}C for 20 sec, 50^{0}C for 20 sec, 72^{0}C for 30 sec, and final extension at 72^{0}C for 3 min. The PCR product was identified in 1% agarose with Tris acetate-electrophoresis buffer (TAE).

DNA *Pol1* Amplification

Amplification of DNA *Pol1* was done to identified the presence of gene encoding polymerase in the selected isolates. The primers used for this assay was Pol1-Fint (forward primer) 5'-GAYCCHAACYTS CARAAYATHCC-3' and Pol1-Rint (reverse primer) 5'-KASSAKYTCRTCGTGNACYTG-3' (8). The PCR program for DNA *Pol1* amplification was performed in 30 cycles by following conditions: pre-denaturation at 95^{0}C for 5 min followed at 95^{0}C for 20 sec, 49^{0}C for 20 sec, 72^{0}C for 30 sec, and final extension at 72^{0}C for 3 min. The PCR product was then visualized under UV illumination in 1% agarose gel with TAE buffer.

RESULTS

Isolation and Bacterial Growth

The bacterial strains from six different biotopes around ijen crater were isolated and cultured on NA medium with 70^{0}C incubation for 72 hours. The single colony that grew after incubation was then streaked to observe the morphological characterization, Gram staining, and further thermal screening. As many as 12 selected isolates were found in this assay.

Morphological characterizations and Gram Identification of Bacterial Isolates

To describe the morphology of 12 selected isolates, several characters such as shape, border, elevation, and color were characterized. The result (Table 1) showed that 12 selected isolates have same colony color in white and mostly in the shape of irregular except isolate 1. While the border and elevation were found to vary. There were found four different colony borders, isolate 1, 6, 8, 10, 11, 12 belong to entire border, isolate 2 belong lobate border, isolate 3 and 4 belong to curled border, while isolate 5, 7, and 9 belong to undulate border. For the colony elevation, there were four different types which are raised (isolate 1, 8, 10, 11, and 12), convex (isolate 2, 5, and 7), flat (3, 4, and 6), and umbonate (isolate 9). For further identification, 12 selected isolates were assayed for gram staining to determine positive and negative gram.

The result in Figure 2 indicated the different gram identification of 12 selected isolates. Gram positive bacteria was formed purple colony color after treated with gram staining bacteria, while gram negative bacteria was formed red colony color. The isolate 5, 6, 7, 8, 9, 10, 11 belong to gram positive bacteria, while 1, 2, 3, 4, 12 belong to gram negative bacteria.

Bacterial Screening

16S r DNA Amplification

16S ribosomal DNA sequences have been used extensively in the classification and identification of *bacteria.* 4 isolates that had been screened at 90 ^{0}C were isolated by Genomic DNA and then used as PCR templates (figure 3). The PCR amplification results of 16s rRNA gene with primary pairs 27F and 1492R showed positive results, namely there was a DNA band with a size of 1465 bp (figure 4). These results are in accordance with the primary F / R measure used. Based on the results above, it shows that the isolates obtained from the Ijen crater are included in the prokaryotic group. To find out the name of the species, it is necessary to have sequencing analysis and blast it with the gene bank.

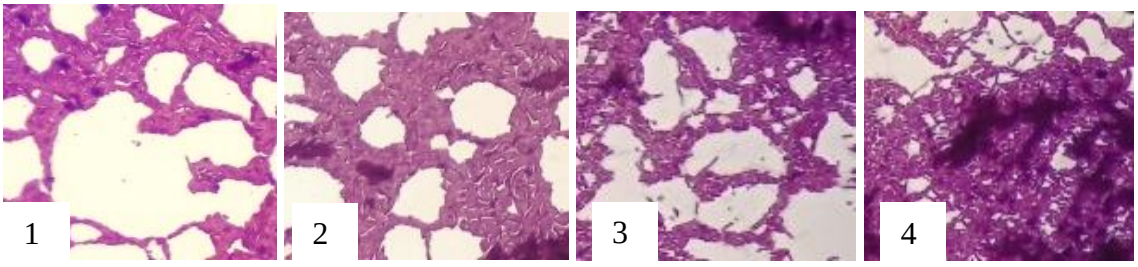
DNA *Pol I* gene

Isolates that have been screened at 90 ^{0}C indicate that these isolates have thermostable character. To confirm the presence of the polymerase coding gene, molecular identification was carried out by PCR. The results of *pol 1* gene amplification using F / R primers showed positive results, indicated by the presence of a single band approximately 600 bp.

Table 1. Morphological characters of thermophilic bacteria isolates

Isolate	Shape	Border	Elevation	Color
1	Circular	Entire	Raised	White
2	Irregular	Lobate	Convex	White
3	Irregular	Curled	Flat	White
4	Irregular	Curled	Flat	White
5	Irregular	Undulate	Convex	White
6	Irregular	Entire	Flat	White
7	Irregular	Undulate	Convex	White
8	Irregular	Entire	Raised	White
9	Irregular	Undulate	Umbonate	White
10	Irregular	Entire	Raised	White
11	Irregular	Entire	Raised	White
12	Irregular	Entire	Raised	White

Figure 1. Sampling site and Map showing the location of hot spring in Ijen Crater, East Java



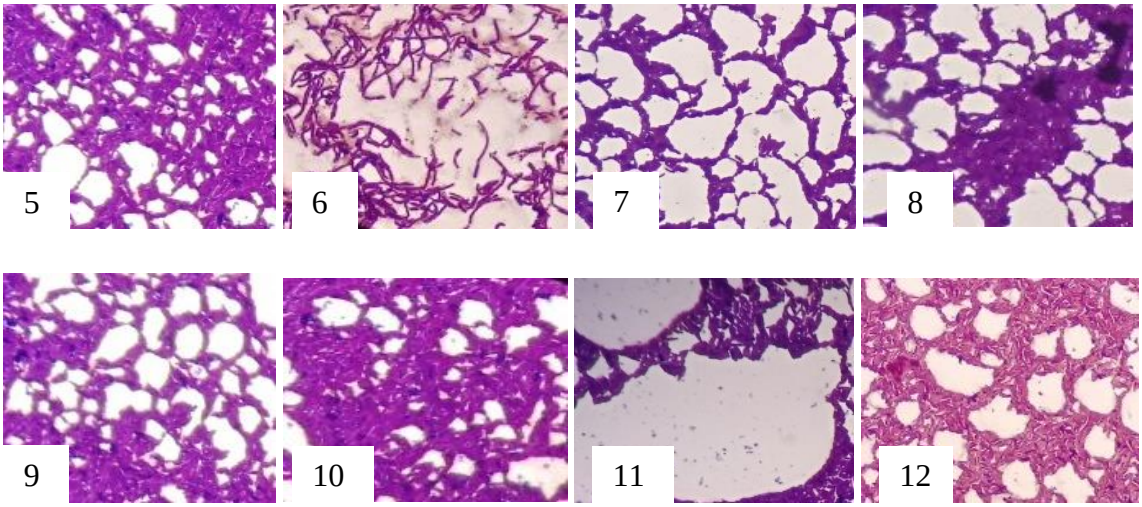


Figure 2. Gram stained of selected bacterial after 70^0C incubation for 72 hours. The number on the pictures show the isolate number. Purple colony color (5,6,7,8,9,10,11) indicated the positive bacteria and red colony color (1,2,3,4,12) indicated negative bacteria.

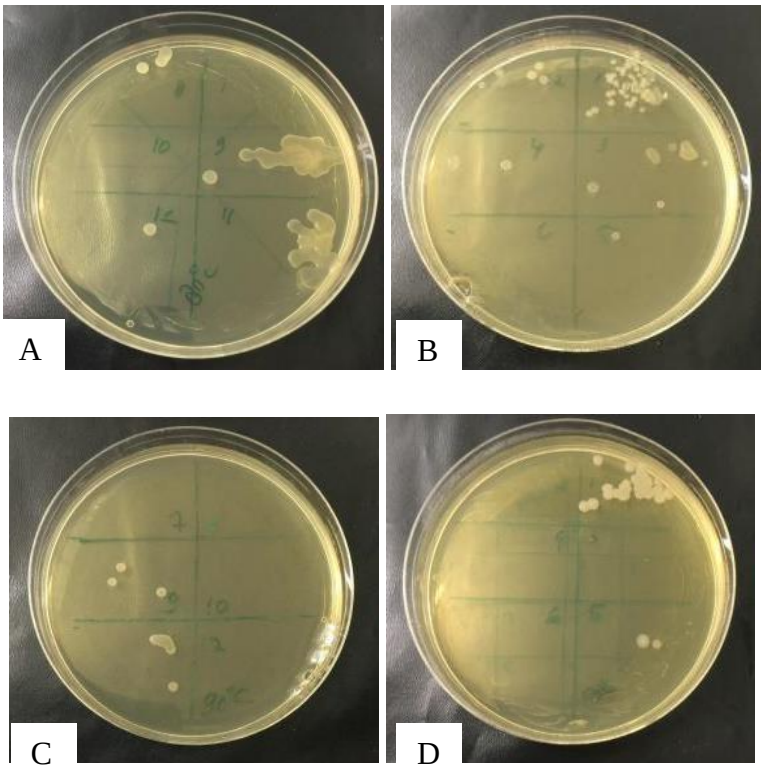


Figure 3. The selected Isolates which grown on NA medium after thermal screening on 80^0C (A and B) and 90^0C (C and D) for 30 minutes. Number 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 on plate shows the isolate number.

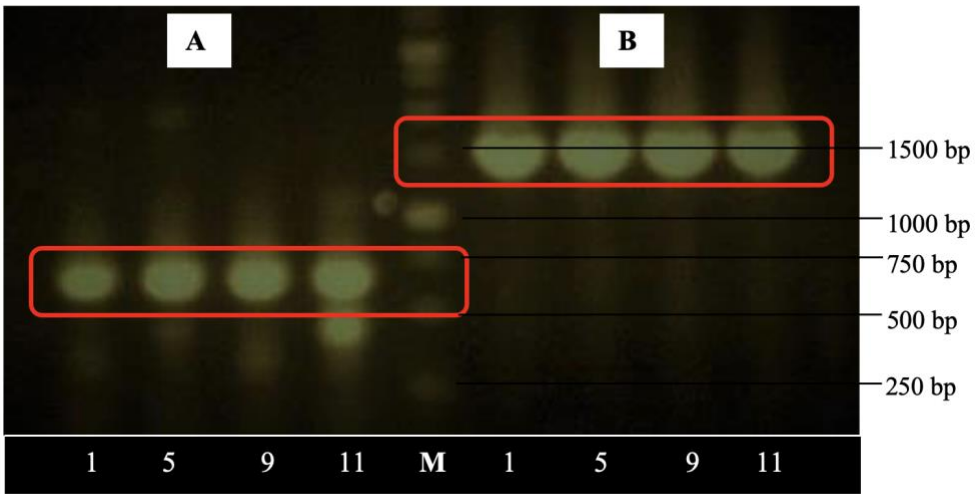


Figure 4. Visualization of DNA *Pol1* (A) and 16S rDNA (B) amplification in 1% Agarose gel under UV exposure. M: 1kb DNA ladder (Promega); 1, 5, 9, 11 : isolate number

DISCUSSION

Ijen Crater is an acidic and hot crater lake, which has one hot spring at one location. the temperature condition is 70 ^{0}C with an acidity of pH 1. Solid samples around the crater in the form of sand, rock, pebble and sulfuric pebble are isolated into a sterile falcon laboratory to prevent contamination. The temperature treatment of 70 ^{0}C in solid samples before serial dilution has the purpose of screening the thermophile bacteria. Liquid samples of hot springs and surface water stored in a thermos, in the laboratory the temperature decreased to 36 ^{0}C – 45 ^{0}C. However, isolation of thermophilic bacteria was successfully performed and bacterial isolates were able to live on a laboratory scale up to 50-90 ^{0}C. Thus, there are thermophilic bacterial isolates that live around crater and crater water. The degree of acidity in the crater and around the crater is very low, approximately pH 1-2, so the bacteria that have been isolated are classified as acidophiles.

Screening of thermophilic bacterial isolates from 6 types of samples resulted in twelve thermophilic isolates that survived at a temperature of 50 ^{0}C. As the screening temperature increased up to 90 ^{0}C, the obtained isolates became four (1, 5, 9 and 11). these four isolates are called hyperthermophiles (9). The initial temperature conditions in the laboratory samples of 45 ^{0}C crater water, each containing thermophilic bacteria that are able to live at a constant temperature of 90 ^{0}C. Thus, the decrease in temperature in the laboratory compared to the temperature of crater water in situ does not kill the thermophilic bacteria present in the sample.

Thermophilic bacteria were successfully isolated with nutrient agar medium with pH 7.0 - pH 7.2. All isolates obtained were identified and characterized by morphology, microscopic, and gram staining. From the test results, it is suspected that the isolates obtained are genus *Bacillus* sp. Genus *Bacillus* sp. has the characteristic of a straight rod-shaped cell, measuring between 0.5-2, 5 x 1.2-10 ¼m and often clustered (10).

The results of *pol 1* gene amplification using F / R primers showed positive results, indicated by the presence of a single band approximately 600 bp. These results are in accordance with the primers sequence used (8). Based on the results above, the 4 isolates found can be used as candidates for polymerase gene sources in producing polymerase enzymes for molecular analysis. In this study, 4 thermophile isolates from Ijen crater that had the *pol 1* gene were the first findings. Previous studies have isolated thermostable bacteria from Central Java (8), West Java (11), North Sulawesi (10) and India (12). Several *pol 1* genes from different thermophile bacteria have also been constructed on both vector cloning and expression (13).

CONCLUSION

Total of 4 bacterial strains (isolate 1, 5, 9, and 11) taken from surface water, pebble, rock, and sand around Ijen Crater were indicated to be hyperthermostable bacteria after selected on 90^0C thermal screening. Among four selected isolates, only isolate 1 was found to be Gram positive bacteria, while other were gram negative bacteria. Isolate 1, 5, 9, and 11 had different morphological characters in shape, border, and elevation, but same in color (white). All 4 isolates were identified to had DNA polymerase which indicated with the presence of DNA *Pol1* as the gene encoding internal polymerase in bacteria. In the next research, researchers would like to do sequenced analysis to found specific species of *Bacillus* sp.

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