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Isolation and Molecular Identification of Protease Producing Bacterium Associated with the Brown Algae *Hydroclathrus* sp. from Hoga Island of Wakatobi District

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ABSTRACT
Advances in fermentation technology, genetic engineering, and enzyme application technology have increased the use of enzymes. Enzymes can be produced by utilizing a source of microorganisms such as bacteria. Proteolytic bacteria or protease enzyme-producing bacteria are found in foods or plants that contain protein such as the brown seaweed *Hydroclathrus* sp. This study aimed to obtain protease-producing bacterium associated with marine algae *Hydroclathrus* sp. from waters around Hoga Island of Wakatobi District and identify the organism based on its 16S rRNA gene sequence. Isolation of bacteria from algae samples was carried out with nutrient agar (NA) media, while proteolytic bacteria selection was carried out on skim milk agar (SMA) media. The bacterial isolates producing proteolytic-clear zone on SMA media were then identified targeting the 16S rRNA gene using the PCR (Polymerase Chain Reaction) method with 27F-1492R primers. Based on the isolation results, there were 3 unique colonies of bacteria that could be cultured from algae samples and coded HIHA-1 to HIHA-3 (HIHA stands for Hoga Island *Hydrolathrus* macroalgae). The selection process for protease-producing bacteria on SMA media resulted in 1 isolate of proteolytic bacteria, namely HIHA-1. Molecular identification by PCR on HIHA-1 isolate resulted in a single DNA band on the electrophoresis gel sized ~1500bp. The sequencing results showed a DNA sequence with the size of 1421bp sharing the highest similarity with the bacterium *Exiguobacterium aestuarii* strain TF-16 (homology level of 99,93%). In conclusion, the proteolytic bacterial isolate HIHA-1 associated with marine brown algae *Hydroclathrus* sp. was obtained and identified as *Exiguobacterium aestuarii* strain HIHA-1.

INTRODUCTION

Advances in fermentation technology, genetic engineering, and enzyme application technology have led to an increased use of enzymes **(Ramadhani *et al.*, 2015)**. Enzymes function as biocatalysts, namely accelerating the rate of a chemical reaction without being

involved in the reaction. The nature of the enzyme is specific to the substrate so that the chemical reaction that occurs will produce a product according to the specificity of the enzyme with the substrate **(Susanti & Fibriana, 2017)**.

Protease plays an important role in biocatalyst reactions causing protein breakdown. This enzyme will catalyse hydrolysis reactions, namely reactions involving the element water (H_2O) with specific bonds of the substrate. Protease enzymes are among enzymes with sales levels reaching up to 60% of the total sales in the world on an industrial scale **(Yuniati *et al*., 2015)** both in the food and non-food industries. Proteases can be produced by utilizing natural resources, including animals, plants, fungi, and microorganisms **(Susanti & Fibriana, 2017)**.

The use of microorganisms such as bacteria is more beneficial because of their fast growth and cheap substrate requirement, with results that can be increased through regulation of growth conditions **(Said & Likadja, 2012)**. One way that can be done to obtain protease enzymes is to isolate proteolytic bacteria **(Puspitasari, 2012)**. Proteolytic bacteria are usually found in foods or plants containing proteins **(Japri *et al*., 2019)**. *Hydroclathrus* sp. is a species of brown macroalgae from the *Phaeophyceae* group growing in the Indonesian marine waters with a protein content of 9.0% and is still rarely exploited commercially. Thus, this brown algae can be used as a source to isolate protease producing bacteria.

Proteases from bacteria can be obtained by isolating bacteria using skim milk agar (SMA) media which contains casein as a substrate for protease enzymes **(Putri, 2012)**. The presence of bacteria is indicated by the formation of a clear zone. Proteolytic bacteria are good if a clear zone is obtained with a diameter of 12mm or more **(Pamaya *et al*., 2018)**. Identification of proteolytic bacteria can be done using the polymerase chain reaction (PCR) technique and DNA sequencing analysis **(Japri *et al*., 2019)**. PCR is a technology used to amplify DNA fragments enzymatically **(Budiarto, 2015)**. Sequencing can be used to determine the identity of a gene or other DNA fragment by comparing the sample sequence with other known DNA sequences. The 16S rRNA gene is another marker gene that is useful in species identification **(Aprilia *et al*., 2014)**.

Isolation of protease enzymes from marine bacteria still needs to be done since the high potential of Indonesia's marine biodiversity has the potential to produce new enzyme findings **(Fuad *et al*., 2021; Ayanti *et al*., 2022)**. Based on this, the aim of this study was to isolate and identify bacteria in the brown algae *Hydroclathrus* sp. to obtain protease enzyme-producing bacteria and to determine their identity based on their 16S rRNA gene sequence.

MATERIALS AND METHODS

This study aimed to obtain protease producing bacteria associated with brown algae *Hydroclathrus* sp. The sampling of brown algae was carried out in the Wakatobi districts, Southeastern of Sulawesi, Indonesia. The living material used in this study was a sample

of the brown algae *Hydroclathrus* sp. collected from the coastal waters of Hoga Island. This research was conducted at the Microbiology Laboratory and Molecular Biology Laboratory Universitas Muhammadiyah Semarang in June 2021.

The work started with weighing the sample of *Hydroclathrus* sp. and then serially diluting it from 10^{-1} to 10^{-5} with 9ml of physiological NaCl. The dilution results were inoculated on NA and media incubation at 37°C for 24h. Any unique grown bacterial colonies were observed and identified. Gram staining was performed to determine the nature and morphology of the bacteria. Subsequently, colony purification was carried out to obtain bacterial pure colonies. After obtaining pure isolates, proteolytic bacterial activity was tested using SMA media based on the formation of a clear zone indicating the presence of protease enzyme-producing bacteria **(Hidayati *et al.*, 2021)**.

The bacterial isolates obtained were isolated using the Quick-DNA™ Miniprep Kit (Catalog No. D6005). The extracted DNA was subjected to the quantification and purity assays of the DNA extract using a Nanodrop spectrophotometer. Bacterial DNA was amplified targeting 16S rRNA gene using universal primer 27F-1492R **(Frank *et al.*, 2008)**, and the product was visualized with electrophoresis with the help of UV Transluminator. Sequencing analysis was carried out by PT Genetika Science using Sanger method. The obtained 16S rDNA sequence was further analysis by BLAST from GenBank at www.ncbi.nih.nim.gov **(Boratyn *et al*., 2013)**.

RESULTS

The sample used in this study was the brown algae *Hydroclathrus* sp. originating from the waters surrounding Hoga Island of Wakatobi District (Fig. 1). The sample was placed in a tightly closed conical tube, and then was stored in a refrigerator at a temperature of 4^{o}C. Isolation was initiated by grinding the samples of *Hydroclatrus* sp. using a mortar, followed by dilution from 10^{-1} to 10^{-5} with a ratio of 1:9 into 0.9% physiological NaCl, which helps maintain the ion balance of bacterial cells **(Lestari, 2014)**. The purpose of dilution is to dissolve or release microorganisms from their substrates into NaCl to reduce the density of bacteria to be planted **(Setyati & Subagiyo, 2012)**. The diluted sample was then isolated by inoculation into NA (Nutrient Agar) media to see bacterial colonies.

Fig. 1. Sample of brown algae *Hydroclathrus* sp. originating from the waters surrounding Hoga Island of Wakatobi District

After dilution and inoculation, colony morphology were observed, and the results of the 10^{-2} dilution were selected from the samples grown on NA media. A total of three colonies having different morphology coded HIHA-1- 3 could be obtained. After the morphology of the bacterial colonies was determined (Table 1), the 3 colonies were purified. Colony morphology characteristics of each isolate can be observed in Fig. (2) and Table (1).

A. B. C.

Fig 2. Results of purified bacterial colonies isolated from the algae *Hydroclathrus* sp. coded as A. HIHA-1 isolate B. HIHA-2 isolate. C. HIHA-3 isolate. HIHA referred to Hoga Island *Hydroclathrus* algae

The purified bacterial isolates were then identified for their colony shape (Table 1). Furthermore, identification by Gram staining on bacterial cells was also carried out to see the nature and morphology of the bacteria. The results are shown in Fig. (3). Isolates that have been purified were tested for protease-producing enzymes on SMA media. Of the 3 pure isolates that could be cultured from algae samples, only one isolate showed protease enzyme activity, namely isolate coded HIHA-1 with a clear zone diameter of 14mm. The test results of bacterial isolates can be seen in Fig. (4).

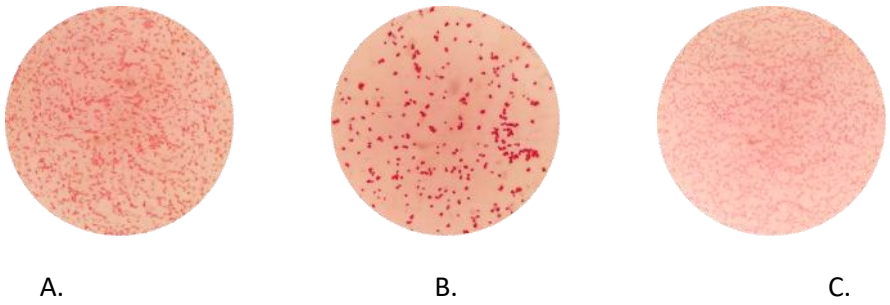

Fig. 3. Results of purified bacterial colonies isolated from the algae *Hydroclathrus* sp. showing: A. Colonies of HIHA-1 bacterial isolate B. Colonies of HIHA-2 bacterial isolate. C. Colonies of HIHA-3 bacterial isolate.

After inoculation, the next step was to identify the morphology of the colonies growing on NA media. The characteristics seen include shape, color, size, elevation, edge, and consistency. Isolation and identification of bacteria is one way to determine the type of bacteria to obtain pure cultures of target bacteria **(Nguyen *et al.*, 2018; Tveit *et al.*, 2019)**. Based on observations in the study, plate dilution-2 obtained three bacterial colonies with different morphology, namely HIHA-1 colonies, HIHA-2 colonies, and HIHA-3 colonies. Furthermore, purification was carried out to obtain pure isolates by inoculating three colonies obtained onto new NA medium. Purification is typically performed three times **(Mandakhalikar *et al.*, 2018)**. The pure bacterial isolates were then carried out with Gram staining to see the nature and morphology of bacterial cells **(Lestari *et al*., 2018)**.

Gram staining by Danish scientist Hans Christian Gram (1853–1938) aimed to distinguish bacterial species into two groups, Gram positive and Gram negative, based on the chemical and physical properties of the cell wall. Bacteria were given the main color of crystal violet and given an iodine solution and then washed off by alcohol. Gram-positive bacteria are purple because the cells bind to the crystal violet-iodine compound and Gram-negative bacteria lose their main color so they take the second color of red safranin **(Yusmaniar *et al*., 2017)**.

After staining the three different isolates, the results obtained were that HIHA-1 and HIHA-3 isolates were Gram-negative (-) coccobacilli, indicated by the shape of a short rod or an intermediate form between cocci and bacilli which was red. Meanwhile, the HIHA-2 isolates obtained Gram negative (-) tetra-cocci as shown in the shape of a square attached to each other, consisting of 4 red cells viewed through a microscope with a magnification of 100x.

Next, the activity of bacteria producing protease enzymes was tested on SMA (Skim Milk Agar) bacterial isolates dotting and incubating for 24 hours at 37°C. SMA media contains casein which functions as a substrate for protease enzymes, the formation of a clear zone around the colony indicates that bacteria can degrade protein and produce protease enzymes **(Pamaya *et al*., 2018)**. After incubation, only one isolate formed a

clear zone, namely HIHA-1 isolate with a clear zone diameter of 14mm and was selected for the next process, namely molecular-based bacterial identification.

Table 1. Bacterial colony morphology

Code	Shape	Color	Size (mm)	Elevation	consistency	Shape	Arrangement	Result
HIHA-1	Round	Yellow	1.5	Flat	Smooth	coccobacilli	Clustered	Gram negative
HIHA-2	Round	White	1.0	Convex	Smooth	Tetra-cocci	Clustered	Gram negative
HIHA-3	Round	Transparent	1.0	Flat	Smooth	Coccobacilli	Clustered	Gram negative

A. B. C.

Fig 4. Results of proteolytic activity test on bacteria isolated from brown algae *Hydroclathrus* sp. on SMA (Skim Milk Agar) displaying: A. Isolate HIHA-1 with clear proteolytic zone observed. B. Isolate HIHA-2 C. Isolate HIHA-3

Isolate showing a clear zone on SMA media, indicating the ability to produce proteases, were then molecularly identified by PCR. Bacterial genomic DNA extraction was carried out to obtain the template needed in PCR to amplify the 16S rRNA gene. The DNA extract obtained at the DNA isolation stage was measured for absorbance at 260 and 280nm. The maximum value of DNA can be absorbed with a wavelength of 260nm and can be used to estimate the DNA concentration.

DNA isolation was carried out to obtain DNA extracts from bacteria. Furthermore, the purity test, DNA is considered to be pure if the OD260/280 ratio is between 1.8 and 2.0 **(Ratnasari & Faridah, 2019)**. A purity level above 2.0 indicates that the DNA sample may be impure due to the presence of residual ethanol or residual bacterial secondary metabolites extracted. However, if during visualization of DNA extracts thick bands are still observed despite the ratio being above 2.0, the DNA isolates can still be used as PCR templates. While the purity of less than 1.8 indicates the possibility of the DNA extract contaminated with protein **(Sandhyco, 2020)**. It was observed that the absorption ratio of 260/280 bacterial genomic DNA extract HIHA-1 recorded a value of 1.95, thus it can be considered to be pure and ready to be used in PCR.

The amplification of the 16S rRNA gene serves as a molecular marker because it is universal and performs identical functions in all prokaryotic organisms. The pure bacterial DNA isolate was then electrophoresed using 1% agarose gel to determine the shape and size based on the ability to move, and the electrophoresis results showed that the bacterial DNA band was at 1421bp. The 16S rRNA PCR product against the selected isolates was then sent to PT Genetika Science Indonesia. Sequencing analysis was carried out by PT Genetika Science using Sanger method.

The DNA extract was then used as a template for amplification of the 16S rRNA gene. The method used was PCR using universal primers gene 16S rRNA 27F and 1492R which resulted in an amplification with a length of ~1500bp **(Amar, 2019; Cuscó *et al.*, 2019; Hassan *et al.*, 2019)**. After the amplification's results from the PCR were completed, it was followed by the process of electrophoresis using agarose 1%. The results obtained from the amplification showed that the DNA had a size of approximately 1500bp (Fig. 5).

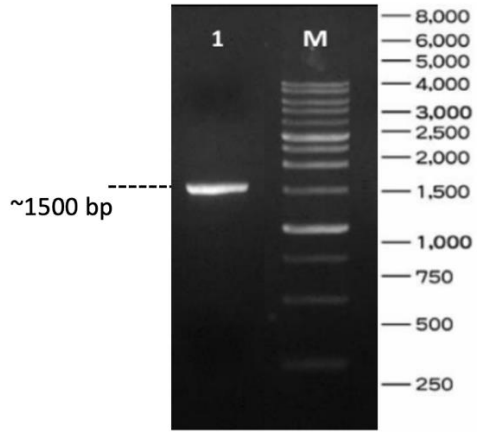

Fig 5. Amplification product of 16S rRNA gene

DNA PCR products from bacterial isolates were sequenced using the Sanger method resulting in 1421bp DNA fragment. Sequencing products using the Sanger method were obtained in the form of double helix format file Fasta forward and reverse. The two reverse and forward primary reading sequences were then combined to obtain consensus sequence using the Geneious program **(Jardim *et al.*, 2021; Shobayo *et al.*, 2021)**. The sequence of bases from the sequencing was then compared with the 16S rRNA DNA sequences in the NCBI database (short for) with the BLAST (Basic Local Alignment Search Tool) program. The results of sequence alignment with the BLAST bioinformatics tool showed that the HIHA-1 isolate had the highest similarity with *Exiguobacterium aestuarii* strain TF-16, with the accession number MZ276306.1 and the homology level of 99.93%. Based on the results of this bioinformatics analysis, the isolate HIHA-1 was then given the name *E. aestuarii* HIHA-1 (Hoga Island *Hydroclathrus* sp. a-1).

DISCUSSION

Obtained PCR product sequence was then aligned to compare its similarity with 16S rRNA sequence data from other bacteria available in the Genbank database **(Sandycho, 2020)**. This is done to determine the identity of gene fragments that can be done with BLAST program. The results of the BLAST program showed that the HIHA-1 isolate *Exiguobacterium aestuarii* was 99.93%. A genus is considered to be similar if it has 95% similarity and is considered to be a species if it has 97% similarity **(Kepel & Fatimawali, 2015)**.

First described by **Collins (1983)**, *Exiguobacterium aestuarii* is a Gram-positive bacterium, growing in an aerobic or anaerobic environment. It has a wide range of habitats, including air, land, and sea **(Astuti *et al*., 2016)**. Optimum growth is at a temperature of 30– 37°C, and the optimal pH for growth is at a range from 6.5– 8.5 . Hypoxanthine is not hydrolyzed, and the bacteria are urease-negative. The peptidoglycan type is L-Lys-Gly. Microscopically, this bacterium has a short rod shape with a circular tip in the early growth phase then becomes shorter almost in the form of a rod-oval in the exponential and stationary growth phase **(Kim, Kodama & Moeller, 2005)**.

However, research conducted by **Kim (2005)** using sediment samples from the tidal plains of Daepo Beach (Yellow Sea) in Korea, and by **Astuti (2016)** using samples of the green algae *Halimeda macroloba* from Menjangan Island in Jepara, revealed that *E. aestuarii* became red or a mixture of red and purple upon Gram- stain treatment. Therefore, this species was categorized as Gram-variable bacteria. Variable Gram bacteria are bacteria that have intermediate properties between Gram positive and negative. Hence, it is sometimes Gram positive, or Gram negative. According to **Kurniati (2018)**, there are several factors that can cause variations in Gram staining, namely changes in acidity; if the pH drops, it is likely that Gram positive bacteria can turn into Gram negative, and vice versa. Irregularities of staining paint, for example in washing for too long, can cause Gram-positive bacteria to give Gram-negative results. The medium factor also affects, Gram- positive bacteria are weak if they are grown for too long in a medium containing easily fermentable materials, so they can turn into Gram negative; in addition to the age of the bacteria, old positive bacteria will lose nutrients so that their ability to absorb crystal violet dye is reduced, and bacteria can turn into Gram negative because they absorb the last color, namely safranin.

E. aestuarii has bioremedial properties, namely the ability to tolerate heavy metals, such as Ni2+ and Cr6+ with bioremediation techniques, which are techniques for using microorganisms to reduce pollutants in the environment. On the other hand, *E. aestuarii* has a sensitivity to several antibiotics such as ampicillin, tetracycline, norfloxacin, trimethoprim, rifampin and chloramphenicol. This is explained in a study by **Gupta (2012)** using samples isolated from the soil rhizosphere of the Kestopur Canal on the northern outskirts of Kolkata, India. Exiguobacterium was found in the *Caulerpa racemose* algae sample CR1-AIK **(Kartika *et al.*, 2021)**.

The genus *Exiguobacterium* is known to be protease-producers species. However, studies regarding the species of *E. aestuarii* isolated from the sea are still few. In addition, the presence of *E. aestuarii* which is capable of producing proteases has never been reported. The results of this study suggest that, *E. aestuarii* can be isolated from samples of brown marine algae *Hydroclathrus* sp., exhibiting the ability to produce protease enzymes. This finding indicates the potential for further development of this plant-like protist in the future, potentially enabling its utilization on a larger scale as a source of new protease enzymes.

CONCLUSION

Based on the research conducted, it can be concluded that, from the sample of *Hydroclathrus* sp., three bacteria with different colony morphologies were successfully cultured, namely hiha-1, hiha-2, and hiha-3. One of the three isolates was a proteolytic bacterium, namely isolate hiha-1, with a proteolytic index of 2, 3. The results of the identification carried out molecularly based on the 16s rRna gene showed that the isolate was *exiguobacterium aestuarii* hiha-1. This indicates that the marine brown algae *hydroclathrus* sp. is a source of protease-producing bacteria *e. Aestuarii* hiha-1. Based on the results obtained from this study, it is recommended to conduct further characterization of the protease enzyme produced by the bacterium *e. Aestuarii* strain hiha-1, such as its activity, substrate specificity, and molecular weight.

REFERENCES

Amar, Ć. (2019). Southeast Europe journal of soft computing investigation of 16S rRNA gene and gene segments for the determination of probiotics. Southeast Europe Journal of Soft Computing, 8(1): 32–34.

Aprilia FE.; Soewondo, A.; Widodo; and Toha AH. (2014). Amplification of COI and 16S rRNA genes from the marine invertebrate *Plakobranchus ocellatus*. Journal of Biotropics. 2(5): 276-278.

Astuti, W.; Radjasa, OK.; Karwur, FF; and Rondonuwu, FS. (2016). Identification of carotenoids in *Halimeda macroloba* reef associated bacteria. Marine Science. 21(4): 151-160.

Ayanti, B.P.; Ethica, S.N.; Sulisytaningtyas, A.R.; Dewi, S.S; and Zilda, D.S. (2022). Prospective purification and assay of thrombolytic protease from *Bacillus* sp. HSFI-10 isolated from Sand Sea Cucumber for antithrombotic agent development. In *7th International Conference on Biological Science (ICBS 2021)*.Atlantis Press. (pp. 404-415).

Boratyn, G.M.; Camacho, C.; Cooper, P.S.; Coulouris, G.; Fong, A.; Ma, N.; Madden, T.L.; Matten, W.T.; McGinnis, S.D.; Merezhuk, Y. and Raytselis, Y. (2013). BLAST: a more efficient report with usability improvements. *Nucleic acids research*, 41(W1), pp.W29-W33.

Budiarto, BR. (2015). Polymerase Chain Reaction (PCR): Its development and role in health diagnostics. BioTrends. 6 (2): 29-38

Cuscó, A.; Catozzi, C.; Vones, J.; Sanchez, A.; Francino,O. (2019). Microbiota profiling with long amplicons using Nanopore sequencing : full-length 16S rRNA gene and the 16S-ITS-23S of the rrn operon. F1000Research .7(1755): 1–29.

Frank, J.A. Reich,C.I.; Sharma,S.; Weisbaum,J.S.; Wilson, B.A. and Olsen,G.J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology*, 74(8): 2461–2470. Available at: https://doi.org/10.1128/AEM.02272-07.

Fuad, H., Hidayati, N.; Darmawati, S.; Munandar, H.; Sulistyaningtyas, A.R.; Ernanto, A.R.; Muchlissin, S.I.; Zilda, D.S.; Nurrahman, N.; and Ethica, S.N. (2021). Exploration of bacteria isolated from" rusip" fermented tissue of sand sea cucumber *Holothuria scabra* with fibrinolytic, anticoagulant and antiplatelet activities. *Aquaculture, Aquarium, Conservation & Legislation*.

Gupta, K.; Chatterjee, C. and Gupta, B. (2012). Isolation and characterization of heavy metal tolerant Gram-positive bacteria with bioremedial properties from municipal waste rich soil of Kestopur canal (Kolkata), West Bengal, India. Journal of Biology. 5(67): 827-836.

Hassan, M. M.; Abdal-Aziz, A.A.; Megahed, A.M.; Rassoul, H.A.A.; Mabrouk,M.Y. (2019). Molecular identification of Listeria species from some processed meat products using specific Iap gene and 16S rRNA gene. Alexandria Science Exchange Journal. 40(2): 333–339.

Hidayati, N.; **Fuad,H.; Munandar,H.; Zilda,D.S.; Nurrahman.; fAttah,M.; Oedjijono.; Semiasih, A.; Ethica,S.N**. (2021). Proteolytic and clot lysis activity screening of crude proteases extracted from tissues and bacterial isolates of *Holothuria scabra*.

Japri, NA.; Sulistyaningtyas, AR.; Darmawati, S. and Ethica, SN. (2019). Isolation and molecular identification of proteolytic bacteria *Staphylococcus cohnii* strain IRLV5 on white shrimp rusip (*Litopeaneus vannamei*) after 120 hours of fermentation based on gene analysis of 16s rRNA. Proceedings of the Student National Seminar Semarang. 2: 325-333

Jardim, B.R.; Kinoti,W.M.; Tran-Nguyen,L.T.T; Gambley,C.; Rodoni,B.; Constable,F.E. (2021). Candidatus *Phytoplasma stylosanthis*, a novel taxon with a diverse host range in Australia, characterised using multilocus sequence analysis of 16S rRNA , secA , tuf , and rp genes. International Journal of Systematic and evolutionary microbiology, 71.

Kartika, A. I., Fitria, M. S. and Oktaviola, V. (2021). Molecular identification of pathogenic bacteria causing foodborne disease in *Caulerpa racemose*. Jurnal Teknologi Laboratorium. 10(1): 31–39.

Kepel, B. and Fatimawali. (2015). Species determination by analysis of the 16SrRNA gene and reduction power test of mercury resistant bacteria isolated from the feces of patients with mercury amalgam patches at Bahu Manado Health Center. Yarsi Medical Journal. 23 (1): 45-55.

Kim, B.; Kodama, K. P.; and Moeller, R. E. (2005). Bacterial magnetite produced in water column dominates lake sediment mineral magnetism : Lake Ely , USA. Geophys. J. Int. 163: 26–37.

Kurniati, TH.; Indrayanti, R.; Muzajjanah.; Rustam, Y. and Sukmawati, D. (2018). Microbiology Practicum Guide. Department of Biology Education, FMIPA. State University of Jakarta.

Lestari, DA.; Muchlissin, SI.; Mukaromah, AH.; Darmawati, S. and Ethica, SN. (2018). Isolation of protease enzyme producing bacteria (*Bacillus megaterium*) IROD3 from red oncom after 72 hours fermentation. National Seminar on Education and Technology. pp.31–39.

Mandakhalikar, K. D.; Rahmat, J.N.; Chiong, E.; Neoh, K. G.; Shen, L.; Tambyah, P.A. (2018). Extraction and quantification of biofilm bacteria : Method optimized for urinary catheters. Scientific Reports. 8(8069): 1–9.

Nguyen, T. M.; Seo, C.; Ji, M.; Paik, M. J.; Myung, S. M.; Kim .J. (2018). Effective soil extraction method for cultivating previously uncultured soil bacteria. Applied and environmental microbiology, 84(24).

Pamaya,D.; Muchlissin, S.I.; Maharani, E.T.W.; Darmawati,S.; Ethica, S.N. (2018). Isolasi bakteri penghasil enzim protease *Bacillus amyloliquefacienns* IROD2 pada oncom merah pasca fermentasi 48 jam. National Seminar on Education and Technology. pp.31–39.

Puspitasari, FD, Shovitri, M. and Kuswytasari, ND. (2012). Isolation and characterization of proteolytic aerobic bacteria from septic tanks. ITS Journal of Science and Arts. 1(1): E1-E4.

Putri, ALO and Kusdiyantini E. (2018). Isolation and identification of lactic acid bacteria from fish-based fermented food (Inasua) traded in Maluku-Indonesia. Journal of Tropical Biology.1(2): 6-12

Ramadhani, P., Rukmi, I., and Pujianto, S. (2015). Production of protease enzymes from *A. niger* PAM18A with variations in ph and incubation time. Journal of Biology. 4(2): 25-34.

Ratnasari, YA and Faridah, IN. (2019). Optimization of Fta cards sample DNA isolation method using purelink ®Genomic DNA kits and Chelex-100, Bachelor Thesis, (1). Available at: http://eprints.uad.ac.id/id/eprint/14764.

Said, M.I, Likadja, J.C. (2012). Isolation and identification of bacteria potentially producing protease enzymes in the leather tanning industry of Pt. Adhi Satria Abadi (Asa), Yogyakarta. JITP. 2(2): 121-128.

Sandycho N. (2020). Isolation and molecular identification of bacteria producing protease enzymes in mullets (*Moolgarda seheli*) gene sequences of 16S rRNA. Muhammadiyah University of Semarang.

Setyati, WA, and Subagiyo. (2012). Isolation and selection of extracellular enzyme producing bacteria (proteolytic, amylolytic, lipolytic and cellulolytic) from mangrove sediments. Marine sciences: Indonesian Journal of Marine Sciences. 17(3): 164-169.

Shobayo, B.;Mishra,M.; Sameroff,S.; Petrosov, A.; Ng,J.; Gokden, A.; Macauley, J.; jain, K.; renken, C.; Duworko, J.T.; Badio, M.; Jallah, .; Hensley, L.;Briese,T.; Lipkin, W.L.; and Mishra,N. (2021). SARS-CoV-2 sequence analysis during COVID-19 Case Surge, Liberia, 2021. *Research Letters*. 27(12): 3185–3188.

Susanti R., dan Fibriana F. (2017). Teknologi Enzim. Penerbit Andi; Semarang

Tveit, A.T.; Hestnes,A.G.; Robinson,S.L.; Schintlmeister, A.; Dedysh,S.N.; Jehmlich, N.; Bergen,M.V.; Herbold,C.; Wagner,M.; Richter,A.; Svenning,M.M. (2019). Widespread soil bacterium that oxidizes atmospheric methane. *PNAS*. 116(17): 8515–8524.

Yuniati R., Nugroho T.T., dan Puspita F., (2015). Uji aktivitas enzim protease dari isolat *Bacillus sp.* galur lokal Riau. Jurnal Online Mahasiswa. 1 (2): 116-122.

Yusmaniar., Wardiyah., and Nida, K., 2017. Ministry of Health of the Republic of Indonesia. Microbiology and Parasitology.