

Isolation and Identification of Protease Enzyme Producing Bacteria from Fermentation of Gonad Sea Urchin (*Echinothrix calamaris*)

Siani La Jamaludin^{1*}, Johanis Fritzgal Rehena², Cecilia Anna Seumahu³ and Dominggus Rumahlatu²

¹Postgraduate Biology Education Study Program, Pattimura University
Jl. Dr. Tamaela, Ambon 97116 Indonesia

²Biology Education Study Program, Faculty of Teacher Training and Education Science, Pattimura University
Jl. Ir. M. Putuhena, Ambon 97233 Indonesia

³Biology Department, Faculty Mathematics and Science, Pattimura University
Jl. Ir. M. Putuhena, Ambon 97233 Indonesia
Email: siani.laj@gmail.com

Abstract

Bekasang of gonad sea urchin is one of the traditional fermentation products which generally involves microorganisms spontaneously. Fermented paste products have a long shelf life and are processed quite easily using protease enzymes. Good exploration of producing protease from bakasam is needed to obtain the protease enzyme-producing microorganism with different characters. The method used in this research is screening with clear zone, measuring the activity of crude extract of protease enzyme characterization of bacteria through gram staining. Identification of PCR microorganisms with 63F primer and 1387r primer and sequencing gen target. The results showed that there were eight isolates of protease enzyme-producing bacteria (G1, G2, G3, G4, G5, G6, G7, and G8) indicated by clear zones around single-colonic bacterial streaks. Only five bacterial isolates (G1, G4, G6, G7, and G8) were tested for the enzyme activity. These isolates have characteristics of positive gram bacteria. The interpretation of the results of molecular analysis using PCR obtained that the target gene shown by the band at a distance of 1300 bp. The results were then sequenced and BLASTN sequences of 16S rRNA gene from five bacterial isolates, namely: G1 was *Staphylococcus piscifermentans* strain CIP103958 with 99% similarity; Isolate G4 was *Staphylococcus saprophyticus* strain ATCC 15305 with 99% similarity; Isolate G6 was *Staphylococcus condimentii* F-2 strain with 99% similarity; Isolate G7 was *Bacillus amyloliquefaciens* subsp. *plantarum* strain FZB42 with 99% similarity; And G8 isolates was *Lactobacillus plantarum* strain JCM 1149 with 99% similarity.

Keywords: bekasang, protease, gonad sea urchins

Introduction

Sea urchins (phylum Echinodermata) is an international mainstay commodity as well as foodstuffs which have important economic value. There are about 84 species of sea urchins found in Indonesian waters, and five of them, namely, *Diadema setosum*, *Arbacia punctulata*, *Salmacis bicolor*, *Echinometra mathaei*, *Tripneustes gratilla* and *Echinothrix calamaris* can be consumed and have economic value. The habitat of sea urchins is in seagrass beds to corals. *Diadema setosum* also becomes an indicator of the health of coral reefs. One cause of coral bleaching is the population of *setosum Diadema* that eats algae which is symbiotic zooxanthellae (Qiu et al., 2014)

The gonads of Sea urchin have long been used as raw food as well as cooked food, daily known by the name of sea urchin eggs. Analysis of

gonad protein of several species of sea urchins show that the gonad of sea urchins contains around 28 species /about 32.1% of the total amino acids, which are dominated by phenylalanine, lysine and valine (Amarowicz et al., 2012). In addition, the sea urchin gonads also contains vitamin A, B complex, and minerals that are essential for growth (Kalogeropoulos et al., 2012). Complete nutritional composition and supported by delicious taste makes the sea urchin gonads as the favoured dishes of communities in many countries.

The maturity time of urchin gonad is influenced by hydrodynamic conditions of location. Maturation period of urchins between April and June for *Paracentrotus lividus* population in Tunisia, although the gonad index also seems to vary in different locations (Navarro and Guirado, 2011). Two aquatic ecosystem factors that may affect the quality of fatty acids of gonadal tissue are water

temperature and food availability. A decrease of temperature will increase a large amount of unsaturated fatty acids to maintain optimal functional membrane fluidity (Pita *et al.*, 2009).

The demand of urchin gonads (*roe*) continues to increase, creating opportunities for aquaculture of urchins, especially in countries that still rely on the export of catches of sea urchins from the wild (Purbiantoro *et al.*, 2014). Urchin gonad is marketed as fresh products, frozen products, salted products, dried products, fermented products and canned products in overseas. Today about 50.000 tons of sea urchins are landed worldwide per year, and the Japanese state dominates both domestic landing and imports of sea urchin gonad. Japan is the largest country producing and consuming sea urchin gonads or called *neriuni* 3-5 ton.day⁻¹, as the component of *sushi*. Gonad of sea urchin and its processed products have not been popular in the wider community, and it is a commodity that is consumed only by certain circles. Sea urchins are believed to be used as a rheumatic medicine and a tonic for men vitality (Pringgenies *et al.*, 2013; Catts and Zurr, 2013). Most people of Mollucas know sea urchins as fresh products, and it is mostly distributed in the area of coral reefs in Indonesia. One of the species is *Echinothrix calamaris* along the coastal area of Ambon (Br Silaban and Srimariana, 2014).

The diversification process of these products through fermentation process needs to be done to increase public consumption and nutritional value of the products. The production of urchin gonad fermentation paste uses salt as a preservative, in addition to monosodium glutamate, sugar and sake into the material (Br Silaban and Srimariana 2014). The addition of salt in the process of paste fermentation is expected to stimulate microbial growth. The combination of salt and acid can give a better effect in protecting the fermentation product from the effects of spoilage microorganisms, compared to using salt solely (Lee *et al.*, 2010).

Protease enzyme producing bacteria play a role in producing good fermentation products. Protease can be isolated from various organisms such as bacteria, fungi, plants and animals. Protease from bacteria is the most number compared to other sources, namely the protease from plants and from animals. Protease can be isolated from the extracellular and intracellular parts (Fatoni *et al.*, 2008). Development of fermentation, genetic engineering and enzyme application technology is because enzymes are biocatalysts that can reduce environmental impact and replace chemical compounds in industry. Enzymes work very efficiently with high accuracy (precision), versatile

and economical (Naiola and Widhyastuti, 2002). Protease is an important enzyme (65% of the world's total enzyme market) and widely used (Huang, 2006) in industrial applications, such as detergents, pharma-ceuticals, leather products, meat cooking, protein hydrolysates, food products, and processing industrial waste (Nascimento and Martins, 2006). The selection of microbes as the source of enzyme rather than plants or animals is because the microbial cells are relatively easily grown, the growth rate is relatively fast, the cell production scale is more easily upgraded. When greater production is desired, the production costs are relatively low, the conditions during the production does not depend on the change of seasons, and the time required in the production process is shorter (Vishwanatha *et al.*, 2010). Therefore, it is necessary to conduct a research related to the isolation and identification of protease enzyme producing bacteria from the results of the fermentation of urchin gonads, especially *Echinothrix calamaris*.

Materials and Methods

Isolation, screening, and enzyme activity test

A total of 10 ml (fermentation samples of *Echinothrix calamaris* urchin gonads) in 100 ml of enriched medium with the medium composition: 0.5% yeast extract, peptone 1%, and NaCl 1%. After that, the sample was incubated in the shaking incubator at 50°C and speed 200 rpm for 48 hours. A total of 0.2 ml of culture was disseminated in the selection medium (liquid medium agar (LA) x skim milk 1%, then incubated at 37°C for 1 day. The growing isolates were scraped into quadrants until pure isolate was obtained.

Bacteria that produce clear zones around the colony signify a protease producer because it can hydrolyze its substrate around the colony. Furthermore, the isolates which are pure and have a clear zone were totaled on LA medium for stock +1% skim milk and LA modification to know proteolytic index (IP) (Fitriani *et al.*, 2013). The measurement of Protease Activity was from the bacterial culture incubated for 2 days. Crude extract protease enzyme activity was measured by Bergmeyer method (Baehaki *et al.*, 2011) using 2% Hammerstein casein substrate (b/v). The test procedure of the crude extract activity of protease is: reacting 0.2 ml of enzyme with 1 ml of Hammerstein casein substrate and 1 ml buffer. The reaction mixture was incubated at 37°C for 10 min, then 0.2 M TCA was added. After that, the solution is incubated at 37°C for 10 min, followed by centrifugation at a speed of 9000 rpm 10 min. From the mixture of the the resulting centrifugation, supernatant was taken and added to

a test tube containing Na_2CO_3 0.4 M then added Ciocalteau Folin reagent (1:2) and incubated at 37°C for 20 min, and the absorbance was measured at a wavelength of 578 nm. To measure the activity of the protease enzyme, Walter method was used (Fitriani *et al.*, 2013), which had been modified. The activity of protease enzyme was calculated by the formula:

$$UA = \frac{Asp - Abl}{Ast - Abl} \times P \times \frac{1}{T}$$

Note: UA= Unit of enzyme activity; Asp= Absorbance sample value; Ast= Absorbance standard value; Abl= Absorbance blanko value; P= Dilution factor; T= Incubation time

Enzyme characterization

Characterization of bacterial isolate aims to determine the nature of morphology. The morphology observed include colony morphology and cell morphology using Gram staining. The shape of the bacterial cell of the isolated bacterial was observed microscopically, and then Gram staining was done. Aseptically a thin layer was made from bacterial suspension on the glass object and fixation was carried on the open air. This thin layer was dyed violet crystal dye and left for 1 min, then rinsed with tap water by holding the glass object in a tilted position. The remaining water left on the glass object was removed and spilled with lugol and left for 1 min. After it was washed again using water, the color was removed by using 96% alcohol and left for 10-20 seconds. After washed briefly using water, it was stained using safranin and left for 10-20 seconds. Glass object was then rinsed using water and dried with absorbent paper (*tissue*). These preparations were observed under a microscope using an objective lens that had been immersed with immersion oil. With microscopic observation, the shape of the bacterial cell and its Gram reaction can be determined. Positive Gram bacteria will be shown in purple, while negative Gram bacteria will be marked in red or pink.

Identification of bacteria using 16S rRNA

Five selected isolates which were capable of producing clear zones were identified morphologically, and biochemically. Morphological observation include the shape, edges and colors of the colony (Hadioetomo, 1985). Molecular identification was performed using PCR and 16S rRNA gene sequencing (Marchesi, *et al.*, 1998). Genomic DNA was isolated from the culture by a modification of the method by Ausubel *et al.*, (1989). Cells were precipitated by centrifugation. After that the cell pellets were washed and resuspended with Tris-EDTA, and then added to 100 ml of the enzyme

lysozyme and proteinase K ($18\text{mg}\cdot\text{ml}^{-1}$; Sigma), the mixture was incubated further for 1 h at 37°C , after that the cells were lissed with CTBA and SDS 10%, the further centrifugation was performed and the supernatant was taken. The supernatant was then washed using phenolchloroform, (25:24) the top phase was taken and added with 7.5 M ammonium acetate, and the mixture was centrifuged at $11.220 \times g$ for 20min at 4°C . Removed the supernatant, washed the pellet using alcohol. The supernatant was dried and the pellet was dissolved with TE pH 8 and the isolated genome was used as a template. The amplification process by PCR used a beginning universal primer 63f and primer 1387r (Marchesi *et al.*, 1998).

The primary pair used was 63f 5'-CAG GCC TAA CAC ATG CAA GTC-3' and primer 1387r (5'GGG CGG WGT GTA CAA GGC-3'). The mixture of PCR 25 μL contained 20 pmol from each appropriate primer, 200 μM deoksinukleotida (dNTP), 0,5U Taq Polymerase, PCR buffer (Stratagene Ltd.). The concentration of template used was 200-300 ng. PCR was performed for 30 cycles consisting of denaturation stage 95°C , 1 min annealing /adhesion primer 55°C 1 min and elongation 72°C for 1.5 min followed by a final step of 72°C for 5 min. The PCR product was reconstituted in agarose gel electrophoresis 1%, with V: 60v, for 45 min with TAE buffer 1 x and visualized with the use of ETBR above UV. PCR products were then sequenced using DNA sequencer. Data from subsequent sequencing were compared with data in GenBank using BlastN program (<http://www.ncbi.nlm.nih.gov/>). Sequence 16S RRNAs from relatively closely related species with fermented gonad fermentation bacteria were extracted and analyzed for their kinship level through alignment steps using CLUSTALW. Furthermore, the reconstruction of the phylogenetic tree *neighbor-joining plot* method used MEGA6 program.

Data analysis

The obtained research data were analyzed descriptively to describe the morphological and physiological observations protease enzyme-producing bacteria and interpretation of the results of molecular analysis using the 16s rRNA early primary universal 63F and 1387r. Primer.

Results and Discussion

Type of isolate of protease enzyme producing bacteria

From the results of isolation and screening of the results of the fermented sea urchin gonads with

the addition of 20% salt in the liquid medium agar (LA) x 1% skim milk were obtained 8 (eight) isolates of protease enzyme producing bacteria. The clear zone formed on the medium showed that the isolates were able to produce protease enzymes, namely, isolate G1, G2, G3, G4, G5, G6, G7, and G8 (Figure 1). Nursyirwani *et al.* (2012) stated that the isolation was carried out by using a medium containing casein, which is a good substrate for isolating protease enzyme producing bacteria and induce the synthesis of alkaline protease enzyme.

Protease is a proteolytic enzyme that catalyzes the termination of peptide bonds in proteins. Proteases are physiologically needed for living organism in plants, animals and microorganisms. Protease is an enzyme that catalyzes the breakdown of peptide bonds in peptides, polypeptides and proteins by using hydrolysis reaction into simpler molecules as short-chain peptides and amino acids (Naiola and Widhyastuti, 2002).

In this research, as much as 20% of salt was added into the gonad sample of fermented sea urchins (*Echinothrix calamaris*). According to Pham *et al.* (2005) and Hidayat *et al.* (2006), the addition of salt 20% - 30% allow all proteins in the gonads of sea urchins to break down into dissolved nitrogen. Thus, it will be obtained 56% of dissolved nitrogen after fermentation. Furthermore, Hidayat *et al.* (2006), explain that with the salting treatment, water withdrawal will occur, in that the degraded protein is degraded in the network will be separated and dissolved into the liquid salt. In addition, according to Abdel-Rahman *et al.* (2011), in the

fermentation process using high salt levels, it is estimated that the kind of BAL will be able to grow and develop. The result of this fermentation also depends on the type of substrate and its surrounding conditions that affect the growth and metabolism of microbes. The changes during fermentation, microbes can convert carbohydrates and their derivatives primarily to alcohol, acid and CO₂.

This research was conducted by adding 5 ml starfruit water into sea urchin gonad samples (*Echinothrix calamaris*) as much as 100 grams. The use of starfruit water serves as a preservative in fermentation. These results are consistent with research by Rahayu (2013) showing that the starfruits contain oxalate, phenol, flavonoid and pectin compounds. Furthermore, according to (Rahayu, 2013), flavonoids are the largest of phenolic compound which has the properties of effectively inhibiting the growth of viruses, bacteria, and fungi or flavonoid compounds which are active as antimicrobials.

Isolate of the pure cultures obtained, only five isolates (G1, G4, G6, G7, and G8) were tested for enzyme activity. at the age of two-day culture and incubated for 20 min at temperature of 37°C. The test results of protease enzyme activity of 5 isolates can be seen in Table 1. The test of protease activity of crude extract enzyme in bacterial supernatant shows that isolat G8 had the highest enzyme activity of 0.08587 IU.ml⁻¹. This means that at that time, every 1 ml of protease enzyme is capable of breaking down casein 0.08587 μmol of tyrosine per minutes.

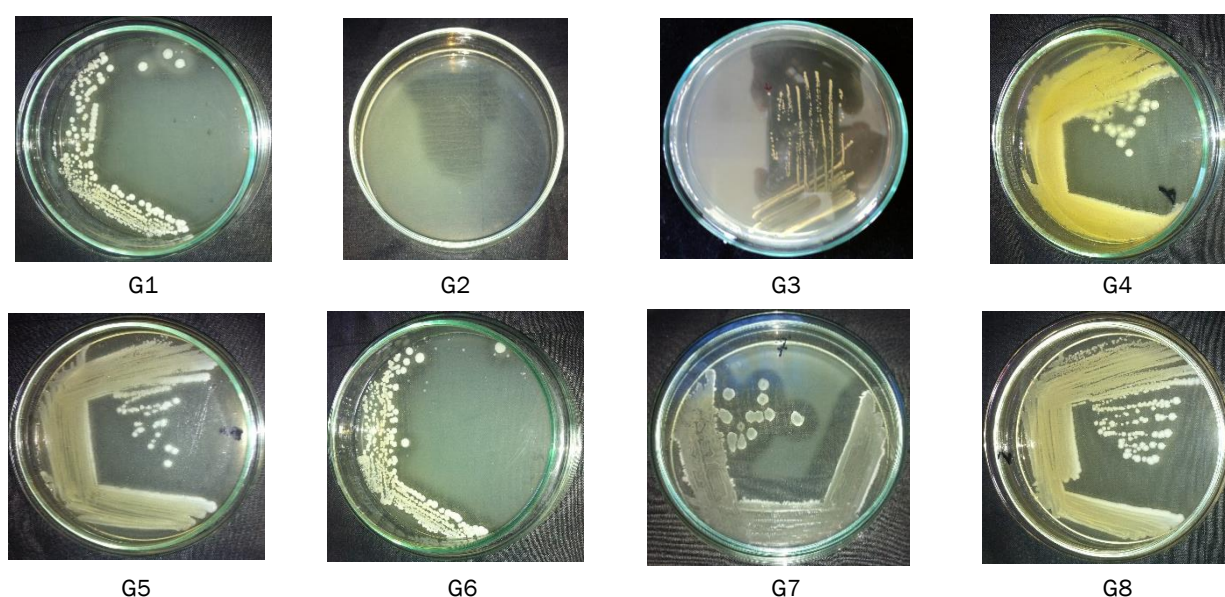


Figure 1. Clear zone on isolation and screening of isolates of protease enzyme-producing bacteria

According to Fadda *et al.* (2010), protease activity occurs because of an increase in the kinetic energy of the enzyme molecule and the increased movement of substrate molecules that cause collisions between enzyme molecules and the increasing substrate. The activity of the protease enzyme will decrease at a temperature above the optimum temperature. This is because high temperatures break up secondary bonds such as hydrogen bonds that keep the enzyme in its natural structure, so that the secondary, tertiary structure of the enzyme is partially damaged followed by a decrease in activity. Temperature is one of the factors that influence enzyme activity. The higher the temperature, the more increased the enzyme activity until the optimum temperature occurs. The activity of protease enzyme reaches optimum activity at 50°C. This is in line with what is proposed by Baehaki *et al.* (2011) that at a lower temperature than the optimum temperature, the enzyme activity is also low, due to the low activation energies available. The energy is needed to create an active complex level condition, both from enzyme molecules and from substrate molecules. Increased temperatures also have an effect on the substrate conformation change, so that the substrate active side experiences barriers to enter the enzyme's active side, and causes the decrease in enzyme activity. Secondly, the increase in thermal energy of molecules that make up the structure of the enzyme protein itself causes the breakdown of non-covalent interactions (hydrogen bonds, van der Waals bonds, hydrophobic bonds and electrostatic interactions) that keep the enzyme 3D structures together, so that enzymes are denatured. Denaturation causes the enzyme-folding structure to open up on its surface, so that the active side of the enzyme changes and results in a decrease in enzyme activity.

Characterization of protease enzyme producing bacteria

The appearance of colony morphology is generally round (circular) and the gram color is purple. Isolate G1 in colony morphology is large, cream pigmentation, rounded edges (round), slightly convex elevation (convex). Isolates G4, morphology of medium colonies, yellow pigmentation, spherical (Circular), rounded edges (Entire), slightly convex

elevation (convex), Isolate G6, medium colony morphology, Cream pigmentation, rounded edges (Entire), slightly convex elevation (convex). Isolate G7, large colony morphology, cream pigmentation, rounded (Circular), slightly dull edge (Undulate), and thin flat elevation (Flat) and Isolate G8, small colony morphology, White pigmentation, optical appearance features transparent, circular, circular edge (entire), flat elevation (Flat). The morphological observation of cells through gram staining, all bacterial isolates showed gram positive and the forms of bacterial cells were in the form of coccus and bacil (Table 2.).

The five bacterial isolates are then characterized by the gram staining method. The research results prove that Aal isolates have morphological characteristics of the cell colony, that is, positive gram bacterial cells shown in purple. The gram staining method is based on differences in the composition of the cell wall they have. Gram-positive cells have walls with thicker peptidoglycan layers than gram-negative cells. Gram-negative bacteria contain lipids and fats in a higher percentage that that on gram-positive bacteria.

Abel *et al.* (2014) explains that the cell wall of Gram-negative bacteria consists of 5-20% peptidoglycan, the rest is polysaccharide, whereas gram-positive bacteria contains of 90% peptidoglycan, the rest is teikoat acid. The cells of gram positive bacteria appear purple, because it can form complex ties with the first color, purple complex of iodine crystals. In gram-negative bacteria, 95% alcohol can increase the porosity of the cell wall by dissolving the lipid in the outer membrane, so that the purple crystals of iodine will be released and the cell becomes colorless. Furthermore, the cell will be red because it is colored by a the comparison color, which is safranin.

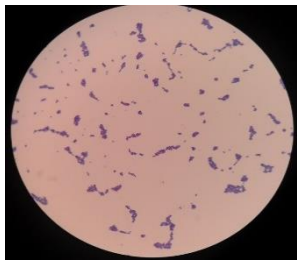
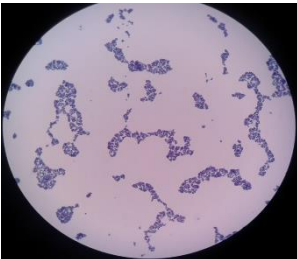
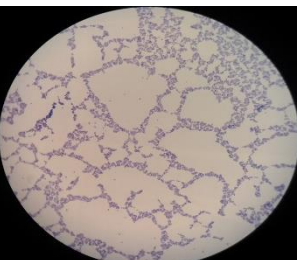
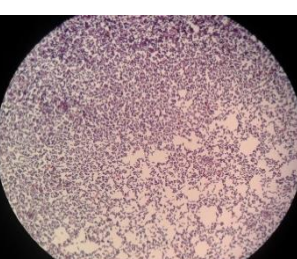
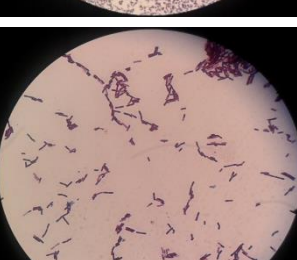
Identification of protease enzymes producing bacteria amplification of 16S rRNA

Amplification of the 16S rRNA gene of five bacteria isolates used PCR with primers 63F and 1387r. The use of primer is a universal primer capable of amplifying the 16S rRNA gene in protease enzyme-producing bacteria which can be seen in the following Figure 2.

Table 1. Culture protease activity 48 hours of five isolates at 37°C

| Sample code | Absorbance U1 (average) | Absorption U2 (average) | Protease Enzyme Activity (IU.ml ⁻¹) |
|-------------|-------------------------|-------------------------|---|
| G1 | 0.729±0.109 | 0.369±0.017 | 0.00174 |
| G4 | 0.772±0.151 | 0.358±0.008 | 0.00079 |
| G6 | 0.665±0.025 | 0.372±0.026 | 0.00493 |
| G7 | 0.813±0.060 | 0.400±0.009 | 0.00517 |
| G8 | 0.674±0.029 | 0.819±0.020 | 0.08587 |

Table 2. Results of Gram staining of protease enzyme bacteria which have the highest activity (magnification 1000 x)

| Isolates | Colony morphology | Cell Morphology |
|---|--|-----------------------------|
| G1  | Large, cream pigmentation, rounded (Circular), rounded edges (Entire), slightly convex elevation (convex) and gram purple | Gram positive/ coccus |
| G4  | Medium, Yellow pigmentation, round shape (Circular), rounded edges (Entire), slightly convex elevation (convex) and gram purple | Gram positive / Coccus |
| G6  | Medium, Cream pigmentation, circular (Circular), rounded edges (Entire), slightly convex elevation (convex), Gram purple | Gram positive / Coccus |
| G7  | Large, cream pigmentation, rounded (Circular), circular edge slightly dull (Undulate), thin flat elevation (Flat) gram purple | Gram Positive / Short Trunk |
| G8  | Small, White pigmentation, features of optical appearance transparent, rounded (Circular), round edges (Entire), thin flat elevation (Flat), gram purple | Gram Positive / Long Trunk |

The 5 sample isolates were amplified by PCR method with primer 63F and primer 1387r (Figure 2.), after electroforensised on a 1% agarose was successfully obtained a *band* (thin ribbon) which is a DNA fragment size of about 1300 bp which is 16S rRNA genes. These results were subsequently sequenced to determine the base sequence contained in order to determine the type of bacteria

in fermented sea urchin gonads (*Echinothrix calamaris*).

Sequencing

The results of gene 16S rRNA sequence of the species which are relatively closed with bacteria fermented sea urchin gonads (*Echinothrix*

calamaris) and analyzed the level of the kinship through the stages of alignment (*alignment*) using CLUSTALW. Fermentation of urchin gonads (*Echinothrix calamaris*) of the five selected Isolates based on 16S-rRNA sequences that are capable of producing five species of bacteria with each similitas 99%, namely: (G1: sample code) *Staphylococcus* strains *piscifermentans* CIP103958 , (G4) *Staphylococcus saprophyticus* strain ATCC 15 305, (G6) *condimenti Staphylococcus* strain F-2 (G7) *Bacillus amyloliquefaciens* subsp. *plantarum* strains FZB42 and (G8) *Lactobacillus plantarum* strain JCM 1149. The results of BlastN program of 16S rRNA gene sequences of the protease enzyme producing bacteria from fermentation gonads of sea urchins (*Echinothrix calamaris*) can be seen in Table 3.

Phylogenetic analysis

Reconstruction of phylogenetic tree is structured data that describes the data that can be interpreted as an associated hierarchy associated and represented by each branch in the form of a phylogenetic tree of species of bacteria, Figure 3 shows and describes the relationship between the five isolates based on phylogenetic tree which is only distinguished on the substitution of nucleotides per site (site) up to a distance of 0.01. This means that it distinguishes the isolates from the nucleotide compositions at each site from each isolate G1, G4, G6, G7, and G8.

Figure 3 shows that it comes from a class of bacilli and two orders, namely ordo Bacillales and ordo Lactobacillales. Ordo Bacillales consists of 3 families, namely Staphylococcaceae, Bacillaceae, and Lactobacillaceae. Staphylococcaceae family consists of three species, namely *Staphylococcus piscifermentans* strains CIP103958, *Condimenti Staphylococcus* strain F-2, and I *Staphylococcus saprophyticus_strain* ATCC 15 305, Bacillaceae family consists of one species namely *Bacillus amyloliquefaciens* subsp. *plantarum* strain FZB42, and Lactobacillaceae family consists of one species, namely *Lactobacillus plantarum* strain JCM 1149.

Interpretation of the results of molecular analysis of the five isolates of protease enzyme producing bacteria from the fermentation of sea urchin gonads (*Echinothrix calamaris*) based on 16S- r RNA sequences, and when compared with the 16S-rRNA gene sequences in *Genebank* database, the strain of the bacteria was found to have the degree of similarity of 99%, namely: (1) the sample code G1 with 99% similarity is *Staphylococcus piscifermentans* strain CIP103958 ; (2) The sample code G4 is *Staphylococcus saprophyticus* strain ATCC 15305 with 99% similarity; (3) The sample code G6 is *condimenti Staphylococcus* strain F-2 with 99% similarity; (4) The sample code G7 is *Bacillus amyloliquefaciens* subsp. *plantarum* strains FZB42 with 99% similarity; and (5) sample code G8 is *Lactobacillus plantarum* strain JCM 1149 with

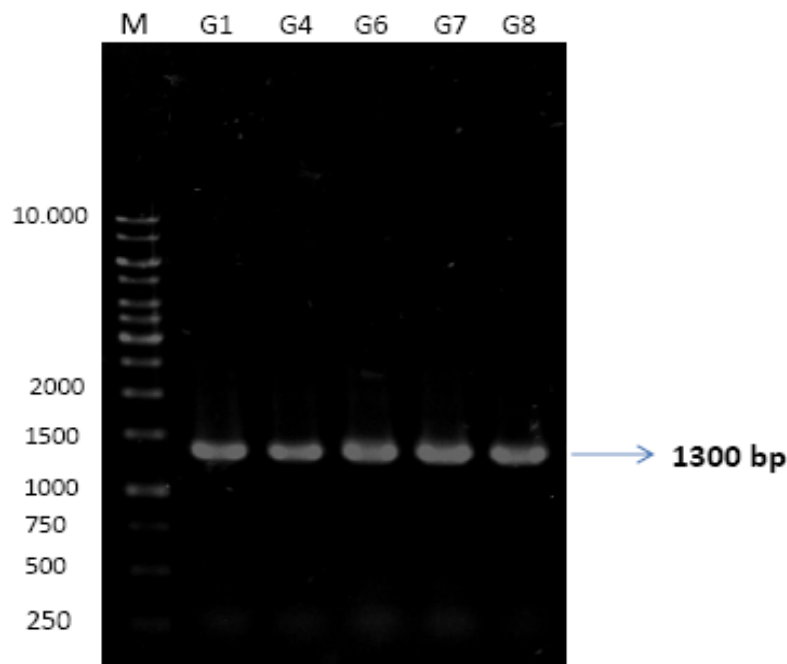


Figure 2. PCR amplification of the 16S rRNA gene with Primer 63f and primer 1387r; M = 1 marker 1 Kb ladder; G1, G4, G6, G7 and G8 = PCR product of bacteria samples

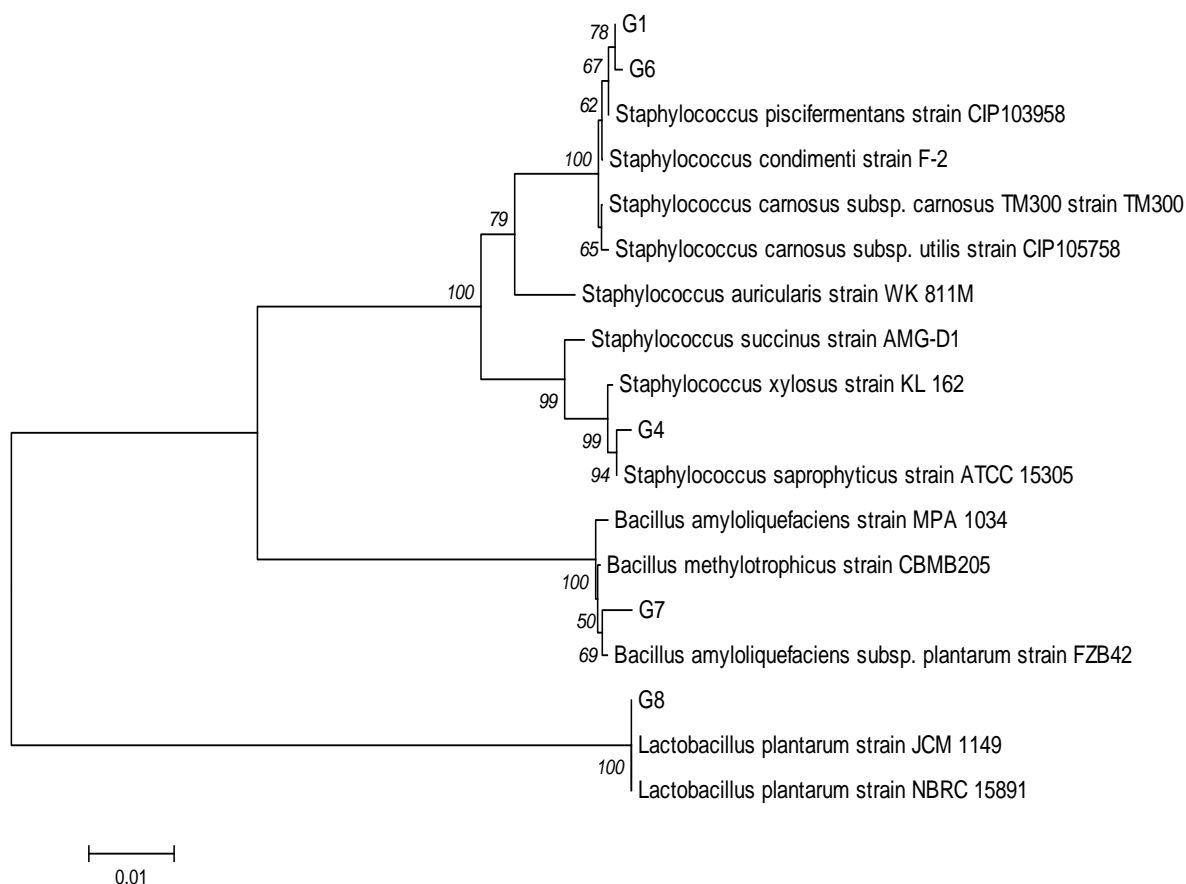


Figure 3. Phylogenetic trees proximity isolates, G1, G4, G6, G7 and G8 against other bacteria within one *clade* or against another *clade* (outer group). This construction was done based on *Neighbor Joining Tree* method with *bootstap* value of 1000 x replay.

99% similarity. These results indicate that of the five isolates were found five strains of bacteria that are all gram-positive bacteria and protease enzyme producer. Five bacteria, the highest enzyme activity of the five isolates was from the isolate G8 namely the *Lactobacillus plantarum* strain JCM 1149, which is one of LAB bacteria (Lactic Acid Bacteria).

This is in line with Sheeladevi and Ramanathan (2011), that *Lactobacillus* is often referred to as lactic acid bacteria group. This is due to its ability to convert lactose and other sugars into lactic acid. According to Sumardiono (2017), *Lactobacillus Plantarum* as one of LAB homofermentatif which has optimal temperatures lower than 37°C. In general the morphology of *Lactobacillus Plantarum* is rod-shaped cells (0.5-1.5 s/d from 1.0 to 10 m) and not moving (non-motile). These bacteria possess catalase-negative, aerobic or facultative anaerobe nature, can dissolve the gelatin, digest protein quickly, does not reduce nitrate, acid tolerant, and capable of producing lactic acid. Colony size ranges from 2-3 mm, white opaque, convex, and the final result of metabolism is lactic acid.

Furthermore Ridwan *et al.* (2005) explain that *Lactobacillus plantarum* acts as agents to preserve food. The ability of these bacteria to alter various compounds which are present in the media into other simpler compounds gives typical flavor and aroma of food and has the ability to enhance the taste and value of fermented food products. Ernawati (2010) describes that *Lactobacillus. plantarum* is included in gram-positive rod-shaped bacteria in pairs or short chains. *Lactobacillus plantarum* is a member of *Lactobacillus* genus, which is often found in fermented food products. *L. plantarum* is one of the largest species among lactic acid bacteria, and it is very beneficial for life, especially in the fermentation of food.

In line with the above opinion, Ernawati, (2010) explains that *L. plantarum* is a Gram-positive bacterium aerotolerant bacteria that grow at 35°C but not at 45°C. This species is still a family with *lactobacilli*. This is unique because it can breathe oxygen but does not have the respiration reaction sequence or cytochrome, that is, oxygen reactions in which the final outcome of the reaction is hydrogen peroxide. According to Afriani (2009), *Lactobacillus*

plantarum has the ability to be able to live in low acid conditions. It is also able to produce antimicrobial bacteriocins that play a role in suppressing the growth of microbial pathogens. Meanwhile, according to Wagih *et al.* (2012), *Lactobacillus plantarum* is capable of producing plantaricin bacteriocin that can act as an antibacterial and antifungal.

The activity of the highest protease enzyme producing bacteria from the results of fermentation of sea urchin gonads (*Echinothrix calamaris*) is *Bacillus amyloliquefaciens* subsp with a value of 0.00517 IU/ml. This research is in line with the research conducted by Novita *et al.* (2006) on the partial characterization of protease enzyme crude extract of *Bacillus amyloliquefaciens* NRRLB-14 396. The research results show that the protease was isolated from the bacterium *Bacillus amyloliquefaciens* NRRL B-14 396 to an average value of protease enzyme activity of 0.431 units.ml⁻¹ at 40°C and pH 7.0. Furthermore, according to Choi and Kim (2013), the production of serine proteases uses *Bacillus amyloliquefaciens* DJ-4 by adding NaCl and heat. In addition, Novita *et al.* (2006) also conducted research using the bacterium *Bacillus amyloliquefaciens* S-94 using PMSF inhibitor, it was known that the produced protease was included as serine protease. Asokan and Jayanthi (2010) also explain that the bacterium *Bacillus amyloliquefaciens* is widely used because it has good characteristics to produce alkaline protease. *Bacillus amyloliquefaciens* can produce several other enzymes that have commercial potential such as α -amylase, β -glucanase, *hemicellulase* and neutral protease.

The bacteria which are identified in this research as a producer of protease enzyme of the results of the fermentation sea urchins gonads (*Echinothrix calamaris*) (next is from the *Staphylococcus* genus comprising three strains. The strains of bacteria code G1 is the bacterium *Staphylococcus piscifermentans* strain CIP103958 ; code G4 is *Staphylococcus saprophyticus* strain ATCC 15 305; and code G6 is *Staphylococcus condimentii* strain F-2. the results of the research are consistent with research conducted by Fatoni *et al.* (2008), that one of the microorganisms from liquid waste of tofu which can produce protease extracellular is thought to be *Staphylococcus* sp. The highest specific activity of protease purification is obtained in 60% ammonium sulfate fraction (FS-60%) amounted to 68.22 U.mg⁻¹ protein with a purity level of 19.24 times of crude enzyme extract. The produced protease has an optimum pH of 8.0 and an optimum temperature of 40°C. According to Milicevic *et al.* (2014), the bacteria of the genus *Staphylococcus* spp can be isolated from the

fermentation of sauce with low frequency (low salt content) as follows: *Staphylococcus xylosus*, *S. equorum*, *S. saprophyticus*, *S. carnosus*, *S. equorum*, *S. succinuss*, *S.warneri*, *S. vitulinus*, *S. pasteuri*, *S. epidermidis*, *S. lentus*, *S. haemoliticus* *S. intermedius*, *S. saprophyticus*, *S. hominis*, *S.auricularis* .

According to Zell *et al.* (2008), strains of *Staphylococcus* such as *Staphylococcus condimentii*, *Staphylococcus piscifermentans*, *Staphylococcus equorum* and *Staphylococcus succinus* are usually obtained from the isolation of fermented foods which is traditionally done. Furthermore, Zell *et al.* (2008) explain that a large number of bacteria such as *S. piscifermentans*, *S. condimentii*, *S. equorum*, and *S. succinus* subsp. *casei*, are consistently found in large amounts in fermented foods, and also acts as a *starter* in the manufacture of bread and food.

Based on the characterization of gram staining which is done on protease enzyme producing bacteria in this research, the morphological features of the colonies are generally spherical (circular) and gram-purple on the genus *Staphylococcus*. The bacteria *Staphylococcus piscifermentans* strain CIP103958 on G1 isolates with large-sized colony morphology, cream pigmentation, rounded edges (entire), elevation slightly convex (convex). The bacteria *Staphylococcus saprophyticus* strain ATCC 15 305 in isolates G4, with a medium colony morphology, yellow pigmentation, globular (Circular), rounded edges, slightly convex elevation. The bacteria *Staphylococcus condimentii* strain F-2 shows the G6 isolates, moderate colony morphology, cream pigmentation, rounded edges (Entire), elevation slightly convex (convex). The observations of cell morphology through Gram staining, three species of protease enzyme producing bacteria showed gram-positive and form cocci-shaped bacteria cells. The culture age 2 days of incubation for 20 min at 37°C. This is in line with what is stated by Mathema *et al.*, (2009) that *Staphylococcus* is gram-positive cocci-shaped bacteria, with a diameter of about 0.5-1.5 μ m, occur in single, in pairs, tetrads, short chains, or like a cluster of grapes. These bacteria are nonmotile, not forming spores, and not having a capsule, although in some strains such as *S. Aureus* has a shape like a capsule which is unusual and capable of producing a biofilm on the prosthetic group,. Most of these bacteria are able to grow on a medium containing 10-15% NaCl. *Staphylococci* can grow at temperatures as low as 15° C and as high as of 45°C, especially in some bacteria, including *S. aureus*. At the macromolecular level, *staphylococci* has a genome size of 2-3 Mb, with a GC composition in general is 30-39%, some containing few and moderate plasmids. The structure of the cell walls is

resistant to the action of lysozyme, but it is susceptible to lysostaphin, where there are metalloendopeptidase glycylglycine which is specifically shaped cross between pentaglinin and residual peptidoglycan.

Furthermore, Zell *et al.* (2008), explains that in general the genus *Staphylococcus* is a Gram positive cocci shaped/spherical (rounded) bacteria, the formation is irregular like grapes. It grows up in a variety of media, fermentation of carbohydrates and produce white pigment to the old yellow (golden). *Staphylococcus* which is pathogens is capable of hemolyzing blood, coagulazing plasma, and producing various enzymes and toxins. Genus *heat-stable staphylococcal enterotoxin* can cause food poisoning (*food poisoning*). This genus quickly forms resistant strains of various antimicrobial and becomes difficult to treat.

The research results which were conducted subsequent alignment analysis were then performed phylogenetic tree reconstruction using the *neighbor-joining plot* on MEGA6 program. It can be seen the relationship between the bacteria found. It shows that the degree of the relationship between isolates G1, G6, that is with bacteria *Staphylococcus piscifermentans* strain CIP103958 and *Staphylococcus condimentii* strain F-2. Isolates G4 has the relationship with the bacteria *Staphylococcus saprophyticus* strain ATCC 15 305, G7 is with the bacterial isolates of *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 strains and isolates G8 is with the bacteria *Lactobacillus plantarum* strain JCM 1149. This is consistent with what has been stated by Crossley and Kent (2009) based on the phylogenetic tree.

Conclusions

The isolation of bacteria fermentation urchin gonads (*Echinothrix calamaris*) resulted in 8 (eight) bacteria that are protease producers. Protease enzyme activity of 5 isolates (G1, G4, G6, G7 and G8) ranged from 0.00079 to 0.08587 IU.ml⁻¹, in which isolates G8 has the highest enzyme activity. Observations of cell morphology by gram staining, all isolates showed gram-positive bacteria and the shape of the bacteria is cocci and bacil. Identification of the protease enzyme producing bacteria based on the results of molecular analysis using PCR technique with universal primer 63F and primer 1387r in which the results are further sequenced and performed BlastN sequences of 16S rRNA genes of five isolates namely: G1 is *Staphylococcus piscifermentans* strain CIP103958 with the similarity of 99%; G4 isolates namely *Staphylococcus saprophyticus* strain ATCC 15 305

with the similarity of 99%; Isolates G6 namely *Staphylococcus condimentii* strain F-2 with the similarity of 99%; Isolates G7 namely *Bacillus amyloliquefaciens* subsp. *plantarum* strains FZB42 with the similarity of 99%; and isolates the G8, namely *Lactobacillus plantarum* strain JCM 1149 with the similarity of 99%.

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