Isolation and characterization of a newly keratinase producing
*Bacillus* sp N1 from tofu liquid waste

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**Abstract.** Keratinase is one of important industrial enzyme. The enzyme is produced by wide range of microbes. Microbial diversity offers keratinase diversity as well. Tofu liquid waste is rich with protein and a good natural environment for microorganism which produced extracellular protein hydrolases. This work reports our work on an isolation of a newly keratinase producing bacterium from tofu liquid waste. A keratinase producing bacterium was isolated in four steps: microbial enrichment using chicken feather to elevate population of keratinase producing microorganism, selection of protease producing microorganism by growing it on skim-milk agar medium following by securing colonies with proteolitic index above 3, selection of keratinase producing microorganism in chicken feather liquid medium, and selection of keratinase producing microorganism with highest keratinase activity. Species of a selected microorganism was identified base on 16S rRNA gene sequence. the isolate was identified as *Bacillus* sp with maximum sequence identity of 89% compare to the available sequence on the data base. *Bacillus* sp is capable to produce extracellular keratinase under 1% feather keratin substrate. Furthermore characterization showed that the enzyme exhibits potential ability to dehair goat skin with better dehaired surface profile.

1. Introduction

Keratin is a group of insoluble and filament-forming proteins produced in certain epithelial cells of vertebrates; they belong to the super family of intermediate filament proteins. Keratin is the main component of the epidermis and the epidermal appendages such as skin, callus, hair, nails, horns, and feathers. These keratinous materials contain high cysteine residues which distinguishes them self from other proteins. Thiol groups of cysteine residues form intra- and intermolecular disulfide bonds that make keratin as one of the toughest biological materials [1, 2]. Based on its sulphur content keratin is classified into soft and hard keratin. Hard keratin found in appendages such as horn, feather, nails, and hoofs. This type of keratin contains high disulphide bonds, tough and inextensible. Soft keratin is found in skin and callus have low content of disulphide bonds and pliable [3].

Efficient disposal of keratin waste is a big concern particularly for poultry industry due to large volume of waste produced. In Indonesia, 42 million chickens was slaughtered everyday in the year of 2015 [4]...
which generated tons of feather waste. This bio-waste is a substantial source for animal feeds, organic fertiliser, and feed supplement. Hydrothermal method is used to degrade keratin. However, this method destruct essential amino acids such as cysteine, arginine, and threonine [5]. Enzymatic degradation offers alternative methods due to specific activity of enzymes.

Keratin is difficult to be hydrolyzed by common proteases such as trypsin, pepsin, and papain [4] due to the unique architecture of keratin. Keratin degradation requires a specific class of proteases known as keratinase [6]. Currently, treatment of feather by microbial keratinases draws wide attention. Keratinase most likely become one of important industrial enzymes [7, 8]. In addition to its potential in feather degradation, keratinase is a potential enzyme to replace chemical dehairing process which normally is done using mixture of sodium sulfide and lime [9, 10]. This chemical process generates hydrogen sulfide gas, obnoxious odor and toxic gas[11]. Many keratinases have been isolated and studied [12-15]. Different bacterial keratinase may have different specificity toward keratin substrate. Tofu liquid waste contains relatively high protein of which naturally provides a good environment for microorganism to grow. In order to use protein, the microorganism must produce extracellular protease. The protease might be able to degrade keratin. Keratin producing microorganism in the environment might be enriched by addition keratin in the environment. This manuscript presents our work on the isolation and characterization of a bacterium from tofu liquid waste and we named the isolate as Bacillus sp. Although the enzyme activity is lower compare to the activity of Bacillus sp MD24 keratinase [16], Bacillus sp N1 produced keratinase faster which might offer more economic benefit. We also tested the capability of keratinase from Bacillus sp N1 to remove hair from goat skin.

2. Material and Methods

2.1. Materials

Bacterial screening was done using skim milk and keratin media. The skim milk medium contain 0.5% NaCl, 0.1% MgSO4, 5.0 % skim milk, and 1.5 % bacto agar while in keratin medium skim milk was replaced by keratin powder. Enzyme production was done using a cultivation medium containing 0.5% NaCl; 0.1 %MgSO4 0.1 %; 0.05% K2HPO4; and 0.1% chicken feathers. Tofu liquid waste was collected from local tofu industry at Bunulejo, Malang, East Java Province and the liquid waste directly was used without any specific treatment.

2.2. Methods

2.2.1. Microbial enrichment and Screening

Chicken feather was sterilized and decomposed by mixing 100 g chicken feather in 1L tofu liquid waste and incubated for approximately 1 month under aerobic condition. One mL of the liquid tofu waste containing decomposed chicken feather was moved to 100 mL of 0.85% NaCl and serial dilution of 1000-fold was created from the suspension using the same physiological salt solution. 100 µL of each diluted suspension was plated on a skim-milk medium and incubated at 37°C for 2 days. Colonies with the ratio clear zone and colony diameter (proteolitic index) above 3 were transferred to fresh skim milk medium and tested for their ability to degrade chicken feather. A colony with highest keratinase activity was secured for next experiment.

2.2.2. Strain identification

Strain analysis was done through comparative genomic method using 16S rRNA gene sequence. Gene sequencing was performed by Macrogen Inc (South Korea). The 16S rRNA gene amplification was done using 785F (5’GGATTAGATACCTGGTA) and 907R (5’CCGTCATATTCTTTAGTTT) primers. Resulting sequence was used as query sequence for searching nucleotide data base using BLASTN program provided by National Center for Biotechnology Information homepage. Sequence alignment was
performed using Clustal X2.1 and phylogenetic tree was constructed using MEGA 6 with the nearest neighbor-joining method. In addition gram staining was done to determine the gram type of the isolate and biochemical test was performed using Microbact Gram-Negative System 12A dan 12B assigned for gram-positive.

2.2.3. Crude extract and hydrolysate productions
Preculture was prepared by inoculating isolated bacterium in 20 mL of sterile NB medium and incubated at 37 °C with aeration speed at 100 rpm for 16 h. Subsequently, 1 mL pre-culture was added to 100 mL of cultivation medium and then incubated at 37 °C, 100 rpm. Culture was centrifuged at 6000 rpm and supernatant was treated as crude extract and hydrolysate. In order to find optimum incubation time to produce optimum keratins, the enzyme was harvested at various time of incubation (1, 2, 3, 4, and day(s)) and keratinase activity was measured.

2.2.4. Enzyme activity measurement
Keratinase activity was measured by following the change of tyrosine concentration after enzymatic hydrolysis. A reaction mixture containing 1 mL of 1% keratin or casein, 1 mL of 50 mM Tris-HCl buffer pH 8 was added with 1 mL of crude extract keratinase. The mixture was mixed thoroughly and incubated at 37 °C for 30 minutes. The enzymatic reaction was stopped by adding 1 mL of 10% TCA. Subsequently, the reaction mixture was incubated on ice for 20 minutes. The mixture was then centrifuged at 5000 rpm for 5 minutes and the absorbance of supernatant was measured at 280 nm.

2.2.5. Miscellaneous
Quantification of amino acids was done using reverse-phase high-performance liquid chromatography at Univeritas Gajah Mada Laboratory Services. Goat skin dehairing was performed in 5 mM phosphate buffer pH 8 and dehaired skin surfaces were analyzed using Scanned Electron Microscopy (SEM).
3. Results and Discussion

3.1. Strain identification

A colony with proteolitic index of 4.2 was chosen as bacterial model. Biochemical test showed that the bacterium was capable to degrade starch and casein. Gram staining indicated that isolate is gram-positive bacterium which ferments glucose xylosa, mannitol, and arabionose, but it does not ferment lactose, sucrose and maltose. Species analysis was analysis genetically based on the 16S rRNA gene. The gene was amplified resulting 1392 bp gene fragment. The gene fragment was aligned against available 16S rRNA gene sequence in The National Center for Biotechnology Information database. Twenty five 16S rRNA sequences identity minimum of 87% were selected and a phylogenetic tree was constructed. All of those sequences were identified as *Bacillus*. Figure 1 shows branch position of the 16S rRNA gene from the isolated bacterium. The nearest branch is *Bacillus tequilensis strain 10b* with sequence identity of 89%. A prokaryotic species is considered to be a group of strains if it has over 97% of 16S ribosomal RNA (rRNA) gene-sequence identity [17, 18]. Based on the 16S rRNA gene sequence homology the isolate only share of maximum 90% identity to the available 16S rRNA gene sequences in the database, therefore the isolate revealed itself as a new strain of *Bacillus* and we named the isolate as *Bacillus sp N1*. 

![Figure 1. Phylogenetic tree of isolated bacterium from tofu liquid waste and selected bacteria. Selection was done base on similarity of 16S rRNA gen sequence.](image-url)
3.2. Optimal growth conditions
Under experimental condition, optimum keratinase activity was observed at day 2 (Figure 2A). At the fifth day more than 50% weight loss was observed (Figure 2B). The weight loss increased sharply from day 1 to day 3 and became slower after day 3. This is most likely due to the low enzyme concentration as cell concentration decreased due to limiting micronutrient in the medium. Keratinase *Bacillus sp N1* could grow from 20-45°C and pH 7.0-9.0 (Figure 3). Optimum keratinase was produced at pH 8.0 (Fig. 3A) and temperature of 37°C (Figure 3B). Optimization of medium is needed to be done to increase enzyme production.

**Figure 2.** Keratinase production (A) and chicken feather weight loss (B) during growth of keratinolytic *Bacillus sp N1*.

**Figure 3.** Effect of pH (A) and temperature (B) on keratinase activity.
3.3. Amino acids concentration in the hydrolysate

Chicken feather hydrolysate was subjected to amino acids analysis. Table 1 shows amino acids composition in the chicken feather hydrolysate. Seventeen amino acids were determined while 3 essential amino acids were not. The hydrolysate poorly contain L-asparagine, L-histidine, and L-serine and rich of L-glutamic acid and L-aspartic acid.

| Amino acids                        | Concentration µg/mL |
|------------------------------------|---------------------|
| L-aspartic acid                    | 79.80               |
| L-glutamic acid                    | 106.40              |
| L-asparagine                       | <0.04               |
| L-glutamine                        | 65.60               |
| L-threoine                          | 69.60               |
| L-glycine                           | 60.20               |
| L-arginine                          | 30.80               |
| L-alanine                           | 54.20               |
| L-tyrosine                          | 64.40               |
| L-tryptophane and L-Methionine      | 78.60               |
| L-valine                            | 26.40               |
| L-phenylalanine                     | 29.80               |
| L-isoleucine                        | 18.80               |
| L-lysine                            | 51.60               |
| L-histidine + L-serine              | <0.01               |

3.4. Application trial of keratinase crude extract on goat skin dehairing

Keratinase crude extract was tested on goat skin dehairing. Figure 4 A and B shows a comparison between undehaired and partially dehaired goat skin by keratinase from Bacillus sp N1. Goat hairs became brittle and easily removed from the skin after immersion for 24 h in 1.5 U/mL keratinase for 24 h. Figure 4C and D show surface of goat skin. Figure 4C shows a hair still attach to the skin and Figure 4D shows holes where hairs are already removed from the skin. Complete dehairing would need higher units of keratinase. Optimization of enzyme/leather is needed to be done to get the best dehairing condition. At experimental condition showed a good trace of dehaired skin which showed removal of complete and leaving good holes (Figure 4D) without damaging surrounding area. This data indicating the keratinase is free from kolagenase.
Figure 4. Skin dehairing by keratinase from *Bacillus* sp N1: A) Undehaired goat skin; B) partially dehaired goat skin; C) Scanned electron micrograph of goat skin with a hair attached to the skin; and D) Scanned electron micrograph of dehaired goat skin.

4. Conclusion
A newly isolate bacterium, *Bacillus* sp N1, was isolated from tofu waste. The isolate exhibits a quite distance strain to the known isolates, which might reflect different metabolic profile. The isolate is capable to produce keratinase using chicken feather as sole carbon and nitrogen source and the enzyme showed a potential use in dehairing process. Fermentation hydrolysate showed relatively high amino acids concentration which is potential to be applied as nutritional animal feed additive.

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