Proceeding of 5th International Seminar on
New Paradigm and Innovation on Natural Sciences
and Its Application (5th ISNPINSA)

ISSN: 978-602-71169-7-9

Growth Optimization of Thermophilic Bacteria *Bacillus thermoamylovorans* and *Brevibacillus* sp. in Producing Keratinolytic Enzyme

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Abstract. Some thermophilic bacteria have been isolated from Tanjung Sakti hot spring. Two isolates which qualitatively showed keratinolytic activity, identified as *Bacillus thermoamylovorans* and *Brevibacillus* sp., were optimized the growth in producing keratinase enzyme. Keratinase is a group of proteolytic enzyme which able to hydrolyze insoluble protein (keratin). In this research, whole chicken feathers were used as keratin substrate. The growth curves and measurement of keratinase enzyme activities were determined to the isolates, with a span of measuring 4 hours during 48 hours incubation, and gained the growth and optimum enzyme activity at 28 h incubation. The isolates were then observed the growth and keratinase activity at several different temperatures and pH. The results showed that the optimum temperature and pH for both isolates were same, i.e. at temperature 70° C and pH 7. The effect of adding a source of carbon and nitrogen into the medium were also observed. The growths of both isolates were increased on the addition of 1% glucose and 0.4% casein.

Keywords: keratinase, thermophilic bacteria, *Bacillus thermoamylovorans*, *Brevibacillus* sp.

Introduction

Keratinases [E.C.3.4.21/24/99.11] is a unique group of proteases that degrade insoluble and very complex proteins, called keratin. eratin is a major component of skin, nails, hair, and feather. Keratin is a class of fiber protein that are very stable due to strong packaging into α-helix and β-sheets by disulfide and hydrogen bonds [1] so it is not susceptible to normal protease such as pepsin, trypsin and papain [2]. Recalcitran keratin can cause environmental problems. The use of keratinolytic thermophilic bacteria through the fermentation process can be effectively used to treat keratin wastes [3].

Keratinase has been reported to be obtained from different microbes such as bacteria, fungi and actinomycetes [4]. Some bacteria of the *Bacillus* group are reported as producing enzyme keratinase [2] including thermophilic bacteria such as *Bacillus licheniformis, Bacillus* sp. and *Brevibacillus thermoruber* [5, 6, 7]. Thermostabil keratinase from other bacteria also have been reported [8, 9, 10]. In this research, we reported optimization of thermophilic bacteria growth in producing keratinolitic enzyme as an early attempt to handling chicken feather waste. The bacteria were isolated from Tanjung Sakti hot spring and have been identified as *Bacillus thermoamylovorans* and *Brevibacillus* sp.

Methodology

Keratinolytic activity assay. Keratinolytic activities were qualitatively tested using the agar diffusion method. Agar medium containing 1% chicken feather powder, 0.05% NH4Cl, 0.05% NaCl, 0.03% K2HPO4, 0.04% KH2PO4, 0.01% MgCl2.6H2O, and 1.5% bacto agar. Keratinolytic activity was characterized by the formation of a clear zone around the bacterial culture on paper disc.

Keratinase activity was assayed quantitatively by adding 200 uL of enzyme into 8 mg chicken feather powder in 800 uL phosphate buffer solution pH 7.5. The mixture was incubated at 60 ° C for 60 minutes. The enzymatic reaction was stopped by addition of 1 ml trichloro acetic acid (TCA) 10%, then left at room temperature for 30 minutes and centrifuged at 10,000 rpm for 10 minutes. 500 uL the supernatant was added with 2.5 mL of sodium carbonate and then incubated for 10 minutes. 500 uL Folin reagents was added and then

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incubated for 30 minutes. Absorbance of the solution was measured at a wavelength of 660 nm. One unit of keratinase activity was defined as the amount of the enzyme required to release 1 μmol of tyrosine per minute under the conditions used.

Optimization of incubation time. Keratin Media which contains 1% chicken feather powder, 0.05% NH4Cl, 0.05% NaCl, 0.03% K2HPO4, 0.04% KH2PO4, 0.01% MgCl2.6H2O were inoculated with 5% (v/v) bacterial cultures. The cultures were incubated at temperature 55°C with shaking 150 rpm. Culture samples were taken periodically every 4 hours for 48 hours of incubation and growth of bacteria was determined by the total plate count with serial dilution method.

The effect of temperature and pH. Influence of temperature and pH were observed respectively by growing bacteria at temperatures 50°, 60°, 70° and 80° C and at pH 6, 7 and 8, and then the growth and keratinolytic activity were observed. Phosphate buffer was used to adjust the pH of the media.

The effect of adding a source of carbon and nitrogen. The effect on the growth of bacteria due to the addition of carbon sources 1% glucose, 1% sucrose and 1% galactose and nitrogen source 0.4% yeast extract, 0.4% tryptone, 0.4% peptone, and 0.4% casein, respectively, into keratin media, were measured simultaneously in producing keratinolytic enzyme.

Results and Discussion

Keratinolytic activity of *Bacillus thermoamylovorans* and *Brevibacillus* sp. were indicated by the formation of a clear zone around the bacterial culture on keratin agar media. This showed that the keratin (feather) was able to be hydrolyzed to short-chain peptides, dissolved and absorbed by the cells. Quantitative measurement of bacterial growth and their keratinolytic activity during the incubation period 0 to 48 hours was shown in Fig. 1. Based on the growth curve in Fig. 1, the growth profile of *B. thermoamylovorans* was different from *Brevibacillus* sp. *Brevibacillus* sp. showed a higher growth rate than *B. thermoamylovorans*. However, keratinolytic activity profiles showed similarity. After incubation of 28 to 48 hours, there was no increase in bacterial growth. This showed the early stationary phase. On a longer incubation period, the growth and activity of keratinolytic increased (data not shown in Fig. 1) which showed more hydrolyzed keratin that could be digested by bacteria. It suggested that the keratinase was an enzyme for the primary metabolite, used by microorganisms for cell growth and can be secreted into the environment.

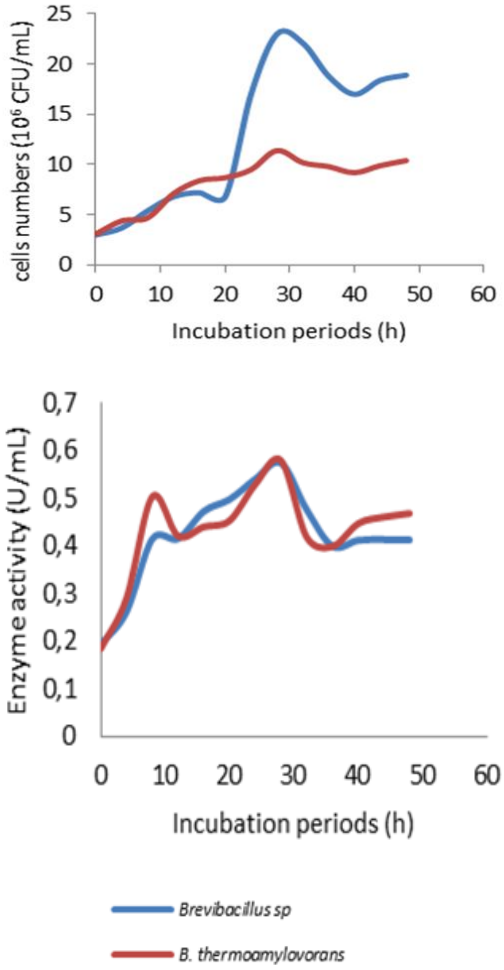

Fig.1 Growth curve of *Brevibacillus* sp and *B. thermoamylovorans* (A) and the keratinolytic activities (B) during incubation period of 0 to 48 hours.

In addition to the growth curve, the effect of differences in temperature, pH, carbon sources, and nitrogen sources on the growth and activity of keratinolytic were also determined. The results were shown in Fig. 2.

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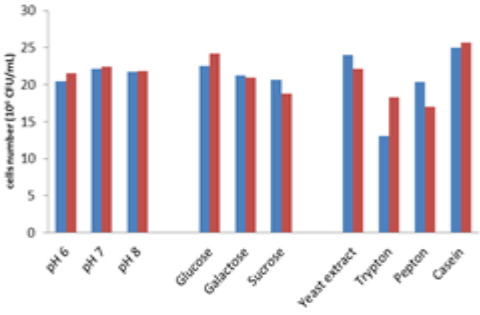
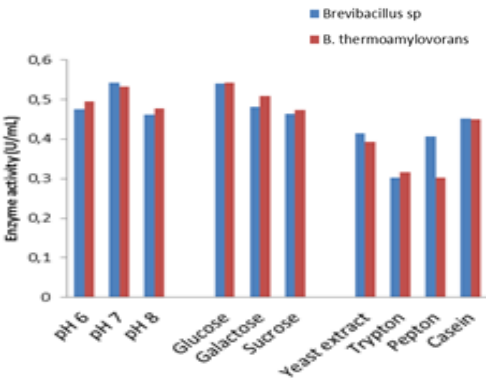
ISSN: 978-602-71169-7-9

The growth and keratinolytic activity of both bacteria at temperatures 50°, 60° and 70° C tended to slightly increase. The growth was observed decrease at temperature of 80° C (data not shown in Fig. 2). pH differences in keratin media did not significantly affect the growth of bacteria, but keratinolytic activity observed higher at pH 7. The addition of a carbon source galactose and sucrose had no effect on the growth and activity of keratinolytic, but the addition of glucose slightly increased the growth of bacteria, especially in *B. thermoamylovorans.* The addition of a nitrogen source yeast extract, tryptone, peptone, and casein into the media produced different effects on bacterial growth and keratinolytic activity. Tryptone and peptone tended to reduce the growth and keratinolytic activity of bacteria, whereas yeast extract and casein could promote growth but not for their keratinolytic activities.



a.



b.

Fig. 2 The effect of pH, carbon and nitrogen sources on the growth (A) and keratinolytic activity (B) of the *Brevibacillus* sp. and *B. thermoamylovorans*

Summary

Brevibacillus sp. and *B. thermoamylovorans* were able to produce keratinase enzyme. The optimum culture conditions for the production of keratinase were 28 hours incubation period, at temperature 70°C and pH 7. Addition of 1% glucose and 0.4% casein could increase the growth but did not affect the keratinolytic activities of the bacteria.

Acknowledgment

This research was funded by Hibah Fundamental Dikti 2015.

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