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Preliminary Study on Keratinase Fermentation by *Bacillus* sp. MD24 under Solid State Fermentation

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Abstract. *Bacillus* sp. MD24 has been reported to produce keratinase under submerged fermentation (SmF) using chicken feathers as sole carbon and nitrogen sources. However, the enzyme production was not preferable for industrial purpose due to relatively low enzyme yield. Solid state fermentation (SSF) was developed to improve keratinase productivity. Water content was varied to achieve optimum keratinase production. The effects of additional agricultural wastes to keratinase production were tested. The results showed that SSF improved keratinase production and optimum keratinase activity was achieved at the water content of 500%. Agricultural wastes (rice husk, sugar cane bagasse, and solid tofu-waste) significantly improved keratinase production. However, the production was delayed when rice straw and sugar cane bagasse were used. The highest activity at the fifth day was achieved when sugar cane bagasse was added to chicken feather medium.

Keywords: Chicken feathers, *Bacillus* sp. MD24, solid-state fermentation.

1. Introduction

Chicken slaughterhouse produces chicken feathers which are widely discarded to the environment. The crude protein content in chicken feather is about 82% of total dry weight [1], which makes chicken feathers as one of the highly potential amino acid sources. The main constituent protein in chicken feathers is keratin, a highly fibrous protein which is insoluble in water and organic solvents. Keratin was built by α -helix (α -keratin) and β -sheet (β -keratin) structures which are super-coiled into polypeptide chain [2]. The structure is stabilized by hydrogen bonds, disulfide bridges as well as hydrophobic interactions. The presence of disulfide bonds made keratin as a tough protein which is not degraded by a general protease such as trypsin, pepsin, and papain [3].

Chicken feather generally is converted into feather meal through the rendering process. Feather meal is used in the formulation of animal feed and organic fertilizer. The process included steam pressure cooking over 140 °C followed by cooling, drying and grinding. However, In addition to high energy consumption, the process produces limited and varying amino acids composition [4]. The fact that feathers in nature do not accumulate is an indicator that feather is broken down into simpler molecules. Many studies have shown that microbes isolated from soil produce specific protease called keratinase which is capable of degrading keratin in feathers. Keratinase is capable of breaking the peptide bonds and the disulfide bridges in keratin to shorten polypeptide and convert it to amino acids.



This specific protease offers an efficient and eco-friendly alternative to process chicken feathers into feather meal [5–7].

Some fungi have been reported to produce keratinase; e.g. *Aspergillus fumigates* [8], *Aspergillus niger* [9], and *Aspergillus flavus* [10]. Among bacteria, keratinases exclusively are produced by *Bacillus* species, e. g. *Bacillus subtilis* [11], *Bacillus licheniformis* [12], and *Bacillus halodurans* [13]. *Bacillus* sp MD 24 is an indigenous Indonesian keratinolytic bacterium isolated from Malang, East Java. *Bacillus* sp. MD24 has been reported to produce keratinase under submerged fermentation (SmF) using chicken feathers as sole carbon and nitrogen sources. However, the fermentation method required a high amount of water and yielding low keratinase activity and a low amount of degraded chicken feather. Solid-state fermentation (SSF) is a fermentation involving solids in the near absence of free water; however, the substrate must be surrounded by enough moisture to support growth and metabolism of microorganism [14]. As chicken feathers are a solid substrate, SSF might be better fermentation technique to produce keratinase. This study reported the production of keratinase by *Bacillus* sp. MD24 under Solid-State Fermentation.

The growth of microbes is affected by energy source availability. When a cell is grown using the chicken feather as a sole carbon and nitrogen sources, keratinase is produced by microbes to degrade keratin into absorbable molecules, amino acids served as a carbon source for energy as well as a nitrogen source. Although under this condition, cell concentration is not always linear to keratinase concentration during fermentation; at certain condition cell concentration, it is directly related to keratinase production. Hence, the addition of another carbon source might help to increase cell concentration and subsequently would increase keratinase production level. Substrate selection for SSF is mainly important for cost and substrate availability [15]. Rice straw, sugar cane bagasse, and solid tofu-waste are carbon-rich materials available for a reasonable amount for industry, and they might be a suitable carbon source for *Bacillus* sp MD24. Those foods and agricultural wastes were tested as additional carbon source to chicken feathers.

2. Methods

2.1. Microorganism and Chicken Feathers

The bacterial strain, *Bacillus* sp. MD24, was isolated from soil in Malang, Indonesia, as reported in [16]. Broiler (*Gallus gallus domesticus*) chicken feathers were collected from poultry slaughterhouse home industry in Malang. The chicken feathers were washed with tap water 5-7 times and dried under sunlight.

2.2. Preparation of *Bacillus* sp. MD24 preculture

Regeneration of *Bacillus* sp. MD24 was done by inoculating pure culture of the bacterium on skim milk-agar medium. The inoculum was incubated overnight at 37 °C. Colonies with clear zone confirmed the production of protease by the bacterium. Subsequently, the cells were inoculated into 50 mL of nutrient broth (NB) liquid medium and incubated on the shaking incubator at 37 °C for 24 hours with an aeration speed of 100 rpm.

2.3. Submerge Fermentation and Solid-State Fermentation

Submerged fermentation was carried out according to [16]. A flask containing 100 mL of medium (5.0 % NaCl; 1.0% MgSO_4 , 0.5% K_2HOP_4 ; and 0.1% of chicken feather feathers) was inoculated with 1 mL of preculture. The inoculum was incubated at 37 °C for five days with an aeration speed of 100 rpm. The crude extract keratinase was separated from degraded chicken feathers by filtration.

Solid state fermentation was undertaken according to [17] with modifications. Five grams of chicken feathers were mixed with various percentages of moistening solution. The moistening solution contained 0.2 (g/L) of KCl, 0.2 (g/L) of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 01 (g/L) of $(\text{NH}_4)_2\text{PO}_4$; 20 (g/L) of CaCO_3 ; 0.5 (g/L) of KH_2PO_4 ; and 0.5 (g/L) of K_2HPO_4 , (pH 8). The percentage of moistening solution is the

percentage of the volume of medium solution to the mass of chicken feathers (v/w) in the fermentation medium. The moistening percentages were calculated by using the following formula:

$$\text{Moisture} = \frac{\text{volume of medium solution (mL)}}{\text{mass of chicken feathers substrate (g)}} \times 100\%$$

The fermentation flasks were incubated at 37 °C for five days. The fermentation results were added with 50 mL of water and filtered. The filtrates were centrifuged at 5000 g and the supernatants were treated as keratinase crude extracts. Enzyme activity was measured every day for five consecutive days.

2.4. Keratinase fermentation with agricultural wastes as additional carbon source

Rice straw, sugar cane bagasse, and solid tofu waste were tested as an additional carbon source to chicken feathers. The agro-wastes were sun dried and grounded into powder using kitchen blender. The trial was carried out at a ratio of chicken feather to those agro-wastes of five to one. The water content was set at 250 %.

2.5. Enzyme Assay

A vial containing 2.5 mL of 1% casein (dissolved in 50 mL of Tris-HCl buffer solution pH 8), and 0.5 mL of keratinase crude extract was incubated at 37 °C for 30 minutes to allow casein hydrolysis. The enzymatic reaction was stopped by adding 1.0 mL of 10% trichloroacetic acid (TCA). The mixture was then incubated at -20 °C for 30 minutes, and subsequently centrifuged for 10 minutes at 5.000 g to separate the precipitated proteins. Tyrosine concentration in the supernatant was measured by measuring the absorbance at 280 nm.

3. Results and Discussion

3.1. Production of keratinase under SSF at a various water concentration

SSF has been reported to have many advantages compared to SmF; e.g. It has high fermentation productivity especially for cultivation of microorganisms for water-insoluble substrates and high concentration of end product [9]. Table 1 shows keratinase activity in the crude enzyme extract produced during SmF and SSF for five consecutive days. The highest activity under both fermentation methods was observed on day 3. SSF yielded lower activity compared to SmF at the water content of 100%. SSF at the water content of 300% produced was slightly higher activity compared to SmF. The maximum activity was observed at the water content of 500% (Figure 1).

Table 1. The comparison of keratinase production between SmF and SSF using chicken feathers as a sole carbon and nitrogen sources.

Day	Keratinase Activity (U/mL)				
	SmF	Water Content under SSF			
	1% substrate	100%	300%	500%	700%
1	2.03 ± 0.2	5.99 ± 1.0	34.03 ± 2.8	187.14 ± 10.3	29.04 ± 5.2
2	62.16 ± 3.1	23.78 ± 2.4	70.82 ± 8.6	378.65 ± 16.3	310.66 ± 8.2
3	69.79 ± 4.2	30.75 ± 7.8	88.16 ± 7.9	527.19 ± 23.2	336.22 ± 9.04
4	29.33 ± 4.2	2.46 ± 0.7	64.43 ± 2.1	424.12 ± 12.5	298.89 ± 17.0
5	27.16 ± 3.2	2.31 ± 0.3	64.4 ± 2.1	409.09 ± 16.4	160.14 ± 17.7

Keratinase production under SSF using poultry feathers as sole carbon and nitrogen sources was reported from *Aspergillus niger* [18], *Paenibacillus woosongensis* [19], *Bacillus weihenstephanensis* [20], and *Myrothecium verrucaria* [21]. It is hard to make conclusion among those reported experiments since they all had different experiment set up. However, all those experiments were done

at lower water content compared to this work ($< 100\%$). The water activity (a_w) in the substrate influences the microbial growth. The mass transfer of water and solutes in microbial cells depend on the water activity of medium. Therefore, metabolic production or excretion of microorganisms can be modified by controlling the water activity [22]. The fermentation of keratinase using chicken feathers as sole carbon and nitrogen seems to need higher water to compare to the reported works, which reflects requirement of higher free water in the medium. Interestingly, *Bacillus* sp MD24 requires more water compared to traditional SSF condition.

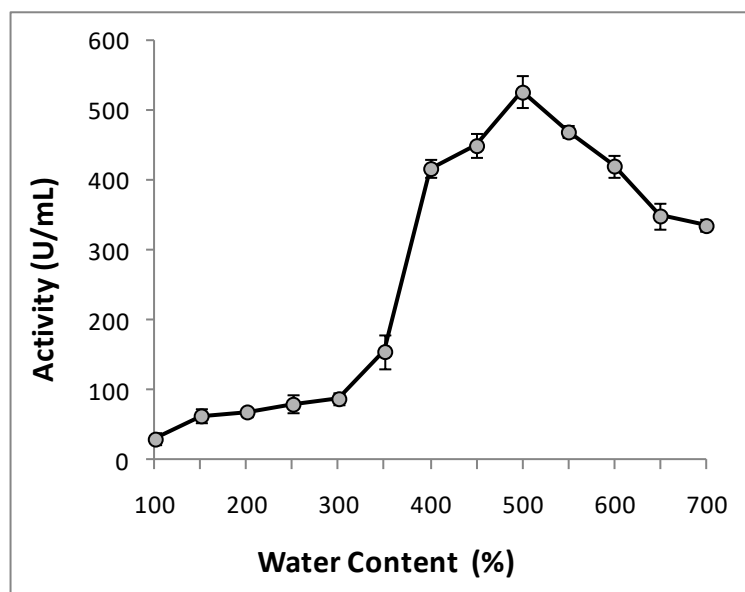


Figure 1. The effect of water content to keratinase production under SSF using chicken feathers as sole carbon and nitrogen sources

3.2. The Effect of Additional Agricultural Wastes on Keratinase Production

Bacterial growth can be influenced by several factors, including energy source. Figure 2 shows initial study on keratinase production in the presence of rice husk, sugar cane bagasse, and solid tofu-waste as additional carbon sources. All three carbon sources increased the production, but rice straw and sugar cane bagasse delayed keratinase production. On day 5, the highest keratinase activity was observed when sugar cane bagasse was added to chicken feathers. The previous trial showed that simple carbohydrates such as glucose and sucrose inhibited keratinase production in SmF. Readily adsorbed energy source might repress keratinase expression due to cell efficiency and limited amount keratinase are needed mostly only to provide nitrogen supply. Simple carbohydrates were not yet tested on SSF; however it most likely would give similar result to SmF. All provided additional carbon sources provided complex carbohydrates which showed better additional carbon sources compared to simple carbohydrates. However, further study need to be done to find the best chicken feather and additional carbon source ratio and incubation time. Chicken feather to agricultural waste ratio was reported as an important condition for keratinase production [23]. An optimum water content also need to be evaluated, since the need of free water might also shift due to water requirement for carbohydrate hydrolysis.

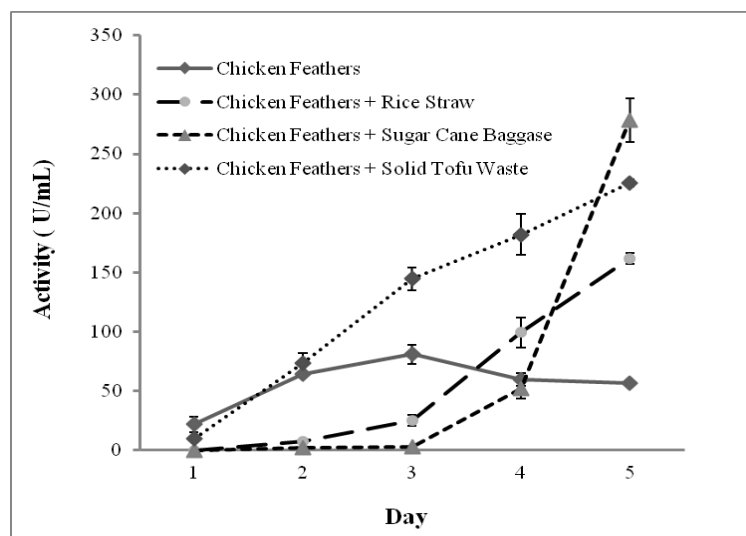


Figure 2. The effect of rice straw, sugar cane bagasse, and solid tofu-waste on keratinase production

4. Conclusion

Keratinase fermentation by *Bacillus* sp. MD24 under SSF was preferable compared to SmF. An optimum keratinase was achieved at the water concentration of 500% when chicken feathers were used as sole carbon and nitrogen sources. This condition was rather unusual compared to traditional SSF condition. Under traditional SSF condition which had very low water content, the keratinase was produced at very low concentration. Since water content was relatively high, the aeration might influence to have enough soluble oxygen for cell respiration. The study also revealed that keratinase production was highly influenced by additional complex carbohydrate, most probably it influenced on cell biomass which subsequently produced keratinase to provide enough nitrogen supply for the cells.

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