Halophilic Bacteria Producing Protease from Salted Fish in Ponrang, Luwu Regency, South Sulawesi

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Abstract

The need for protease enzymes for medical and industrial purposes. The need for proteases in the world reaches 65% of the total sales of enzymes, and in Indonesia can reach 2.500 tons every year, and 99% percent of the enzyme needs are still imported from abroad. Salted fish is one of the foods that contain a lot of protein, which is about 42% in 100 g of salted fish. It allows the presence of proteolytic bacteria that have halophilic properties in salted fish. This study aims to explore the presence of proteolytic bacteria with halophilic properties in salted fish. Proteolytic isolates were isolated using SMA media from salted fish from Ponrang District, Luwu Regency, South Sulawesi. A qualitative test was carried out by measuring the clear zone formed in Skim Milk Agar (SMA). From the isolation process, 51 isolates were obtained. However, after the screening, only 25 pure isolates were found that formed a clear zone, followed by a quantitative test to see which isolates had stable activity at incubation times of 24, 48, and 72 hours using Tryptic Soy Broth (TSB) media. The results obtained four superior isolates, P1A1K, P2B2PS, P3C6PS, and P3C6P, then continued to quantify halophilic bacteria properties by administering NaCl with 15% and 20% concentration into TSB media for 24 and 48 hours incubation. Two isolates with the highest protease activity were P3C6P isolates with the activity of 43.23 ± 7.11 $U.mL^{-1}$ at 15% salt concentration and 42.83 ± 3.04 $U.mL^{-1}$ at 20% salt concentration and P2B2PS isolates of 38.05 ± 4.05 $U.mL^{-1}$ at 15% salt concentration and 38.15 ± 1.47 $U.mL^{-1}$ at 20% salt concentration. The two isolates were then tested for pathogenicity on blood agar media. It was found that only P3C6P isolates did not have pathogenic properties, so P3C6P isolates were continued with catalase, oxidase, and gram staining tests, which showed negative catalase and oxidase results and were gram-positive, followed by identification based on sequences. 16S rDNA and phylogenetic tree construction where isolate P3C6P was identified as *Bacillus cereus* with a 100% similarity level to the WHX1 strain.

Keywords: Enzyme, Protease, Proteolytic, Salted Fish, 16s-rDNA.

INTRODUCTION

Enzymes are one of the essential ingredients needed in the industrial sector. Aside from being a raw material and biocatalyst in chemical reactions in industrial activities, enzymes are also widely used as materials in industrial waste treatment processes [1]. The need for protease enzymes in the world ranks first in the trade of enzymes, namely around 65% of the total sales of enzymes in the world. Global market demand for protease enzymes has increased by 7% from 2015-2020 and is expected to continue to increase [2]. In Indonesia, the need for enzymes, especially proteases for industrial use, can reach more than 2.500 tons annually and continue increasing [3].

To meet this need, Indonesia must import 99% of its enzyme needs from several countries, such as China, Japan, India, and parts of Europe. Today, BPPT (Agency for the Assessment and Application of Technology) is Indonesia's only enzyme production unit with one of the enzymes being developed, namely the protease enzyme [4].

Protease enzymes function to decompose proteins in living things into simpler amino acids. Proteases in Indonesia are widely used in the health sector for producing chitosan [5]. In the industry, the enzyme is used in the leather, textile, food, protein hydrolyzate industries, milk processing, beer, film, and waste treatment [6]. Bacteria are the most widely used enzyme producers compared to animals and plants because the growth of bacteria is fast and can be genetically engineered for benefit production activities [7]. Every 100 g of salted fish contains 42% protein, 1.50% fat, calcium, and phosphorus [8]. They were making salted fish very suitable as a growth medium for proteolytic bacteria that have halophilic properties or are resistant to salinity conditions. Although research related to the exploration of proteolytic bacteria has yet to be widely carried out, several studies have found the presence of proteolytic bacteria in fish, which are also preserved by the salting process where proteolytic bacteria are found in salted fish [9].

Salted fish from Ponrang District, Luwu Regency, South Sulawesi, is one of the food ingredients that have the potential to be explored in order to obtain superior proteolytic

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bacterial isolates. It is because the manufacture of salted fish in the area is still done traditionally, so it is still very easily damaged due to contamination by microorganisms. This research is very important considering the increase in the need for protease in Indonesia, which is experiencing an increase while Indonesia must depend on imports to meet this need [3].

MATERIAL AND METHODS

Isolation of Proteolytic Bacteria

The samples used in this study were three types of salted fish, namely the first sample of salted tembang fish (*Sardinella fimbriata*), the second—sample of yellow-tailed salted fish (*Ocyurus chrysurus*), and the third sample of salted red snapper (*Lutjanus campechanus*) obtained from Ponrang District, Luwu Regency, South Sulawesi Province. The bacteria were isolated using skim milk agar (casein 0.5 %, yeast extract 0.25%, dextrose 0.1%, skim milk 2.8%, agar 1.5%), and pH 7 [8]. Incubation was carried out at 37^{o}C for 24 hours. Bacterial colonies growing with clear zones were then purified to obtain a single colony and stored on a slanted agar for further testing [10].

Qualitative Test of Proteolytic Activity

The protease enzyme was detected using skim milk agar (SMA) media. The disc paper was immersed in the inoculum for 24 hours and left for 15 minutes. After that, it was taken and placed on SMA medium, incubated at 37°C for 48 hours, and observed the clear zone formed. The proteolytic hydrolysis index was calculated by dividing the clear zone's diameter by the paper disc's diameter [11].

Quantitative Test of Proteolytic Activity

The starter was made by taking as many as five loopful pure isolates inserted into 20 mL of TSB media and incubated for 24 hours at 37°C. After that, the bacterial cell density was equalized using a hemocytometer to obtain a cell count of $2x10^{8}$ cells.mL^{-1}.

Furthermore, 2 mL of starter inoculum, whose density has been equalized to 10^{8} into 18 mL of sterile TSB media, was then incubated using a shaker incubator at room temperature for 24, 48, and 72 hours. Then 2 mL of 24, 48, and 72 hours inoculum were taken and centrifuged at 10,000 rpm for 10 minutes at 4°C to separate the liquid and *crude extract*.

The crude extract was taken as much as 1 mL and then mixed into the 5 mL of 0.2 M phosphate *buffer solution* containing 1% casein, then incubated for 10 minutes at 37°C using a water bath after which 5 mL of 5% TCA (trichloroacetic acid) was added to stop the reaction that occurred. Then incubated again at room temperature for 30 minutes then centrifuged at 10. 000 rpm at 4°C for 15 minutes.

Crude enzyme extract or supernatant was the material to be used in the protease activity test [12]. The crude enzyme extract formed was then taken as much as 2 mL, added with 5 mL of Na_2CO_3 as much as 5 mL, and incubated at room temperature for 30 minutes. Furthermore, 1 mL of Folin Ciolcateu's (Merck) reagent 0.5 M was added and incubated for 30 minutes at room temperature. A blue color gradation was formed due to the reaction between the folin reagent and free tyrosine in the sample [13]. Furthermore, the samples were sampled in a UV-Vis *spectrophotometer* with a wavelength of 660 nm to determine the optical density (OD). Each sample isolates with stable proteolytic activity were continued to the next test.

Protease Activity Test in Salt Stress

A total of 1 mL of crude extract was dissolved in 5 mL of 0.2 M phosphate buffer solution with 1% casein as a substrate, then incubated for 10 minutes at 37°C using a water *bath*. Then it was mixed with 5% TCA (*trichloroacetic acid*) as much as 5 mL to stop the reaction. The samples were then incubated at room temperature for 30 minutes and centrifuged at 10.000 rpm for 15 minutes at 4°C.

The resulting crude extract or supernatant was then taken as much as 2 mL, mixed with 5 mL of Na_2CO_3, and incubated at room temperature for 30 minutes. The sample was then added with 1 mL of Folin Ciolcateu's (Merck) reagent 0. 5 M and incubated for 30 minutes at room temperature. Adding folin formed a blue color because of the reaction between folin and free tyrosine in the sample. The darker the blue color that appears, the higher the tyrosine concentration in the sample. The samples were then sampled in a UV-Vis spectrophotometer with a wavelength of 660 nm to determine the optical density (OD). Then the results of the OD measurement were converted into tyrosine levels and analyzed for protease activity. Each isolate with stable protease activity from each sample will be continued to the next test.

Pathogenicity Test

One loopful of pure culture aged 24 hours was inoculated on blood agar (5% sheep blood) using the streak plate method and then incubated at 37°C for 48 hours [14]. There is a

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clear zone around the inoculated isolate. The isolate was classified as a pathogen because the isolate was capable of lysing red blood cells on blood agar media.

Identification of Bacteria Based on 16S rDNA Sequences

The selected isolates with the highest protease activity were cultured with Nutrient Agar (NA) medium and incubated at 30°C for 24 hours. Chromosomal DNA from selected isolates was extracted according to standard protocols/Bacterial Miniprep KIT (Zymo Research Corp, Tustin, USA). The 16S rDNA was amplified using 27f primer (5' –AGAGTTTAGTCCTGGTCCAG-3) and 1492r primer (5'CTACGGCTACCTTGTTACGA-3'), which are complementary to the 16S rRNA gene ends of all bacterial strains [15]. Spindown suspension was conducted to homogenize the composition of the solution. After that, the PCR tube was inserted into the PCR machine and run according to the PCR program. The PCR program used for the 16S rDNA amplification process is predenaturation, denaturation, annealing, extension, and post-extension. The PCR reaction begins with an initial denaturation at 94°C for 5 minutes. It continues with 35 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 60 seconds), elongation (72°C for 90 seconds), and final elongation at 72°C for 5 minutes.

Amplicons of 16S rDNA were confirmed using 1.5% agarose gel electrophoresis—alignment 16S rDNA acidity. DNA bands with a molecular weight of ± 1500 bp indicate that the 16S rDNA has been amplified. Amplicons were then sequenced at 1st BASE DNA in Malaysia using the Sanger sequencer. Sequencing was carried out using BigDye(R) terminator V3.1 cycle sequencing kit. DNA sequences were BLAST with sequence references in the NCBI database. Based on the Neighbor-Joining Algorithm and the Tamura Nei-Model, the phylogeny tree was constructed using Mega 6 software for Windows.

Data Collection

The protease activity data were analyzed by two-way ANOVA using SPSS 20.0 program for windows. If there is an interaction, it is continued with Tukey's real difference test.

RESULTS AND DISCUSSION

Proteolytic Bacteria Isolation

At the isolation stage, 51 isolates were growing on the media used, where fish samples 2 and 3 were the samples with the highest colony distribution (Fig. 1). After observing, there were about 25 isolates that formed a clear zone on SMA media seven isolates from salted fish 1, seven isolates from salted fish 2, and 11 isolates from salted fish 3. It became the basis for determining the 25 isolates that formed a clear zone on SMA media which entered the next stage.

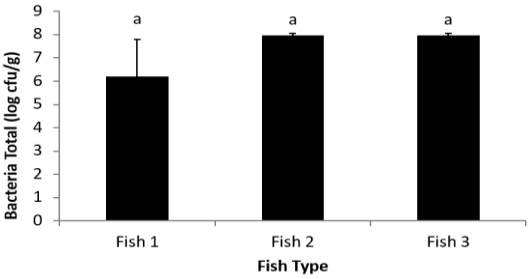

Figure 1. TPC calculation results from three salted fish samples. Data are mean average, standard deviation data from three replications, and values followed by letter indicate no significant difference (p <0. 05). Data were analyzed by one-way ANOVA and Tukey.

The peptone and skim milk content in the media serves as the primary carbon source needed in the metabolic process of bacterial cells. In addition, the protein contained in skim milk agar selective media also functions as an inducer for protease enzymes. The hydrolysis of casein content in skim milk will form a clear zone around the colony due to the ability of bacterial isolates to produce and secrete extracellular protease enzymes. Thus, a hydrolysis reaction occurs in skim milk into peptide-peptide bonds and amino acids, expressed by forming a colorless zone around the colony [16].

The white color on the SMA media itself was formed because the casein in milk contains phosphor protein, which is a type of protein that contains phosphorus; where this phosphoprotein will bind to calcium calcenate, which is soluble in water, to form a white colloid on the agar media [17]. The formation of a clear zone on the media is the main indicator that these bacteria are proteolytic because they can utilize casein as a source of nutrition [18].

Qualitative Test of Protease Activity

This test was conducted to determine the ability of the selected isolates to produce extracellular protease enzymes. A total of 25 isolates tested at this stage were confirmed as proteolytic bacteria. The positive test was indicated by the appearance of the skim *milk agar media,* which was initially white to clear. It indicated that all isolates could hydrolyze protein

on SMA media by changing the casein substrate (protein) contained in the medium [19]. Skim milk, the essential ingredient in SMA media, is the primary carbon and nitrogen source for proteolytic microorganisms [20].

Based on this confirmation test, 25 isolates were selected with the highest clear zone diameter to enter the next stage (Fig. 2). The protease activity of all isolates of proteolytic bacteria from salted fish showed that all isolates had varied activities during the incubation period. Based on the clear zone, the highest proteolytic activity in SMA media was P3B4B and P2B2PS isolates. The lowest activity was P3C6PC2 and P3C22PC isolates at 24 hours incubation period.

Protease Activity Quantitative Test Results

This test showed that the protease activity tested on three types of salted fish with incubation times of 24, 48, and 72 hours had quite a variety of activity variations (Fig. 3). It can be seen that the highest and lowest protease activity in each salted fish sample tends to be different. However, the highest activity occurred on average at 24 hours of incubation and the lowest at 48 hours, then increased again to 72 hours of incubation. Isolates with stable protease activity from each sample were salted fish 1 with isolate code P1A1K, salted fish sample 2 was P2B2PS, and salted fish sample 3 were P3C6PS and P3C6P.

Micro protease enzymes are constitutive or partially inducible enzymes that always available in microbial cells in relatively constant amounts. These enzymes will be synthesized when induction occurs, for example, on substrates in the media.

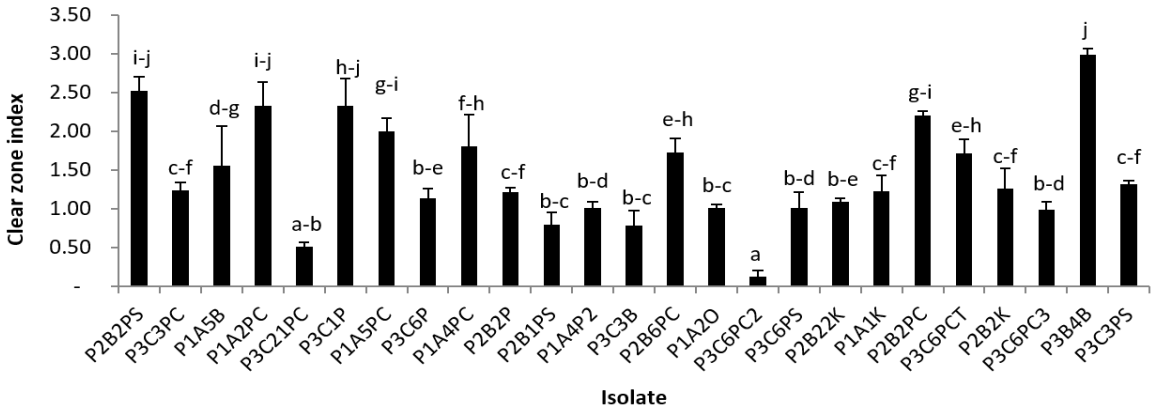

Figure 2. Diameter of the clear zone of proteolytic bacterial isolates on SMA media. Data are mean and standard deviation from three replications, and scores followed by different letters indicate a significant difference ($p < 0.05$).

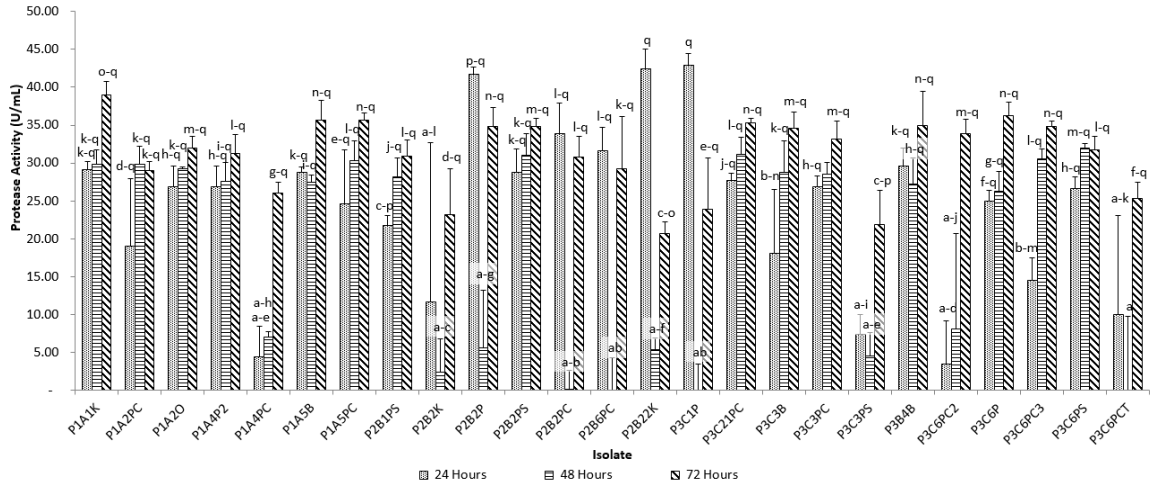

Figure 3. Protease activity at an incubation time of 24 hours, 48 hours, and 72 hours. Data show the mean and standard deviation of three replications. The letter notations indicate differences in interactions between isolate and incubation times on protease activity ($p < 0.05$).

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The higher concentration of the substrate will be in line with the increased synthesis of the enzyme. It allows the high activity of the enzyme at 24 hours of incubation [21]. While the protease activity, which decreased at 48 hours of incubation, probably occurred due to a decrease in the amount of substrate in the media, especially if the substrate was the main carbon source [22]. If the protein concentration decreases in the medium, the enzyme activity will also not reach its maximum activity because the enzyme cannot find the substrate to react [23].

The decreased number of bacterial cells is because the bacterial cells cannot survive in conditions of minimal carbon sources in the media after 48 hours. It will indirectly affect the incubation time of 72 hours, where there will be a balance between the number of bacterial cells that survive and the amount of substrate available in the media. The activity of the protease enzyme will increase along with the amount of nutrient preparation on the substrate, so the reaction rate will also increase [24].

Protease Activity in Salt Stress

This test was conducted to see whether the isolates with stable protease activity in the previous test could still produce protease enzymes when under stress by adding salt to the test media used. The addition of salt content in the media was 15% and 20%, where this test also confirmed whether all the isolates tested had halophilic abilities, namely the ability to tolerate salt conditions. The salt content addition to the media is based on the average salt content of salted fish. According to SNI (Indonesian National Standard), it is determined that the salt content of salted fish is allowed with a maximum limit of 20%, where this level is still included in the tolerance range of halophilic bacteria, which is in the range of 2 to 30%.

The observations on the changes in the media found four isolates tested: salted fish 1 with isolate code P1A1K, salted fish sample 2 P2B2PS, and salted fish sample 3 P3C6PS and P3C6P. It could grow under salt-stress conditions, even though some isolates could not grow well under salt stress. After incubation for 24 hours, two isolates were estimated to have higher activity than the other isolates. These two isolates changed the medium from clear to turbid on the media, indicating bacterial growth on the medium. In addition to P2B2PS isolates, the same thing was found in P3C6P isolates. This isolate also changed the media with a 15% salt concentration to become turbid at 36 hours of incubation. It also changed the turbidity media with a 20% salt concentration. After being observed, the longer the incubation time, the media became more turbid. From the results of the enzyme activity test, it was found that the highest activity occurred at 24 hours of incubation (Fig. 4).

This test found that based on the incubation period of 24 hours, isolates with the code P3C6P had the highest protease activity, namely 43.23 ± 7. 11 U.mL^{-1} at a salt concentration of 15% and 42.83 ± 3.04 U.mL^{-1} at a salt concentration of 20% higher than P2B2PS. It also shows the turbidity of the media used, namely 38.05 ± 4.05 U.mL^{-1} at 15% salt concentration and 38.15 ± 1.47 U.mL^{-1} at 20% salt concentration.

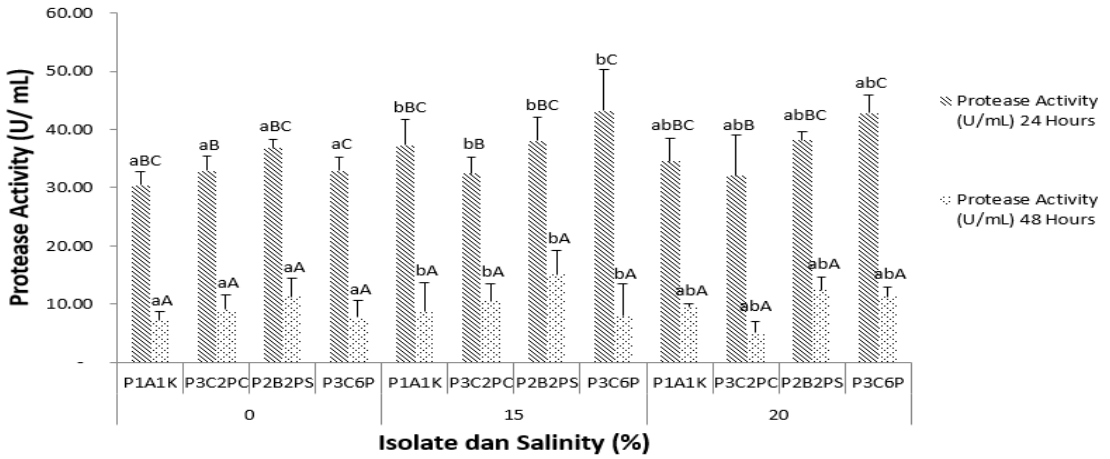

Figure 4. The activity of protease enzymes aged 24 and 48 hours at 0, 15, and 20% salinity. Data show the mean and standard deviation of triplications. Capital letter notation indicates the effect of salinity on protease enzyme activity ($p < 0.05$), and lowercase notation indicates the interaction of isolates and incubation times on protease enzyme activity ($p < 0.05$).

A pathogenicity test was carried out to select the isolates to be identified. This result is slightly lower than the *Bacillus cereus* VITSN 04 strain, with its activity reaching 167.07 ± 0.38 $U.mL^{-1}$ from protein-rich soil samples [25] and higher when compared to *Bacillus cereus* originating from hot springs with an activity of 0.691 ± 0.67 $U.mL^{-1}$ [26].

The influence of salinity still limits this research. The stability of the enzyme produced still needs to be studied further regarding the stability of the protease enzyme produced at various temperatures and pH. The stability of the enzyme produced still needs to be studied further regarding the stability of the protease enzyme produced at various temperatures and pH. Considering that each enzyme requires an appropriate pH and temperature because the enzyme will not be able to work optimally and can damage the enzyme's structure if the pH and temperature do not match the needs of the enzyme. Several studies have reported that proteases will generally be optimal at pH 7 to 8.5, and temperatures from 25°C to 55°C will experience denaturation at pH and temperature above these limits [27].

Pathogenicity Test Results

The results of the pathogenicity test on four superior isolates using blood media to obtain the results of isolates with the code P1A1K, P2B2PS, and P3C6PS. The results showed a Beta hemolysis reaction where these bacteria were able to lyse red blood cells, marked by the presence of a clear zone around the media. So, it is indicated as a pathogen in living things.

The isolate with the code P3C6P showed a Gamma hemolysis reaction where it was unable to lyse blood cells on blood media, so it was possible that this isolate did not have pathogenic properties against living things. Blood agar media can be used as a medium to test the ability to lyse blood [28]. Bacteria that can lyse blood will produce a clear zone around the colony, indicating beta hemolysis, or a green color indicating alpha hemolysis [29]. Beta hemolytic bacteria are the general rule in bacterial pathogen billing and highly predict pathogenicity. Non-pathogenic bacteria are very rare that have the ability to betamolyze [30].

Blood agar can also be used in testing rhamnolipid biosurfactant production, characterized by forming a clear zone around the colony. The size of the clear zone produced depends on the concentration of the biosurfactant produced by the bacteria [31]. Rhamnolipid is one of the virulence factors in *Pseudomonas aeruginosa*, which plays a role in pathogenesis in humans [32].

Identification Results Based on 16S rDNA Sequences

Based on the identification of 16S rDNA sequences aligned with reference stains from the database Genbank and the results of the analysis using MEGA 11 on isolate P3C6P showed that the isolate was an isolate of *Bacillus cereus* WHX1 with a similarity index of up to 100%, which can be seen in the construction of the phylogeny tree (Fig. 5).

Bacteria from the genus *Bacillus* are one of several genera often used as producers of enzymes such as chitinase, amylase, and proteases. This genus is a gram-positive, rod-shaped bacterium where this *Bacillus species* can be obligate aerobes or facultative anaerobes, making it easier to breed.

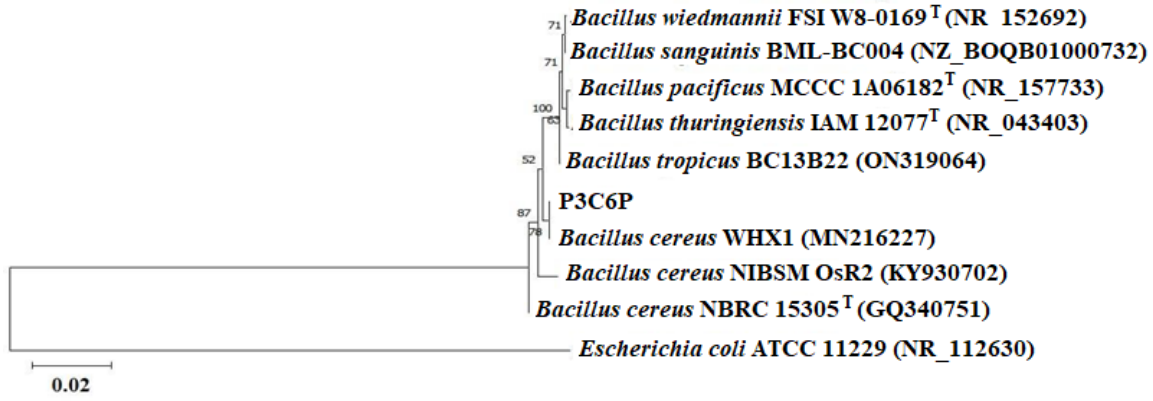

Figure 5. Bacterial P3C6P phylogeny tree for the potential to produce halophilic protease enzymes based on 16S rDNA sequence data constructed using the Neighbor-Joining algorithm and bootstrap 1000. The number on each branch indicates the branch bootstrap value.

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CONCLUSION

This study found 25 isolates with proteolytic abilities, and P3C6P isolate (*Bacillus cereus WHX1*) isolated from a salted red snapper (*Lutjanus campechanus*) has the potential to be a superior isolate because the enzyme produced is relatively stable and has non-toxic-properties. Pathogens to living things. So that this isolate can be developed and further researched for use in the industrial sector.

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