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ISOLATION AND CHARACTERIZATION OF PROTEASE-PRODUCING BACTERIA FROM PUFFER FISH SKIN WASTE

RADEN LUKAS MARTINDRO SATRIO ARI WIBOWO[1,2,3], ZAENAL BACHRUDDIN[4], NANUNG AGUS FITRIYANTO[2], TOMOYUKI NAKAGAWA[5], TAKASHI HAYAKAWA[5] AND AMBAR PERTIWININGRUM[2*]

[1]Postgraduate School of Animal Science, Faculty of Animal Science, Gadjah Mada University, Jl. Fauna No. 3, Bulaksumur, 55281 Yogyakarta, Indonesia
[2]Department of Animal Products Technology, Faculty of Animal Science, Gadjah Mada University, Jl. Fauna No. 3, Bulaksumur, 55281 Yogyakarta, Indonesia
[3]Department of Leather Tanning Technology. Politeknik ATK, Jl. Ringroad Selatan, Glugo Panggungharjo, Sewon, Bantul, 55281 Yogyakarta, Indonesia
[4]Department of Animal Nutrition and Feed Science, Faculty of Animal Science, Gadjah Mada University, Jl. Fauna No. 3, Bulaksumur, 55281 Yogyakarta, Indonesia
[5]Departement of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, Gifu, Japan

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Abstract – Puffer fish is one of the waste fisheries catch. It is not only can be a waste of fisheries that is difficult to degrade, but also can be used as a source of proteolytic bacteria. Thus, this innovation, i.e. enzymes produced by bacteria which cause pufferfish's skin to decay, can be used as a substitute for chemicals in tanning processes. The objective of this research was to obtain bacterial isolates from the waste of puffer fish skin (*Arothon reticularis*) as one of the sources of proteases. Materials used in the study were 30 pieces of 30-days fermented pufferfish skin. The results of the isolation showed that 6 of 10 kinds of strains performed caseinolytic activity by showing the clear zone in the media of Skim Milk Agar. Based on the results of SEM test, the morphological form of two types of microbes were short Bacillus, while four types of microbes were long-shaped Bacillus. The proteolytic enzymes assay showed that bacteria strain Kt had the highest specific activity (70.32 U/mg), while bacteria strain Pb had the lowest activity (32.49 U/mg). All bacteria obtained were the superior bacteria that can be used for the removal of thorns/spines from pufferfish skin in the tanning process. The proteolytic enzyme test showed that BRAW_Kt bacterial strain had the highest specific activity (70.32 U/mg) and BRAW_Pb bacterial strain had the lowest specific activity (32.49 U/mg).

INTRODUCTION

The potential of marine fisheries in Indonesia reaches 6.4 million tons per year spread over Indonesia territorial waters and Exclusive Economic Zone with allowable catch up to 5.12 million tons per year or about 80%. This potential is an opportunity to increase fish production, both for capture fisheries and aquaculture. It is estimated that there are 28,400 species of fish in the world, and in Indonesia produces more than 25,000 species. However, few of them are worth consuming, i.e. 1-5% and used as ornamental fish, i.e. less than 1%, while the rest are predicted to play a role in the food chain system in aquatic ecosystems. Pufferfish (*Arothon reticularis*) is not a popular fish in fishing. When it gets caught, it is generally put back in the sea. If it is taken to land, it is because it gets caught among other fishes. It is considered useless because only a few people use it (CBS, 2016). Reecntly, pufferfish is only fishery waste and it is only dumped and left to decompose. It is a pity because it damages the environment. Therefore, if the resulting bacteria from the decomposed pufferfish (especially proteolytic bacteria) can be isolated, and it will benefit the environment (Wibowo *et al*., 2017).

***Corresponding author's email**: artiwi@mail.ugm.ac.id

Proteases are widely-used in industries such as pharmacy, detergents, food, waste industry, and leather tanning industries (Ward, 1985; Pastor *et al.*, 2001; Brandelli *et al.*, 2010). Fisheries waste offers a renewable source of these useful enzymes. Extraction of enzymes from fisheries waste may improve the economics of the fisheries industry while minimizing the environmental impact of waste disposal (Daboor *et al.*, 2012). On the other hand, the use of protease for tanning is suitable because it has very low collagenase activity so the enzyme can thoroughly remove hair without affecting the tensile strength of the hide (Prakash *et al.*, 2010).

Recently, protease enzymes can be produced easily from microorganism, such as bacteria and fungi (Macedo *et al.*, 2005). Microbial proteases represent a good source of enzymes due to a number of characteristics like their broad biochemical diversity, their rapid growth, the limited space required for cell cultivation and ease with which the enzymes can be genetically manipulated to generate new enzymes for various applications (Aftab *et al.*, 2006). Protease can be isolated from the extracellular and intracellular parts (Rao *et al.*, 1998). Sharmin *et al.* (2004) has been reported that the production of extra cellular protease by different microorganisms can be strongly influenced by culture condition Protease-producing bacteria, particularly the ones that produce extracellular proteases.

The importance of proteases and high marketability of these enzymes encourage the scientists to look for new productive sources of proteases that have high activity level. Currently, scientists start to use waste as source of protease-producing microorganisms (Marzam *et al.*, 2004). As protease enzyme producer, microorganism is preferable because it can quickly and easily produce the protease enzyme, although it needs to be controlled carefully in the application. In an effort to develop clean products or cleaner production, it is necessary to look for alternative solutions, or at least to reduce the industrial impact (Moon *et al.*, 1993). The indicated-toxic-fish, pufferfish, whose spiny skin is discarded as waste, can be used to take helpful bacteria from the decay process (Sharmin *et al.*, 2004).

Considering all the things described above, it is important to perform protease hydrolysate production from fish waste source enzymatically as an effort to handle environmental problem and to increase economic value. Therefore, this study aimed to obtain bacterial isolates from the waste of pufferfish (*Arothon reticularis*) skin as one of the sources of enzyme protease.

MATERIALS AND METHODS

Isolation of the Proteolytic Bacteria

The pufferfish skin was fermented for 30 days and used as isolate sources. Fish skin was cut with scissors into small pieces then washed several times with distilled water. It was used as raw material for isolation and identification of microorganisms. Primary isolation was conducted on pufferfish powder. Pufferfish skin was dried in a Memmert oven at 60°C for 48 hours, then mashed by KIRIN KBB-315SG food processor (Kate and Pethe, 2014). Pufferfish skin powder was taken as much as 1 g and dissolved in 10 mL of sterilized water (Sundararajan *et al.*, 2011).

The bacteria was inoculated in petri dish then incubated at 30°C for 48 hours in an aerobic incubator (Heraeus, UK). Every single colony that showed morphological differences was taken to be moved onto the specific media which contained pufferfish skin powder as substrate. This step was repeated several times to obtain pure isolates. The selection for isolates which showed high protease activities was conducted by growing the isolates on agar medium of casein at 37°C for 24-48 hours. The further selection of bacteria was, according to Gupta and Ramnani (2006), determined by the ratio of comparison between the clear zone and the largest colony diameters. The difference of casein hydrolysis ability depends on the species and the isolation environment of the bacteria (Wibowo *et al.*, 2017).

Enzyme Production

The enzyme production was based on the method of Hoq *et al* (2005) One purely-cultured isolate was inoculated into 5 mL of sterile pre-culture media, and it was then incubated at 30°C for 48 hours in an incubator shaker at 120 rpm. A total of 3 mL of media was inoculated into 100 mL of fermentation media which included pufferfish powder. The fermentation was then carried out using flask of 250 ml. It was constantly shaken at 120 rpm using an incubator at 30°C for 48 hours, and centrifuged in centrifugation machine (Rotofix-32, USA) at 4500 rpm at 4°C for 20 min. The supernatants were

preserved at 4°C and assayed for protein and enzymes.

Protease Activity

The advanced protease activity was carried out in accordance with the method of Bergmeyer *et al*. (1983) which had been modified by mixing 3 ml of 1.5% casein with 0.5 mL phosphate buffer at pH 7 and 1 ml of enzyme, and they were then incubated at 37°C for 10 minutes. Three milliliters of 5% Trichloro acetic acid was added and incubated at room temperature for 10 minutes. Furthermore, the solution was filtered using Whatman No. 1 filtrate, taken by 0.75 mL, and added with 2.5 mL of Na_2CO_3 (0.5M) and 0.5 mL of Folin reagent. It was then incubated at room temperature for 15 minutes. The supernatant absorbance of each solution was measured with spectrophotometer at λ = 578 nm and calculated for its concentration and activity.

Scanning Electron Microscopy of Microbial Cells

The making of fixation solution was conducted using 0.2145 g of sodium cacodylate, 1.0081 g of NaCl, 630 µL HCl 0.2 M, 1 mL of glutaraldehyde and was added with aquades to make a volume of 100 mL. Agar medium was made using 3.5 g of agar, 0.1 g of gelatin, and added with 1/100 stock solution. Then it was boiled on the stove and stirred constantly until it dissolved evenly. The steriled agar medium was then poured into the petri dish. A special filter paper that had been cut to the size of 5x5 mm was put on the un-ossified agar, and we waited until it was cool and ossified. The isolate was taken 1 µL and dripped on filter paper that had been placed on the agar, then it was incubated for four days. Filter paper that had been overgrown by isolate on the agar was then taken and put in Eppendorf tube, added with 1 ml of fixation solution and incubated at 4°C for 30 minutes. The fixation solution was then taken using a pipette until it was empty, added with 1 mL of 60% acetone and incubated at room temperature for 15 minutes, then continued by 80% and 100% acetone (repeated twice) with the same method. It was then coated and ready to be observed using a Scanning Electron Microscope (Fitriyanto, 2008).

Isolate Growth Measurement

The pre-culture was taken 2 mL and put into 100 mL of stock liquid medium with the addition of pufferfish skin flour as a source of carbon and nitrogen. Based on the method of Sivakumar *et al*., (2012) which had been modified, the time required to get a stationary growth was every 2 hours. 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, and 36 hours.

Molecular Identification

The molecular identification was conducted using the 16S rRNA gene sequence (Benson, 2010). The sequencing of 16S-rRNA gene consisted of several stages, including DNA extraction, amplification between 16S-rRNA gene and PCR, and sequencing using a sequencer machine.

Amplification of 16S rRNA gene was conducted using a thermal cycler. The Primers used are the couple of 8F (5' - AGAGGTTGATCCTGGCTCAG-3'), primer 1492R (5' - GTTTACCTTGTTACGACTT-3'). The PCR process began with initial denaturation stage at the temperature of 94°C for 5 minutes, and continued with the process of 30 cycles consisted of denaturation process at the temperature of 94°C for 1 second, primer attachment at 55°C for 1 minute, and elongation at 72°C for 1 minute. After the 30 cycles had finished, it was followed by the lengthening process at the temperature of 72°C for 10 minutes, and the PCR process stopped at the temperature of 12°C. PCR results were then viewed by electrophoresis on 0.8% agarose gel.

Phylogenetic Analysis: The identical 16S rDNA sequences were identified by phylogenetic tree analysis and manual comparison, where the sequences with a similarity of more than 90% were described as identical, and these sequences were used for further phylogenetic analysis as Operational Taxonomic Unit (UTO). The evolutionary tree was based on distances compiled using the nearest neighbor algorithm.

Data Analysis

Bacterial Isolation and Identification were conducted with descriptive method, a research method to find a fact with correct interpretation. The data of microbial activity obtained was described as the Mean±SD. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple-RangeTest (DMRT) using software SPP Inc. (Chicago, IL, USA). Differences considered significant when the probability was less than 5%.

RESULTS AND DISCUSSION

There were approximately 10 bacterial colonies

grown on the basis media that were added with pufferfish flour. The flour was obtained by drying the 5-time-washed fresh pufferfish which was then put into the oven at 50°C and blended using a blender.

Caseinolytic Activity

Figure 1 showed that on the third day of observations, BRAW_KT strains showed the largest colony diameter (3.70 mm), followed by BRAW_PT strain (3.06 mm). The third largest diameter was BRAW_PI strain (2.40 mm), BRAW_ST strain (2.15 mm), BRAW_KM strain (1.75 mm) and BRAW_PB strain (1.34 mm). The statistical analysis was conducted with variance analysis that showed that there were significant differences among the treatments. A further test was conducted using Duncan's Multiple Range Test (DMRT). It showed that BRAW_PT and BRAW_KT strains did not have significant difference (P> 0.05); BRAW_PI and BRAW_ST strains did not have significant difference (P> 0.05); and BRAW_KM and BRAW_PB strains also did not have significant difference (P>0.05). However, BRAW_KT and BRAW_PT strains had a significant difference (P<0.05) with BRAW_PI, BRAW_ST, BRAW_KM and BRAW_ PB strains. Based on these results, BRAW_PB, BRAW_PT, BRAW_KM, BRAW_KT, BRAW_PI and BRAW_ST isolates were able to degrade the casein.

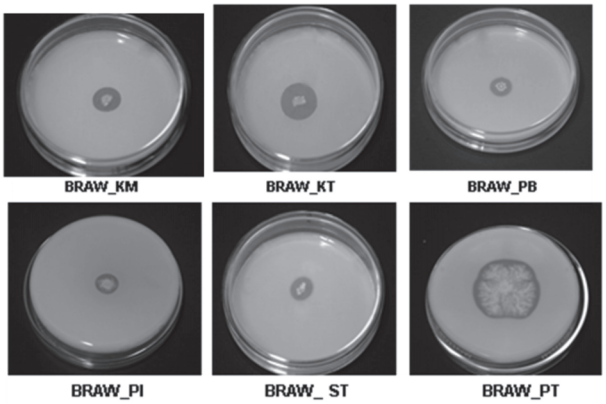

Fig. 1. Clear zone indicates caseinolytic activity

Proteolytic Activity

Protease is also called peptidase or proteinase. It is a hydrolase-class enzyme that will break down protein into simpler molecules such as short oligopeptides or amino acids, with hydrolysis reaction on the peptide bond. From the proteolytic enzyme test, the results showed that BRAW_KT coded bacteria had a specific activity of 70.32 U/mg. This was the highest activity and was equal to the diameter of clear zone in the casein test. On the other hand, BRAW_PB strain exhibited the lowest activity (32.49 U/mg), and this result was equal to the diameter of the clear zone that was shown by BRAW_PB strain bacteria on the casein plate.

Based on the Unidirectional Pattern Complete Randomized Design test, there were significantly different results among the treatments. A further test using Duncan's Multiple Range Test showed that BRAW_KT bacteria strain isolate had a significantly different proteolytic activity (P <0.05) that was greater than other isolates' proteolytic activity. The other three isolates, BRAW_KM, BRAW_PT and BRAW_PB, were not significantly different to each other (P>0.05), but they were significantly different (P<0.05) to BRAW_PI and BRAW_ST isolates. BRAW_PI and BRAW_ST isolates also showed no significant difference to each other (P<0.05).

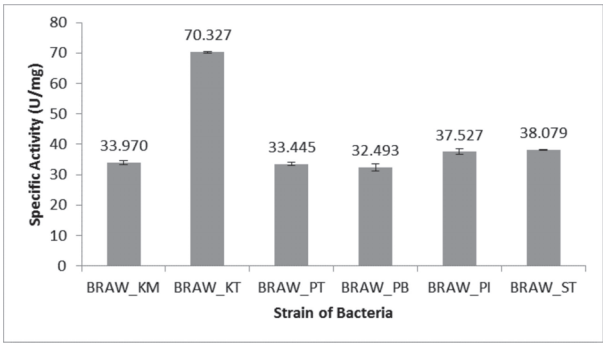

Fig. 2. Proteolytic Enzyme Activity

Molecular taxonomy, sequencing and phylogenetic analysis

The phylogenetic tree analysis results are shown in Figure 4. Based on the formed phylogenic tree, all isolates had a very close genetic relationship with different species of Bacillaceae Family.

DISCUSSION

The next selection stage was the test on the ability to degrade casein and skim milk media. Skim milk contains casein, a milk protein which will be broken down by proteolytic microorganisms into dissolved nitrogen compounds so the colony will be surrounded by a clear area. It was already shown that these microbes have proteolytic activity (Sivakumar *et al.*, 2012). Based on this test, there were only six isolates that had the ability to degrade

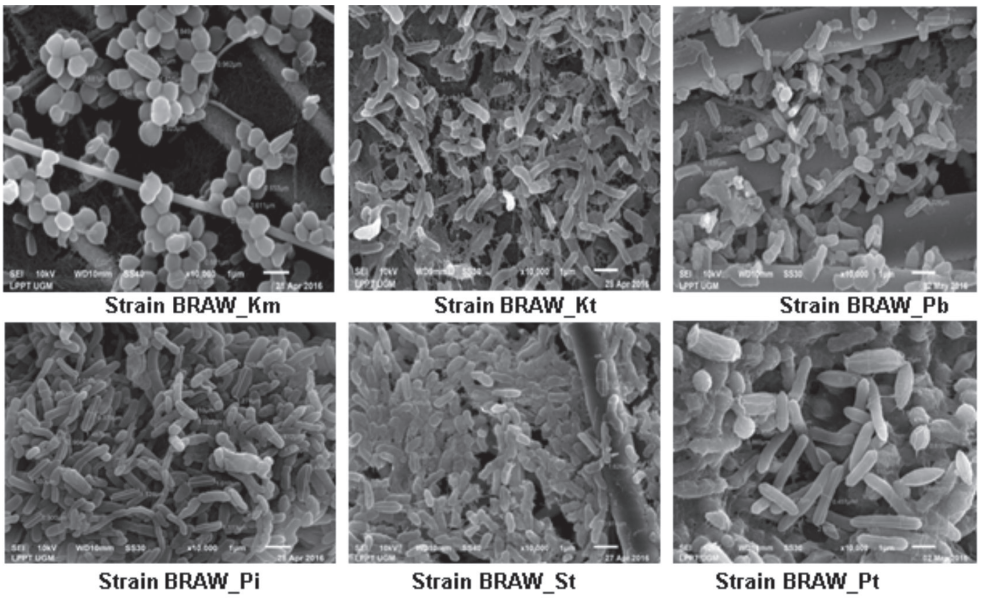

Fig. 3. The Morphologies of Six Isolates Seen from SEM

casein isolates while the other four did not.

Sivakumar *et al.*, (2012) and Benson (2001) confirmed that the clear zone formed around colonies on the casein plate was due to the formation of casein degrading enzyme, so we observed the casein degrading abilities of the isolates on the casein agar plate. The isolates, which were grown at 30°C for 48 hours, showed enough clear zones (Figure 1).

It was consistent with the previous finding of Gupta and Ramnani (2006) that most reports of natural protease are based on its caseinolytic activity. Therefore, bacteria which have caseinolytic activity should be studied further.

On the other hand, it has been reported that most proteinolytic bacteria also have a caseinolytic activity. Thus the caseinolytic ability of bacteria can be used to select the initial proteinolytic bacteria. From these facts, it was presumed that the six strains had protease together with caseinase. It was in line with the study of Pillai and Archana (2008) who made a selection of 20 isolates of bacteria from volcanic sediments in Mumbai, India, based on the comparison of clear zone diameters and the diameter of the colonies on skim milk agar media.

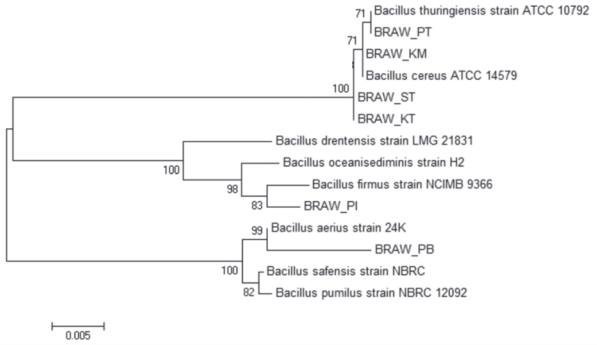

Fig. 4. The Phylogenic Tree of Six Isolates

From the results of SEM, there were two types of morphological forms of microbes in the form of short bacilli, while four types of microbes were long-shaped bacilli. SEM was useful to clarify the simple coloring using safranin dye, and Gram and Zn staining indicated that all of the isolates belonged to Bacilli class.

The value of bootstrap is indicated by the numbers found on the branches of phylogenetic trees. According to Hall (2013) the higher the bootstrap value then the more reliable or trusted. The phylogenetic tree of the analysis is presented in the figure 4. BRAW_KM, BRAW_ST, and BRAW_KT showed 99% similarity with *Bacillus cereus*. BRAW_PT was close to *Bacillus thuringiensis* with 99% similarity, BRAW_PB was close to *Bacillus aerius* with 99% similarity, and BRAW_PI showed 99% similarity with *Bacillus firmus*.

CONCLUSION

The results of the isolation obtained 10 types of strain, and only 6 out of 10 bacterial strains presented caseinolytic activity using agar skim milk media by showing the clear zone. The proteolytic

enzyme test showed that BRAW_Kt bacterial strain had the highest specific activity (70.32 U/mg) and BRAW_Pb bacterial strain had the lowest specific activity (32.49 U/mg).

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