Purification of Keratinase from *Bacillus* sp. MD24 using Ammonium Sulfate Fractionation

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Abstract. A keratin degrading bacterium, *Bacillus* sp MD24, was isolated from soil. The crude keratinase produced by the bacterium has been reported to dehair goat skin. However, the dehairing process took 72 hours. In order to shorten the dehairing time it is necessary to increase the keratinase concentration. This could be done by optimizing keratinase production either finding the best fermentation media or optimizing fermentation condition. Another way to increase the concentration could be done by partially purifying the enzyme. Keratinase from *Bacillus* sp MD24 had been produced under submerged fermentation, however, it produced a relatively low amount of enzyme. Although an effort to increase enzyme production had been reported by solid state fermentation, the enzyme concentration was not enough for industrial purposes. This work aimed to increase enzyme concentration by partial purification through enzyme precipitation using ammonium sulphate. The research was conducted in three stages: (1) regeneration the bacterium, (2) production of keratinase, and (3) purification of keratinase with ammonium sulfate fractionation. Keratinase activity was measured using Anson method and protein concentration was measured by Lowry method. Enzyme purity was clarified using a combination of specific activity, purity level, and SDS-PAGE analysis. Based on the analysis result, ammonium sulphate did not act as a good precipitation agent for the keratinase. Two major bands were suggested as keratinase with an estimated molecular weight of 25 and 66 kDa as monomer and dimer form, respectively.

1. Introduction

Industrial process in which using chemicals has been affecting the environmental quality and health of people around the world. Enzymatic processes offer to replace these noxious process for a better life. In the leather industry, various enzymes specifically from microbial proteolytic are the most essentially used. Dehairing is an important step during leather processing. Hair is composed primarily of strong fibrous proteins named as keratin which contains a large amount of cysteine residues. The cysteine residues form a strong covalent disulphide bond. Enzymatically this strong chemical bond can be disrupted by a class of protease named as keratinase.

Microbial keratinases had been reported to be promising enzymes for application in dehairing process. Keratinolytic enzymes or called keratinase (E.C 3.4.21 / E.C 3.4.24 / E.C 3.4.99) is one of the serine- or/and metallo-hydrolase groups that disrupt the peptide and disulfide bond in the keratin. Various extracellular keratinase showed capability to degrade keratin, such as keratinase produced by *Bacillus amyloliquefaciens* S13 [1], *B. amyloliquefaciens* TCCC11319 [2], *Bacillus cereus* [3], *Bacillus cereus* PCM 2849 [4], *Bacillus sp* JM7 [5], *Bacillus sp* [6], *Bacillus licheniformis* [7], *Bacillus licheniformis* N5 [8], *Bacillus subtilis* LFB-FIOCRUZ 1266 [9].
Keratinases have a very high diversity in term of biochemical and biophysical properties among microorganisms isolated from different environmental. In addition, the needs of the global markets are increasingly becoming the main reason for the leather industry to produce leather quickly, the use of minimum materials, and good quality products. Therefore, the production of keratinase from new bacteria with better dehairing capabilities is a considerable prospect for industrial progress. A new *Bacillus* sp. was isolated from decomposed chicken feather containing soil, *Bacillus* sp. MD24 produces keratinase and the enzyme showed activity in dehairing of goat skin [10]. Based on the result of Scanning Electron Microscope Analysis, enzymatically and chemically dehaired goat skin were showed similar or improved surface of the skin, which makes the keratinase from *Bacillus* sp. MD24 as potential candidate for application specifically in industrial process such as leather industry to avoid problems due to the use of chemicals. However, the dehairing process took 72 hours and was reported that the enzyme concentration was still relatively low (0.3 U/mL). In order to shorten the dehairing time, it is necessary to increase the keratinase concentration.

The optimization of keratinase producing either finding the best fermentation media or optimization of fermentation condition had been attempted. The keratinase had been produced under Submerge Fermentation (SmF) with variations in additional carbon sources, pH, and temperature. The fermentation result showed that the activity of the enzyme was still low (6.09 U/mL). The Solid State Fermentation (SSF) method also had been applied, but the enzyme concentration produced by this method was not enough for industrial purposes [10][11]. Another alternative to increase the keratinase concentration is preconcentration of the enzyme by enzyme purification methods. The enzyme purification is a method for obtaining pure enzyme without the presence of contaminants such as residual metabolites from microbes, residual isolation media and other mineral salts. The loss of contaminants that will increase the concentration of the enzyme so that the enzyme activity increases. Contaminants are likely to interfere with the enzyme stability, so that reduce or eliminate its activity. This paper reports our work on keratinase purification from *Bacillus* sp. MD24 using precipitation method by partial ammonium sulfate fractionation.

2. Materials and methods

2.1. Keratinase production

*Bacillus* sp. MD24 was isolated from decomposed chicken feather containing soil [10]. The fermentation method was undertaken according to [11] procedures with modification. The bacteria was grown under solid state fermentation media at water concentration 500% with chicken feather as sole carbon and nitrogen sources, and maintained at 37 °C for three days. The crude extract keratinase was filtered and recovered in the culture filtrate by centrifugation at 10.000 rpm for 10 minutes at ± 4 °C.

2.2. keratinase Assay

Keratinase activity was assayed by the modified protease colorimetric method using tyrosine as the standard. One unit (U) of protease activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine at the defined assay conditions.

2.3. Protein Assay

Protein concentration was measured by the modified method of Lowry et al. [12] using bovine serum albumin (BSA) as the standard.

2.4. Keratinase purification

All operation was performed at ± 4 °C. The crude extract of keratinase was precipitated by the gradual addition of solid ammonium sulfate with gentle stirring in the following saturation ranges 0-25%, 25-50%, 50-75%, and 75-100%. The precipitate was collected by centrifugation at 10.000 rpm for 10 minutes. The precipitate was then dissolved in a minimal volume of 50 mM Tris-HCl CaCl$_2$ 2 mM
buffer (pH 8.0) and desalted by dialysis against the same buffer. Keratinase activity and protein concentration from each fraction were assayed.

2.5. Electrophoretic methods
Sodium dodecyl sulphate – polyacrylamide gel electrophoresis 12% was carried out according to the Laemmli method [13] and stained with Coomassie brilliant blue.

3. Result and discussion

3.1. Purification of extracellular keratinase
A keratinase crude extract was purified from the fermentation filtrate of Bacillus sp. MD24. The crude extract was purified by precipitation using (NH₄)₂SO₄ fractionation (0-25%, 25-50%, 50-75%, and 75-100%). The activities and yield during the purification processes are summarized in Table 1. All fractions showed almost no specific activity and purity difference compared to the crude extract. Total activity of all fractions is 735.20 U, it is about 14.36% of total activity in the crude extract. Furthermore, in the remaining supernatant, it still contained keratinase activity of 4200 U or about 84.17% of total activity in the crude extract. It means ammonium sulphate did not act as a good precipitation agent for keratinase from Bacillus sp. MD24. However, ammonium sulphate might act as a good precipitation agent of impurities protein which lead to increasing purity of keratinase.

| Fraction     | Total Enzyme Activity (U) | Total Protein (mg) | Specific Activity (U/mg of Protein) | Purity Level (fold) | Yield (%) |
|--------------|---------------------------|--------------------|------------------------------------|-------------------|-----------|
| Crude Extract| 5111.74                   | 1493.80            | 3.42                               | 1.00              | 100.00    |
| 0-25 %       | 45.63                     | 12.87              | 3.54                               | 1.03              | 0.89      |
| 25-50 %      | 184.44                    | 53.53              | 3.44                               | 1.00              | 3.61      |
| 50-75 %      | 290.69                    | 75.66              | 3.84                               | 1.12              | 5.69      |
| 75-100 %     | 213.46                    | 59.40              | 3.59                               | 1.05              | 4.17      |

The profile of protein content in crude extract and all fractions was analyzed by SDS-PAGE is shown in Figure 1. The profile showed strong bands were estimated molecular weight of 25 and 66 kDa. This is almost similar to the keratinase produced from Bacillus amylo liquefaciens strain S13, it has appeared two protein bands at 28 kDa and 47 kDa. It was reported that the two protein bands are monomers [1]. Compared to keratinase from the literature, the first protein band of keratinase from Bacillus sp. MD24 has an almost similar molecular weight, but the second protein band is different, which was higher than of other keratinases secreted by Bacillus amylo liquefaciens strain S13 (28 kDa and 47 kDa) [1], Bacillus amylo liquefaciens strain TCCC11319 (28 kDa) [2], Bacillus subtilis strain SCK6 (30.95 kDa) [14]. However, it is possible that two protein bands of keratinase from Bacillus sp. MD24 are monomeric and dimeric form respectively. In addition, other literature keratinase from Bacillus subtilis MTCC (9102) reported that has a molecular weight range between 64-69 kDa [15]. Thus, the two bands suggested as keratinase. However, further procedure must be done such as chromatography or organic solvent precipitation, especially on the remaining fraction. SDS-PAGE also must be done to see the protein profile of the remaining fraction.
4. Conclusion
A new keratinase produced by *Bacillus sp.* strain MD24, newly isolated from soil, was partially purified. Purification of the keratinase using ammonium sulfate fractionation methods. Based on the result, ammonium sulfate did not act as a good precipitation agent for the keratinase. Enzyme and protein assay were showed almost no specific activity and purity difference compared to the crude extract. The protein profile showed strong bands estimated molecular weight of 25 and 66 kDa.

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