

# Keratinase Production by *Bacillus* sp. MD24 in Sub-merge and Solid State Fermentation

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**Abstract** Chicken feathers are valuable solid waste that mainly contains insoluble proteins called keratin. Naturally, keratin is degraded by microbes that produce a specific protease called keratinase. Many reports have been addressed to study *Bacillus* species as keratin-degrading microbes. However, most reported works were using pathogenic soil *Bacillus*. Our work has been concentrated on keratinase produced by a soil bacterium, *Bacillus* sp. MD24 and we confirmed it as a non-pathogenic bacterium. This report describes the different profiles of keratinase fermentation between sub-merged fermentation (SmF) and solid-state fermentation (SSF) using chicken feathers as a sole carbon and nitrogen source. Keratinase production and weight loss were followed for 10 consecutive days, the keratinase molecular weight was investigated, the type and concentration of 18 amino acids were determined using LC-MS/MS after 10 days to seek its potential as the amino acid source, and protein by-product concentration was measured. The results showed that keratinase production as well as weight loss in SmF was better compared to SSF with different optimum production times. Surprisingly, L-arginine was a dominant amino acid in SmF hydrolysate which contained 42.06% of total amino acids and made *Bacillus* sp. MD24 is a potential organism for L-arginine production. As protein by-product concentration was relatively low in both conditions showing effective utilization of chicken feathers as matter and energy source, consequently, cell-free keratinase should be applied to degrade chicken feathers into valuable materials.

**Keywords:** Keratinase, *Bacillus*, chicken feathers, fermentation, amino acids.

## Introduction

Chicken feathers are a major by-product of the poultry industry. The global poultry market indicates a 4.1% growth between 2021 and 2025, reaching a production of 100.9 million metric tons in 2025 (Saenz 2022). Depending on the breed, sex, age, and muscularity, chicken feathers make up from 3 to 6% of the total live weight (Hardiman and Katanbaf 2012). Ironically, compared to egg white which contains only about 57% protein, chicken feathers contain 85% protein in the form of fibrous protein. While protein is an important nitrogen supply for humans and animals, the chicken feather, as solid waste, is insoluble in water and organic solvent and it is hardly degraded by protein digestive enzymes such as trypsin and chymotrypsin due to its complex structure which is stabilized by crosslinker disulfide bound.

Polypeptides and amino acids derived from chicken feathers have high potential for many applications such as animal feed (Adelina *et al.* 2021; Eaksuree *et al.* 2016; Machado *et al.* 2021; Adelina *et al.* 2021) biofilm (Nurkhasanah. 2020; Tesfaye *et al.* 2018), fertilizer (Rai and Mukherjee 2015), and wood-adhesive (Jiang *et al.* 2008).  $\beta$ -keratin, a structural protein constructing feathers, has natural characteristics such as providing waterproofing and preventing desiccation, thus providing bioactivity, biocompatibility, and good physical properties for preparing hydrogel for wound repair (Kushibiki *et al.* 2021; Wang *et al.* 2017). Especially preparation of soluble keratin for biofilm and wood adhesive will need a high concentration of soluble proteins rather than amino acids. This condition is hard to achieve

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under chemical degradation due to its random cleavage of peptide bound. On the contrary, enzymatic degradation of chicken feathers provides a homogenous length of polypeptides due to its specific cutting site of keratinase of peptide bound. Besides, enzymatic degradation is more favorable for reducing chicken feathers due to its negative impact on human health and the environment which is one of the important issues in sustainable development goals, an urgent call for action by all countries.

Keratin in chicken feathers can be degraded by specific microbial protease so-called keratinase. Much research has been focused on finding keratinase-producing microbes, screening and optimizing fermentation conditions, and characterizing the enzyme. Depending on the substrate phase and the amount of water, two types of fermentation conditions have been studied. Those are sub-merge fermentation (SmF) and solid-state fermentation (SSF). SSF refers to microbial fermentation with a solid substrate and a limited amount of water, generally, the solid substrate and water ratio is set between 1:1 and 1:10. While SmF was carried out in a huge amount of water with solid substrate concentration is set between 0.5 and 5% (w/v) of the total volume of the medium. For solid substrates, SSF was thought to give more advantages compared to SmF. Among those, SSF reduces contamination possibilities, applies a simple reactor design and less spatial requirements, provides all necessary for cell growth, requires low energy which reduces the production cost at the industrial level, and high product concentration which leads to low-cost downstream processing, and might produce the slightly different character of the desired product (Nkemnaso 2019). An advanced study on the life cycle impact assessment (LCIA) using ReCiPe 2016 methodology which applied indicator scores to express the relative severity of an environmental impact category showed that SSF has 13% lower impact in the SSF process than the SmF process for enzyme recovery and mainly due low electricity consumption (Catalán and Sánchez 2020). Some studies showed different product profiles between SSF and SmF conditions. Comparing transcriptomic and metabolomic profiles between SSF and SmF showed that the metabolite production and its corresponding gene expression levels in *P. expansum* 40815 were significantly influenced by the fermentation approach (Kim *et al.* 2016). This finding validated different metabolic pathways expressed by organisms directly related to different ecological states, physiologies, and growth conditions. Therefore, advanced studies must be carried out before bringing a protocol at the industrial level to find the best condition and provide the greenest process and benefit to humans and the environment.

Mazotto *et al* compared chicken feather degradation profile between SSF and SmF conditions by *Aspergillus niger* (Mazotto *et al.* 2013). The keratinase production was 7 times higher when the *A. niger* strains were cultivated under SSF condition rather than SmF condition. *A. niger* is a filamentous fungus that naturally uses hyphae to absorb nutrients from the local environment and transport them. During fermentation under SmF conditions, the cells filamentous were constantly introduced to high shear stress due to constant stirring which caused hyphae fragmentation and disturbed cell growth and it might reduce metabolite productivity. On the other hand, *Bacillus* which also produced keratinase when it is grown using chicken feather substrate, has a different morphological structure which is motile and swims around the environment which most likely has a different profile between both fermentation conditions. Some *Bacillus* species have been studied under sub-merge fermentation (SmF) (Mazotto *et al.* 2013; Suharti *et al.* 2018) and solid-state fermentation (SSF) using chicken feathers as a sole carbon source as well (Mazotto *et al.* 2013; Tuly *et al.* 2022). However, no report on profiling keratinase fermentation for *Bacillus* sp. between SSF and SmF was found. Production of  $\alpha$ -amylase by *Bacillus* sp. KR-8104 produced a slightly different enzyme character but no further study of such activity and product profile was reported (Hashemi *et al.* 2013). We have done SmF and SSF studies to explore chicken feather degradation (a solid-state substrate), keratinase production, and by-product profiles using chicken feathers as the sole carbon and nitrogen source by *Bacillus* sp. The studies employed *Bacillus* sp. MD24 as an organism model.

We had previously reported our work on the fermentation of keratinase by *Bacillus* sp. MD24 under SmF and the enzyme has been tested to remove hair from goat skin (Suharti *et al.* 2018). *Bacillus* sp. MD24 was characterized as a Gram-positive bacterium that has only 89% sequence identity compared to the available 16S rRNA sequences in the NCBI database reflecting as a newly isolated microorganism that offer opportunities for further investigation. Keratinase from *Bacillus* sp. MD24 has been initially characterized and it has optimum activity at pH 8 and an optimum temperature of 37°C. Furthermore, we have communicated our initial work on degrading chicken feathers using the bacterium under SSF

(Nurkhasanah and Suharti 2019) and the initial characterization of its solid by-product (Suharti *et al.* 2019) as well as its possible application for biofilm preparation (Nurkhasanah *et al.* 2020) and biofertilizer (Andriyani *et al.* 2021). This paper reports our work on the confirmation of *Bacillus* sp. MD24 pathogenicity, keratinase production and soluble by-product profiles under SmF and SSF condition profiles.

## Materials and Methods

### Hemolysis Assay

A hemolysis assay was done to detect whether *Bacillus* sp. MD24 isolate produces protease that damages hemoglobin. This information is important if the bacterium were used for any application which has public health importance such as industrial application. The assay was done using a blood agar plate. The plates contained 8 g/L nutrient agar powder (Merck) and 5% (v/v) defibrinated cow blood. Sterile nutrient agar solution and sterile defibrinated blood were mixed at  $\pm 50^\circ\text{C}$ . *Bacillus* sp. MD24 was inoculated on the blood agar plate with a quadrant streak and incubated at  $37^\circ\text{C}$  for 24 - 48 h.

### Chicken Feathers Preparation

Broiler chicken (*Gallus gallus domesticus*) feathers were obtained from local chicken slaughterhouses at Malang, East Java, Indonesia which have ages between 2 and 3 months. Chicken feathers were washed with soap solution, rinsed with tap water 5 times, and dried by sunlight, and cut  $\pm 0.5$  cm before use it for fermentation.

### Regeneration and starter preparation of *Bacillus* sp. MD24

*Bacillus* sp. MD24 was grown in Skim Milk Agar (1.5% bacto agar, 0.5% NaCl, 0.1%  $\text{MgSO}_4$ , 5.0% skim milk at pH 8) and then incubated for 24 hours at  $37^\circ\text{C}$ . The fresh inoculum was taken and inoculated in Luria Bertani (LB) liquid medium (1% peptone, 1% NaCl, and 0.5% yeast extract). The inoculum was then incubated for 16 hours at  $37^\circ\text{C}$ , and culture was used for further experiment.

### Fermentation Keratinase under SSF and SmF

The starter medium was made with LB medium and incubated for about 6 hours in the shaker water bath at  $37^\circ\text{C}$  until  $\text{OD}_{600}$  about 0.5. The culture was then used for fermentation. For SmF 1 mL of the starter of *Bacillus* sp. MD24 was inoculated in a 100 mL fermentation medium containing 0.5% NaCl; 25 mM  $\text{K}_2\text{HPO}_4$ , 0.2%  $\text{MgSO}_4$ ; and 1% chicken feathers in 50 mM Tris-HCl pH 8. Whereas for SSF, 1 mL of starter was added to the mixture of 1 g chicken and fermentation medium at a ratio of 1 to 5 or 1 to 10. For SmF, the inoculum was then incubated in a shaker incubator at  $37^\circ\text{C}$ , 100 rpm for 10 days, while for SSF inoculum was incubated at the same condition without agitation. After 10 days of incubation, the enzyme crude extract was separated. Because of water limitation, 50 mL water was added to SSF broth before the separation. The remaining chicken feathers were filtered and dried under sunlight for 2 days and the filtrate was centrifugated to separate the cells and cell debris at 10.000 rpm and the supernatant was freeze-dried for 24-37 hours. Keratinase activity, chicken feathers and weight loss were measured every day for 10 consecutive days.

### Determination of Protein Concentration

Protein contents in the keratin hydrolysate were determined by BCA Protein Assay Kit (Pierce™) using the BSA standard curve. The amount of 0.1 mL crude extract was added to 2 mL of BCA reagent (Reagent A: Reagent B = 50:1). The mixture was incubated at  $37^\circ\text{C}$  for 30 minutes and cooled at room temperature for 15 minutes and the absorbance was measured at 562 nm. Amino acids analysis was done based on raw data previously published by (Andriyani *et al.* 2021; Nurkhasanah *et al.* 2020).

### Enzyme Activity Measurement

Protease enzyme activity was carried out based on the Anson method. The tested samples of 0.2 mL were mixed with 0.5 mL of 1% (w/v) casein and then incubated for 5 minutes at  $37^\circ\text{C}$ . The enzymatic reaction process was stopped by adding 1 mL of 10% TCA. The mixture was incubated at room temperature for 5 minutes and centrifuged at 10000 rpm for 5 minutes. One mL of supernatant was taken and 2.5 mL of 0.5 M  $\text{Na}_2\text{CO}_3$  and 0.5 mL of Folin-Ciocalteu reagent (1:1) were added, and then incubated in the dark for 30 minutes. The blue solution was measured with a UV-Vis spectrophotometer at a wavelength of 660 nm.

### Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Zymography

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using the method of Laemmli with 12% separation gel and 4% stacking gel. Zymography was performed by reactivating enzymes in

12% SDS-PAGE. After the completion of electrophoresis, the gel was soaked for 30 min in 2.5% (v/v) Triton X-100 with constant shaking to remove SDS. The gel was washed three times with 100 mM glycine-NaOH buffer pH 10.0 to remove Triton X-100. The gel was then overlaid onto a 1% casein. Enzyme activity was visualized by incubating the gel overnight at 37°C. The reaction was stopped by flooding with 5% (w/v) TCA. The band of the corresponding peptidase appeared as clear zones.

### Amino Acid Determination of The Hydrolyzed Soluble Proteins in Liquid Fermentation Product

After 10 days of incubation, enzyme activity dropped up to 5 times compared to its optimum production. Soluble proteins were completely hydrolyzed using 6N HCl and autoclave at 110°C for 12 h and filtered through 0.22 µm membrane. The filtrate was subjected to amino acids determination using liquid chromatography-tandem mass spectroscopy (LC-MS/MS). Acquity UPLC beh C18 column with the dimension of 1.7µm, 2.1x50 mm was used with a mobile phase A consisting of 99.5% of 0.1% Pentadecafluorooctanoic Acid (PDFOA) and 0.5% CH<sub>3</sub>CN with 0.1% formic acid and mobile phase B consisting of 10% of 0.1% PDFOA and 90% of CH<sub>3</sub>CN with 0.1% Formic acid at a flow rate of 0.6 mL/min. Amino acids standard consisting of 1000 ppb of each of 18 types of amino acids.

## Results and Discussion

### Characterization of *Bacillus* sp. MD24 as a Non-pathogenic Bacterium

The hemolysis assay showed no clear (Figure 1) zone surrounding the bacterium colonies which confirms the bacterium is not hemolytic. Blood agar plates generally are used to distinguish pathogenic bacteria based on their hemolytic activity on red blood cells (Yao *et al.* 2012). Some bacillus strains which have keratinase activity were reported to have hemolytic protease activity (Tiwarly and Gupta 2010). It is important to clarify the pathogenicity of the microbe before any industrial application. Most studied keratinase were reported from widely identified human pathogens such as *Bacillus pumilus* (Habbeche *et al.* 2014), *Bacillus liceniformis* (Abdel-Fattah *et al.* 2018), and *Bacillus subtilis* (Mazotto *et al.* 2011). The experiment showed that *Bacillus* sp. MD24 is safe for further application with benefits to the environment and (bio) economy.

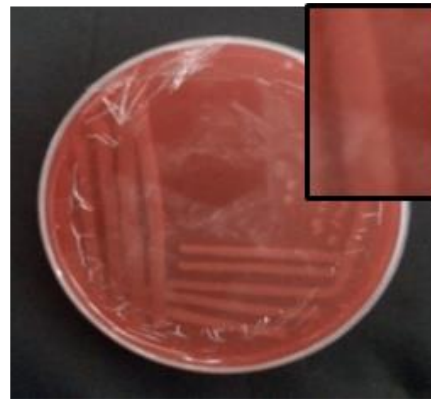
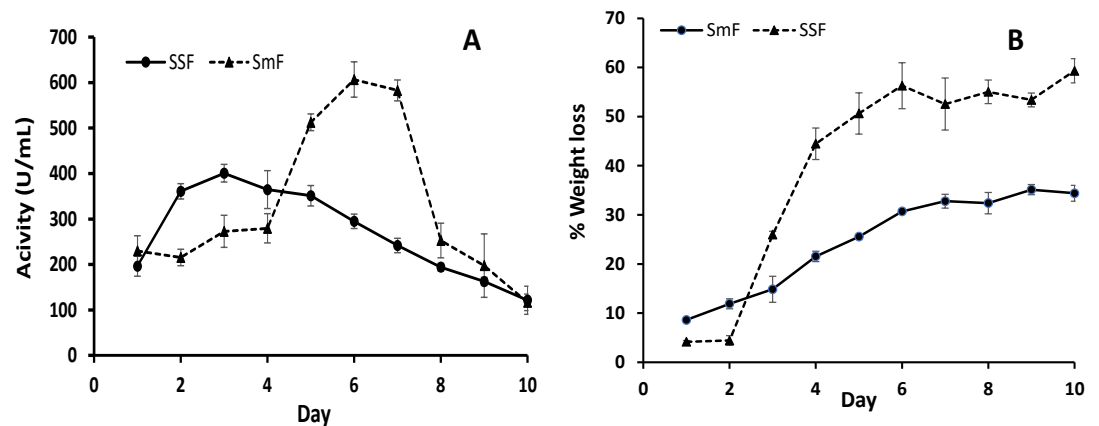


Figure 1. Blood agar plate culture of *Bacillus* sp. MD24

## Analysis of Soluble Product

### 1. Keratinase



**Figure 2.** Keratinase production and weight loss of chicken feathers during 10 days of incubation. A. Fluctuation of enzyme activity. B. Weight loss

Keratinase fermentation under SmF and SSF conditions exhibited different keratinase activity, keratinase-specific activity, and chicken feather weight loss. Keratinase activity and keratinase-specific activity are shown in Figure 2A. Keratinase production during SSF was optimum on day 3 while during SmF optimum on day 6. Although the production was faster under SSF, the activity was lower, therefore, SmF condition is better for keratinase production by *Bacillus* sp MD24. Chicken feather degradation under SmF is also better (Figure 2B). Consistent with our previous finding, after 10 days of incubations, chicken feathers degraded up to 60% under SmF and only 35% of them were degraded under SSF. The speed of degradation was quite good compared to *Pseudomonas microphilus* which only degraded chicken feathers up to 20% for 10 days (Kani, *et al.* 2012). Although other *Bacillus* strains degraded chicken feathers by up to 78% (Nagal and Jain 2010), *Bacillus* sp. MD24 is superior for further applications due to health issues related to pathogenicity.

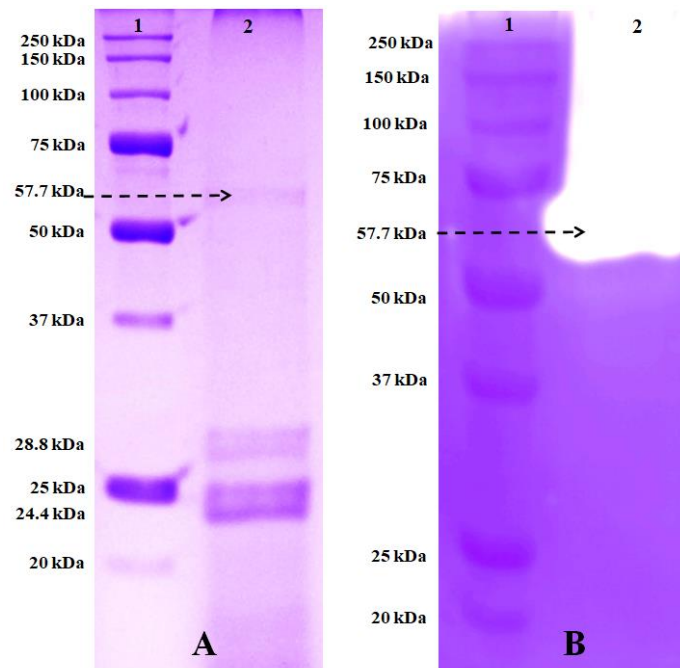
SmF medium contains more water, which led to higher keratinase activity, keratinase-specific activity, and chicken feather weight loss. This phenomenon might reflect the natural character of *Bacilli*. *Bacillus* have different characteristics compared to filamentous fungi which have unique morphological characteristics including the formation of hyphae, which allow these microorganisms to colonize and penetrate the solid substrate in search of nutrients, and therefore they are excellently suitable for SSF. Bacteria have flagella, a locomotion organ that acts like a propeller, to provide mechanical action to perform chemotaxis. Water provides a medium for bacteria for swimming. Agitation also provides fluid flow which subsequently helps the whirls of the media and increases the motion of bacteria (Mendelson *et al.* 1999). Bacteria also possess pili, hair-like structures on the surface of the cell which have a role in movement and are involved in adherence to surfaces. Both flagella and pili are important for bacteria to hunt nutrients such as carbon and nitrogen sources when *Bacillus* sp. MD24 was grown under the chicken feathers as the sole carbon and nitrogen source.

We further check the effect of agitation on keratinase under both conditions. SmF fermentation typically was done with agitation at 100 rpm while SSF was done without agitation. When fermentation under SSF was agitated at 100 rpm the activity was nearly zero during fermentation and no increasing activity was observed until day 10. It seems water content is a more dominant factor compared to oxygen concentration for keratinase production using solid-chicken feathers.

In-depth analysis of water, behavior was studied using Low field nuclear magnetic resonance (LF-NMR) on dynamic state and microstructure distribution of water within cornmeal-SSF by *Cordyceps militaris*, a fungus used for medication, during the fermentation process. Fermentation not only utilized water in the hydrolysis of the substrate but also introduced water into the substrate matrix by the formation of micropores which was detected by increasing the spin-spin relaxation time and the mobile bulk water spin-spin relaxation time which shows the water availability during fermentation was also increased during fermentation. This finding explained how the presence of bulk water is important during fermentation. In *Bacillus* sp. MD24 water is even more important as a medium for cell motility, therefore,

for the bacterium the bulk water existence might be the best fermentation condition.

Depending on the fermentation purposes, one must make the best decision for the keratinase harvesting step. Keratinase production purposes must be harvested on day 3 for SSF and day 6 for SmF. However, the highest specific activity in both SmF and SSF was achieved on day one. Keratinase production under SSF by *Bacillus* sp.MD24 decreased when chicken feather was used as substrate with the addition of other carbon, both complex and simple carbon sources (Suharti *et al.* 2019) making chicken feathers the bests substrate for keratinase production by *Bacillus* sp. MD24.



**Figure. 3** Image of SDS-PAGE and zymography assay. A. SDS-PAGE. B. Zymography assay. Line 1 is the molecular weight standard and line 2 is the soluble product

SDS and zymography were only done using SmF product on day 10. Figure 3 shows electrophoresis profiles of SDS-PAGE and zymography assay. The soluble product contained 3 bands with calculated molecular weights of 24.4 kDa, 28.8 kDa, and 57.7 kDa, and only the highest molecular weight exhibited gel protease activity using casein substrate. Studies showed various sizes of keratinase between 26 and 130 kDa (Qiu *et al.* 2020). Further SDS and zymography should be done to investigate whether SSF produced different keratinase. A crude extract of keratinase produced by *A. niger* exhibited different clear zone when zymography was performed in different copolymerized protein substrates which informed the presence of different proteases in the keratinase crude extract with specific substrates. More zymography should be carried out using different copolymerized substrates to know whether keratinase crude extract contains more protease bands or not, especially using soluble keratin from chicken feathers.

## 2. Amino acid analysis

LC-MS/MS results, which is the most reliable technique for analyzing a mixture of amino acids, showed that the amino acid composition in the hydrolysates from SmF and SSF products was different (Table 1). To observe the effect of water on the amino acid profile, water content varied with chicken feather and water ratio of 1:5 and 1:10 in SSF, and 18 amino acids were analyzed. Interestingly L-arginine content increased following water amount. At the same chicken feather substrate in SmF contained an abundant amount of water, and L-arginine was far more compared to it in hydrolysate produced by SSF with feather and water ratio of 1:10. On the contrary, other minor amino acids such as L-tyrosine, L-glutamic acid, L-aspartic acid, L-cysteine, L-threonine, L-alanine, L-glycine, L-tryptophan content decreased following increasing of water content. The rest of the amino acids did not show consistency in decreasing or increasing following the amount of water in the fermentation system. The experiment shows that water plays an important role in fermentation.

**Table 1.** Amino acids profile in hydrolysates of liquid products

Amino acids	Percent composition		
	SSF (37°C; 1:5*)	SSF (37°C; 1:10*)	SmF
L-Arginine	5.32	14.14	42.06
L-Histidine	0.00	26.55	8.44
L-Lycine	29.53	47.81	26.58
L-Phenylalanine	2.75	1.03	8.93
L-Isoleucine	0.92	0.60	4.19
L-Leucine	2.83	0.69	9.23
L-Tyrosine	0.67	0.34	0.00
L-Methionine	0.92	0.94	0.01
L-Valine	3.41	14.65	0.54
L-Proline	3.91	6.26	0.00
L-Glutamic acid	6.16	1.97	0.00
L-Aspartic acid	6.57	2.57	0.00
L-Cysteine	5.66	2.57	0.00
L-Threonine	14.06	2.74	0.00
L-Serine	0.58	0.60	0.00
L-Alanine	6.24	1.46	0.00
L-Glycine	2.16	0.77	0.00
L-Thryptophan	8.32	0.94	0.03

\*chicken feathers and water ratio

Amino acids are the final product of keratin's complete degradation. Acid degradation produces all types of amino acids that make up keratin while the liquid product of the SmF and SSF contained a mixture of free amino acids, keratinase, and polypeptide as the product of chicken feather degradation and other extracellular protein proteins produced by the microbe. Table 2 shows the amino acid content in chicken feather hydrolysate that was obtained from acid degradation, and SmF by *Chryseobacterium aquifrigidense* and *Pseudomonas aeruginosa*. Acid degradation of chicken feathers enhance by microwave irradiation produced a high concentration of L-glycine while L-histidine and L-lysine are missing (Chen *et al.* 2015), theoretically, glycine is a major amino acid that makes up chicken feathers. Each microbe produced different compositions of amino acids. *C. aquifrigidense* produced 18 amino acids at percentages below 10% (Bokveld *et al.* 2021) and *P. aeruginosa* produced 16 amino acids with the major component of L-serine and L-methionine (Bokveld *et al.* 2021).

The amino acid existence was influenced by many factors. Firstly, in an environment with limited carbon and nitrogen source in which only chicken feathers were available, keratinase was produced by the cell-degraded chicken feather into amino acids that were readily uptake by the microorganism to be converted into cell biomass and energy to carry out all metabolite biosynthesis. The amino acids would be present in the fermentation soup at any given time. Secondly, keratinase and other extracellular enzymes also had a limited lifetime in the fermentation soup which showed activity change during fermentation. At day 10 both in SmF and SSF the activity decreased as shown in Figure 2A The enzymes denatured and eventually degraded into amino acids and added amino acids availability in the soup. Thirdly, different fermentation conditions influence different gene expression and yield different settings of proteome profiles including extracellular proteins which eventually degraded into amino acids. Fourthly, in batch fermentation on day 10, most of the cells likely are already lysis due to environmental stress and limited medium, especially minerals, and release proteins into the medium and eventually also degraded into amino acids. Therefore, the presence of amino acids in this experiment is the sum of all four sources: direct degradation of keratin in chicken feathers by keratinase, degradation of any soluble extracellular enzymes or proteins produced by the cell, and degradation of proteins from lysed cells.

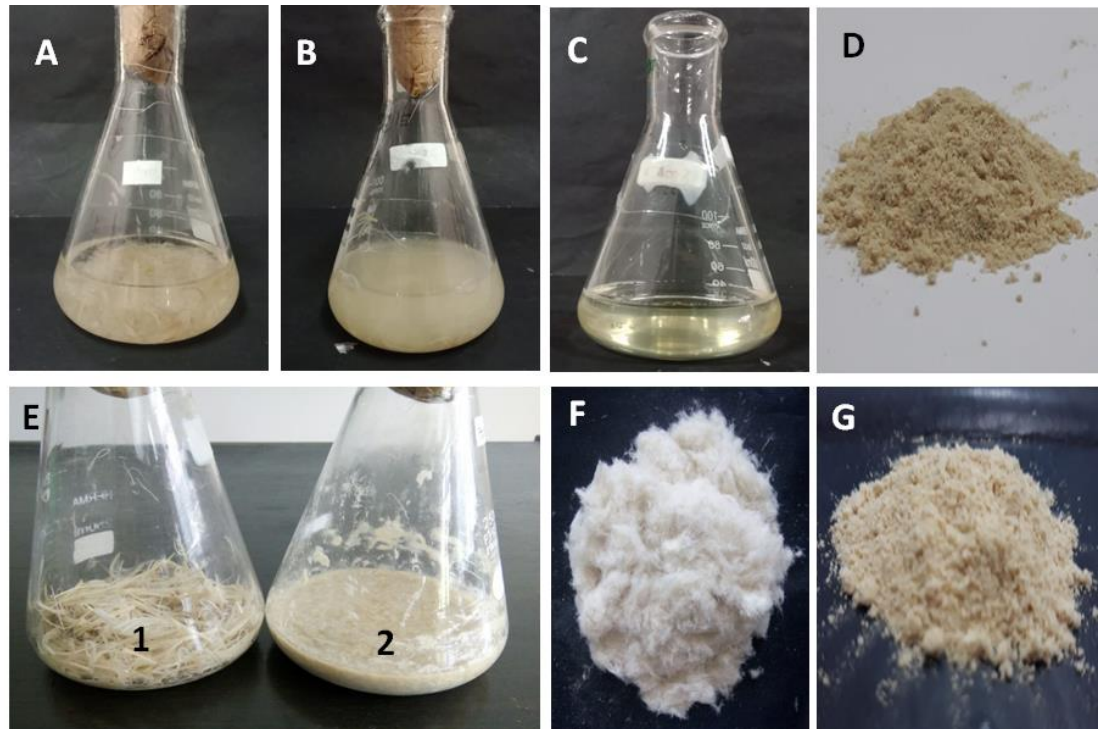
**Table 2.** Profile of amino acids content of chicken feather hydrolysate products

No	Amino acids	% Composition		
		Acid digestion	<i>C. aquifrigidense</i> FANN1	<i>P. aeruginosa</i>
1	L-Arginine	4.30	9.80	2.46
2	L-Histidine	n.d	5.62	23.54
3	L-Lycine	n.d	6.14	4.20
4	L-Phenylalanine	0.86	4.58	2.08
5	L-Isoleucine	3.32	4.58	1.55
6	L-Leucine	2.62	7.45	3.07
7	L-Tyrosine	1.00	2.96	4.50
8	L-Methionine	1.02	0.44	2.23
9	L-Valine	1.61	7.28	2.35
10	L-Proline	12.00	7.36	0.00
11	L-Glutamic acid	7.62	7.19	8.52
12	L-Aspartic acid	6.00	4.40	7.23
13	L-Cysteine	8.85	3.14	0.00
14	L-Threonine	4.00	4.84	4.35
15	L-Serine	16.00	8.85	23.54
16	L-Alanine	3.44	4.84	4.01
17	L-Glycine	n.d	8.41	4.13
18	L-Thryptophan	n.d	2.14	2.23

### 3. Protein content

Soluble proteins are another by-product of keratinase fermentation using protein-rich-chicken feathers as a sole substrate. Figure 4 shows chicken feathers' degradation under SmF and SSF conditions. Figure 4A shows the medium was still clear and the chicken feather was solid in the medium. After 8 days of incubation, the medium became turbid due to the presence of growing cells, and the SmF on day 8 (Figure. 4B). Figure. 4C shows the supernatant of a soluble product with a slightly yellowish solution and Figure. 4D shows the lyophilized soluble product (freeze-dried) for 24 h. Figure 4E shows chicken feather degradation under SSF conditions. The remaining insoluble by-product was filtered, washed, and oven-dried (Figure 4F), while the filtered soluble product was lyophilized (Fig 4G). Protein concentration was determined and yielding 4.8 mg and 4.1 mg protein per gram chicken feather substrate. The powder was very hygroscopic and sticky upon air introduction to air. The protein concentration was relatively low compared to chicken feather weight loss after 10 days of incubation which was 61.84 % and 36.19% for SmF and SSF, respectively. Degraded chicken feathers were effectively absorbed by cells for promoting cell metabolism and division yielding cell biomass. Cell biomass was not determined since it was difficult to measure due to the presence of insoluble by-product which required certain technique to separate cells and insoluble product especially insoluble product that has the same size or mass as the cells. Our previous report showed that keratin biofilm from SSF hydrolysate showed better character compared to acid-hydrolyzed (Nurkhasanah *et al.* 2020), however, it would need plenty amount of hydrolysate to make sufficient biofilm at the industrial level. Therefore, cell-free keratinase must be used to degrade chicken feathers to produce more soluble keratin and amino acids which are very important for further applications such as animal feed, biofilm, keratin hydrogel, fertilizer, and wood adhesive. Further investigation should be done to improve keratinase production and optimized significant factors. In addition, medium-chain length polyhydroxyalkanoate (mcl-PHA) production was linked to keratinase production and chicken feather degradation by cell biomass of *Pseudomonas putida* (Pernicova *et al.* 2019). Investigation of other biomaterial production might be done to explore other benefits of *Bacillus* sp. MD24





**Figure 4.** Chicken feather degradation by *Bacillus* sp. MD 24 under SmF and SSF conditions: A-D are chicken feather degradation under SmF condition: sterilized medium (A), culture on day 8, filtrate on day 10 before freeze-dried, and powder of the solidified soluble by-product (D). E-G are chicken feather degradation under SSF conditions at substrate and salt solution ratio of 1:10: medium before (1) and after 10 days fermentation (2) (E), dried insoluble by-product (F), and powder of freeze-dried liquid by-product (G).

## Conclusions

*Bacillus* sp. MD24 with chicken feathers as a sole carbon and nitrogen source was produced in SmF and SSF conditions with different optimum activity, time production, and speed of chicken feather degradation. Compared to fungal keratinase production *Bacillus* sp. MD24 produced preferred to produce keratinase under SmF condition rather than SSF condition. Amino acid by-product type and concentration were different between SmF and SSF conditions with the highest concentration was L-arginine obtained under SmF condition which a potential organism to produce L-arginine using chicken feather substrate. Further study should be done regarding optimizing keratinase production and investigating keratinase character in both fermentation conditions.

## Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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