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Short Communication

Identification of Protease-Producing Halophilic Bacteria Isolated from Salt-Pond Soil

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Abstract

Protease is an important enzyme for various industries, such as pharmaceuticals, leather production, meat processing, protein hydrolyzate, food products, and waste processing industries. This study aimed to isolate and identify protease-producing bacteria isolated from salt-pond soil at the Tuban Regency, East Java, Indonesia. Bacterial communities in the soil samples were firstly isolated from the soil samples by culture-dependent technique on Luria Bertani agar enriched with 5% NaCl. Thereafter, bacterial colonies that grew on the media were purified and screened for their protease production using a skim-milk agar. The bacterial colony which produced protease was further identified using phenotypic (gram staining) and genotypic assays (the 16S rDNA sequence). The result showed that one isolate out of six (isolate C2) obtained from the soil sample was observed to produce a protease enzyme. Based on its 16S rDNA sequence, the isolate was identified as *Bacillus amyloliquefaciens* strain UBC. These results suggest that *B. Amyloliquefaciens* strain UBC is a salt-tolerant bacterium (halophilic bacteria) which has the potential to be further developed for protease-producing biological agents.

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1. Introduction

The fermented fish products generally use a high concentration of salt to prevent the growth of pathogenic microorganisms. The pathogenic microorganisms can only live at a low level of salts, 0 -1%, while generally dying at high salt concentration. In addition, high salt concentration (>10%), will improve sensory quality and increase shelf life of food products. In a fermentation process, microorganisms are commonly needed as a starter to initiate the fermentation process. Several studies indicate that it is generally better to start by adding halophilic microorganisms that can produce an enzyme required for the fermentation process (Kumaunang *et al*., 2019).

Soil is involved in biological cycles and fertility development. As an important soil biochemical factor, enzyme activity is used to breakdown the adverse effects of various pollutants on soil health (Sun *et al*., 2014) because enzymes are easily influenced by soil physical, chemical and biological factors (Ma *et al*., 2015). Land that is used for salt production has a high salt content. Microorganisms found in the soil will also adapt to such a harsh environment (high salt concentration). Therefore, microorganisms found in the salt-pond soil will have the ability to survive in the high salt concentrations.

Protease is an important enzyme (65% of total enzymes), which has been used widely for industrial applications, such as detergent pharmaceuticals, skin products, processed meats, protein hydrolysates, food products, and the waste treatment industries (Nascimento and Martins, 2006). In its development, especially in the field of biotechnology, enzyme application technology is a biocatalyst that can reduce environmental impacts and replace chemical compounds in industries. Enzymes work very efficiently wit high accuracy (precision), versatile, and economical (Naiola and Widhyastuti, 2002). The choice of microbes as a source of enzymes over plants or animals is because microbial cells are relatively easy to grow, fast growth, and easy to be scaled up. When produced in a large scale, it would become much cheaper, production does not depend on seasonal changes, and the time needed in the production process in shorter (Vishwanatha *et al*., 2010). Thus, this study aimed to isolate and identify salt-resistant bacteria that can produce protease enzyme from salt-pond soil in Tuban Regency, East Java Province, Indonesia.

2. Materials and Methods

2.1 Materials

Materials which were used in the present study were: Luria Bertani (LB) agar, Skim Milk Agar (50 g/L), casein 5 gr/L, yeast extract 2,5 g/L, glucose 1 g/L, agar 12.5 g/L) pH 7, NaCl, Aquades, Qiagen Extraction kit (Qiagen, USA), Iodin, Cristal Violet, alcohol 95%, dan safranin.

2.2 Sampling

A soil sample was collected randomly at ~5cm depth with a sterile iron pipe from a salt pond at the Tuban region, East Java province, Indonesia. The soil was then put in a polyethylene bag and placed in a cool box containing ice to keep the temperature at 4ºC. Subsequently, the sample was transported to the laboratory for further analysis.

2.3 Isolation and purification

Bacteria from the sample was isolated using a spreading method. In brief, 1 g soil sample was diluted in a physiological saline solution (0.9%) with serial dilutions (10^{-1} – 10^{-3}). Then, 100 µl solution was pipetted out from each serial dilution and subsequently spread on a modified LB agar. The modified LB agar contained: 5% NaCl, 1 % polypepton, and 0.5% yeast extract. After that, bacterial colonies that grew on the agar plate were purified using a streak plate method. Then, the pure bacterial colonies were preserved in LB media and stored at freeze (4ºC) until further analysis.

2.4 Screening of protease activity

The pure bacterial isolates were screened for their extracellular protease activity using skim milk agar. Each isolate was inoculated on the skim milk agar and incubated at 35ºC for 24 hours. Colonies which produced protease indicated by the formation of clearance zone around the growing colonies were directly transferred into the LB agar. The colony which produced the widest clearance zone was selected for species identification.

2.5 Gram-Staining

Gram staining method was carried out to strengthen the identification process beside molecular analysis. In brief, a sterile loop was touched to the bacterial colony and mixed with two drops of sterile water previously prepared on an object-glass. Thereafter, the mixture was spread in the middle of the object-glass to form a thin and fixed layer. After dry, the smear of the bacteria was flooded with cristal violet for two min and subsequently washed with running tap water. Then, iodine was added for two min and washed again with the running water, followed by air-drying. Then, the object-glass was flooded with 95% alcohol until te purple dye is no longer

visible, washing with the running water and air-dried. The object-glass was flooded again by safranin for three sec, washed again, and followed by air-drying. After that, the object-glass was observed under a microscope at 40x magnification.

2.6 Partial sequence of 16s rDNA

The protease-producing isolate was further analyzed for species identification based on its partial 16S rDNA sequences according to a protocol of Nursyam and Prihanto (2018). Firstly, DNA was extracted using a commercial kit for DNA extraction (the Qiagen extraction Kit). Then, 16S rDNA was amplified by the Polymerase Chain Reaction technique using 27F primers (5'-AGAGTTTGATCATGGCTCAG-3 '), and 1492R (5'- TACGGCTACCTTGTTACGA-3') (Lane, 1991). One µL isolated DNA sample was used as a template and was dissolved in an 18.5 µL PCR mixture containing: 2.5 µL Buffer B with Mg2 + 10x, one µL dNTPs, one µL forward primer, one µL reverse primer and 0.2 µL *Taq polymerase*. The mixture was then put into a PCR machine. The process begins with denaturation at 94 ° C for 45 sec, annealing temperature at 61 ° C for 45 sec, and an elongation temperature of 72°C for 2 min. The program was carried out as many as 35 cycles. The PCR process ended with a final elongation at 72 ° C for 10 min.

The next step was to purify the PCR product using a DNA fragment extraction kit (Qiagen, USA). The purified PCR product was mixed with a buffer solution namely Hi-DiTM Formamide (Genetic Analysis Grade-Applied Biosystem) and sequenced using ABI-PRISM® 310 Genetic Analyzer. Sequencing results were used to determine the similarity of the partial 16S rDNA sequences with other bacterial DNA sequences that present in GenBank using the help of the Basic Local Alignment Search Tool (BLAST) program at http://www.ncbi.nlm.nih.gov. Bacterial relatedness was presented in the form of a phylogenetic tree made with the Phylogeny.fr program rock. This program was available online at the site http://www.phylogeny.fr (Dereeper *et al.*, 2008).

3. Results and Discussion

3.1 Bacterial isolation from salt-pond soil

The research results showed that seven bacteria were isolated using the LB agar media with 15% NaCl and followed by purification. Then, the purified isolates were grown on the LB agar media and stored in a freeze at 4 ° C. Each isolate was coded to distinguish it (Figure 1).

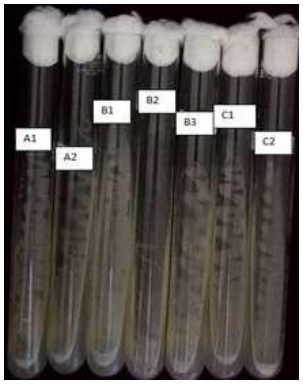

Figure 1. Seven proteolytic bacteria isolated from salt-pond soil. A1 = Proteolytic bacterium isolated from 10^{-1} dilution, A2 = Proteolytic bacterial isolated from 10^{-1} dilution, B1 = Proteolytic bacterial isolated from 10^{-2} dilution, B2 = Proteolytic bacterium isolated from 10^{-2} dilution, B3 = proteolytic bacterium isolated from 10^{-2} dilution, C1 = isolate of proteolytic bacterium isolated from 10^{-3} dilution, C2 = isolate of proteolytic bacterium isolated from dilution of 10^{-3}

3.2 Proteolytic bacteria

Proteolytic activity of bacteria isolated from the salt-pond soil indicated by the formation of a clearance zone around the bacterial colony. The results showed that only three isolates were able to grow on the skim milk agar with 5% NaCl. Based on the analysis, it was found that only one isolate (C2 isolate) produced the protease enzyme (Figure 2).

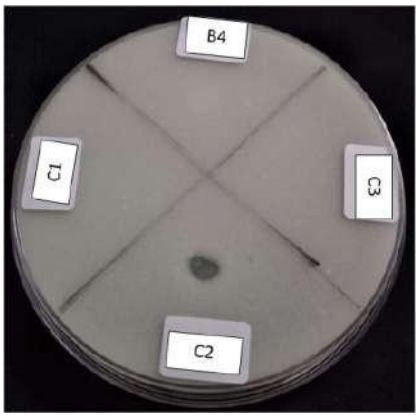

Figure2. Screeningofprotease-producingbacteriaonskim milkagar.C1,C2,C3,B4=bacterialisolatestobescreened.

The presence of proteolytic activity was detected by the formation of a clear hydrolysis zone around the bacterial colony. Several researchers have also used a similar method to screen protease-producing bacteria. The identification method of proteolytic bacteria

using skim milk agar was also carried out by Sinha *et al*., (2013). The present study was able to isolates seven bacterial strains but found out only a single strain (C2) could produce protease indicated by the formation of a clearance zone around the bacterial colony. Similarly, Choudhary and Jin (2012) explained that a hydrolysis zone around a bacterial colony grown on skim-milk agar media coud be used as an indicator for protease production. The formation of the clearance zone suggests that extracellular produced of the bacterial strain could hydrolyze casein and skim milk content in the agar. According to Sarker *et al*., (2013) protease production was generally influenced by nutritional factors such as carbon and nitrogen sources and environmental conditions including pH, temperature and incubation period.

Skim milk agar is a medium commonly used to isolate and identify protease-producing bacteria. The ability of the media to be used as a protease screening medium is due to its nutritional composition. Skim milk in the media, for instance, was the most effective nitrogen source to increase protease production. In addition, there were several other sources of nitrogen, such as casein and gelatin (Anbu, 2016). Several studies that identified proteolytic bacteria from several sources aimed at the application of these microbes in scientific development activities and biotechnology (Ebrahempour and Ashraf, 2015).

Bacterial isolate that had protease activity was C2 isolate. Whereas the other isolates did not form a clearance zone. The wider the clear zone which was formed, the higher the ability of bacteria to produce protease enzymes. Thus, based on these results, C2 isolate was selected for further identification.

3.3 Gram staining

The morphological observation of cells through Gram staining showed that the bacterium was included in the group of Gram-positive bacteria, and the shape of cells was round. This was indicated by the color of bacterial cells which were purple. The Gram staining method was based on differences in cell wall composition. Gram-positive cells have walls with a thicker layer of peptidoglycan than Gram-negative cells. The Gram staining results of proteolytic bacteria can be seen in Figure 3.

3.4 Molecular identification

PCR product which was run on the electrophoresis agar, showed that the size of amplified gene from the C2 was ~1.5 kbp, Figure 4. This result was similar to a study performed by Prihanto *et al*. (2019) in which t the amplicon was 1.6 kbp in size. Meanwhile, an amplicon of 1.4 kbp was also demonstrated by the results of research by Gani *et al*., (2019).

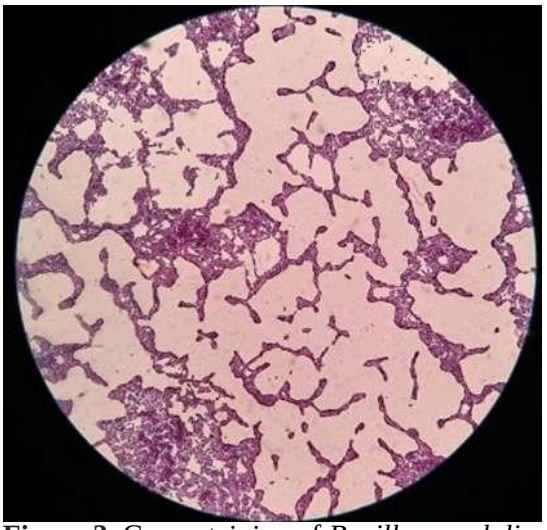

Figure 3. Gram staining of *Bacillus amyloliquefaciens* strain UB-C viewed under a light microscope

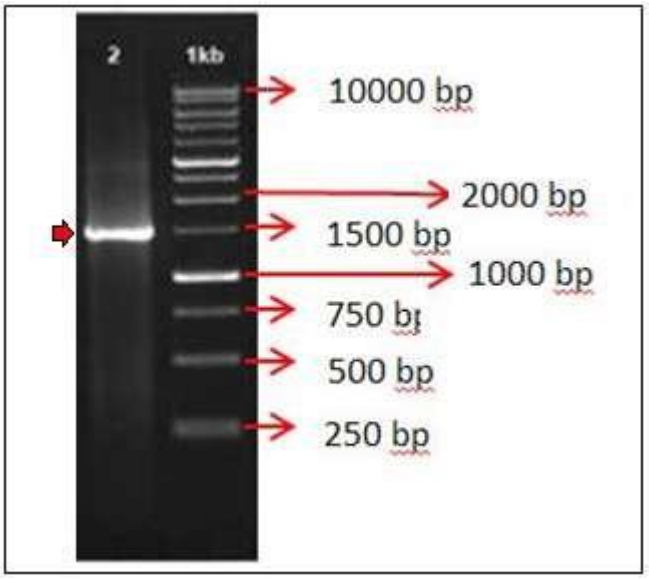

Figure 4. 16s rDNA amplified from a protease producing isolate. Band on the right side is DNA from bacterial isolate and the band on the right side is series of DNA makers to indicate the size of the amplified gene from the bacterial sample.

The results of molecular identification using phylogenic tree analysis showed that C2 isolate had a very close relationship to *B. amyloliquefaciens* NBRC 15717 (Figure 5). Furthermore, *B. substilis* strain 12118 had a relative relationship with C2 isolate. The second group is divided into two branches, where one branch is only filled with *B. megaterium* strain

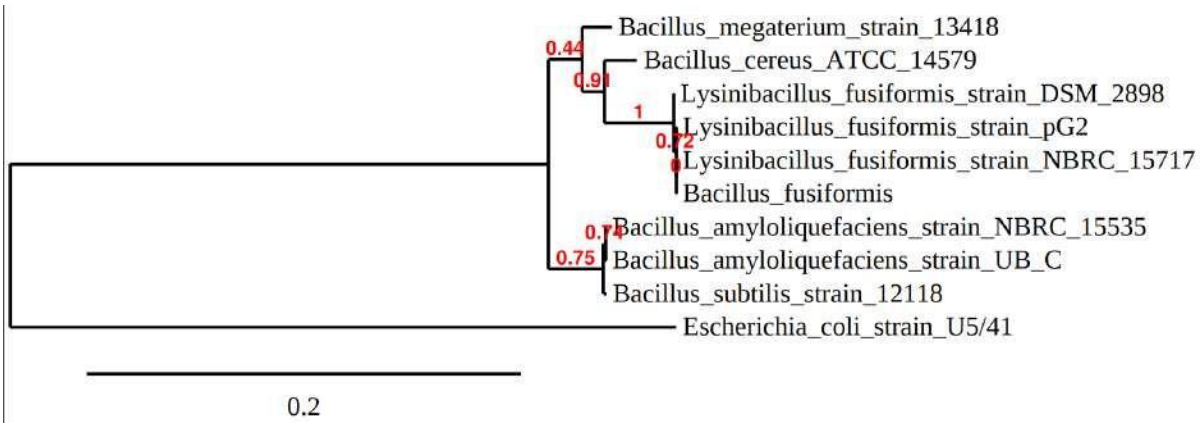

Figure 5. A phylogenetic tree showing the position of bacterial isolate C2. *E. coli* was used as a bacterial species from another bacterial group.

13418. The results of the analysis using the analysis tree strengthen the conclusion that the C2 isolate bacteria was *B. amyloliquefaciens*. Considering that this isolate bears 99% similarity with *B. amyloliquefaciens* NBRC 15717, we named the bacterial strain, *B. amyloliquefaciens* strain UBC.

If the sequencing result has similarities more than 98% to a certain bacterium that has been known before in the gene bank, then the species should be considered as the same species. However, the DNA sequence has a similarity of less than 97%; then, it can be considered as a new species (Janda and Abbot, 2007). Phylogenetic tree reconstruction described data that can be interpreted as related hierarchies and represented by each branch in the form of a phylogenetic tree of bacterial species (Kim *et al*., 2011).

B. amyloliquefaciens has an average value of protease enzyme activity of 0.43 unit.ml^{-1} at 40^{o}C and pH 7. Protease production frequently uses *B. amyloliquefaciens* DJ-4 by adding NaCl and heat (Choi *et al*., 2013). Furthermore, Asokan and Jayanti (2010) explained that *B. amyloliquefaciens* was widely used in biotechnology activities because it had good characteristics to produce alkaline proteases. *B. amyloliquefaciens* can provide several other enzymes that have commercial potentials such as α-amylase, β-glucanase, hemicellulose, and neutral protease.

The genus Bacillus is an extracellular protease producer. *Bacillus* sp. is a neutral protease group with a pH of 7 (Sandhya *et al*., 2005). *B. amyloliquefaciens* produces higher proteases than *B. subtillis*. Production of high-level extracellular enzymes makes *B. amyloliquefaciens* attractive, especially to industrial microbiologists (Wang *et al*., 2016).

4. Conclusion

This study concludes that protease-producing microorganisms can be isolated from salt pond soil in the Tuban Regency, East Java Province, Indonesia. Based on the sequence of the 16S rRNA gene, the bacterial isolate producing protease was identified as *B. amyloliquefaciens*. The bacterial strain was a potential isolate as a protease-producing biological agent.

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Authors' Contributions

Author 's Contribution All authors have contributed to the final manuscript. The contribution of each author as follows, AAP, conceptual, and idea, collected the data, drafted the manuscript, and designed the figures. RN; drafted the manuscript, critical revision. HM and JA; collected the data, analyzed the data. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests

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