Antibacterial and Antioxidant Activity of Newly Keratinolytic Bacteria, *Azotobacter chroococcum* B4

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**Abstract**: Keratinolytic bacteria of *A. chroococcum* B4 was evaluated for its potential of antibacterial and antioxidant activity. Kirby-Bauer method was used to know antibacterial potential of B4 against *S. aureus, S. epidermidis, Bacillus cereus, Bacillus pumilus, Bacillus subtilis, Listeria monocytogenes, Proteus sp., enteropathogenic*Escherichia coli, *Enterobactersakazakii*, and *Salmonella enterica*. Antioxidant test was done using DPPH radical scavenging activity assay with ascorbic acid as a control. In this study, hydrolysate of pellet, dialysis, and fraction 25 of B4 keratinase purification of previous study was used for antibacterial and antioxidant test. The result showed that B4 hydrolysates inhibited Gram positive pathogenic bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*, and Gram negative *Enterobacter sakazakii*. All hydrolysates showed to have antioxidant properties in which fraction 25 showed higher compared to that of others. This study showed poultry waste-derived keratinase of B4 might be useful as supplementary protein, antibacterial, and antioxidant in the animal feed formulations.

**Keywords**: Antibacterial, antioxidant, *Azotobacter chroococcum*, keratinase.

**Introduction**

World wide, poultry-processing industries generate more than 4 billion pounds of feathers annually as waste. Feather weight accounts up to 5-7% of mature chicken. Chicken feather is a material that contains about 90% of its protein as keratin¹. Microbial degradation is an economical method for the conversion of keratin waste into useful products. As previously reported, keratinase can be utilized as biocatalysts in fields of leather, textile, and waste recycling². Keratinous wastes are used as source of amino acids, soluble peptides, and proteins, which are typically utilized as animal feed additives as well as nitrogen source for plants³.

Dwi Suryanto et al /International Journal of PharmTech Research, 2020,13(2): 123-127.

DOI= [http://dx.doi.org/10.20902/IJPTR.2019.130215](http://dx.doi.org/10.20902/IJPTR.2019.130215)
Antioxidants play an important role in human health and food processing. Many antioxidative peptides have been identified and characterized as well as antimicrobials. Such peptides, encrypted in protein sequences, only demonstrate biological activities after their release from the native protein such as keratin. Recently, we isolated a newly *Azotobacter chroococcum* B4 producing keratinase. The purpose of this study was to examine the antibacterial and antioxidant potential of feather hydrolysates of B4 for its possible use in animal feed formulation.

### Materials and Methods

#### Strain and growth medium

*Azotobacter chroococcum* B4 was grown on Feather Meal Broth (FMB) composed by chicken feather powder (15 g), K$_2$HPO$_4$ (0.7 g), KH$_2$PO$_4$ (0.4 g), NaCl (0.5 g), MgSO$_4$ (0.1 g) in 1000 mL distilled water. Chicken feather waste was collected from slaughterhouses. Feathers were washed using detergent-water and cut into small pieces (± 2 cm), prior soaking in acetone for 24 h. Washed feathers were dried in oven at 40°C for 72 h to yield chicken feather powder. Growth medium was sterilized at 121°C, 1 atm for 15 min.

#### Antibacterial activity of keratinase B4

Hydrolysate of pellet, dialysis, and fraction 25 solution of B4 keratinase of previous study were used for antimicrobial and antioxidant test. Antibacterial activity was tested against *Staphylococcus aureus*, *S. epidermidis*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Proteus* sp., enteropathogenic *Escherichia coli*, *Enterobacter sakazakii*, and *Salmonella enterica* using Kirby-Bauer method. Ten microlitres of sample solutions was put on sterile disc paper. The discs were placed on top of bacterial lawns grown on nutrient agar (Oxoid UK). The culture was incubated at 28°C. Diameter of clear zones or inhibition zones around the discs was measured after 24 h.

#### Antioxidant activity of feather hydrolysate

Scavenging of 2,2 diphenyl-1-picrylhydraclyl (DPPH) radical assay was used to measure antioxidant activity of supernatant of feather hydrolysates. Ascorbic acid was used as the positive control. Each 20 µL sample was added to 180 µL DPPH in methanol solution, making it a final total volume of 200 µL. The mixture was maintained for 30 min in the dark at 37°C, and then DPPH radical reduction was measured the using UV–Visible microplate reader (Thermo Scientific) at 517 nm. In addition, the following formula was used to determine the DPPH radical (scavenging activity) inhibition percentage:

\[
\text{Scavenging activity (\%) } = 1 - \frac{(A_{\text{sample}} - A_{\text{sample blank}})}{A_{\text{control}}} \times 100
\]

\(A_{\text{control}}\) is the absorbance of DPPH solution (without hydrolysate), \(A_{\text{sample}}\) is the sample absorbance (DPPH solution with hydrolysate) and \(A_{\text{sample blank}}\) is the sample absorbance only (extract without DPPH solution). The test was conducted in triplicate using ascorbic acid as positive control of antioxidant activity. Ascorbic acid was used as a control.

### Results and Discussion

#### Antibacterial activity of keratinase B4

Hydrolysate samples obtained from precipitation with ammonium sulphate, dialysis, and column fractionation of keratinasewere used in testing antimicrobial activity against Gram-positive and Gram-negative bacteria. Inhibition to bacterial growth were shown as clear zone around paper disc (Figure 1.)
Figure 1. Antibacterial activities of B4 hydrolysate against pathogenic bacteria *S. aureus* ATCC 6538, *L. monocytogenes* and *E. sakazakii* shown as clear zone around paper dics (arrowed)

Table 1. Antibacterial activity of B4 hydrolysate

| Bacterial isolates               | Inhibition zone (mm ± SD) | Precipitation | Dialysis | Fraction 25 |
|----------------------------------|---------------------------|---------------|----------|-------------|
| *S. aureus* ATCC 6538            |                           | 0             | 0        | 11±0.6      |
| *S. epidermidis*                  |                           | 0             | 0        | 9±0.3       |
| *B. cereus*                      |                           | 0             | 0        | 8±0.3       |
| *B. pumilus*                     |                           | 0             | 0        | 0           |
| *B. subtilis*                    |                           | 0             | 0        | 9.7±0.3     |
| *L. monocytogenes*               |                           | 0             | 8±0.3    | 10±0.4      |
| *Proteus* sp.                    |                           | 0             | 0        | 8±0.0       |
| *E. coli* (EPEC)                 |                           | 0             | 9±0.3    | 9.8±0.3     |
| *E. sakazakii*                   |                           | 0             | 9±0.3    | 10±0.3      |
| *S. enterica* serovar *typhimurium* |                       | 0             | 8±0.3    | 8±0         |

Hydrolystae inhibition to pathogenic bacterial isolates varied to some extent (Table 1).

It was observed that higher inhibition zone was found in Gram-positive bacteria *S. aureus* by 11 ± 0.6 mm, *L. monocytogenes* by 10 ± 0.4 mm, and Gram-negative bacteria *E. sakazakii* by 10 ± 0.3 mm.

Interestingly, ammonium sulphate precipitation showed no inhibitory activity, dialysis fraction showed low inhibitory activity against *L. monocytogenes*, *E. coli* (EPEC), *E. sakazakii*, and *S. typhimurium*, while high antibacterial activity was observed in fraction 25 to all pathogenic bacterial isolates (Table 2). Sequential purification steps resulted more purified protein/keratinase, which showed to have antibacterial activity by disturbing cell wall or membrane structure at variable degrees. The bacterial damage by proteases like papain is due to a broad proteolytic activity, resulting in the rupture of the cell wall or membrane and subsequent loss of cell integrity.8
This work indicated that keratinase B4 might be potentially to inhibit pathogenic bacterial growth as reported previously. An antimicrobial activity of some microbial keratinases, such as those produced by B. subtilis CH-1 inhibiting the growth of S. aureus and E. coli\(^7\), Nocardiopsis sp. 28ROR inhibiting S. aureus\(^9\), and B. safensis LAU 13 inhibiting E. coli\(^10\).

**Antioxidant activity of keratinase B4**

In this study three hydrolysate of keratinase purification of A. chroococcum B4 was also used for its antioxidant properties. The results indicated that all hydrolysates presented antioxidant activity as concentration dependent (Figure 2).

B4 hydrolysates showed to have varied in antioxidant activity, in which 200 µg/ml of pellet was lower antioxidant activity compared to others. Fraction 25 of 1000 µg/ml was found to have relatively high antioxidant activity. At that concentration, fraction 25 and ascorbic acid have scavenging activity of 88% and 91.27, respectively.

Antioxidant activity is likely to occur through various mechanisms\(^4\). The ability of hydrolysates might due to composition of bioactive peptides that contribute to antioxidant activity\(^11\). As previously reported, this study showed similar result in that protein and peptide hydrolysates have an effective antioxidant activity in vivo and in cellular cultures\(^12,13\). Several studies on bioactive peptide was reported from feather hydrolysates from B. pumilus A1\(^14\), Chryseobacterium sp. kr6\(^15\), and B. subtilis S1-4\(^5\).

This study of antioxidant property of feather hydrolysate of A. chroococcum B4 was firstly reported. The insight of antioxidant assay of bacterial hydrolisate of enzyme purification is an important and necessary step before being applied in humans and animals, so it is very important to evaluate peptide activity of A. chroococcum B4 antioxidant using various methods in the future.

**Conclusion**

Hydrolysates of B4 keratinase purification showed antibacterial and antioxidant activities. Relatively high antimicrobial activity was observed against pathogenic Gram positive S. aureus and L. monocytogenes, and Gram negative E. sakazakii by fraction 25. DPPH radical scavenging of fraction 25 of 1.000 µg/ml activity reached 88%. This was closed to ascorbic acid which was its radical scavenging activity reached 91%. Thus,
biodegradation of feathers by A. chroococcum B4 keratinase represented a beneficial biotechnological process for handling feather waste by having proteolytic/keratinolytic, antimicrobial, and antioxidant activities used for animal feed formulation.

Acknowledgement

This work was supported by grants from the Directorate Research and Community Services Ministry of Research, Technology, and Higher Education Jakarta, Indonesia through PMDSU (Program Magister menuju Doktor untuk Sarjana Unggul).

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