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ISOLATION, IDENTIFICATION AND DEHAIRING ACTIVITY OF INDONESIAN NATIVE KERATINOLYTIC BACTERIA *EXIGUOBACTERIUM* SP. DG1

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ABSTRACT

The objective of this work was to isolate and identify keratinolytic bacteria from soil containing sheep hair and sheep tannery waste, which has the potential of removing hair in the tanning process. The bacterial identifications were conducted using 16S rRNA gene sequencing method. Also the bacteria were examined using morphological and biochemical studies. To determine the ability of the enzymes, the study used casein hydrolysis, keratinase, collagenase, and dehairing activity test. The experimental research was conducted in the laboratory applying a completely randomized design and was analyzed using Duncan's New Multiple Range Test. The results showed that the isolate number one has the highest casein hydrolysis (2.88) and keratinolityc activity (2.87 U/ml) but performed the lowest collagenase activity (0.59 U/ml). This isolate was identified as *Exiguobacterium* sp. DG1 (LC008359), which has potential use for biotechnological process to remove hair in the leather tanning process.

Keywords: Isolation, identification, dehairing, keratinolytic bacteria, *Exiguobacterium* sp. DG1

INTRODUCTION

Keratin is the main protein element in the hairs. Keratin is difficult to degrade because it has a dense arrangement of polypeptides which is stabilized by several hydrogen bonds and hydrophobic interactions as well as a disulfide bond. Composition and molecular configuration of amino acids make keratin structure strong and rigid. Keratin polypeptide chains form α-helices in the hair and wool as well as β-sheet in a feather. Cross-linking of the protein chains by cystine bridges lead to increase keratin mechanical stability and could not be hydrolyzed by regular proteolytic enzyme (Kreplak et al., 2004).

Keratin does not accumulate in nature even though it has strong stability. In nature, keratin can be degraded by microorganism such as keratinolytic bacteria (Brandelli, 2008). These bacteria generally produce extracellular keratinase enzyme, if they are grown on keratinous substrates. Keratin substrate is hydrolyzed by the keratinase enzyme of microbes due to the reduction of disulfide bonds in keratin (Brandelli et al., 2010). Keratinase hydrolyze hair substrate started by breaking disulfide bonds. Then disulfide bonds (SS) is converted into a thiol group (SH) and dithio compound such as dithiodiglycolic acid and cystine (Ogawa et al., 2008).

Keratinase enzymes are widespread in nature which generally produced by bacteria isolated from poultry waste. Some feather degrading bacteria which have been isolated from soil and poultry waste are largely classified into *Streptomyces* and *Bacillus* group. However, a variety of bacteria which could degrade feather have been reported by researchers, such as a gram-positive bacteria isolates like *Arthrobacter* sp. (Lucas et al., 2003), *Microbacterium* sp. and *Kocuria rosea* (Bernal et al., 2003). In addition, some bacteria have been isolated from the hair contains environment such as *Bacillus subtilis* isolated from cow hair, leather waste, and soil (Macedo et al., 2005) as well as *Stenotrophomonas* sp. D1 which was isolated from soil containing deer hair (Yamamura et al., 2002).

In this study, a novel keratinolityc bacteria was isolated from soil containing Garut sheep hairs at traditional Garut sheep farms and sheep tannery waste. Garut sheep is a native sheep in Indonesia which are kept for fancy and meat production. The sheep are produced in semi-intensive and intensive production systems. The sheep

hair substances in Garut Sheep farm areas are abundant because the farmers usually cut their sheep' hairs in farm areas.

The present study described the characteristics of the bacteria based on molecular identification by 16S rRNA method, morphology and biochemical reactions. The studies also determine the abilities of the enzyme which were measured using casein hydrolysis, keratinase, collagenase, and dehairing activities test.

MATERIALS AND METHODS

Isolation and Characterization of Bacteria: Keratinolityc bacteria were isolated from soil containing Garut Sheep hairs and waste of sheep tannery. The samples were collected in Garut District, West Java Province, Indonesia. In order to select the keratinolityc bacteria, samples were suspended and cultivated in a sheep hair meal (SHM) medium ($10gl^{-1}$ SHM as the sole carbon and nitrogen source; $0,5gl^{-1}$ NaCl; $0,3gl^{-1}$ K_2HPO_4; $0,4gl^{-1}$ KH_2PO_4; pH 7,5). They were incubated in refrigerated incubator at 25°C for 7 days with constant shaking at 180 rpm.

SHM agar plates medium spread with 0,1ml of each dilution from the proper concentrations, plates were incubated at 25°C for 48 hours. SHM agar contains $10gl^{-1}$ SHM; $0,5gl^{-1}$ NaCl; $0,3gl^{-1}$ K_2HPO_4; $0,4gl^{-1}$ KH_2PO_4; and $1,5gl^{-1}$ bacteriological agar. Singles colonies that showed different appearance were isolated and plated on SHM agar plates. These steps were repeated until pure isolates obtained. Pure isolates transferred to media contain skim milk $15gl^{-1}$, $0,5gl^{-1}$ NaCl; $0,3gl^{-1}$ K_2HPO_4; $0,4gl^{-1}$ KH_2PO_4; bacteriology agar $15gl^{-1}$. It was incubated at 25°C for 48 hours. Colonies showing protease activity then tested for further analysis.

Taxonomic Studies: The taxonomic studies used molecular analysis based on 16S rRNA. The genomic DNA was extracted from the cells after cultivation of 24 hours using Genomic DNA Mini Kit (Geneaid Cat.No.GB100, Genaid Biotech Ltd, Taiwan) according to manufacturing instructions. The 16S rRNA gene was amplified by PCR using the primers 9F (5'-GAG TTT GAT CCT GGC TCA G-3'), and 1541R (5'-AAG GAG GTG ATC CAG CC-3'). Then the amplified DNA was purified by Gel/PCR DNA Fragments extraction kit. The Big Dye Terminator Cycler Sequence Kit and a 310 DNA sequencer were used for sequencing 16S rRNA. The homologous sequences in Gene Bank were analyzed by Basic Local Algorithm Search Tool (BLAST). Molecular phylogenetic studies of the 16S rRNA were conducted using Molecular Evolutionary Genetic Analysis (MEGA) (Zhang et al., 2009). The mor-phological and biochemical characteristic of the isolated bacterium were examined to confirm the test result.

Production and Enzyme Assay: Keratinase production was carried out in the following basal medium containing NaCl, $0.5gl^{-1}$, K_2HPO_4, $0.3gl^{-1}$, KH_2PO_4, $0.4gl^{-1}$, and SHM $10gl^{-1}$ as sole source of nitrogen and carbon. Then the bacteria were removed by centrifuged at 10,000g for 15 min at 4°C. The supernatants were used for crude enzyme preparation.

The Keratinolytic activities were determined using modified method of Wang et. al. (2008) by which keratin azure (K-800, Sigma Aldrich, USA) was used as insoluble substrate. The enzyme samples (500µl) were incubated in a solution of 5mg of keratin azure in 500µl of 50mM sodium phosphate buffer for 30 min at 30°C. Reaction mixture was stopped using 10% TCA. Subsequently, the tubes were centrifuged at 13.000g for 5 min and the absorbance of the supernatant was measured at 595nm. Control samples were prepared in a similar manner except the enzyme was replaced by sodium phosphate buffer. The assays were conducted in four replicates. One enzymatic unit was defined as the amount of enzyme resulting in an increase in absorbance at 595nm of 0.01 after reaction at 30°C for 30 min with keratin azure.

The collagenase activities were determines by modified method of Pillai and Archana, (2008) using pure collagen as substrate prepared in 50mM Tris-Cl buffer pH 8.0. The enzymes samples (0.2ml) were incubated in a solution of 0.4ml substrate in 0.4ml buffer for 30 min at 30°C. Reaction mixture was stopped using 10% TCA. Absorbance of supernatant collected after centrifugation at 12.000g for 10 min was measured at 520nm. One unit was defined as the amount of enzyme that increased in

0.1 absorbance under the condition described.

Dehairing assay of sheep skin: Dehairing of sheep skin was performed using the dip method, modified from Shrinivas and Naik (2011). Skin pieces of approximately 5X5cm (18-19g) were dipped in the crude enzyme preparation and incubated at room temperature for 24 hours. Skins were also incubated in aquades under the same condition as control. Skins were removed and analyzed for dehairing activity by gently scraping with forceps and change of color after incubation was observed.

Data analysis: This research was conducted using descriptive and experimental methods. Experimental research in the laboratory used a completely randomized design (CRD) and effect of treatment was performed using analysis of variance and the differences between treatments were analyzed using Duncan's Multiple Range Test. All the experimental research was repeated four times.

RESULTS AND DISCUSSION

Isolation of Keratinolytic Bacteria: In this research, the keratin utilizing bacteria were isolated from soil in Garut Sheep farm and waste in sheep tannery. Fifty six bacteria colonies were isolated in which 34 colonies came from soil and 22 colonies came from waste. The selection based on the appearance of the colonies obtained 16 different colonies. The bacteria colonies had different colors, predominantly white color, while some bacteria produce pigments of other colors such as yellow, orange, and beige. Bacterial colonies were mostly round with a convex elevation, whereas the colony size ranged from 0.25mm to 2.00mm. Observations using a microscope with a magnification of 1,000 times showed that these bacteria were rods and cocci.

Skim milk agar with minimal mineral was chosen as a medium to test the ability of the bacteria to hydrolyze casein. The test showed that four isolates of these bacteria were capable of degrading casein. Casein hydrolyze ability test could be used as indicator to determine whether the bacteria has caseinolytic activity. This test was used as first keratinolytic bacteria selection because many scientific reports have been reported that keratinolytic bacteria derived from a nature have a good caseinolytic activity (Gupta and Ramnani, 2006). Selection of wild-type keratinolytic bacteria could be determined by counting the ratio of the clear zone diameter with colony diameter on skim milk agar. The four isolates produced clear zone ratio between 2.2 to 2.8. Isolate number one produced the highest ratio (2.88), it was significant difference ($P<0.05$) compared to other isolates (Fig 1).

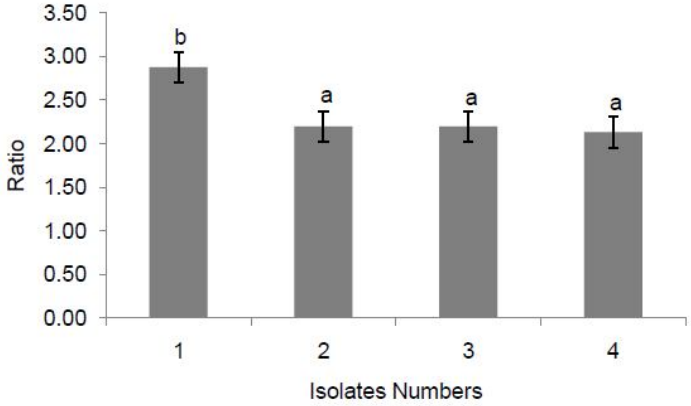

Figure -1: The ratio of clear zone diameter and colony diameter on skim milk agar, incubated at 25^{o}C for 48 hours. *Bars* are standard error of the mean. Mean with the different letters are significantly different according to Duncan's multiple range test at $P = 0.05$.

The average of clear zone ratio produced by this research showed higher results compared to Pillai and Arcana (2008) were smaller compared to bacterial study who produced clear zone ratio as much as 5.0 to 9.5 from animal feces in the Beijing zoo, China (Zhang et. al. 2009). Casein is the main protein in milk and it

can be dissolved by eksoenzim caseinase so the media became clear (Benson, 2001).

Keratinase Activity: The data in Fig 2 showed that the four isolates produced keratinase activity as much as 1.79 to 2.87. Isolate number one had the highest keratinolytic activity (2.87U/ml) and showed significant difference (P<0.05) compared to other isolates. Keratinase activities generated by the four isolates were similar with keratinase activities produced by *Streptomyces albidus* E4 and *streptomyces griseoaurantiacus* E5 (Kansoh et al, 2009).

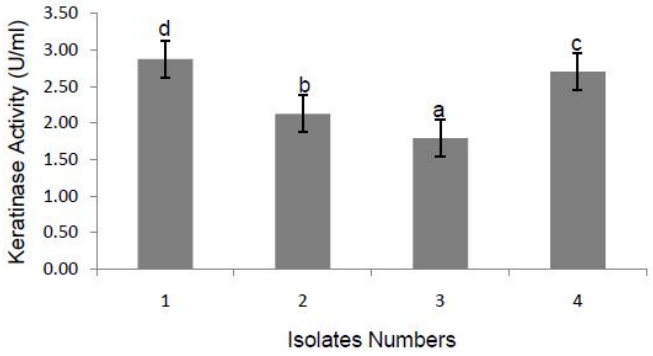

Figure- 2: Various isolate keratinolytic production in sheep hair meal substrate that incubated at 25°C, 48 hours, 180 rpm. *Bars* are standard error of the mean. Mean with the different letters are significantly different according to Duncan's multiple range test at *P* = 0.05.

The results showed that isolates derived from the same place could have different enzyme activity. This could happen because each isolate has a different ability to produce keratinase enzyme. This result was in line with Anbu et. al., (2006) study who showed that microorganisms isolated from the same place and have the same genus but in different species could produce different keratinolytic activities.

Collagenase Activity: Collagenase is one of the enzymes that capable of hydrolyzing collagen substrate. The study showed that the isolate number one had the lowest collagenase activity (0.59U/ml) and had significant difference (P<0.05) compared to others, while the isolate number four had the highest collagenase activity (0.96U/ml) and had significant difference (P<0.05) compare to others (Fig 3).

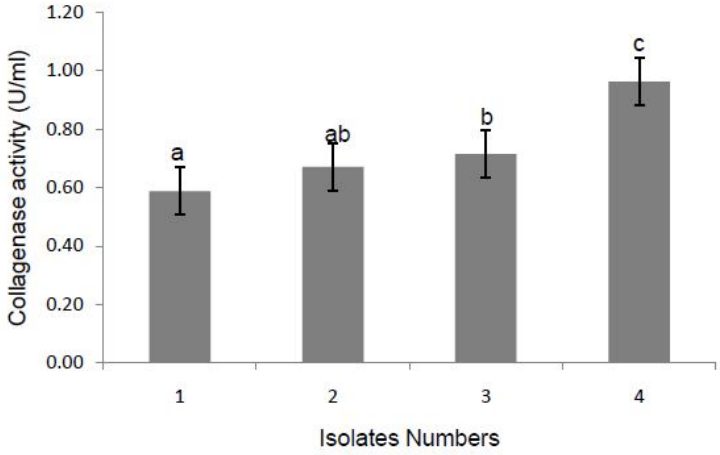

Figure-3: Various isolate collagenase production in sheep hair meal substrate that incubated at 25°C, 48 hours, 180 rpm. *Bars* are standard error of the mean. Mean with the different letters are significantly different according to Duncan's multiple range test at *P* = 0.05.

Isolate number one could be selected for leather processing because it had the smallest collagenase activity. This is because the enzyme for leather processing should have small collagenase activity due to collagen is the major protein constituent of skin that will be degraded by collagenase (Srinivas and Naik, 2011).

Identification of selected bacteria: Based on the test results (caseinolytic, keratinase, and collagenase activity), then the isolate number one was selected. This isolate was isolated from a soil containing hair sheep in Garut sheep farm. The identifications of this isolate were based on several morphological and biochemical tests, also 16S rRNA sequence data. The results of characteristics studies on the isolate number one were summarized in Table 1.

Table-1: Morphological and physiological characteristics of keratinase-producing bacteria isolate number one.

Morphological characteristics	
Gram stain	Positive
Form	Small rods
Spore forming	Non spore forming
Culture Characteristics	
Sheep Meal agar colonies	Yellow color, round shape, shiny, convex elevation, average ledges, and diameter 1-2mm
Physiological characteristics	Positive
Catalase	Positive
Oxidase	Positive
Motility	

The isolate one had a yellow typical colony color and microscopic observation of the isolate showed that this isolate was a gram positive bacterium with small rods.

Gram stain causes the color difference on gram-positive and gram-negative, this happens because the gram stains using multiple dyes. Crystal violet was used as first entry into the cytoplasm of the cell so that the color become purple, the addition of lead crystal violet iodine reacts with iodine to form large crystals that could not exit the cell wall. Effects of alcohol on gram-negative bacteria causing the outer cell membrane dissolved to form small holes in the peptidoglycan, causing the crystal violet iodine diffused out of the cell that lead colorless cells, and the addition of a safranin cause cells become pink (Tortora et al., 2001). There were many gram-positive bacteria that had keratinase activity such as *Bacillus subtilis* SLC (Cedrola et al., 2011); *B. subtilis* 1271, *B. licheniformis* 1269 and *B. cereus* 1268 (Mazoto et al., 2011), *Streptomyces* sp. Strain AB1 (Jouadi et al., 2010).

The genus determination based on phylogenetic analysis of the 16S rRNA gene. The 16S rRNA sequence of isolate one showed high similarities to the group consisting of several *Exiguobacterium* strain (similarity 98%). The Isolate shared 98% sequence similarity with *Exiguo-bacterium* Sp MH3, *E. acetylicum* N5*, E. acetylicum* DSM20416, *E. indicum* strain BR18, and so on. Bootstrap analysis resulted in relatively high values for the branching of the isolate within the *Exiguobacterium* cluster (Fig 4).

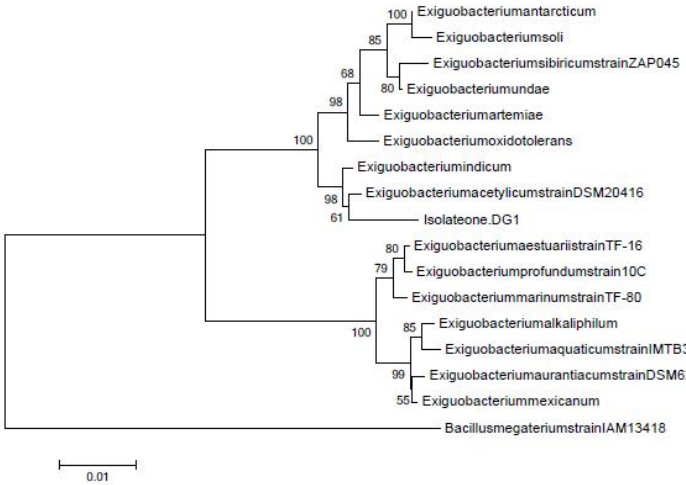

Figure-4: Phylogenetic position of strain Isolate one .DG1 within the genus *Exiguobacterium*. The branching pattern was generated by the neighborhood-joining method. Bar jukes-cantor distance of 0.01. *Bacillus megaterium* was used as out of group.

Based on phylogenetic, the isolate position had closer relationship with *E. acetylicum* strain DSM 20416, therefore it could be named as *E. acetylicum* DG1, but based on submission result from DNA Data Bank of Japan (DDBJ/EMBL/GenBank) with accession number LC008359 the isolate number one was named as *Exiguobacterium* sp. DG1. The results of the morphological and biochemical test also had similarities with *E. acetylicum* PTCC1756 that isolated from soil, which the bacteria had characteristics a yellow pigmented colony, gram-positive, small rod cells, motile, catalase and oxidase positive, and could hydrolyze casein (Aliabadi et al., 2014).

Dehairing Activity: Incubation of the keratinase crude enzyme with sheep skin for dehairing activity showed that after 24 hours incubation at room temperature, hair was remove very easily compared to the control. In the control sample hair loosening was not observed event by mechanical means such as plucking by forceps (Fig 5c). Enzyme treated sheep skin pieces was white, smooth and silky (Fig 5b), mean while chemical treated sheep skin was grey-blue, and rough (Fig 5a).

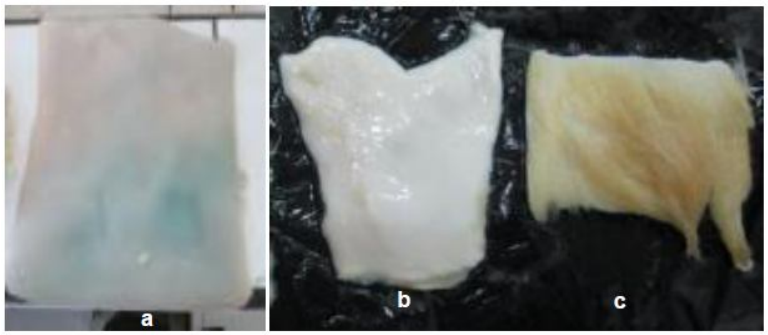

Figure-5: Dehairing of sheep skin **a**. conventional/chemically dehaired with 6% lime and 2,5% Na_2S, **b**.enzymatically dehaired with crude enzyme preparation from *Exiguobacterium* sp. DG1, **c.** control, incubated in aquades under same condition.

Keratinase enzyme was produced by *Exiguobacterium* sp. DG1 had potential biotechnology application to dehairing process in cleaner leather processing. The used of hazardous chemical like Na_2S in dehairing process could be minimized by replacing it with this enzyme. Many keratinase produced by microbial has potential application on dehairing process such as *Pseudomonas aeruginosa* MCM B-327 (Zambare et al, 2011), *Bacillus halodurans* JB 99 (Shrinivas and Naik,

2011), *Bacillus subtilis* DM-04 (Rai and Mukherjee, 2010), *Bacillus pumilus* CBS (Jaouadi et al, 2009), and *Bacillus subtilis* (Pillai and Archana, 2008).

CONCLUSION: In conclusion, *Exiguobacterium* sp. DG1 derived from Sheep farm soil produced extracellular enzyme that has highest caseinolytic (2.88) and keratinase (2.87 U/ml) activities, but has lowest collagenase (0.59 U/ml) activity, also has dehairing activity. Enzyme produced by this bacterium has the potential of removing hair on the leather tanning process.

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