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## Research Article

# Screening and Characterization of Keratinolytic Bacteria from Puffer Fish Skin Waste

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## Abstract

**Background:** Puffer fish skin tannery is an alternative to substitute the production of hide and animal skin in Indonesia that has been decreasing. To improve the quality of puffer fish leather, keratinase was needed by removing the thorns. **Objective:** The aim of this study was to screen bacteria that show keratinolytic activity. **Methodology:** The isolated bacterial strains were screened for the production of extracellular keratinase using skim milk agar. After incubation, the formations of a clear zone around the bacterial growth were observed. They were identified based on morphological enzyme activity and molecular identification. Bacterial screening and identification were conducted with descriptive method. The microbial activity tests were analyzed using a completely randomized design. **Results:** The result of the screening was that 3 of 5 kinds of strains exhibited caseinolytic activity (by showing the clear zone). Three *Bacillus* bacteria that were newly isolated from puffer fish waste-using a feather enrichment technique-were identified on the basis of 16S ribosomal RNA gene sequence analysis, physiological and carbohydrates assimilation tests. They were revealed as the strains of *Bacillus thuringiensis* for BRAW\_PT isolate, *Bacillus aerius* for BRAW\_PB isolate and *Bacillus firmus* for BRAW\_PI isolate. The results of proteolytic enzymes assay showed that *Bacillus firmus*BRAW\_PI has the highest protease and keratinase activity, which was  $37.52 \pm 0.96$  and  $6.781 \pm 0.479$  U mg<sup>-1</sup> consecutively. **Conclusion:** All bacteria obtained were the superior bacteria that can be used for the removal of thorns from puffer fish skin in the tanning process.

**Key words:** Characterization, enzyme, keratinolytic, microbia, pufferfish, screening, skin waste

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Leather and its processing industry is one of the leading industries in Indonesia<sup>1</sup>. It is reflected in the large contribution of the industry to national fund. Although the leather industry is considered as important field, there are many issues, which have to improve by the government, business people and other stakeholders, including higher education institutions.

One of the issues in leather industry is limited fresh leather material. Indonesia has a shortage of material supply to fulfill domestic demands and then the amount of import of raw- and finished-leather material keeps increasing in order to fulfill domestic demands of them<sup>2</sup>. The rarity of leather material is experienced by industry players since Decree of The President of The Republic of Indonesia No. 46/1997, which states that raw material can only be imported from countries free from contagious animal diseases included in the A list of Office International des Epizootis. Since the regulation was implemented, an animal product imported to Indonesia has been under strict monitoring to prevent contagion or entry of contagious diseases, such as foot and mouth disease and rinderpest. Government Regulation of The Republic of Indonesia No. 82/2000 Article 30 on Animal Quarantine authorizes rejection if an animal comes from forbidden country or area<sup>3</sup>. Considering the size of Indonesia's waters is bigger than the land, which is around 5.8 million km<sup>2</sup>, fishery production in Indonesia is quite large and so is the number of fish type (commodity) caught<sup>4</sup>.

Puffer fish (*Arothron reticularis*) is an underutilized fish in any fishing. It can be superior product for the surrounding communities. One of the utilization of puffer fish skin is for tannery business. The tanning of puffer fish leather can be an alternative to tanning industry that is currently limited to the production of cattle leather. Fish tanning business doesn't only give value added to leather waste but also become an alternative in fulfilling leather material in the leather industry in Indonesia. Additionally, puffer fish also has a uniqueness that lies in its rounded body shape<sup>5</sup>.

The quality of leather from puffer fish can be improved by removing its spines. One of the ways to destroy the spines is to degrade the protein keratin in it with the keratinase enzyme. Keratinase is important for the pre-tanning process in leather industry so the skin tanning can be an eco-friendly process by reducing the use of sodium sulfate<sup>6</sup>.

Keratinase that is produced by microbes is an enzyme capable of degrading the structural protein that is generally found in feathers, hair and wool. The types of bacteria that have been screened to produce extracellular keratinase that can degrade fur and keratin, are for example *Bacillus*

*licheniformis*, *Bacillus subtilis*, *Bacillus cereus* and *Bacillus pseudofirmus*<sup>7</sup>. Therefore, this study was performed to screen and to characterize the keratinolytic bacteria as one of the innovative solutions in tannery processing and become an interesting major to be developed and studied in depth.

## MATERIALS AND METHODS

**Materials:** Materials used in this research were 30 pieces of 14-days-rotten puffer fish skin. The skin was taken randomly from the fish market in TPI Tangjungsari of Marine and Fisheries Agency of Rembang. It is used as raw material for the isolation and identification of microbes.

The growth medium for the isolation process was the same as the one used by Macedo *et al.*<sup>8</sup> with modification: Puffer fish skin flour as the sole carbon and nitrogen sources as much as 10 g and as mineral sources are: 0.5 g of NaCl, 0.3 g of K<sub>2</sub>HPO<sub>4</sub> and 0.4 g of KH<sub>2</sub>PO<sub>4</sub>. The medium for the stock solution includes 1 g of yeast extract, 1 g of biological peptone, 0.5 g of NaCl and 100 mL of H<sub>2</sub>O.

The materials for microbial identification are: (a) Simple staining including 70% alcohol, safranin, Gram staining including, gram A (crystal violet), gram B (Lugol iodine), H<sub>2</sub>O, gram C (acetone alcohol washing solution), gram D (safranin), Ziehl Neelsen's stain includes ZN A carbol fuchsin solution as the primary stain, ZN B acid alcohol solution (37%) as a laxative and ZN C Loeffler's methylene blue solution. The materials for molecular identification consist of tris-HCl, NaCl, EDTA, SDS, proteinase K, phenol, chloroform, TE, ethanol, RNAase, PCR extraction kit (Genaid), sequencing kit, primary ABI PRISM 3730xl Genetic Analyzer develop by Applied Biosystems, USA. Kit that was used for the sequencing purpose is BigDye<sup>®</sup> Terminator v3.1.

The equipments used in this research are: HIRAYAMA Vertical Autoclaves autoclave (Tokyo, Japan), LABCONCO Horizontal laminar air flow (Kansas, USA), MEMMERT oven (Schwabach, Germany), MEMMERT water bath (Schwabach, Germany), SOCOREX macro and micropipettes (Socorex Isba S.A. Switzerland), HANNA pH meter (Michigan, USA), THERMOLYNE stirrer (Atlanta, USA), OHAUS analytical balances (Shanghai, China), MOTIC test tubes, loopful (Wertheim-Mondfel 97877, Germany), microscopes MOTIC Wertheim-Mondfel 97877, SNE 4500M SEM microscope-Pleasanton, CA, PERKIN ELMER Lambda 25 UV/Vis Spectrophotometer (Waltham, MA 02451, USA), ROTOFIX 32 coolbox and centrifuge (California, USA), EPPENDORF 5804R refrigerated centrifuge (California, USA), PCR BIO-RAD T100 Thermal Cycler sequencer Applied Biosystems, (USA), STUART shaker (Staffordshire, UK), Glove Box (China),

MEMMERT (Schwabach, Germany) and HERAEUS incubators (UK/Ireland), IWAKI glasswares, (Fukushima, Japan) and HERMAGlasswares, (Germany) glasswares, such as Erlenmeyer, petri dishes, glass slide, bunsen, glass beaker, measuring glass and flask. All reagents were of analytical reagent grade.

**Screening of keratinolytic bacteria:** The isolated bacterial strains were screened for the production of extracellular keratinase using skim milk agar medium. The pure cultures were streaked on the skim milk agar plates and the plates were incubated at 37°C for 48 h. After incubation, the formations of a clear zone around the bacterial growth were observed<sup>9</sup>.

**Caseinolytic activity:** Skim milk agar medium was sterilized at 121°C for 15 min at 15 lbs pressure. The isolates were streaked on the medium. The zone formed around the colonies due to the production of caseinase enzyme was considered as a positive result. The organisms screened with skim milk agar medium were subcultured by growing the bacterium in nutrient broth medium at 37°C for 24 h<sup>10</sup>.

**Morphological test:** The identification of bacteria can be conducted by viewing the colony morphology both macroscopically and microscopically, including simple, Gram, Zn and spore staining as well as viewing on the bacterial biochemical test. The morphology of bacteria includes the shape, size, texture, colony color and motility. The biochemical test was conducted to ensure the species of bacteria, it included the test of catalase, oxidase and fermentation of carbohydrates.

**Production of keratinolytic enzymes:** The enzyme production was based on the method of Hoq *et al.*<sup>11</sup> with slight modification. Each of the isolates was cultivated in a basal medium (per liter of the solution: NaCl, 0.5 g; 0.06 g; K<sub>2</sub>HPO<sub>4</sub>, 0.3 g; K<sub>2</sub>HPO<sub>4</sub>, 0.4 g) containing keratinous substrates: (10.0 g of puffer fish, pH 7.5) as the only source of nitrogen, carbon and sulfur. The supplementation of 1.0 g of nitrogen (yeast extract) and 1.0 g of carbon sources (bacteriological peptone) together was also tested in presence or absence of keratinous substrates under identical conditions. Cultivation was done with 5 mL of 24 h grown inoculum (in nutrient broth) of the respective bacterial cultures (100 mL) on the liquid medium in a 500 mL Erlenmeyer flask at 37°C under shaking (120 rpm) for 24 h. The samples were selected after 24 h and centrifuged at 4500 rpm at 6°C for 20 min. The supernatants were preserved at 4°C and assayed for protein and enzymes.

One pure isolate was cultured in 5 mL of sterile Pre-culture medium (Stock solution) and incubated at 30°C for 48 h in a shaker incubator at 120 rpm. After incubation, the broth was centrifuged at 4500 rpm for 20 min and the supernatant was used to study the keratinolytic activities.

**Protease activity:** Protease activity was determined with the method of Bergmeyer *et al.*<sup>12</sup> One unit (U) of proteolytic enzyme activity is defined as the amount of enzyme that releases 1 μmol tyrosine per milliliter per minute at standard test condition. Specific activity is stated in enzyme activity unit per milligram of protein.

**Keratinolytic activity:** Keratinolytic activity was determined with the method of Wang *et al.*<sup>13</sup>. The substrate used was keratin azure. The keratinase enzyme activity was measured by using several types of keratin substrates such as feather flour,<sup>14-16</sup> and keratin azure.<sup>17,18,13</sup> Keratin azure is pure keratin derived from wool that is stained with azo dyes. Keratin azure that is added with water and is reacted with specific enzyme will produce a blue-colored reaction product. The amount of keratinase enzyme activity is determined by Unit, where one unit (U) of keratinase activity is defined as the amount of enzyme required to improve the absorbance of 0.01 between the sample and the control at a wavelength of 595 nm in accordance with the test conditions<sup>19,13</sup>

**Scanning Electron Microscope (SEM):** The fixation solution was made by using 0.2145 g of sodium cacodylic, 1.0081 g of NaCl, 630 μL of HCl 0.2 M, 1 mL of glutaraldehyde and was added with H<sub>2</sub>O for a volume of 100 mL using a Volumetric flask. After all of the ingredients were dissolved and mixed together until the mixture was homogenous, the solution was put into a dark bottle and stored in the refrigerator.

Agar medium was made using 3.5 g of agar, 0.1 g of gelatin and added with 1/100 stock solution for dilution that consisted of 1 mL of stock solution added with 99 mL of H<sub>2</sub>O. The 100% stock solution was made by putting 1 g of meat extract, 1 g of microbiological peptone, 0.5 g of NaCl and 80 mL of H<sub>2</sub>O into a glass beaker. It was then stirred well until it dissolved evenly and the pH was adjusted to 7.2. If it is too acidic, it can be added with 0.1 N of NaOH and if it is too alkaline, it can be added with 0.1 N of H<sub>2</sub>SO<sub>4</sub>. Then, the solution was poured into the Erlenmeyer and added with H<sub>2</sub>O to get 100 mL volume. It was then boiled on the stove and stirred constantly until it dissolved evenly. After that, it was sterilized with an autoclave.

The sterilized agar medium was then poured into the petri dish. A special filter paper that had been cut to the size of 5×5 mm (previously sterilized) was put on the un-ossified agar and we waited until it was cool and ossified. The isolates which had previously been grown on preculture medium and shaken for one night was taken 1 µL and dripped on the filter paper that had been placed on the agar, then it was incubated for 4 days.

Filter paper that had been overgrown by isolates on the agar was then taken and put in microcentrifuge tube, added with 1 mL of fixation solution and incubated at 4°C for 30 min. The fixation solution was then taken using a pipette until it was empty, added with 1 mL of acetone 60% and incubated at room temperature for 15 min. The acetone 60% was then taken until it was empty and replaced by 1 mL of acetone 80%. The acetone 80% was then taken until it was empty and replaced by 1 mL of acetone 100%, the process with acetone 100% was repeated twice. After the acetone had been removed and replaced by 1 mL of pentyl acetate and stored at room temperature for 20 min, it was dried using filter vacuum. When the fluid was gone, the sample had reached the critical point drying. It was then coated and ready to be observed using a SEM<sup>20</sup>.

**Molecular identification:** The molecular identification was conducted using the 16S rRNA gene sequence. The sequencing of 16S-rRNA gene consisted of several stages, including DNA extraction, amplification between 16S-rRNA gene and PCR, then sequencing using ABI PRISM 3730xl Genetic Analyzer developed by Applied Biosystems, USA. Kit that we use for the sequencing purpose is BigDye® Terminator v3.1 .

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

**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. The data of microbial activity obtained were described as the Mean ± SD. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple-Range (DMR) test using software SPSS Inc., (Chicago, IL, USA). Differences considered significant when the probability was less than 5%.

## RESULTS

Totally five isolates were found from puffer fish waste. All the isolates were subjected to primary screening on Milk Agar plate and 3 of 5 isolates formed the clear zone, which supported the degradation and utilization of casein (Skim Milk) by the respective isolates. Those organisms were named as BRAW\_PT, BRAW\_PB and BRAW\_PI strains (BRAW-Buntal Rembang Ari Wibowo). The macroscopic observation on bacterial isolates is shown in Table 1.

They capable of growing and degrading puffer fish skin at 35°C within 14 days. The BRAW\_PT, BRAW\_PB and BRAW\_PI strains which appeared single or in the chain had straight rods. They were Gram-positive, endospore-forming organisms, aerobic, motile, strong oxidase and catalase positive. The additional morphological, physiological and biochemical test are shown in Table 2.

**Caseinolytic activity:** Table 3 shows the results of clear zone diameter in 72 h of observations, BRAW\_PT strains showed the largest colony diameter (3.06 mm), followed by BRAW\_PI strain (2.40 mm). The smallest colony diameter (1.34 mm) belonged to BRAW\_PB strain. The results of clear zone diameter and the diameters of colonies from the largest to the smallest were then sorted and tested to determine the protease activity.

**Proteolytic activity:** The results of proteolytic enzyme assay showed that BRAW\_PI had a specific activity of

Table 1: Observation of keratinase bacterial isolates

Isolate codes	Colony colors	Colony shapes
1	Milk (Ps)	Round, convex, glossy
2	White (Pb)	Round, convex, wavy
3	Sharp-white (Pt)	Round, convex, jaggy
4	Viscous-white (Pk)	Round, convex
5	Pink (Pi)	Round, convex, jaggy

Table 2: Morphology of keratinolytic isolates

Morphology	Bacteria strains		
	BRAW_PT	BRAW_PB	BRAW_PI
Catalase	Positive	Positive	Positive
Oksidase	Positive	Positive	Positive
Upright media	Rhizoid	Beaded	Beaded
Slant media	Echinulate	Echinulate	Echinulate
Elevation	Convex rugose	Convex	Convex papillate
Deep structure	Opaque	Opaque	Finely granular
Colony form	Curied	Circular	Circular
Motility	Positive	Negative	Negative
Spore staining	Positive	Positive	Positive
Gram staining	Positive	Positive	Positive
Acid staining (Zn)	Acid negative	Acid negative	Acid negative
<b>Carbohydrate fermentation</b>			
Glucose	Positive	Positive	Positive
Fructose	Positive	Positive	Positive
Sucrose	Positive	Positive	Positive
Lactose	Positive	Positive	Positive

Table 3: Diameter of clear zone

Bacterial strains	Incubation time		
	24 h	48 h	72 h
BRAW_PT	0.242±0.01 <sup>a</sup>	2.029±0.01 <sup>b</sup>	3.063±0.06 <sup>c</sup>
BRAW_PB	0.000±0.00 <sup>a</sup>	1.018±0.01 <sup>b</sup>	1.339±0.03 <sup>c</sup>
BRAW_PI	0.058±0.07 <sup>a</sup>	1.992±0.01 <sup>b</sup>	2.404±0.04 <sup>c</sup>

<sup>a-c</sup>Different superscripts in the same column indicate significant differences (p<0.05)

Table 4: Proteinase enzyme activity

Strains	Enzyme activity (U mg <sup>-1</sup> )
BRAW_PT	33.44±0.56 <sup>a</sup>
BRAW_PB	32.49±1.18 <sup>a</sup>
BRAW_PI	37.52±0.96 <sup>b</sup>

<sup>a,b</sup>Different superscripts in the same column indicate significant differences (p<0.05)

Table 5: Keratinase enzyme activity

Strains	Enzyme activity (U mg <sup>-1</sup> )
BRAW_PT	4.867±0.326 <sup>a</sup>
BRAW_PB	4.349±0.634 <sup>a</sup>
BRAW_PI	6.781±0.479 <sup>b</sup>

<sup>a,b</sup>Different superscripts in the same column indicate significant differences (p<0.05)

37.52±0.96 U mg<sup>-1</sup> (Table 4). This was the highest activity of the three isolates. The test showed significantly different results among the treatments. A further test using Duncan's Multiple Range Test (DMRT) showed that BRAW\_PI had a significantly different proteolytic enzyme activity (p<0.05) than other isolates. Proteinase enzyme activity of BRAW\_PT and BRAW\_PB had no significant differences (p<0.05).

**Keratinase activity:** Keratinase activity assay was carried out on all of 3 isolates. The test results of keratinase activity are shown in Table 5. Based on the Table 5, BRAW\_PI strain had the highest (6.78 U mg<sup>-1</sup>) specific keratinase activity. The

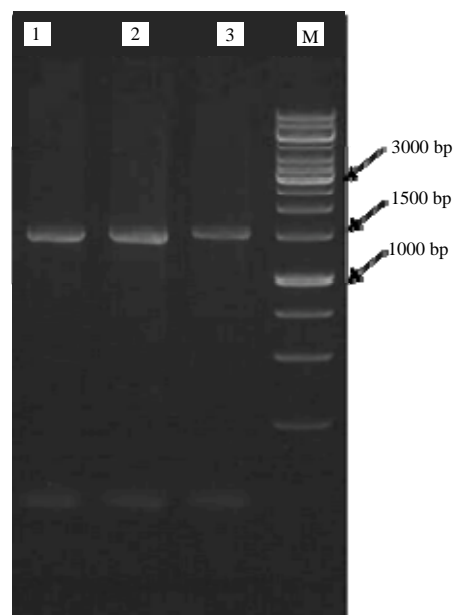


Fig. 1: Agarose gel electrophoresis of amplified products

Lane 1: BRAW\_PI, Lane 2: BRAW\_PB, Lane 3: BRAW\_PT, M: Broad range marker

BRAW\_PI had a significantly different proteolytic enzyme activity (p<0.05) than other isolates. The proteinase enzyme activity of BRAW\_PT and BRAW\_PB had no significant differences (p<0.05).

**Molecular taxonomy, sequencing and phylogenetic analysis:**

Results of PCR with 16S rRNA primer was then viewed by electrophoresis on a 0.8% agarose gel as shown in Fig. 1. The isolates had a very close genetic relationship with *Bacillaceae* family based on the 16S rRNA gene sequencing method. BRAW\_PT was closed to *Bacillus thuringiensis* by showing 99% similarity, BRAW\_PB was closed to *Bacillus aerius* by showing 99% similarity and BRAW\_PI was closed to *Bacillus firmus* by showing 99% similarity. The phylogenetic tree (Fig. 2) was constructed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis 6 (MEGA6) program.

**DISCUSSION**

Skim milk contains casein, a milk protein which will be degraded by proteolytic microorganisms into dissolved nitrogen compounds so the colony will be surrounded by a clear area. It showed that these microbes had proteolytic activity<sup>21</sup>. Based on this test, there were only three isolates that had the ability to degrade casein isolates while the other two did not.

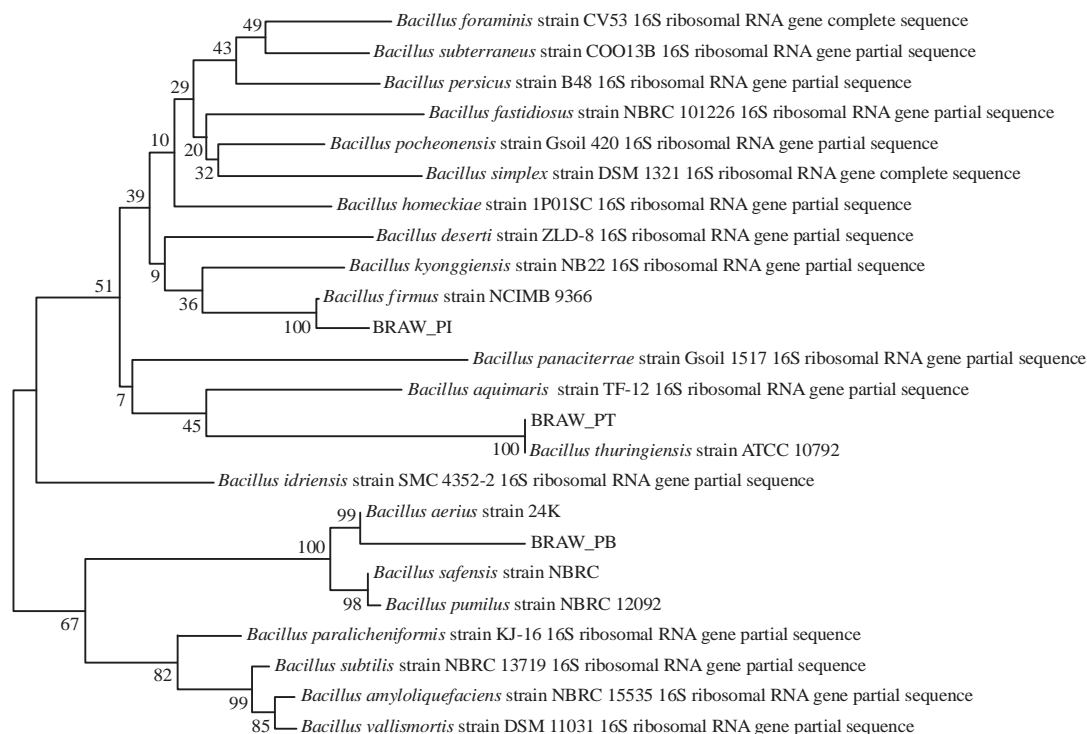


Fig. 2: Phylogenetic tree of three isolates

Three of five isolates that showed protease activity were later corroborated by the skim milk agar and they were used for further research. Sivakumar *et al.*<sup>22</sup> confirmed that the zone formed around colonies was due to the formation of the casein enzyme. It was considered as a positive result. The caseinolytic ability of bacteria could be used to select the initial keratinolytic bacteria because most keratinolytic bacteria that derived from nature also had a good caseinolytic activity. The results obtained showed that the BRAW\_PB, BRAW\_PT and BRAW\_PI isolates were able to degrade the casein because casein is the main protein in milk. Benson<sup>23</sup> stated that the media became clear due to the caseinase exoenzymes produced by bacteria.

Based on the method of Gupta and Ramnani<sup>9</sup>, the chosen casein agar media was related to the most reported keratinase enzyme derived from nature. The isolates were grown in an incubator at 30°C for 72 h. The statistical analysis showed that there were significant differences among the treatments. Duncan's Multiple Range Test (DMRT) showed that the clear zone diameter of all isolates had been significantly affected by the incubation time. All isolates had the largest clear zone diameter in 72 h. It can be seen in Table 3.

Brandelli *et al.*<sup>24</sup> stated that the hydrolysis ability of casein depended on the species and environment of the bacterial isolation place. Furthermore, in order to ensure the ability of

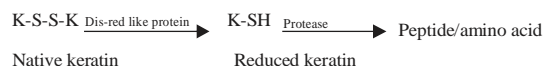
bacteria in hydrolyzing protein, a protease activity test was conducted. The screening was conducted on 3 isolates using qualitative (the formation of the clear zone) and quantitative (enzymes activity) methods<sup>25</sup>.

Protease is also called peptidase or proteinase. It is a hydrolase-class enzyme that will break down proteins into simpler molecules such as short oligopeptides or amino acids, with hydrolysis reaction on the peptide bond. Proteolytic bacteria are the bacteria that are able to produce extracellular protease enzyme. The enzyme breaks protein that is produced in the cell and releases it out of the cell.

Some researcher found keratinase activity in some bacterial enzymes such as *Bacillus subtilis* that has keratinase activity<sup>26</sup> of 1.8 U mL<sup>-1</sup>, *Aspergillus sp.* 1.7 U mL<sup>-1</sup>, *Cladosporium sp.* 1.9 U mL<sup>-1</sup>, *Periconia sp.*<sup>27</sup> 1.5 U mL<sup>-1</sup> as well as *Streptomyces gulbargensis* that has keratinase activity<sup>18</sup> of 1.5 U mL<sup>-1</sup>. Nevertheless, the keratinase activity of those three strains is lower than the one produced by *Bacillus megaterium*<sup>28</sup> F7-1 by 58 U mL<sup>-1</sup>, *Chryseobacterium sp.*<sup>29</sup> by 40 U mL<sup>-1</sup> and *Flavobacterium sp.*<sup>30</sup> by 7 U mL<sup>-1</sup>.

Some keratinolytic microorganisms have been reported, including several species of fungi such as *Microsporium*<sup>31</sup>, *Trichophyton*<sup>32</sup>, *Streptomyces*<sup>33,34</sup> and *Actinomyces*<sup>35,36</sup>. Recently, keratinase activity was also reported for coccus that was rod-shaped Gram-positive.

Yamamura *et al.*<sup>37</sup> reported a joint action between the similar protein disulfide reductase and proteases produced by *Stenotrophomonas sp.* to degrade deer-hair keratin. The mechanisms of keratin degradation occurred as follows:



There were two types of morphological forms of microbes in the form of short bacilli, while four types of microbes are long-shaped bacilli. The 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 results of amplification of the encoding 16S rRNA gene of bacterial isolate were then determined for its DNA base sequences. The process of determining the base sequence of DNA was conducted by 1st BASE, Malaysia. The results of the determination was then read with DNA baser. Then, the DNA's base sequence obtained was used to search for the comparison of DNA sequence in various similar microorganisms or those that have a close genetic relationship to the NCBI (National Center for Biotechnology Information) GenBank through the BLAST (Basic Local Alignment Search Tool) method (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The results of the matching using BLAST method was then selected to find the one that had the closest genetic relationship and sequenced for the phylogenetic tree using the Molecular Evolutionary Genetics Analysis 6 (MEGA6) program. The sequence of 16S rRNA gene has been determined for many strains. Genbank is the largest data bank for nucleotide sequences, saving over 20 million nucleotide sequences and almost more than 90.000 of them are the 16S rRNA gene. It shows that many previously saved nucleotide sequences are compared with the sequence of a newly known strain. In addition, the universal 16S rRNA gene in bacteria can be used to analyze the phylogenetic relationship between the bacteria from the genus level of many phyla to the level of strains that are species and subspecies.

The genetic relationship of bacteria was known from the base sequence analysis of 16S rRNA gene nitrogen. The base sequences of the isolates' nitrogen and the base sequences of the reference strain nitrogen or comparators were used to analyze the genetic relationship in the form of a phylogenetic tree. The bootstrap value was indicated by the number contained in the branches of the phylogenetic tree. According to Hall<sup>38</sup>, the higher value bootstraps, the more reliability or trustworthiness. The results of phylogenetic tree analysis are shown in Fig. 2.

Based on the formed phylogenetic tree, all isolates had a very close genetic relationship with different species of Bacillaceae family. BRAW\_PT was close to *Bacillus thuringiensis* with 99% similarity, BRAW\_PB was close to *Bacillus aerius* with 99% similarity and BRAW\_PI was close to *Bacillus firmus* with 99% similarity.

## CONCLUSION

The results of the screening showed that 3 of 5 bacterial strains presented keratinolytic activity. They are *Bacillus thuringiensis* BRAW\_PT, *Bacillus aerius* BRAW\_PB and *Bacillus firmus* BRAW\_PI. The proteolytic enzyme test showed that BRAW\_PI bacterial strain had the highest protease and keratinolytic specific activity, which was  $37.52 \pm 0.96$  and  $6.781 \pm 0.479$  U mg<sup>-1</sup> consecutively.

## SIGNIFICANCE STATEMENTS

The study managed to discover bacteria with the ability to degrade keratin of pufferfish skin, which is helpful for an environmentally friendly tanning process. The bacteria that discovered in this study was the result of independent isolation. The innovation of bacteria with keratinolytic activities from decaying pufferfish's skin has never been conducted before. It also could use to process on pufferfish skin tanning which also never been conducted before. This study will help the researcher to overcome environmental pollution resulting from tanning processes which have not been widely studied by other researchers. 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.

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