Keratin biofilm from chicken feathers

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Abstract. Keratin is an important biomaterial for industrial applications. About 90% of keratin is found in chicken feathers. Previous study showed that solid-state fermentation of keratinase by Bacillus sp. MD24 using chicken feathers a sole carbon and nitrogen source produced micro-keratin as a solid by-product. However, soluble by-product which most probably contained soluble keratin was not yet studied. This paper reports our investigation in soluble keratin in liquid by-product as possible raw material to generate biofilm. Fermentation of keratinase was done for 10 consecutive days by Bacillus sp. MD24 and liquid by-product was separated from solid by-product by centrifugation at 5000 rpm. The size of soluble keratin was examined by particle size analyser (PSA). The soluble keratin was filmed and the film was characterized using Fourier Transform Infrared Spectroscopy (FTIR) and film surface was analysed using Scanning Electron Microscopy (SEM). Dissolution of keratin using ionic liquids 1-Butyl-3-methylimidazolium chloride ([BMIM]Cl) and 1-Ethyl-3-methylimidazolium acetate ([EMIM]Ac) was done as alternative keratin degradation process. Dissolution was performed under nitrogen atmosphere at 130 °C for 7 hours. Insoluble fraction was recovered by addition of water. Keratin biofilm was generated and FTIR spectra indicated of absorption bands which were assigned to the peptide bonds (-CONH). The bands exhibited the presence of protein. There were no much difference on surface structure between keratin biofilm produced from soluble keratin by-product and dissolved keratin produced by dissolution using ionic liquid.

1. Introduction
Chicken feathers are accumulated from poultry waste and produced in millions of tons annually. Chicken feather disposal leads to environmental pollution. Besides being abundant and cheap, chicken feathers contain about 90% keratin [1]. Keratin is a fibrous protein that insoluble in water and organic solvent, which makes it difficult to recycle. It is a biopolymer built by α-helix (α-keratin) and β-sheet (β-keratin) structure, which stabilized by hydrogen bonds, peptide bonds, disulphide cross-linkages, salt bridges, and other intramolecular force [2].

Keratin is an important raw biomaterial for industry, especially keratin film. Ramakrishnan et al. [3] reported keratin based bioplastic film. Keratin biofilm was used for biomedical application, such as keratin film for ocular surface reconstruction [4]. In order to use keratin from nature such as chicken feathers...
feathers, it requires solubilization process. Traditional methods of keratin hydrolysis were done under strong acid, strong alkali, high pressure and high temperature, which were environmentally unfriendly process. Many solvents had also been used for keratin dissolution, such as urea, thiol and formic acid which were environmentally unfriendly chemicals. Therefore, new solubilization processes need to be developed for the sake of the environment protection.

Enzymatic biodegradation by keratinolytic protease from microorganisms is one of a new biotechnology alternative to solubilize keratin that efficient and eco-friendly. Keratinase capable to break the peptide bonds and the disulphide cross-linkages in keratin to shorten polypeptides. Bacillus sp. MD24 had been shown to produce keratinase and insoluble micro-keratin as by-product under Solid-State Fermentation (SSF) with chicken feathers as substrate [5, 6]. However, the presence of soluble keratin by-product was not yet analysed. Soluble keratin is a potential material for generating new biomaterial products such as bioplastic and biomembrane. This study aimed to analyse the soluble keratin from SSF and its potential to form biofilm.

Another emerging process in solubilizing keratin is using ionic liquids. Ionic liquids are a group of organic salts composed of organic cations and a variety of anions, which are liquid below 100 °C and low vapor pressure [7]. Ionic liquids are thermally stabile chemicals, save solvent, and easily recycle. Imidazole cation has been shows as an excellent dissolution agent for duck feather [8], wool [9], and turkey feather [10]. However, the effect of the alkyl group in solubilizing chicken feathers has not been tested. In this study, chicken feather was dissolved using two difference imidazole based cationic liquids, 1-Butyl-3-methylimidazolium chloride ([BMIM]Cl) and 1-Ethyl-3-methylimidazolium acetate ([EMIM]Ac) and analysing the dissolved keratin as well as its capability to form biofilm. Furthermore, we compared it with soluble keratin and biofilm produced from SSF.

2. Materials and Method

2.1. Degradation of chicken feathers under solid-state fermentation (SSF)
Five grams of chicken feathers in Erlenmeyer flask was added to 120 mL of moistening solution. The moistening solution contained 0.2 g/L of KCl; 0.2 of MgSO$_4$.7H$_2$O; 1 g/L of (NH$_4$)H$_2$PO$_4$; 2 g/L of CaCO$_3$; 0.5 g/L of KH$_2$PO$_4$; and 0.5 g/L of K$_2$HPO$_4$ (pH 8). The mixture was added with 1% (v/v) of Bacillus sp. MD24 pre-culture and incubated at 37 °C for ten days.

2.2. Keratin biofilm formation from SSF soluble keratin
Liquid by-product from Solid-State Fermentation (hydrolysate) was separated from solid by-product and cells by centrifugation at 5000 rpm. The soluble keratin was dialysis using Snake Skin Dialysis Tubing 10K MWCO for 8 hours. The dialyzed solution was poured into a petri dish and dried at 50 °C until biofilm was formed. The soluble keratin size was measured using a particle size analyser.

2.3. Keratin biofilm formation from ionic liquid-soluble keratin
A 1 g of chicken feathers was added to a vial containing 1 gram of [BMIM]Cl and 1.7 g chicken feathers were added to a vial glass containing 1 g of [EMIM]Ac, which were fitted into a heating block at 130 °C for 10 hours under an N$_2$ atmosphere. It was constantly stirred under magnetic stirring condition until they were completely dissolved. Keratin solution was poured into a Petri dish and let it solidify at room temperature. The ionic liquid was recovered by extracting it with water.

2.4. Biofilm characterisation
Fourier Transform Infrared Spectroscopy (FTIR) was used to analyse the functional groups contained in keratin film. The FTIR was performed in the wavenumber range of 400 cm$^{-1}$ to 4000 cm$^{-1}$. Surface topography of biofilm was observed by Scanning Electron Microscopy (SEM).
3. Results and Discussion

3.1. Degradation of chicken feathers under solid-state fermentation (SSF)

After incubation for ten days, whole chicken feathers were degraded into chicken feather pulp. Dried pulp was reported as keratin microfiber [6] (Figure 1A). Soluble hydrolysate in the supernatant was transparent with a brownish yellow colour (Figure 1B). The same colour was observed in [11] which confirmed that the supernatant contained soluble keratin. While the dissolved keratin obtained from keratin dissolution using both ionic liquids had dark brown colour which was similar to dissolved keratin by ionic liquid from duck feather (Figure 2) [8]. The dark colour of soluble keratin depended on the concentration of keratin. The higher the concentration, the darker the colour. Therefore, soluble keratin from the SSF process had less concentration.

![Figure 1. Degradation of chicken feathers on day 10. A. SSF result and B. Supernatant](image1)

![Figure 2. Dissolved keratin. A. Dissolved keratin with [BMM]Cl and B. Dissolved keratin with [EMIM]Ac](image2)
3.2 Determination of keratin particle size using particle size analyser (PSA)

Determination of keratin particle size using PSA was carried out 5 times to each sample, which exhibited various sizes of keratin (Table 1). The average size of dissolved keratin obtained from SSF, [EMIM]Ac-dissolution, and [BMIM]Cl-dissolution were 2677 ± 611 nm, 1085.78 ± 359 nm, and 1311.88 ± 573 nm, respectively. The size of soluble keratin and dissolved keratin were still at μm size, and soluble keratin from SSF has the largest size. Size of molecule related to its capability to dissolve. The small particle will dissolve better. However, protein has different properties related to its capability to dissolve. Hydrophilic protein dissolves better than hydrophobic protein. Keratin contains highly helix structure which made keratin insoluble in water. Disrupting the structure might converted protein structure become less hydrophobic. During ionic removal from dissolved keratin by extracting it with water, dissolved keratin did not dissolve in water which can be concluded that dissolve keratin remains highly hydrophobic, while soluble keratin from SSF process is water-soluble protein and it most likely hydrophilic protein. Enzymatic process and dissolving process are different process. Enzymatic process caused keratin hydrolysis at certain point of peptide bonds which yielding different length of hydrolysate depending on amino acid sequence in keratin. When cationic liquid was used, the solubilization starts with breaking of thioester bonds by nucleophilic attack of chloride caused the removal of lipids from the keratin surface, followed by the penetration of cationic liquid into the cortex leading to swelling and/or dissolution of proteins. Eventually, this process produced various length of peptides. Therefore, with respect to homogenous product, enzymatic process produces better result compared to dissolution using ionic liquids. However, dissolution process produced higher yield compared to SSF.

### Table 1. The results of PSA Analysis

| Measurement | SSF (nm) | [EMIM]Ac (nm) | [BMIM]Cl (nm) |
|-------------|----------|---------------|---------------|
| 1           | 2721     | 1694          | 2327          |
| 2           | 3594     | 1114          | 1185          |
| 3           | 2835     | 961.7         | 1064          |
| 4           | 2027     | 816.1         | 1008          |
| 5           | 2162     | 843.1         | 975.4         |
| Average     | 2677.4 ± 611 | 1085.78 ± 359 | 1311.88 ± 573 |

3.3. Keratin biofilm formation

Keratin biofilm from soluble keratin prepared in different ways had different colors and surface texture (Figure 3). The surface texture of biofilm from soluble keratin (Figure 3A) was smoother compared to dissolve keratin (Figure 3B and 3C). The difference surface texture might be due to speed of solidification process. Soluble proteins need longer time to evaporate water which caused slow biofilm generating and eventually produced a smooth surface.
3.4. Characterisation of keratin biofilm

The FTIR spectra of keratin in the region 400-4000 cm\(^{-1}\) are given in Figure 4. FTIR band showed characteristic absorption bands assigned to the peptide bonds (-CONH). All the biofilm showed similar FTIR main bands. The amide A band was assigned to stretching vibration of N-H bonds (range 3270-3310 cm\(^{-1}\)) [12]. The amide I band was connected mainly with the C=O stretching vibration (range 1700-1627 cm\(^{-1}\)) [13]. The amide II was related to N-H bending and C-H stretching vibration (range 1515-1520 cm\(^{-1}\)) [14]. A weak absorption band was observed amide III which was due to the C-N and C-O stretching, N-H and O=C-N bending vibration (range 1220-1236 cm\(^{-1}\)). Overall FTIR spectra indicated the presence of protein.

![Figure 4](image)
SEM images in Figure 5 showed similar to SEM images in Idris et al. [14]. In contrast to Figure 3, the micro-surface of the keratin biofilm from soluble by-product SSF had wavy structure (Figure 5A and 5D), while images of the surface of keratin biofilm from [EMIM]Ac-dissolved keratin (Figure 5B and 5E and 4e) were smoother and clear. The Figure 4C and 4F show that the surface of keratin film from [BMIM][Cl]-dissolved keratin contains solid debris, possibly trace of ionic liquid. Overall both soluble keratin and dissolve keratin are good raw materials to generate new product such as bioplastic and biomembrane.

![Figure 5](image)

**Figure 5.** The surface images at 10kx magnification of keratin biofilm from SSF by-product (A), [EMIM]Ac-dissolved keratin (B), [BMIM][Cl]-dissolved keratin (C), 50kx magnification of keratin biofilm from SSF by-product (D), EMIM][Ac-dissolved keratin, and BMIM][Cl-dissolved keratin (F)

### 4. Conclusions
In summary, the solubilization of feather keratin using Solid-State Fermentation, [EMIM]Ac, and [BMIM][Cl] produced soluble and dissolved keratin. Degraded keratin prepared through SSF and ionic liquid dissolution produced biofilm with different colours and surface, and it might be developed further to make new products. Therefore, further study must be done to generate novel materials. In addition, degradation chicken feathers should be improved to get more soluble keratin.

### 5. Acknowledgment
This work was supported by PNBP research grant of Universitas Negeri Malang under contract No. 20.3.166/UN32.14.1/LT/2019.
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