Pertanika J. Trop. Agric. Sci. 45 (4): 961 - 972 (2022)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Isolation, Characterization, and Optimization of Keratinase from *Bacillus cereus* BRAW_KM

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ABSTRACT

Indonesia possesses tremendous marine resources. Therefore, their marine products are appropriate for exploration. In the prior study, bacteria generating keratinase enzyme have isolated from local fish market trash. The keratinase may hydrolyze keratin on the skin. Surrounding parameters, such as temperature, pH, and incubation duration, are the factors affecting the activity of the enzyme. This study aims to isolate and characterize keratinase, and optimize its production. The enzyme from *Bacillus cereus* BRAW_KM was the main material utilized in this research. First, the keratinolytic bacterium was isolated and investigated the properties of keratinase using native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE. Then, the ideal conditions of keratinase synthesis were adjusted by temperature, pH, and incubation time on enzyme activity. Of 10 isolations discovered, one isolate shows the potential as a keratinolytic bacterium, which tends to behave like *Bacillus* sp. The molecular weights of keratinase were 130 kDa and 95 kDa. The optimum keratinase enzyme activity from *B. cereus* BRAW_KM was at 29 °C, pH 9, and 90 minutes of incubation.

Keywords: *Bacillus cereus*, characterization, isolation, keratinase enzyme, optimization

ARTICLE INFO

Article history:
Received: 22 April 2022
Accepted: 10 June 2022
Published: 09 September 2022

DOI: https://doi.org/10.47836/pjtas.45.4.07

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INTRODUCTION

Indonesia is the world's biggest archipelagic state, with 54,716 kilometers of coastline and 17,508 islands, and the world's fourth most populated country, with 247.5 million people (Food and Agriculture Organization of the United Nations [FAO], 2019). Indonesia, behind China, is the world's second-largest producer of fisheries and

ISSN: 1511-3701
e-ISSN: 2231-8542

aquaculture. Indonesia's fish production has risen steadily during the last 50 years. Fish supply climbed to 10.7 million tons in 2014, up from 0.8 million tons in 1960 (Tran et al., 2017). The catch is dried, fermented, salted, boiled, or smoked in various proportions, with 46% consumed fresh from the fish market (FAO, 2019).

Wibowo et al. (2017b) identified the bacteria that produce keratinase from fish market waste, which was later described and optimized by Wibowo and Yuliatmo (2020). This enzyme is employed in the hair removal (unhairing) procedure for eco-friendly fish skin tanning. According to Tamersit and Bouhidel (2020), the unhairing procedure results in a highly polluted solution. Dettmer et al. (2013) also explained that the unhairing process leads to the destruction of hides hairs can use the conventional lime-sulfide method, even though this method causes emissions. High biological oxygen demand (BOD), total suspended solids (TSS), and chemical oxygen demand (COD) are the emission that loads in the effluent leather industry. Furthermore, protein degrading chemical materials, such as calcium carbonate ($CaCO_3$) and sodium sulfide (Na_2S), are used in leather manufacture. It accounts for about 80-90% of the total pollution in leather making (Dettmer et al., 2012). For environmental concerns and to reduce sodium sulfide usage for the tanning process, the keratinase enzyme can be used, which is important for the tanning process and future technology (Kandasamy et al., 2012).

The employment of enzymes in the tanning process is a great future trend. The keratinolytic enzymes have been discovered by researchers. Most of them are derived from *Bacillus* strains, such as *Bacillus* BPKer and BAKer (Gegeckas et al., 2018), *Bacillus aerius* NSMk2 (Bhari et al., 2019), *Bacillus cereus*, and *Bacillus polymyxa* (Laba & Rodziewicz, 2014); *Bacillus subtillis* is among others (Mousavi et al., 2013). Several parameters can impact enzyme production, including pH, temperature, and incubation time. Condition optimization is an important aspect of enzyme production (Mechri et al., 2017). In this research, the isolation, characterization, and optimization of keratinase from *Bacillus cereus* BRAW_KM is expressed as an innovative solution to support cleaner production in leather tanning factories.

METHODS

Isolation of *Bacillus cereus* BRAW_KM

Samples of Buntal fish skin were obtained from a local fish market in Rembang, Center of Java, Indonesia. Nutrient agar (Merck, Germany) was used for inoculation of the sample and incubated for 48 h at 37 °C until colonies appeared. Colonies were selected as representative samples based on morphological and colony color observations. Then, the selected colonies were isolated by transferred to the new NA plates (Wibowo et al., 2017b).

Identification of *Bacillus cereus* BRAW_KM

Proteolytic Activity by Skim Milk Agar Identification. The isolate was then streaked on skim milk agar (Merck, Germany)

(0.8% skim milk, 0.5% sodium chloride [NaCl], 1% meat extract, 1% peptone, 1.5% agar) and incubated for 48 h at 37 °C. After incubation, a clear zone around the bacterial growth was observed (Wibowo et al., 2017a). All chemicals were used in this study purchased from Merck (Germany).

Scanning Electron Microscope. Cells bacteria grown in NA medium were harvested after 72 h of incubation and subjected to scanning electron microscopy (SEM) analysis (PerkinElmer, USA). The 0.22 M sucrose (Merck, Germany) in cacodylate buffer (0.1 M, pH 7.2) (Merck, Germany) was used to wash the cell, which was subsequently fixed cacodylate buffer containing 2% (v/v) glutaraldehyde (Merck, Germany) at 4 °C for 2 h. The suspension was centrifuged before being rinsed in cacodylate buffer once more. The samples were fixed in cacodylate buffer containing 1% osmium tetroxide (Merck, Germany) at 4 °C for 2 h, dehydrated by gradation of alcohol (Merck, Germany) concentration, and dried in hexamethyldisilane (Merck, Germany) and mounted on aluminum stubs. The sample is sputter-coated with gold/palladium, then viewed using SEM.

Morphology Test. Bacterial identification was performed by observing colony morphology such as texture, shape, size, motility, colony color, zinc (Zn) staining, and Gram staining. At the same time, the biochemical test includes oxidase, catalase, and fermentation of carbohydrates. The results were compared to the standard from Bergey's Manual of Determinative Bacteriology (Bergey & Gibbons, 1974).

Keratinase Enzyme Production

Inoculum Preparation for Enzyme Production. The following sources were utilized in this research: *B. cereus* BRAW_KM was isolated from the culture of a previous study (Wibowo et al., 2017a). The fermentation medium contained the following ingredients: 0.5 g/L NaCl, 0.3 g/L dipotassium hydrogen phosphate (K_2HPO_4), and 0.4 g/L potassium dihydrogen phosphate (KH_2PO_4), 1% yeast extract, 0.5% NaCl, 1% peptone, and 100 mL distilled water, 1% ammonium sulfate ($(NH_4)_2SO_4$), 20 mM Tris (hydroxymethyl) aminomethane hydrochloride (pH 8), 12 kDa dialysis sheet, 1 mM ethylenediamine tetraacetic acid (EDTA), 50 mM sodium bicarbonate ($NaHCO_3$), and distilled water. Five (5) mL of preculture medium were inoculated with one dose of pure culture product isolate obtained from agar media and incubated overnight at 120 rpm in a shaker.

Enzyme Production. The method developed by Hoq et al. (2005) was used to produce the keratinase enzyme. Approximately 1.5 mL of the isolate was inoculated into a 50 mL liquid medium and incubated overnight at 120 rpm in a shaker. Yellowing is a characteristic of enzyme production. The isolates were separated from the extracellular enzyme by centrifugation for 15 minutes at 4 °C and 1, 400 × *g*. The generated supernatant was a raw enzyme that enzyme activity could be tested. The

enzyme activity of the collected enzyme was determined.

Enzyme Purification. At 4 °C, a one-liter culture product of fermentation was centrifuged for 15 minutes at 10,000 × *g*. The pellet was separated from the supernatant that had been formed. The enzyme extract present in the supernatant was able to be concentrated due to the concentration method. The enzyme was refined by precipitating it with saturated ammonium sulfate at a concentration of 60% for many hours. Saturation was accomplished using ammonium sulfate (Tatineni et al., 2008). The ammonium sulfate crystals were gently added while constantly swirling until entirely dissolved. The solution was stored at 4 °C for 24 h before being centrifuged at 10,000 × *g* for 15 minutes at 4 °C. Following centrifugation, the pellets and supernatant are separated. The pellets are collected and considered the purified enzyme, while the supernatant is removed and considered as other nutrients such as saccharides and minerals (Yuliatmo et al., 2017).

Characterization of Enzyme. ***Determination of Molecular Weight of Protein by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).*** In addition to ethanol, other SDS-PAGE components include sterile water, acrylamide solution, Tris hydrochloride (Tris-HCl), SDS, deionized water (dH_2O), N'-tetramethyl-ethylenediamine (TEMED), ammonium persulfate, glacial acetic acid, Coomassie blue, and 70% methanol. All the buffers used in the tests, including sodium phosphate, glycine, sodium hydroxide (NaOH), and Tris-acetate, were used. Some of the materials used to assess the activity of the enzyme keratinase were keratin azure, Tris HCl (pH 7.5), and a 10% trichloroacetic acid (TCA) solution, among other things. In the case of protein separation, the SDS-PAGE method is used to determine the molecular weight of the proteins being separated. In SDS-PAGE, the essential concept is that proteins are denatured by sodium dodecyl sulfate, followed by molecular weight separation by electrophoresis using a gel, in this case, polyacrylamide, to separate proteins with varying molecular weights, as described above. On SDS-PAGE, the identification and characterization of protein bands were carried out in comparison to bands that had previously been separated using conventional protein separation methods (Laemmli, 1970).

Protease Activity by Native PAGE. Protease activity of the enzyme was performed by Hiol et al. (1999) using clear native PAGE (CN-PAGE). The 10% concentrated CN-PAGE contained 30% acrylic amide solution; 0.8% bisacrylamide; 1.5 M Tris hydrochloride (pH 8.8); 1.0 M Tris hydrochloride (pH 6.8), 0.8% 1,2-Bis(dimethylamino)ethane, 10% ammonium persulfate, 50% glycerin, 0.1% casein, TEFCO clear dry (Japan), running buffer solution (1.5 g Tris (hydroxymethyl) aminomethane, 7.2 g acetohydroxamic acid ($C_2H_5NO_2$), and 500 mL distilled water),

and 70 mm ADVANTEC filter paper (Toyo Roshi Kaisha, Japan).

Optimization of Enzyme Production. ***Keratinase Activity.*** Keratin azure (Sigma-Aldrich, USA) was used as a keratin substrate to determine the keratinase activity. The keratinase activity test is based on Wang et al. (2009). An incubation period of 30 minutes at 30 °C with the agitation of 180 rpm was carried out in a shaker incubator with a 500 mL enzyme sample in 5 mg keratin azure solution in 500 mL 50 mM sodium phosphate buffer at 50 mM sodium phosphate buffer. The process was stopped by adding 1 mL of 10% TCA solution to the mixture. Centrifuging the solution at 13,000 × *g* for 5 minutes after it had been maintained cool was the first-rate procedure. It was necessary to measure the absorbance of the azo dye extracted from the supernatant at 595 nm to compare it to the absorbance of the control tube. The control tube was subjected to the identical procedures as the experimental tube, with the exception that the enzyme sample was replaced with sodium phosphate buffer instead of phosphate buffer. One unit (U) keratinase activity was defined as the amount of enzyme causing a 0.01 absorbance increase between the sample and control at 595 nm under the conditions given.

The Effects of Temperature, pH, and Incubation Time on Keratinase Activity. The purified enzyme's keratinase activity was determined using the following buffers (sodium acetate [CH_3COONa, pH 4–6], trisodium phosphate [Na_3PO_4, pH 7–8], Tris sodium hydroxide [Tris-NaOH] [pH 9-11]) at pH 6, 7, 8, 9, 10, and 11. By incubating processes at a variety of temperatures, including 25 °C and 27 °C, as well as 29 °C, 31 °C, and 33 °C, the optimal temperature was identified. Additionally, the incubation length for keratinase characteristics varied from 30 minutes, 60 minutes, 90 minutes, 120 minutes, and 150 minutes, according to the experiment results (Nayaka et al., 2013).

Determination of V_{max} and K_m. The enzyme kinetic parameters Michaelis constant (K_m) and maximum rate of reaction (V_{max}) were obtained by analyzing keratinase activity at various substrate concentrations (1-10 mg). With the use of the Lineweaver-Burk plot, the values for K_m and V_{max} were determined. Several researchers (Gupta et al., 2015) have suggested that.

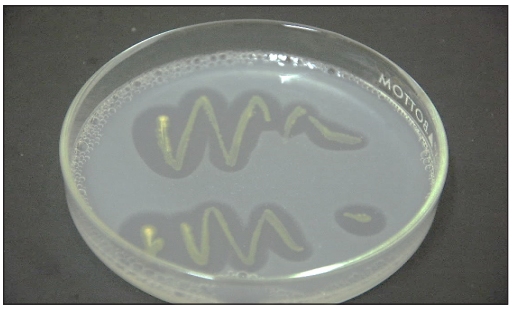
RESULTS AND DISCUSSION

Isolation of *Bacillus cereus* BRAW_KM

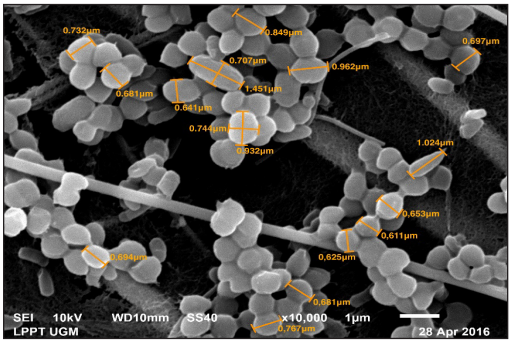
Ten strains of bacteria have been successfully collected. It shows the clear zone on a skim milk agar plate, indicating its ability to degrade casein protein (Figure 1A). In addition, skim milk agar is the best medium for the preliminary screening of keratinase (Balakumar et al., 2013; Selvam et al., 2013).

Identification of *Bacillus cereus* BRAW_KM

Bacteria morphology is essential for identification. It was occurred by SEM. The observations using a microscope showed that the bacteria form is small



(a)



(b)

Figure 1. (a) Bacteria growth on skim milk agar; (b) Scanning electron microscope of isolating *B. cereus* BRAW_KM

Table 1
Morphology and biochemical characteristics

Characteristics	Results
Catalase	+
Oxidase	+
Deep media	Beaded
Slant media	Echinulate
Elevation	Effuse
Edge	Entire
Inner structure	Translucent
Colony form	Circular
Glucose	+
Fructose	+
Sucrose	+
Lactose	+
Motility	-
Spore	+
Gram staining	+
Acid staining (Zn)	-

Note. + = Positive reaction; - = Negative reaction

rod shape, almost round (Figure 1B). Morphological, physiological, and biochemical characteristics are represented in Table 1. The characteristics assay results showed that these bacteria were classified as *Bacillus* sp. (Wibowo et al., 2017a).

Characterization of Keratinase

Molecular Weight Determination Using SDS-PAGE. Electrophoresis is widely used for protein characterization, including the measurement of molecular protein weight. By estimating the molecular protein weight of the enzyme after it has been exposed to SDS-PAGE, the molecular weight of the enzyme may be determined and matching it with the band in standard protein. For example, molecular weights of enzymes from *Bacillus cereus* strain BRAW_KM are between 130 kDa and 95 kDa. It is in line with Mazotto, Coelho, et al. (2011)'s research that generally, all *Bacillus* spp. genus had keratinases with a molecular weight of 13.8 kDa and 140 kDa. Figure 2 shows the molecular weight of the keratinase of *B. cereus* BRAW_KM. Other extracellular keratinases *Bacillus pumilus*, *Bacillus cereus*, and *Bacillus subtilis* KS-1 had molecules weight 65 kDa, 45 kDa, and 25.4 kDa, respectively (Kumar et al., 2008; Mazotto, de Melo, et al., 2011).

Native PAGE. The native PAGE technique was utilized to detect the protein bands and the protease activity of the protein bands, which in this instance was determined to be a specific bacterial strain. According to Sattayasai (2012), enzymatic activity may stain a wide range of proteins in gels.

Wilson and Walker (2010) further stated that the native PAGE procedure does not denature the sample since it has the potential to create bonds with the protein's secondary structure, which would otherwise cause the sample to be destroyed. Native PAGE examination of the enzyme extract from *B. cereus* BRAW_KM using casein substrate (Figure 2) revealed that the enzyme extract could hydrolyze the protein. The protein band of *B. cereus* BRAW_KM could be seen clearly in the image. As a result, the enzyme BRAW_KM from *B. cereus* is capable of degrading protein.

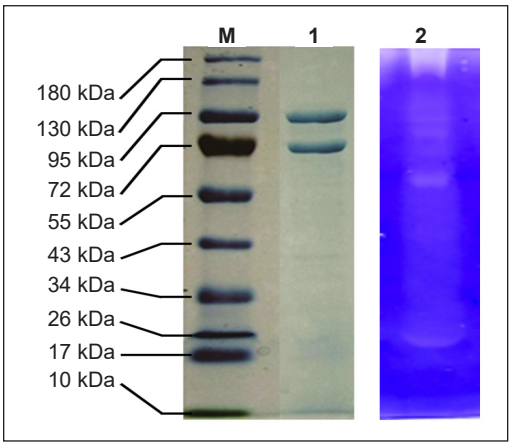


Figure 2. Characteristics of keratinase from *B. cereus* BRAW_KM on SDS-PAGE (1) and native PAGE (2), respectively
Note. M = Molecule weight standard

Optimization of Keratinase Production

The Influence of Temperature on Activity of Keratinase. Figure 3 represents the effect of temperature on enzyme activity. The best condition for keratinase activity was found by incubating the samples at temperatures ranging from 25 °C to 33 °C. *Bacillus cereus* strain BRAW_KM enzyme has an optimal temperature of 29 °C (6.34 ± 0.03 U/mg), and as the temperature increases, the enzyme's activity drops. It is substantially identical to the findings obtained by Balakumar et al. (2013). *Bacillus subtilis* was inoculated into the medium to maximize keratinase production at different temperatures, and they reported that an increase in production was seen at 30 °C.

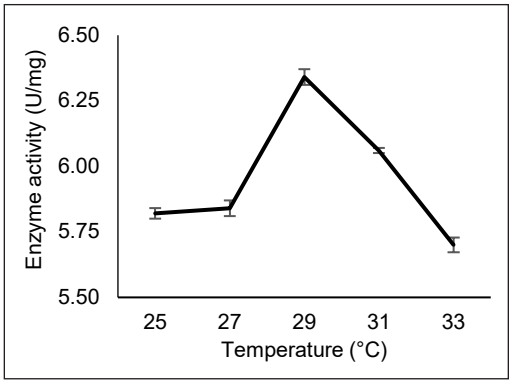


Figure 3. The influence of temperature on the activity of keratinase

The Influence of pH on Activity of Keratinase. The enzyme from *B. cereus* BRAW_KM activities is optimal at pH 8, with the highest activity at 7.13 ± 0.03 U/mg (Figure 4). It is similar to alkaline protease

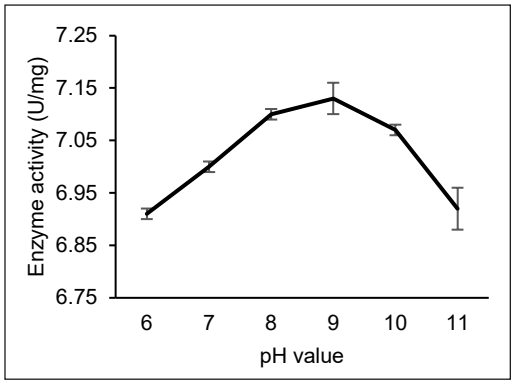


Figure 4. The influence of pH value on the activity of keratinase

from APR-4 *Bacillu*s sp., which has the maximum activity at pH 9 (Kumar et al., 2008), and to keratinase from *Bacillus* sp., which has the highest activity at pH around 7 and 8 (Selvam et al., 2013). Keratinase produced by these bacterial strains may be classified as an alkaline protease because the enzyme's maximum activity occurs at alkaline pH levels. Lin et al. (1996) explained that high pH treatment does not reduce keratinase activity, but low pH does.

The Influence of Incubation Time on Activity of Keratinase. The enzyme activity of *B. cereus* BRAW_KM increases until 90 minutes (6.17 ± 0.02 U/mg), then its activity decrease by 9% after 90 minutes (Figure 5). The optimal incubation period of keratinase from *B. cereus* BRAW_KM almost the same results have been reported by Gupta et al. (2015), who found that keratinase from *B. subtilis* stabilizes up to 90 minutes and drops by 11% after 120 minutes. Other results were seen between the *B. subtilis* proteolytic enzyme BLBc11 (Dettmer et al., 2012) and a commercially available keratinase, with the former demonstrating steady activity for 120 minutes and the latter demonstrating variable activity (Dettmer et al., 2011). According to Gessesse et al. (2003), the enzyme from *Bacillus pseudofirmus* sp. became inactive after 20 minutes of incubation. Apart from that, Ogino et al. (2008) identified proteolytic enzymes that become inactive after just ten minutes of incubation.

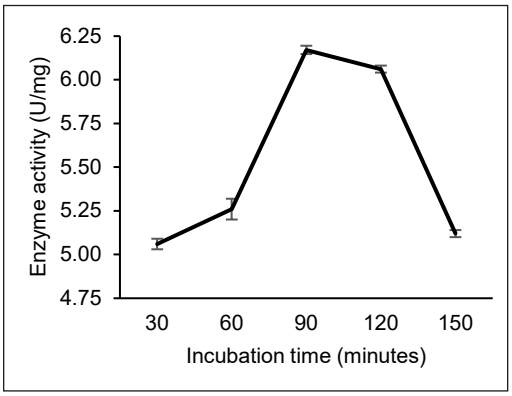


Figure 5. The influence of time incubation on the activity of keratinase

Kinetics of Keratinase

The Michaelis-Menten plot was used to plot Lineweaver-Burk plots against substrates at varying concentrations, and the results were analyzed (Figure 6). According to the

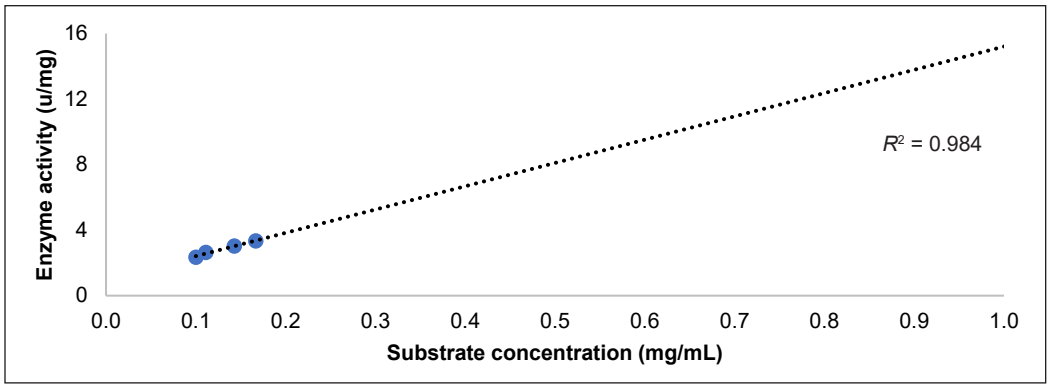


Figure 6. Graph of keratinase enzyme kinetics (Lineweaver - Burk plot)

study's results, the Michaelis constant (K_m) of keratinase from *B. cereus* BRAW_KM was found to be 13.98 mg/mL, and the maximum rate of reaction (V_{max}) was determined to be 1.01 mg/mL/min. The Michaelis-Menten equation was used to estimate the enzyme's reaction at various substrate doses to study enzyme kinetics. Keratinase generated by *Pseudomonas aeruginosa* KS-1 has a higher K_m of 1.66 mg/mL and a higher V_{max} of 3.1 mg/mL/min than previously reported (Sharma & Gupta, 2010). Purified keratinase from *Bacillus thuringiensis* has a greater K_m (5.97 mg/mL) than other keratinases (Sivakumar et al., 2012). The calculated K_m and V_{max} values for keratin obtained from feathers were 6.6 mg/mL and 5.0 mg/mL/min, respectively, for keratin derived from feathers (Gupta et al., 2015).

CONCLUSION

The bacteria isolation resulted in keratinolytic bacteria, *Bacillus cereus* BRAW_KM. The SDS-PAGE and native PAGE investigated enzymatic characterization. It resulted in the molecular weights of keratinase being 130 kDa and 95 kDa. In addition, temperature, pH, and incubation period on enzyme activity were shown to be the most effective factors in determining the optimal conditions for keratinase synthesis. The best conditions were 29 °C, pH 9, and 90 minutes of incubation.

ACKNOWLEDGEMENTS

The authors are thankful to the Laboratory of Microbiology and Enzyme Technology, Department of Leather Processing Technology, Politeknik ATK Yogyakarta for providing the facilities for our study.

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