Screening of Factors Influencing Keratinase Fermentation from *Bacillus Haynesii* BK1H using The Plackett-Burman Design (PBD)

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Abstract. Keratinase is a class of proteases that degrade keratin into polypeptides and amino acids by breaking peptide and disulfide bonds in keratinous proteins. Protease is one of the largest industrial enzymes, the global protease market is increasing rapidly every year. In previous studies, a keratinase-producing microbe was isolated from Bleduk Kuwu which was identified as *Bacillus haynesii* BK1H. However, further study needs to be done to optimize the production by observing microenvironmental factors that influence keratinase production. In this study, screening some microenvironmental factors is reported. Screening factors including carbon sources, type and concentration of metal ions, agitation speed, amount of inoculum, pH, and temperature. Screening for these factors was started with the One Factor at A Time (OFAT) method and followed by the Plackett-Burman Design (PBD) method. In this study, sequential work was done: (1) Regeneration of *Bacillus haynesii* BK1H Microbes, (2) Preparation of Tyrosine Standard Series Solutions, (3) Screening of Factors Affecting Protease Fermentation of *Bacillus haynesii* BK1H using the One Factor at A Time (OFAT) method.), and (4) Maintaining Significantly Influential Factors by Using Plackett-Burman Design (PBD). The results of the OFAT approach showed that the best condition for keratinase production was achieved at rice husk concentration, additional carbon source, of 1%; pH of 7; a temperature of 35°C; the amount of inoculum of 1%; agitation speed of 150 rpm; magnesium sulfate concentration of 0.04 g/mL, and calcium chloride concentration of 0.0005 g/m. Justification of those factors using PBD confirmed that only additional rice husk, magnesium sulfate and calcium chloride concentration, and agitation speed were significantly important toward keratinase production at selected experiment level limits.

1 Introduction

The need for proteases in 2020 reached USD 3,454.3 million and is expected to increase to USD 5,762.7 million in 2030 [1]. Proteases can be applied in waste management, the leather industry, detergent additives, medicines, food, and feed [2]. Proteases can be obtained from plants, animals, and microorganisms such as viruses, fungi, and bacteria [3]. Proteases produced from microbes have several advantages, namely they are easy to produce on a large scale in a shorter time, do not need to use large areas of land [4], have faster growth rates, lower production costs, and are not dependent on seasonal changes [5].

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Previous research has succeeded in isolating of 8 protease-producing microbes from the muddy Bledug Kuwu crater, Central Java. The eight microbes were proven to be able to degrade protein in skim milk agar media as indicated by the formation of clear zones around the microbial colonies. One of these microbes, namely BK1H, was identified genotypically as *Bacillus haynesii* (hereinafter referred to as *Bacillus haynesii* BK1H) which was studied further and reported in this manuscript. *Bacillus haynesii* BK1H showed highest protease activity among 8 isolated microbes using skim milk as a substrate in SSF (Solid State Fermentation) media with a protease activity value of 5.802 U/mL, so it has the highest potential to become a industrial protease-producing microbe [6]. Optimization of fermentation has not been carried out. Fermentation optimization needs to be done to obtain the best fermentation conditions that produce the best amount of enzyme. This can be done by determining the important factors that influence the increase in enzyme production.

Factors that can affect enzyme fermentation include temperature, pH, agitation, amount of inoculum, micro and macro nutrients. Micronutrients are nutrients needed in concentrations of less than 10^{-4} M, including Ca^{2+}, Zn^{2+}, and Mn^{2+}. While macronutrients are nutrients needed in concentrations of more than 10^{-4} M, namely C, N, and O [7]. Temperature and pH greatly influence the three-dimensional structure of proteins due to changes in the acid-base balance side chian of amino acid residues that will affect cell growth [7], [8]. Agitation affects the delivery of oxygen and nutrients to cells [10], and metals are protein cofactors and cell stabilizers. In a previous study [4], *Bacillus haynesii* BK1H produced protease with skim milk as the carbon source, however, skim milk is quite expensive if used on a large scale. Furthermore, chicken feathers which are rich in protein have also been reported to be used as a source of carbon and nitrogen to produce keratinase, therefore this research will focus on keratinase. Since keratinase has both protease and disulfide reductase activity, one might use teh benefit of protease only for application. Additional carbon sources need to be investigated to increase cell productivity, especially as an energy source. Each cell requires these conditions in different types and concentrations, so it is necessary to screen the influence of carbon sources, agitation, temperature, pH, amount of inoculum, and type. as well as metal concentrations that significantly affect keratinase production which can be experimentally followed by measuring keratinase activity.

Generally, the screening of influencing factors begins with testing the conditions of the fermentation environment to select which factors have a positive effect on fermentation. This is done using the One Factor at A Time (OFAT) approach. The use of OFAT has the disadvantage that it doesn not take into account the combined effect of several factors, so it hard to conclude which factor has the most significant effect on the fermentation process [11]. Therefore, it is necessary to screen the environmental conditions of the keratinase fermentation media to determine the factors that have a significant effect prior to optimizing those factors. The Design of Experiment (DoE) with a multivariate factor approach using Plackett-Burman Design (PBD) was chosen as an approach for screening factors that have a linear and significant effect on increasing the productivity of keratinase secreted by *Bacillus haynesii* BK1H.

2 Method

The tools used in this study were Petri dishes, erlenmeyer, beaker glass, measuring cup, stirring rod, spatula, measuring pipette, test tube, test tube rack, measuring flask, watch glass, spirit lamp, match, Schott bottle, and needle. ose, 2 mL microtube, 1000 µL micropipette (Dragon lab); 5000µL, sterile tip, analytical balance (Precisa XT 120 A), microcentrifuge (Tomy MX-105 High-Speed Refrigerated Microcentrifuge), oven (Memmert), vortex (Dragon Lab MX-5), water bath shaker incubator (Memmert), shaker incubator (New

Brunswick), Laminar Air Flow, autoclave (Tomy), UV-Vis spectrophotometer (Spectrophotometer Spectronic 20 50 DA), Hot Plate (Thermo Scientific), and pH meter.
The materials used in this study were microbial isolates of *Bacillus haynesii* BK1H, chicken feathers, rice husks, soybean powder, bagasse, coconut pulp, corncobs, skimmed milk, bacto agar, sodium chloride (NaCl), bacto peptone, yeast extract, sodium hydroxide (NaOH), potassium hydrogen phosphate (K_2HPO_4), casein (Amresco: E666-500G), trichloroacetic acid (TCA), sodium carbonate (Na_2CO_3), Folin-Ciocalteu reagent, tris-base ($C_{14}H_{11}NO_3$), hydrochloric acid (HCl) , sodium hydroxide (NaOH), glycine, sodium citrate, citric acid, $CaCl_2$, $CoCl_2$, $MnCl_2$, $ZnCl_2$, $NiCl_2$, $MgSO_4$, distilled water, and alcohol.

2.1. Regeneration of *Bacillus haynesii* BK1H Microbial Pure Cultures

Regeneration of pure microbial cultures was carried out by aseptic technique. One ose of *Bacillus haynesii* BK1H microbial isolate was taken from the glycerol stock and then inoculated on the surface of skim milk agar (SMA) media. The inoculated SSA media was then incubated in an incubator at 37°C for 48 hours.

2.2. Preparation of Tyrosine Standard Series Solution

A total of 2 mL of a standard solution containing 0 (blank), 10, 30, 50, 70, 90, and 110 µg/mL of tyrosine was added with 5 mL of 0.5 M Na_2CO_3 solution and 1 mL of Folin-Ciocalteu reagent. The solution was homogenized by swirling and then incubated in a dark room for 30 minutes. The absorption of the blue colored complex was measured at a wavelength of 660 nm using a UV-Vis spectrophotometer. Tyrosine concentration data was made using a standard curve and a linear regression equation which was used to determine the concentration of tyrosine produced by the activity of the keratinase enzyme based on its absorption at the same wavelength.

2.3. Screening of Bacillus haynesii BK1H Fermentation Factors with the One Factor at A Time (OFAT) Method

2.3.1. Preparation of Liquid Starter Culture Media

Liquid culture medium was prepared using Luria Bertani (LB) broth (1 % pepton, 0,5 % yeast extract, and 0,5 NaCl).The *Bacillus haynesii* BK1H culture in SSA media was taken with a sterile toothpick and then incubated into liquid LB. The liquid media was incubated in a water bath shaker incubator for 16 hours (overnight) and the Optical Density (OD) was measured at a wavelength of 600 nm. Furthermore, 1% of the culture was transferred to a fresh medium, and then 1% (vthe culture a freshstarter medium and then incubated in a water bath shaker incubator until the OD value reachs 0.3-0.6.

2.3.2. Effect of Aditional Carbon Sources in Fermentation Media

Substrate sources of carbon and nitrogen such as rice husks, corncobs, bagasse, coconut pulp, and soybean powder were mashed using a blender and then sifted using a 60 mesh sieve. Then, 0.25 g and 0.5 g of chicken feathers were added to each of these substrates. Then it was added with 50 mL salt solution containing 0,025 g K_2HPO_4 and 0,25 g NaCl in Tris-HCl buffer pH 8 50 mM. Then the medium was autoclaved and inoculated with 1% (500 µL) starter and added 200 µL of 0.04 g/mL MgSO4. The inoculum was incubated in the shaker

incubator for 3 days at a temperature of 37°C and a speed of 100 rpm and then activity of crude extract was measured.
The addition of C source substrate with the highest enzyme activity was then varied in concentration at, 1% and 5%. A total of 0.5 grams of chicken feathers were added with various substrates of carbon and nitrogen sources with the highest enzyme activity of 0.25 grams for 1% concentration and 0.5 grams for 5% concentration. Then it was added with a salt solution that had been dissolved in 50 mL of Tris-HCl buffer pH 8 50 mM. Then the media was autoclaved and inoculated with 1% (500 µL) starter and added 0.04 g/mL (200 µL) $MgSO_4$. Then the media was incubated in the incubator shaker for 3 days with a temperature of 37°C and a speed of 100 rpm and then continued with the activity test.

2.3.3. Effect of Metal Ions in Fermentation Media

A total of 0.5 grams of chicken feathers were added with a salt solution that had been dissolved in 50 mL of Tris-HCl buffer pH 8 50 mM. Then the media was autoclaved and inoculated with 1% (500 µL) starter and added 0.04 g/mL (200 µL) MgSO4. Then the media was added with various metal ions such as Ca, Co, Mn, Zn, and Ni as much as 250 µL for a concentration of 0.0005 g/mL and 500 µL for a concentration of 0.001 g/mL. Then the media was incubated in the incubator shaker for 3 days at 37°C and 100 rpm and then continued with the activity test.

2.3.4. Effect of Agitation in Fermentation Media

A total of 0.5 grams of chicken feathers were added with a salt solution that had been dissolved in 50 mL of Tris-HCl buffer pH 8 50 mM. Then the media was autoclaved and inoculated with 1% (500 µL) starter and added 0.04 g/mL (200 µL) $MgSO_4$. Then the media was incubated in the incubator shaker for 3 days
at a temperature of 37°C and a speed of 100; 150; and 200 rpm and then proceed with the activity test.

2.3.5. Effect of Inoculum Amount in Fermentation Media

A total of 0.5 grams of chicken feathers were added with a salt solution that had been dissolved in 50 mL of Tris-HCl buffer pH 8 50 mM. Then the media was autoclaved and inoculated with 1% (500 µL); 5% (2500 µL) starter and 0.04 g/mL (200 µL) $MgSO_4$ added. Then the media was incubated in the incubator shaker for 3 days with a temperature of 37°C and a speed of 100 rpm and then continued with the activity test.

2.3.6. Effect of pH in Fermentation Media

0.5 grams of chicken feathers were added with a salt solution that had been dissolved into 50 mL of 50 mM citrate buffer (for pH 6); 50 mM Tris-HCl buffer (for pH 7 and 8); 50 mM Glycine-NaOH buffer (for pH 9). Then the media was autoclaved and inoculated with 1% (500 µL) starter and added 0.04 g/mL (200 µL) $MgSO_4$. Then the media was incubated in the incubator shaker for 3 days with a temperature of 37°C and a speed of 100 rpm and then continued with the activity test.

2.3.7. Effect of $MgSO_4$ Concentration in Fermentation Media

A total of 0.5 grams of chicken feathers were added with a salt solution that had been dissolved in 50 mL of Tris-HCl buffer pH 8 50 mM. Then the media was autoclaved and inoculated with 1% (500 µL) starter and added 0.01 g/mL (50 µL); 0.04 g/mL (200 µL); and 0.08 g/mL (400 µL) $MgSO_4$. Then the media was incubated in the incubator shaker for 3 days with a temperature of 37°C and a speed of 100 rpm and then continued with the activity test.

2.3.8. Effect of Temperature on Fermentation Media

A total of 0.5 grams of chicken feathers were added with a salt solution that had been dissolved in 50 mL of Tris-HCl buffer pH 8 50 mM. Then the media was autoclaved and inoculated with 1% (500 µL) starter and added 0.04 g/mL (200 µL) $MgSO_4$. Then the media was incubated in the incubator shaker for 3 days at 32°C; 35; 37°; and 42°C and 100 rpm speed and then proceed with the activity test.

2.4. Determination of Significantly Influential Factors Using Plackett-Burman Design (PBD)

In this study, there were 7 factors, namely the concentration of carbon sources (%w/v), metal ions (g/mL), agitation (rpm), amount of inoculum (%v/v), pH, $MgSO_4$ (g/mL), and temperature. (°C) was tested at lower (-) and upper (+) limits (Table 3). Furthermore, the seven factors were entered into the Plackett-Burman experimental design using Minitab20 software with 3 repetitions and produced 36 sets of experiments and produced a Responsese in the form of enzyme activity (U/mL).

Table 1. Low and high level of factors used in PBD

No	Factors	Low Level (-)	High Level (+)
1.	Concentration of Carbon Source	1%	5%
2.	Concentration of Metal Ion	0,0005 g/mL	0,001 g/mL
3.	Agitation	100 rpm	150 rpm
4.	Amount of Inoculum	1%	5%
5.	pH	7	8
6.	$MgSO_4$	0,01 g/mL	0,04 g/mL
7.	Temperature	37°C	45°C

Table 2. PBD Experimental Design Matrix

Run	Temperature (°C)	pH	$MgSO_4$ (g/mL)	$CaCl_2$ (g/mL)	Amount of Inoculum (%v/v)	Carbon Source (%b/v)	Agitation (rpm)	Keratinase Activity (U/mL)
1	+	-	+	-	-	-	+	Response 1
2	+	+	-	+	-	-	-	Response 2
3	-	+	+	-	+	-	-	Response 3
4	+	-	+	+	-	+	-	Response 4
5	+	+	-	+	+	-	+	Response 5
6	+	+	+	-	+	+	-	Response 6
7	-	+	+	+	-	+	+	Response 7
8	-	-	+	+	+	-	+	Response 8

9	-	-	-	+	+	+	-	Response 9
10	+	-	-	-	+	+	+	Response 10
11	-	+	-	-	-	+	+	Response 11
12	-	-	-	-	-	-	-	Response 12
13	+	-	+	-	-	-	+	Response 13
14	+	+	-	+	-	-	-	Response 14
15	-	+	+	-	+	-	-	Response 15
16	+	-	+	+	-	+	-	Response 16
17	+	+	-	+	+	-	+	Response 17
18	+	+	+	-	+	+	-	Response 18
19	-	+	+	+	-	+	+	Response 19
20	-	-	+	+	+	-	+	Response 20
21	-	-	-	+	+	+	-	Response 21
22	+	-	-	-	+	+	+	Response 22
23	-	+	-	-	-	+	+	Response 23
24	-	-	-	-	-	-	-	Response 24
25	+	-	+	-	-	-	+	Response 25
26	+	+	-	+	-	-	-	Response 26
27	-	+	+	-	+	-	-	Response 27
28	+	-	+	+	-	+	-	Response 28
29	+	+	-	+	+	-	+	Response 29
30	+	+	+	-	+	+	-	Response 30
31	-	+	+	+	-	+	+	Response 31
32	-	-	+	+	+	-	+	Response 32
33	-	-	-	+	+	+	-	Response 33
34	+	-	-	-	+	+	+	Response 34
35	-	+	-	-	-	+	+	Response 35
36	-	-	-	-	-	-	-	Response 36

2.5. Determination of Keratinase Enzyme Activity

Keratinase enzyme activity was measured using the Anson method. Casein substrate 1% (w/v). 0.5 mL was added with 0.2 mL of centrifuged enzyme. The mixture of substrate and enzyme was vortexed until homogeneous and incubated at 37°C in the bath for 5 minutes. 1 mL of 10% TCA was added to the mixture to stop the enzymatic reaction, then the mixture was vortexed and incubated at room temperature for 15 minutes. The mixture was centrifuged at 10,000 rpm at 4°C for 10 minutes. 1 mL of the resulting supernatant was put into a test tube and then added with 2.5 mL of 0.5 M Na_2CO_3 and 0.5 mL of Folin-Ciocalteu reagent (1:1). The solution was homogenized by vortex and incubated at room temperature for 30 minutes in the dark. The absorbance of the solution was measured at a wavelength of 660 nm. The resulting absorbance value is interpolated on the standard tyrosine curve to get tyrosine concentration data and the concentration was used to calculate the activity of the keratinase enzyme.. Enzyme activity is calculated by Equation 1. Enzyme activity is expressed in Units. The unit is defined as the amount of enzyme that catalyzes the conversion of one micromole of substrate per minute under specified conditions of the assay method.

$$Enzyme\ activity\ \left(\frac{U}{mL}\right) = \frac{Concentration\ of\ tyrosine\ x\ (volume\ substrate+enzyme)}{volume\ enzyme\ x\ incubation\ time} \quad (1)$$

Note = Concentration of tyrosine (µg/mL)
= Volume substrate + enzyme (mL)
= Volume enzyme (mL)
= Incubation time (minute)

3 Result and Discussion

3.1. Regeneration of Pure Microbial Cultures

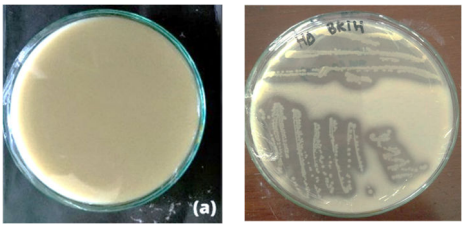


Fig. 1. (left) Skim Milk Agar (SMA) medium before being overgrown with *Bacillus haynesii* BK1H microbes. (right) Skim Milk Agar (SMA) medium after being overgrown with *Bacillus haynesii* BK1H.

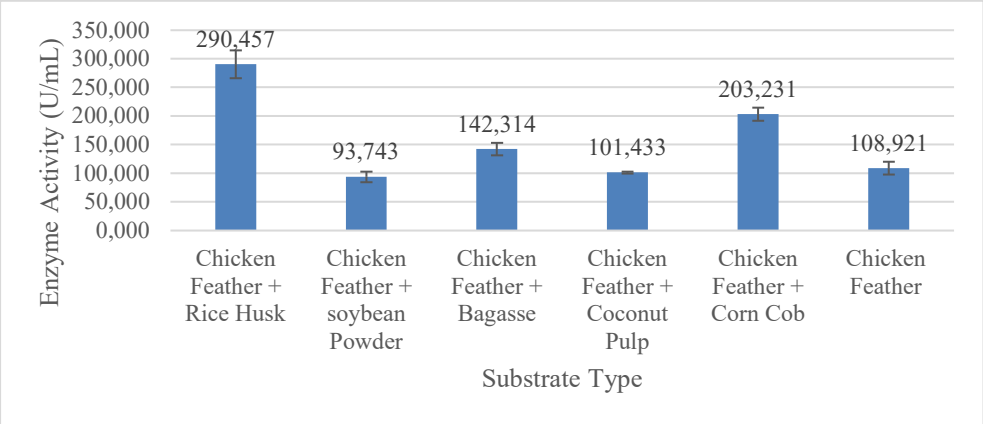
The aim of regenerating a pure culture of *Bacillus haynesii* BK1H is to obtain fresh microbes so as to obtain optimal results. Figure 1a. shows SMA media that has not been overgrown with microbes, while Figure 1b. is SSA media that has been overgrown with *Bacillus haynesii* BK1H. The clear zone that appears on the surface of the SSA media indicates the presence of protease activity produced by microbial colonies that hydrolyze the casein emulsion in milk in the SSA medium. The results of casein hydrolysis are short amino acids and polypeptides which dissolve so that the casein emulsion around the colony disappears and causes a clear zone. These amino acids are a source of energy for bacteria to survive. [44].

3.2. Screening of Bacillus haynesii BK1H Fermentation Factors with the One Factor at A Time (OFAT) Method

3.2.1. Effect of Carbon Sources in Fermentation Media

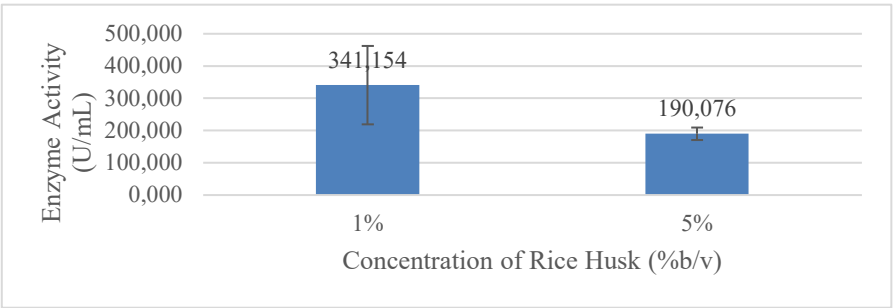
The main carbon source used in this study is chicken feathers which act as nutrients for microbes. Chicken feathers contain about 90% β-keratin which is a very stable and insoluble fibrous structural protein that can be utilized in several applications such as animal feed and fertilizer [45]. To increase the production of the resulting keratinase, other carbon sources are also added. The type of carbon source and optimum conditions greatly affect the production of enzymes so that the activity of these enzymes is also optimal. Therefore, in this study, optimization of the types of carbon sources was carried out, namely rice husk, soybean powder, bagasse, coconut pulp, and corn cobs at a concentration of 1% each. Based on the data in Table 3., it can be seen that the type of carbon source that can produce the highest enzyme activity is chicken feathers added with rice husk, which is equal to 290,46 ± 24,47 U/mL and can increase enzyme activity by 166,67% of the enzyme activity is produced by the carbon source of chicken feathers. So that rice husks will be studied further. The availability of rice husk is quite abundant because rice is the staple food in Indonesia. Rice husk as an alternative feed ingredient has a nutrient content of 12.5% water, 3.1% crude protein, 29.2% non-nitrogen extract material, 35% crude fiber, 2.7% fat, and 17. 5% ash [46].

Table 3. Table of the Effect of Enzyme Activity on Substrate Type



To produce optimal enzyme productivity, it is also necessary to optimize the concentration of carbon sources. The pattern of Keratinase productivity using rice husk with different concentration variations can be seen in Table 4.

Table 4. Table of the Effect of Enzyme Activity on Concentration Variations of Rice Husk



It can be seen from Table 4. rice husk with a concentration of 1% produced the highest activity value of 341.15 ± 121.78 U/mL, while at a concentration of 5% the enzyme activity decreased by 44.28% and produced enzyme activity was 190,08 U/mL. In previous studies, cellulase enzyme activity produced by Aspergillus niger continued to increase up to a substrate concentration of 2.5% rice husk, while at a concentration of 3% there was a decrease in enzyme activity. This is probably caused by the concentration of a carbon source that is too large which can inhibit the formation of the enzyme substrate complex so that the production of the enzyme does not run optimally, causing saturation in the production of the enzyme [31].

3.2.2. Effect of Metal Ions in Fermentation Media

Based on Table 5., the addition of Ca metal at a concentration of 0.0005 g/mL can Based on Table 5., the addition of Ca metal at a concentration of 0.0005 g/mL can increase enzyme activity by 3.44% (103.25 ± 28.20 U/mL) of the enzyme activity produced without the addition of metal ions , whereas the addition of Co, Mn, Zn, and Ni metals at the same concentration reduced the activity of the keratinase enzyme or decreased cell growth. On Table 5, shows that the addition of Ca, Co, Mn, Zn, and Ni at a concentration of 0.001 g/mL cannot increase enzyme activity. This has been reported in Faridah's research (2021), that the

activity of the enzymes produced by *Bacillus haynesii* BK1H increases with the addition of Ca and Mg metals, and there is no visible change in the addition of Co and Zn, but this study has not carried out the addition of Mn and Ni metals. [4]. Enzyme activity can increase because calcium ions have a role as a maintainer of cell structure. The decrease in enzymes may be caused by these metals damaging the integrity of the three-dimensional structure of the protein because it damages the salt bridges as a stabilizer of the three-dimensional structure of the protein. Proteins can be localized outside the cell (extracellular) or on the surface of the membrane/cell wall. So if the metal ions damage the three-dimensional structure of the keratinase secreted by the cell or on the surface of the membrane/cell wall, the activity of the protein can decrease or disappear [47]. Other effects can cause a decrease in the speed of cell growth because cell activity is not optimal [48].

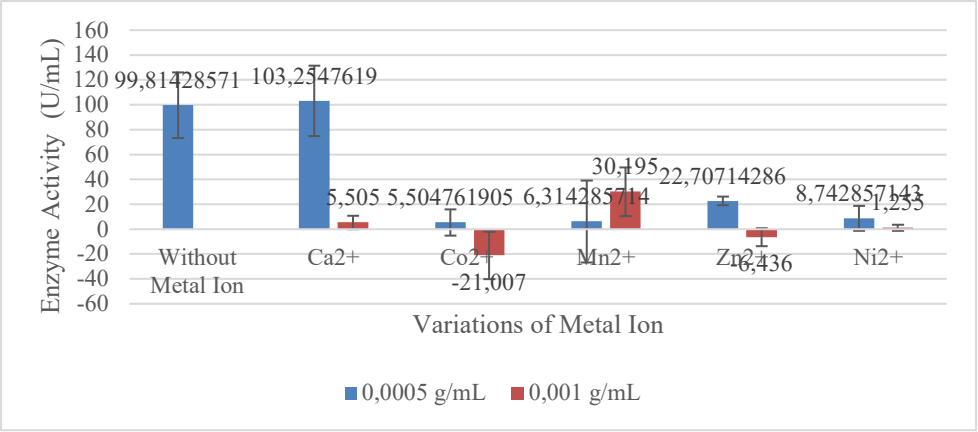
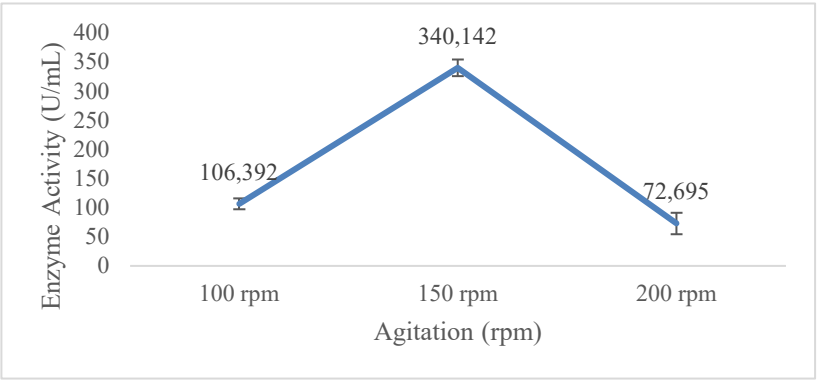


Table 5. Table of the Effect of Enzyme Activity on Variations of Metal Ion 0,0005 and 0,001 g/mL

3.2.3. Effect of Agitation in Fermentation Media

Table 6. Table of the Effect of Enzyme Activity on Variations of Agitation Rate



Based on the data in Table 6., an agitation speed of 150 rpm resulted in the highest enzyme activity of 106.39 ± 9.30 U/mL, but at an agitation speed of 100 and 200 rpm the enzyme activity decreased by 68.72% and 78, 63%. In the study of Patil and Kurhekar (2020), protease activity by *Bacillus isronensis* increased at an agitation speed of 100-200 rpm, but decreased at an agitation speed of 250 rpm [27]. Agitation can play a role in helping to

homogenize the mixture in the media [49]. In addition, agitation functions as a provider of oxygen which is used for cell growth, as well as eliminating exhaust gases produced during the fermentation process. Oxygen is used for microbial growth in aerobic fermentation, but some microbes can be affected by oxygen at excessive concentrations [8].

3.2.4. Effect of Amount of Inoculum in Fermentation Media

Table 7. Table of the Effect of Enzyme Activity on Variations of Amount of Inoculum

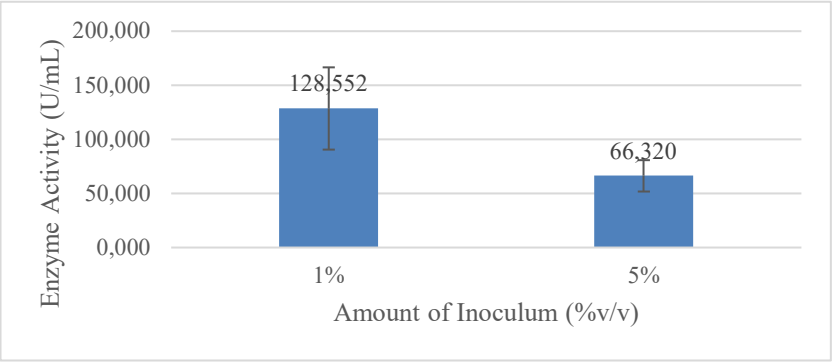


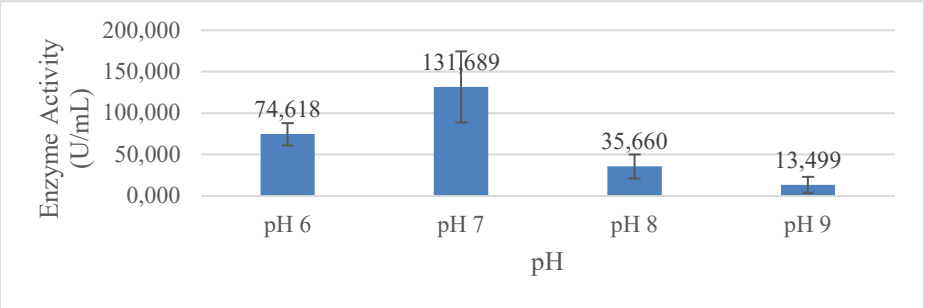
Table 7, shows the effect of variations in the number of inoculums in the fermentation medium of *Bacillus haynesii* BK1H, for the number of inoculums 1% the enzyme activity produced was 128.55 ± 38.07 U/mL, but there was a decrease in activity at the amount of 5% inoculum of 51 .6%. In the study of Nguyen and Tran (2018), there was an increase in enzyme activity when the number of inoculums increased by up to 20% and decreased by 25% [35]. Meanwhile, in a study by Bakry et al (2022) it was reported that the optimum enzyme activity was at 1% inoculum [50]. The number of inoculums affects the presence of nutrients and dissolved oxygen in the media. When the amount of inoculum is too low, bacterial growth will be slow and the enzymatic activity will be low. Increasing the number of inoculums will increase the interaction between substrates, nutrients, and bacteria, thereby increasing bacterial metabolism, protein synthesis, and enzyme activity. However, a high number of inoculums causes competition for nutrients and substrates and a decrease in enzyme activity [35]. In this study, enzyme activity was measured on the 3rd day of production. There is a possibility that at 5% inoculum, there will be an increase in activity on the 1st or 2nd day. So that on the 3rd day the availability of nutrients at the amount of 5% inoculum is different from the amount of 1% inoculum and will cause a decrease in enzyme activity [51]. It is necessary to propose further research to determine the effect of harvest time and the number of inoculums.

3.2.5. Effect of pH in Fermentation Media

Based on Table 8., conditions at pH 7 produced the highest enzyme activity, namely 131.689 ± 42.88 U/mL, but there was a decrease in enzymes at pH 6, 8, and 9, namely 43.34%, 72.92 %, and 89.75. This is the same as the study by Bakry et al (2022) who reported that *Bacillus haynesii* produces optimum xylanase activity at pH 7 [50]. Whereas in Faridah's research (2021), the optimum protease activity of *Bacillus licheniformis* is at pH 9 [4]. Each microbe has its own optimum pH, where under these conditions the performance of enzymes in the process of catalyzing a reaction will work well [52]. pH is an important parameter in microbial growth because active metabolic reactions in microbial cells depend on the

production of keratinase against pH [9]. pH affects the three-dimensional structure of enzymes so that it can affect the speed of enzyme activity in catalyzing a reaction.

Table 8. Table of the Effect of Enzyme Activity on Variations of pH



3.2.6. *Effect of $MgSO_4$ Concentration in Fermentation Media*

Table 9. Table of the Effect of Enzyme Activity on Variations of Concentration of $MgSO_4$

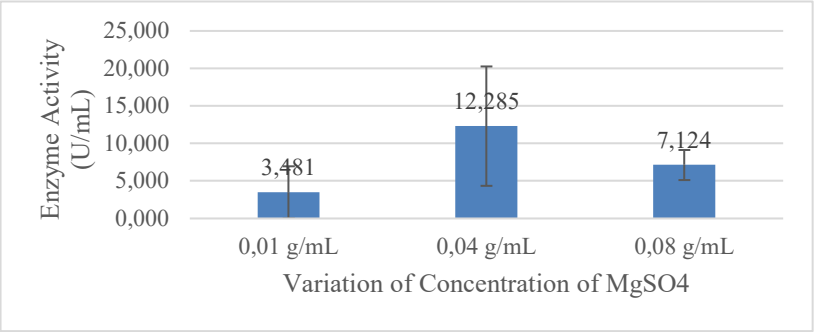


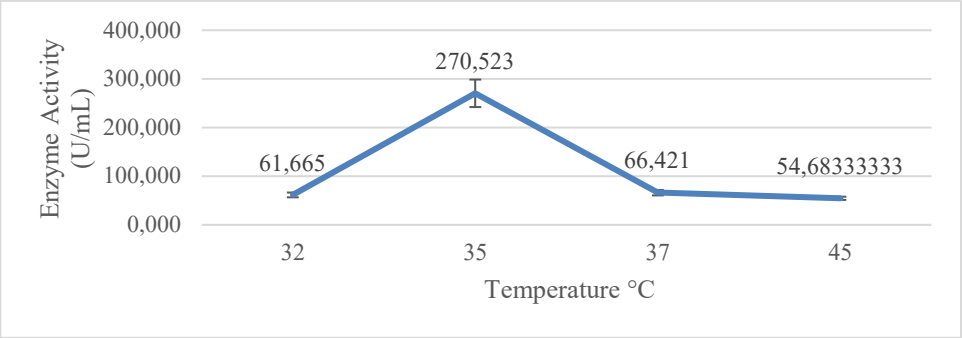
Table 9., shows that at a concentration of 0.04 g/mL $MgSO_4$ produced the highest enzyme activity, namely 12.28 ± 7.96 U/mL, but at $MgSO_4$ concentrations of 0.01 g/mL and 0.08 g/mL decreased enzyme activity by 71.67% and 42.01%. Magnesium ions have as a cofactor to support catalysis in biochemical reactions, to maintain the function of all organisms [53], as a stabilizer of cell membranes [54] and play a role in cell division [55]. Deficiency of magnesium can limit cell growth results in media [56]. Meanwhile, the presence of magnesium in large quantities can affect the structure, stability, and functionality of proteins [57], and can interfere with osmotic processes [58].

3.2.7. *Effect of Temperature in Fermentation Media*

Based on Table 10., at 35°C the highest enzyme activity was 270.523 ± 28.04 U/mL. Whereas at temperatures of 32, 37 and 45°C, the enzyme activity decreased by 77.21%, 75.46%, and 79.79%. The same results have been reported by Irfan et al (2015) that Bacillus subtilis produces an increase in enzyme activity at temperature 35°C and its activity decreased at 45°C [59]. Temperature is one of the important factors that can affect microbial growth and production of keratinase [9]. Microbial growth and metabolism must be at an appropriate temperature in order to produce optimal activity. The optimum temperature of a microbe is often different from the optimum temperature of other microbes. High temperatures can produce high reaction rates so as to accelerate cell growth. However, if the temperature exceeds the optimum limit for microbial growth, inactivation and denaturation

of enzymes will occur [8]. Based on the optimum temperature, *Bacillus haynesii* BK1H can be classified as a mesophilic bacterium. Mesophiles are microorganisms that grow at moderate temperatures, namely between 20°C and 45°C, and their optimum growth temperature is around 30-39°C [60].

Table 10. Table of the Effect of Enzyme Activity on Variations of Temperature



3.3. Determination of Significantly Influential Factors Using Plackett-Burman Design (PBD)

In the PBD model, the factors analyzed were then matched to the multivariate linear regression equation with an expected P value of <0.05. In other words, factors that have a P value <0.05 have a linear relationship to activity, while factors that have a P value > 0.05 do not have a linear relationship, but are still influential but have different relationships, for example having a quadratic or exponential relationship, so temperature pattern, number of inoculums, and pH need to be studied further in relation to activity. From the research data in Table 11., $MgSO_4$ (p-value = 0.019), $CaCl_2$ (p-value = 0.032), carbon source (p-value = 0.019), and agitation (p-value = 0.000) are considered to have a relationship linear to activity, while the variables temperature (p-value = 0.511), pH (p-value = 0.410), and the number of inoculums (p-value = 0.654) do not have a linear relationship.

Table 11. Table Analysis of Variance (ANOVA)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	418328	59761	5,06	0,001
Linear	7	418328	59761	5,06	0,001
Temperature	1	5227	5227	0,44	0,511
pH	1	8265	8265	0,70	0,410
MgSO4	1	73556	73556	6,23	**0,019**
CaCl2	1	60286	60286	5,11	**0,032**
Amount of Inoculum	1	2423	2423	0,21	0,654
Carbon Source	1	73703	73703	6,24	**0,019**
Agitation	1	194867	194867	16,51	**0,000**
Error	28	330473	11803		
Lack-of-Fit	4	268244	67061	25,86	0,000
Pure Error	24	62229	2593		
Total	35	748801			

Linier equation :
Activity= 219 – 3,01 Temperature – 30,3 pH – 3013 $MgSO_4$ + 163689 $CaCl_2$ + 4,10 Amount of Inoculum + 22,62 carbon source + 2,943 Agitation

In PBD also produced a linear equation. The minus sign (-) means that the higher the concentration of the factor, the activity of the enzyme will decrease, while the plus sign (+) means that the higher the concentration of the factor, the higher the activity of the enzyme. The linear equation shows that an increase in temperature is not followed by an increase in enzyme activity at the limit level used in the study, namely in the temperature range of 32-45°C, and an increase in pH is not followed by an increase in enzyme activity at the limit level used in the study, namely in the pH range of 6 -9, and the increase in MgSO4 concentration was not followed by an increase in enzyme activity at the limit level used in the study, namely in the concentration range of 0.01 – 0.08 g/mL. If the model in PBD is appropriate, then the P value of Lack of fit > 0.5. Whereas in research conducted the P value of Lack of fit <0.5 so that the equation is not suitable and it is necessary to review the PBD design of the influencing factors to obtain an acceptable linear equation.

4 Conclusion

In the One Factor at A Time (OFAT) approach, it can be seen that the factors that can affect the fermentation of *Bacillus haynesii* BK1H keratinase with the highest activity are at the condition of the substrate concentration of rice husk as an additional 1% carbon source; pH 7; 35°C; amount of inoculum 1%; 150rpm; 0.04 g/mL $MgSO_4$, and 0.0005 g/mL $CaCl_2$. The factors that significantly influence the fermentation of keratinase by *Bacillus haynesii* BK1H and have a linear relationship using the Plackett-Burman Design (PBD) are $MgSO_4$, $CaCl_2$, carbon sources, and agitation.

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