Isolation of Bacteria Producing Enzyme Collagenase From Waste of Pufferfish (*Arothon reticularis*) Skin

Raden Lukas Martindro Satrio Ari Wibowo[1,2,3], Ambar Pertiwiningrum[2], Zaenal Bachruddin[4], Nanung Agus Fitriyanto[2] T. Nakagawa[5], T. Hayakawa[5]

[1]Postgraduate Animal Science, Faculty of Animal Science, Universitas Gadjah Mada, Jl. Fauna No. 3, Bulaksumur, Yogyakarta 55281 – Indonesia

[2]Department of Animal Product, Faculty of Animal Science, Universitas Gadjah Mada, Jl. Fauna No. 3, Bulaksumur, Yogyakarta 55281 – Indonesia

[3] Department of Leather Tanning Technologies. Polytechnic of ATK, Jl. Ringroad Selatan, Glugo Panggungharjo, Sewon, Bantul, Yogyakarta

[4]Department Nutrition and Animal Feed. Animal Science, Universitas Gadjah Mada, Jl. Fauna No. 3, Bulaksumur, Yogyakarta 55281 – Indonesia

[5]Departement of Applied Life Science Laboratory. Faculty of Applied Biological Sciences Gifu University

Corresponding email: alexius.lucaswibowo@gmail.com

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 collagenolytic bacteria.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 collagenases. Sample from puffer fish skin waste was inoculated in enrichment media and colonies producing clear zone in skim milk agar were selected and identified as *B. cereus* BRAW_KM. Medium optimization to grow the selected collagenase producing bacterial strain was checked with various parameters such as temperature (at 31 and 33ºC), pH (8 to 9), substrate concentration (15 g/L), osmotic pressure (4%), inoculum concentration (8%), and agitation speed (100 to 120 rpm). The bacteria produced extracellularly collagenase enzymes in enrichment media and its collagenase activity was 1,029 U/mg.

Keywords: Bacteria, Collagenase, Isolation, Puffer fish skin, Waste

INTRODUCTION

It is estimated that the potential of Indonesia's marine fisheries to be sustainable reaches 6.4 million tons per year that spread over Indonesian territorial waters and the Exclusive Economic Zone with an allowable catch of up to 5.12 million tons per year or about 80% of the sustainability potential. This potential comprises one of the opportunities to increase fish production, both for capture fisheries and culture fisheries. According to Data from the Directorate of Product Processing (2006), it has been estimated that there are 28,400 species of fish in the world, and in Indonesian founded more than 25,000 species. Nevertheless, only a few of them fit for consumption, i.e. by 1-5% and are used as ornamental fishes, i.e. by less than 1%, while the rest is predicted to play a role in the food chain system in aquatic ecosystems. Puffer fish are one of the fisheries waste from the catch that can still be used because they are rich in proteases, which among others serve as a source of enzyme collagenase.

Collagenases are a hydrolytic enzyme which can be used for multiple purposes and applications for industrial needs, medicine, and research. This enzyme in fisheries processing is used in fish tanning, membrane removal, and protein hydrolysates (Bjarnason 2001). Some researchers have isolated collagenases from fishes, such as from pyloricaeca of yellowtail fish (Shahidi and Botta 1994), mackerel, the pancreas of catfish *Parasilarus asotus* (Kim et al., 2002) and digestive organs found in 19 fish species (Shahidi and Botta 1994).

Among the components of fisheries waste is the skin. In general, the skin contains many proteins in the form of collagen fibers that have substantial grace power. Collagen is a connective tissue consisting of collagen fibers and elastin containing polysaccharides as well as various organic and inorganic components. Collagen has a unique amino acid composition. About one-third of the amino acids contained is glycine, 6-10% of them is hydroxyproline, and 10-12% of them is proline (de Man, 1997). Its main characteristics are its triple-helical conformation and its amino acid content, in which the amount of hydroxyproline residues present is much higher than the amount found in other proteins existing in nature due to their rigid structure, and only a limited number of proteases may cleave collagen, such as collagenolytic proteases or collagenases (Harrington, 1996). Collagenases are a hydrolytic enzyme which can be used for multiple purposes and applications for industrial needs, medicine, and research. This enzyme in fisheries processing is used in fish tanning, membrane removal, and protein hydrolysates (Bjarnason, 2001). Some researchers have isolated collagenases from fishes, such as from pyloricaeca of yellowtail fish (Shahidi and Botta 1994), mackerel, the pancreas of catfish *Parasilarus asotus* (Kim et al., 2002) and digestive organs found in 19 fish species (Shahidi and Botta 1994).

Considering the importance of all of those things describes, collagen hydrolisate production from fish waste source enzymatically as an effort to handle enviromental problem and to increase economicalvalue is important to perform. Therefore, this study was aims to obtain bacterial isolates from the waste of pufferfish (*Arothon reticularis*) skin as one of the sources of enzyme collagenase.

MATERIALS AND METHODS

Bacteria isolation. The materials used in the present study were 30 sheets of pufferfish skin that underwent 30 days of decay. The growth medium for the isolation process was based on Macedo et al. (2005) with several modifications added, namely pufferfish skin flour as a single carbon and nitrogen source by 10 g; minimum minerals that consisted of NaCl by 0.5 g; K_2HPO_4 by 0.3 g; and KH_2PO_4 by 0.4 g. The medium for stock solution consisted of 1 g of Yeast extracts, 1 g of biological Peptone, 0.5 g of NaCl, and 100 ml of Aquadest.

Collagenase activities. Based on the modified method proposed by Pilai and Archana, (2008), collagenase activities were examined using 0.2 ml of coarse enzymes dissolved in the pure collagen solution by 0.4 mg (Sigma-Aldrich, St. Louis, USA) in 0.4 ml of the 50 mM Tris-Cl buffer solution at pH 8. The reaction was incubated at 30°C for 30 minutes. The enzyme reaction was discontinued using 0.6 ml of 15% TCA and afterwards stored in ice for 15 minutes. The solution was centrifuged at 12,000 g for 10 minutes. The absorbance of supernatants was measured at a wavelength of 520 nm. A unit of enzyme collagenase is defined as the quantity of enzymes that can increase absorbance by 0.01 according to the test conditions.

RESULTS AND DISCUSSION

Collagenase Activities in Bacteria

Collagenases are a type of enzymes with the ability to degrade collagen. Generally, they are defined as an enzyme capable of degrading polypeptide bonds. This enzyme is classified into two different types based on its physiological function. Serine collagenases take part in hormonal production and pharmacological activities. These functions include protein digestion, blood clotting, fibrinolysis, complex activation, and fertilization (Neurath 1984; Park et al. 2002). Based some founded isolate from the research, isolates with the highest collagenase activities was BRAW_KM by 1,029 U/mg.

Morphological Identification

Observation made on the morphology of bacterial colonies included their shape, edges, internal structure, elevation, and color. The obtained isolate showed the following properties: nonmotile, oxidase-positive, and catalase-positive. There were numerous Gram-positive bacteria *Bacillus subtilis* SLC (Cedrola et al., 2011); *B. subtilis* 1271, *B. licheniformis* 1269, and *B. cereus* 1268 (Mazoto et al., 2011), and *Streptomyces* sp. Strain AB1 (Jouadi et al., 2010). Based on the observation results, it was revealed that the shape, edges, internal structure, and elevation of the colonies of bacterial isolates were: they had a circular shape, efuse elevation, an entire edge shape, and a translucent internal structure. The cell morphology showed that all bacterial isolates had a gram-positive stem cell shape and were acid negative in acid staining. For the carbohydrate test, the result was positive for glucose, fructose, sucrose, and lactose tests and had spores. The catalase and oxidase tests generated positive results. According to Cappucino and Sherman (1987), bacterial colonies may have a round and irregular shape with a convex, concave or flat surface and flat or wavy edges.

Then, BRAW_KM isolates were observed in terms of their optimum growth based on the number of substrates, growth temperature condition, pH, inoculum concentration, agitation difference, and osmotic pressure. To determine the optimal bacterial growth, the growth was measured using various substrate concentrations. The substrates used were Pufferfish Flour at the concentrations of 5, 10, 15, 20, and 25 g/L. The obtained results suggested that the optimal use f substrates was achieved at the concentration of 15 g/L. The pH treatment in this study was given at pH levels 6, 7, 8, 9, 10, 11 and 12. Based on the obtaine result, it was revealed that the optimal pH ranged from pH 8 to 9. Different organisms showed maximum enzyme production at different pH levels. For example, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus pumilus* produced enzymes maximally at pH f 7.0, 5.9, and 5.6 (Sivakumar et al. (2012), respectively. According to Rochimaa et al. (2015), the optimum pH

of enzyme collagenase from *Basilus subtilis* ranges from 7 to 9 (from 1,298 units per mL to 1,321 units per mL).

The temperature treatment to examine bacterial growth was given at 25, 27, 29, 31 and 33ºC. The result of the study showed that the most optimum bacterial growth occurred at a temperature between 31 to 33ºC. The inoculum concentrations were given at the following percentages: 1, 2, 3, 4, and 5% in growth media. The result suggested that the higher concentration of inoculum given, the higher level of growth. This was because in the growth medium there were more bacteria, thus causing growth competition. The different agitation (60, 80, 100, 120, 140 and 160 rpm) affected to collagenolytic bacterial growth. The optimum growth of it was speeds at 100-120 rpm. The treatment of osmotic pressure difference was the difference of NaCl concentration, such as 1, 2, 3, 4, and 5%. It was revealed that the isolates were able to grow to a concentration of 4%. This was based on the modified method proposed by Rao and Narasu (2007) stating that bacterial growth requires the NaCl concentration by 1, 2, 3, 4, 5, 6 and 7. The maximum enzyme activity was found at 215 U/ml.

Analysis of the Base Sequence of 16S rRNA Genes

According to Clarridge III (2004), the sequence of 16S rRNA genes has been determined for many strains. GenBank as the largest nucleotide sequence data bank has more than 20 million nucleotide sequences and nearly 90,000 of them are 16S rRNA genes. This indicates that many of the previously stored nucotide sequences are used to compare the sequence of a new strain discovered. Besides, 16S rRNA genes have universal properties in bacteria so that they can be used to analyze the family relationship between bacteria from the genus level of various phyla to the strain level, namely species and subspecies. The base sequence is presented in Figure 1.

The nitrogenous base sequence of isolates and that of strains used as a reference or as a comparison were used to be analyzed to determine the family relationship in the form of a phylogenetic tree. The phylogenetic tree obtained from the analysis results is presented in Figure 2.

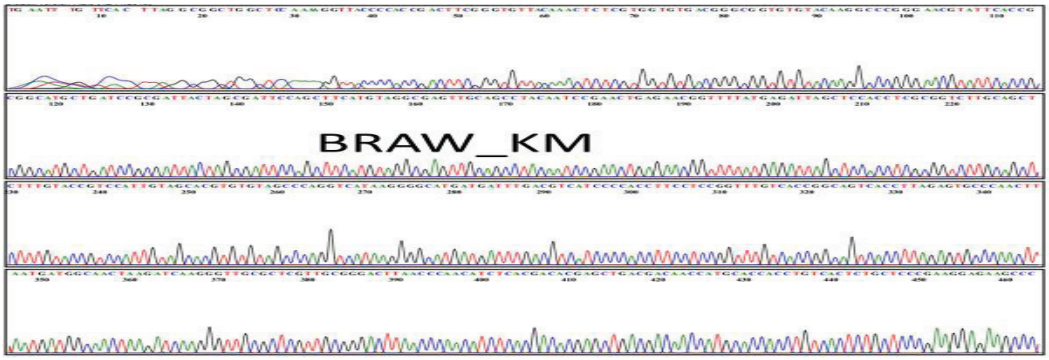

Figure 1. The Base Sequence of the isolates of BRAW_KM

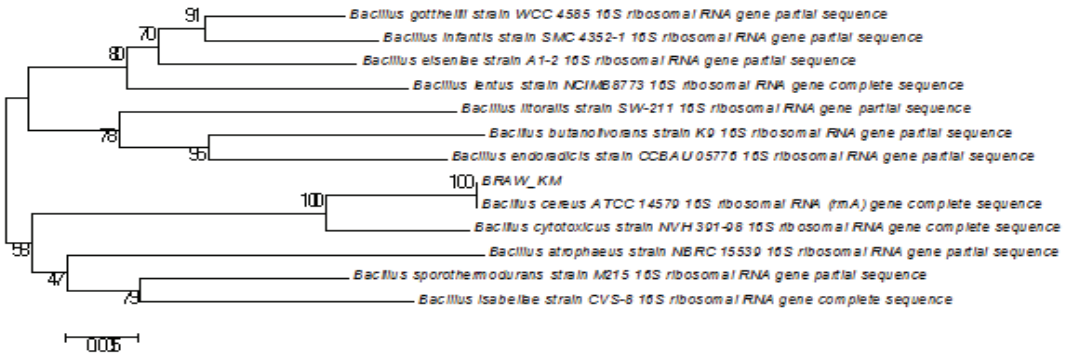

Figure 2. The Phylogenetic Tree of Bacteria BRAW_KM

Based on the obtained phylogenetic tree, it is revealed that BRAW_KM has a very close family relationship with the family *Bacillaceae,* i.e. with *Bacillus cereus*, as indicated by the value of similarities by 100%.

CONCLUSIONS

1. BRAW_KM isolates will have their optimum performance at a temperature between 31 and 33ºC; the optimum pH ranges from 8 to 9; the optimum quantity of substrates is equal to 15g/l; the osmotic pressure is equal to 4%; the optimal speed ranges from 100 to 120 rpm; and the inoculum concentration is equal to 8%.
2. The collagenase activity of bacteria BRAW_KM reaches 1,029 U/mg.
3. They have a very close family relationship with the family *Bacillaceae*, i.e. with *Bacillus cereus*, as indicated by the value of similarities by 100%.

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