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Partial Characterization of Keratinase of Thermophilic Bacteria from Three Hotsprings of North Sumatra

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Abstract: Keratin is insoluble protein and resistant to degradation by most proteolytic enzymes. One major source of keratin is feather of poultry, where this material is usually dumped to the environment. Microbial keratinolytic hydrolysis represents an attractive alternative to improve the nutritional value of feather wastes. In this study, keratinolytic bacteria of Penen, Semangat Gunung, and Sipaholon hotprings of North Sumatra, Indonesia were isolated. Sixteen keratinolityc bacteria were isolated from the hotsprings. Three isolates KW05, SQ04, and WR03 were choosen and partial characterized for their crude keratinase activity. KW05 showed to have high keratinolytic activity. Assay of the crude enzyme in different pH and temperature showed that KW05 was more active in pH 5.0, while SQ04 and WR03 were in pH 6.5. Crude keratinase of all three isolates showed more active in 60°C.
Keywords: feather,keratinase, keratinolyticindex, hotspring.

Introduction

Keratins are the most abundant proteins in epithelial cells of vertebrates and represent the major constituents of skin and its appendages. This insoluble protein fibrous found in skin, nails, hair, fur, feather, and wool[1,2,3,4,5,6]. The main component is a constituent of α-keratin keratin, a protein fiber structure that contains disulfide crosslinking, hydrogen and hydrophobic[1,4,5,7,8]. Therefore keratin is insoluble in water and highly resistant to degradation by proteolytic enzymes such as trypsin, pepsin, and papain[9,10].

Keratin represents nearly 90% of feather weight, which constitute up to 5-10% of the total mature chicken weight[3,5,7,8,11,12]. Tanner fur factory and abattoir throw in a very large number of keratin-containing materials such as wool, feathers, animal hair, horns, etc.[3]. Meanwhile poultry processing plants produce millions of tons of feathers as a by-product resulting an environmeltal problems[3,5,12,13]. One alternative to reduce this pollution is to reuse it as animal feed, while preventing the accumulation in the environment and reduce the progression of certain diseases[3,13,14,15].

Although feathers contain potential protein and amino acids in a very large number, but only a few can be used as animal feed because it can not be digested completely[16]. Feather digestability can be improved by NaOH, steam, and heat treatment[7,15,17,18]. However, the process needs large amount of energy[3,11,14,15,16,18,19], and can damage the amino acid ingredient, produce low quality of feed and only a few are digested[16]. Therefore, it is necessary to find an alternative way of treating waste of this recalcitrant keratin[11,13].

Several microorganisms such as bacteria, actinomycetes, saprophytic fungi and dermatophytes have been reported to degrade keratin[8,20,21]. These keratin degrading microorganisms live in different environment and ecological conditions including hotspring, indicating extensive capabilities of degrading protein including keratin and other solid substrates[8]. Enzymatic hydrolysis carried by keratinolytic microorganisms is one good alternative to improve the nutritional value of feather waste[12,13,16]. In this study, isolation of keratinolytic

bacteria from three hotsprings of North Sumatra and partial characterization of its crude keratinase was conducted.

Materials and Methods

Bacterial Isolation

Water samples were taken from three hotpsings of Penen (KW), Semangat Gunung (SQ), and Sipaholon (WR). Bacterial isolation was conducted using skim milk agar. Colony with clear zone were screened and selected for its proteolytic ability.

Keratin Solution Preparation

A total of 5 g of chicken feathers were cleaned and cut into small pieces soaked in 250 ml dimethyilsulfooxide (DMSO) and heated in a reflux condenser at 100°C for 2 hours. A 500 ml of acetone was added and then stored at -10°C for 2 hours. Suspension was centrifuged at 10,000 rpm for 10 minutes at 5°C. The pellets were then rinsed twice with distilled water on filter paper. The pellets were dried at 40°C and grinded. Keratin solution was prepared by dissolving 1 g of keratin powder with 20 ml of 0.05M NaOH. pH was adjusted to 7 with 0.1 M HCl. The suspension was then redissolved in 200 ml of phosphate buffer of pH 6.5 to make 1% keratin solution.

Relative Keratinolytic Test

Proteolytic isolates were tested for its ability to hydrolyze keratin. Each isolate was grown in feather meal agar containing 1% keratin[13]. Culturer were incubated at 53°C for 48 hours. Keratin hydrolysis zone or keratinolytic index was measured by comparing the diameter of clear zone divided by diameter of bacterial colony. Keratinolytic index is considered as the relative strength of the enzyme. Isolates with the highest keratinolytic indexd were selected for further study.

Keratinase Production

A 1 ml of keratinolytic bacterial cell ($\approx 10^8$ cells/ml) suspension in 0,9% NaCl were grown in feather meal broth containing 1% keratin of pH 6.5. The cultures were incubated in a water bath shaker (150 rpm) at 53°C for 72 hours. Crude enzyme was obtained by centrifuging bacterial culture at 10 000 rpm for 10 min at room temperature. The supernatant was collected as crude keratinase.

Crude Keratinase Assay

A 1 ml of 1% keratin solution was added to a mixture of 1 ml of phosphate buffer (pH 6.5) and 1 ml of crude keratinase. The mixture was pre-incubated at room temperature for 5 minutes followed with incubated for 10 minutes at 53°C. Enzymatic reaction was stopped by adding 2 ml of 10% tricloroacetic acid. This mixture was then added with 0.5 ml of Folin-Ciocalteau reagent and incubated for 30 minutes at room temperature until the blue color is fully developed. The absorbance was measured with a spectrophotometer at 660 nm. One unit of enzyme activity is defined as the amount of keratinase enzyme that hydrolyzes 1 mg of substrate per minute.

Effect of pH on Keratinase Activity

Effect of pH on keratinase activity was measured at varying pH of 4.0, 4.5, 5.0, 5.5 with acidic buffer, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 with phosphate buffer, and 8.5, 9 with Tris-HCl buffer. The reaction was conducted at 53°C for 10 minutes. Keratinase activity was measured as previously described.

Effect of temperature on Keratinase Activity

To determine the effect of temperature on keratinase, crude enzyme was subjected to different temperature of 40 to 80°C for 10 minutes. The reaction was conducted at pH 6.5. Keratinase activity was measured as previously described.

Results and Discussion

Bacterial Isolation

Keratinolytic bacteria have been isolated from poultry waste site[3,13,16,21], others were isolated from inconventional sources such as marine[12], and hot spring and sulfuric soil[20]. In this study keratinolityc bateria

were isolated from three hotsprings of Penen, Semangat Gunung, and Sipaholon, North Sumatra, Indonesia. Isolation was conducted in skim milk agar to obtain proteolytic bacteria. Proteolytic bacterial isolate was indicated to show a clear zone around its colony (Figure 1). Proteolytic bacterial isolates were screened for their ability to degrade keratin in feather agar. (Figure 2). The potensial ones showed to have wider clear zone around its colony grown in feather agar.

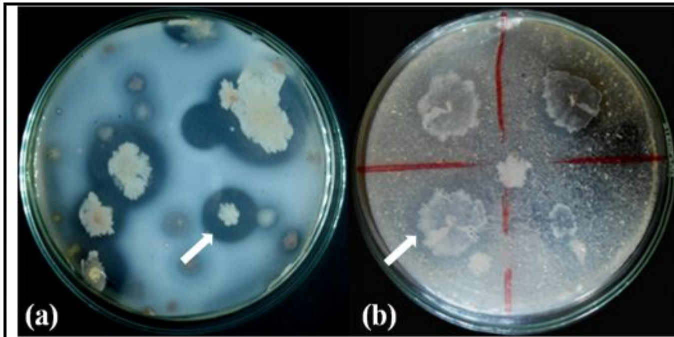

Figure 1. Isolates showing clear zone of hydrolysis (arrowed) on (a). skim milk agar plate and (b). feather meal agar after incubation of 48 hrs at 53°C.

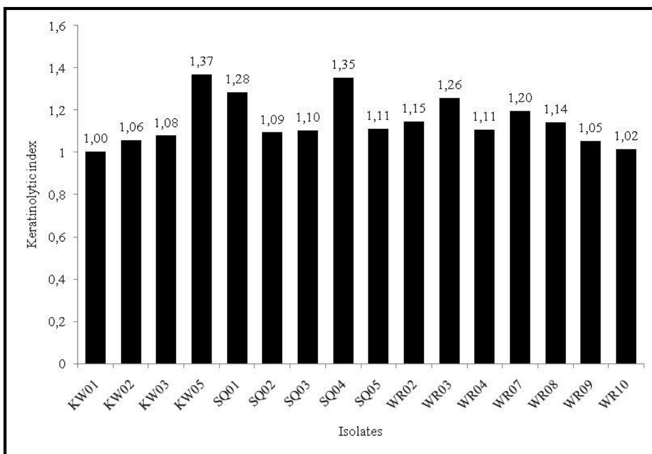

Figure 2. Assay of keratinase as keratinolityc index of bacterial isolates of Penen (KW), Semangat Gunung (SQ), and Sipaholon (WR) hotsprings

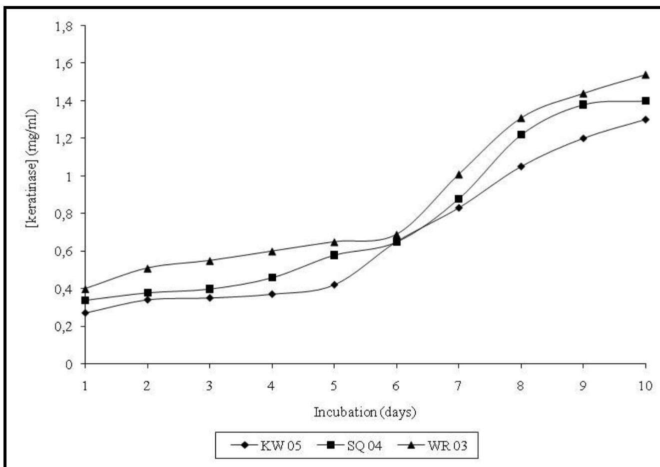

Figure 3. Keratinase concentration in feather broth media in 10 days at 53°C of pH media of 6.5

The results showed that twenty isolates showed to produce keratinase with varying ability to some extent (Figure 3). Three isolates KW05, SQ04, and WR03 seemed to have more keratinolityc ability with keratinolytic index of 1.30, 1.35, and 1.26, respectively. These three isolates were used for further study.

Hydrolytic assay of crude keratinase

Keratinase of KW05, SQ04, and WR03 were produced in feather broth media. Keratinase production was measured for 10 days at 53°C of pH media of 6.5. (Figure 4). It seemed that keratinase still increased up to 10 days of incubation, except that of SQ04. Extracellular keratinase activity of *B. licheniformis* was maximum during stationary phase at 72 h, while *Bacillus sp.* MTS was reached at 48 h[20], while in different media *B. licheniformis* showed to have optimum activity at 6 days of bacterial incubation[13].

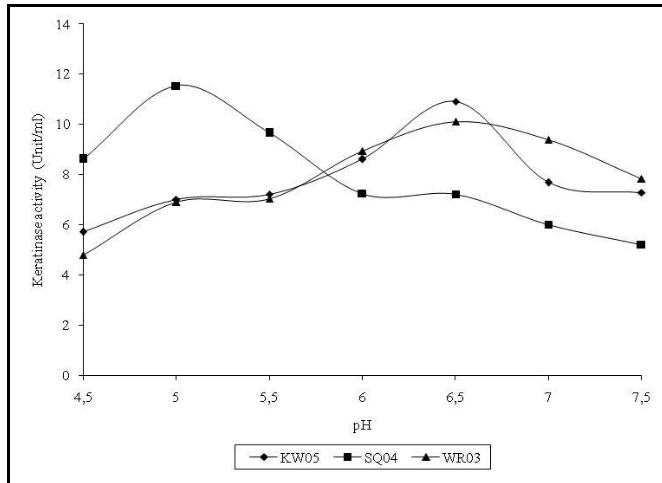

Figure 4. Crude keratinase activity of KW05, SQ04, and WR03 at different pH incubated at 53°C

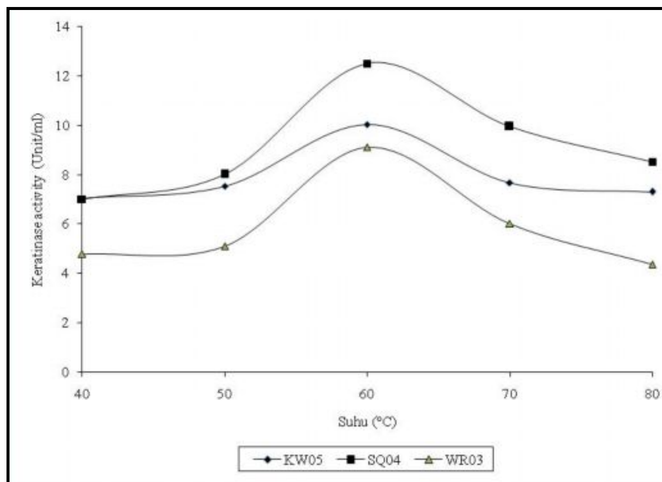

Figure 5. Crude keratinase activity of KW05, SQ04, and WR03 at different temperature of pH media of 6.5

Crude keratinase activity was measured at 53°C of pH media of 6.5. It showed that keratinase of KW05 was more active with specific activity of 839.23 Unit/mg followed by WR03 and SQ04 (Table 1). This result indicated that bacterial isolate from different ecological sites showed to have different potential of keratinase, as also reported by Rahayu et al. (2010).

Table 1. Crude keratinase activity of KW05, SQ04, and WR03 incubated in feather broth media in 10 days at 53°C of pH media of 6.5

Isolates	Total protein (mg/ml)	Activity (Unit)	Spesific activity (Unit/mg)
KW05	1.30	10.91	839.23
SQ04	1.40	7.21	515.00
WR03	1.54	10.09	655.19

Effect of pH and temperature on keratinase activity

To characterize bacterial keratinase, the effect of pH and temperature on enzyme activity was investigated. Crude keratinase was subjected to assay at different pH values (pH 4.5 to 9) at 53°C. The result showed that the activity of crude keratinase of KW05 was optimum at lower pH (pH 5), while SQ04 and WR03 were more active at pH 6,5. Different optimum pH of keratinase activity was reported previously, in wihichmost of optimum activity were at high pH. *Bacillus* sp. MTS isolated from sulfuric soil showed to have optimum keratinase activity at pH 8 and 10[20], *Bacillus* spp. isolated from agroindustrial residues from a poultry farm were active at pH 10[17], and *B. licheniformis* FK14 was optimum at pH 8.5[22].

To know the effect of temperature crude keratinase of KW05, SQ04, and WR03 were subjected to a different temperature between 40-80°C. The result showed that all isolates were more active at 60°C. It can be understood since the water temperature of the hotsprings whre bacterial isolates originated were similar. *Bacillus* sp. MTS keratinase was more active at 55°C[20], *Bacillus* spp. keratinase showed to have high activity at 40°C and 50°C, while thermotolerant *B. licheniformis* FK14 has optimum temperature for the enzyme at 60°C[22].

Acknowledgments

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