Optimization of Protease Activity by *Bacillus haynesii* BK1H Using Response Surface Methodology

Wildanna Rohma Septianingrum[1], *Eli Hendrik* Sanjaya[1], *Mieke* Alvionita[1], and *Suharti* Suharti[1*]

[1]Departement of Chemistry, State University of Malang
Jl. Semarang No. 5, Malang, East Java, 65145, Indonesia.

Abstract. Proteases are enzymes that catalyze the hydrolysis of peptide bonds into oligopeptides and amino acids. Previous bacterial studies reported that eight proteolytic isolates had been isolated from Bledug Kuwu mud, Central Java. One of these isolates is BK1H which has a proteolytic index of 2.960 making it possible to be studied further. In this study, optimization of the protease activity of BK1H was carried out using 3 factors namely temperature, pH, and divalent metal cations. This research was conducted through several stages, (1) Bacterial culture regeneration, (2) Protease fermentation using the SmF method, (3) Determination of the type of protease, (4) One Factor at a Time (OFAT), (5) Plackett-Burman Design (PBD), (6) Response Surface Methodology-Central Composite Design (RSM-CCD), and (7) Confirmation of RSM-CCD optimization results on experimental results. OFAT experiment results showed the lowest activity and the highest activity was obtained at 37°C and 57°C; pH 6 and 8; and $CaCl_2$ 0.5 and 1.0 mM. The significance test of the 2 factors namely temperature and pH has a p value <0.05, which indicates that these factors have a significant effect on protease activity. The optimum factor value in the RSM-CCD has a temperature of 63.10°C and pH 10.82, with the predicted activity of 149.910 U/mL, while the experimental results have activity of 157.630 ± 16.240 U/mL.

1 Introduction

Proteases are enzymes that catalyze the hydrolysis of peptide bonds into oligopeptides and amino acids. Proteases play an important role in various industrial fields such as detergents, animal feed, leather tanning, and adhesives [1]. As much as 62.80% of the proteases used by industry come from microbes [2], proteases from microbes can produce high activity and require low production costs making them suitable for commercial applications. Indonesia has high microbial biodiversity, thus providing a good opportunity to obtain protease-producing isolates with high activity. One source of microbes is the volcanic mud of Bledug Kuwu, Grobogan, Central Java. One of these isolates is *Bacillus haynesii* BK1H which has a proteolytic index of 2.960, so it is predicted to produce high protease activity [3].
Previously, the One Factor at a Time (OFAT) approach was used to optimize the protease activity of *Bacillus haynesii* BK1H, but it did not give optimal results. OFAT has a weakness,

* Corresponding author: suharti.fmipa@um.ac.id

© The Authors, published by EDP Sciences. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).

namely the resulting response will be different if the experimental conditions such as temperature, pH, and the type of metal cation change. So it is necessary to review OFAT to determine the effect of the third factor on a wider range, as well as to determine Low Level (-) and High Level (+) for further analysis.
Determination of the significance of factors affecting protease activity can be carried out experimentally, by combining all factors simultaneously using the factorial approach in Plackett-Burman Design (PBD). Factors that have been proven to be significant can be determined for their optimum value through Response Surface Methodology-Central Composite Design (RSM-CCD) analysis, then confirmed by experiment to determine the accuracy of the RSM-CCD optimization results.

2 Experiment

2.1 Chemicals and Instrumentation

The tools used in this study were a Erlenmeyer, measuring cup, measuring flask, test tube, beaker glass, spirit lamp, petri dish, micro pipette 100µL and 1000µL capacity, tip, wire loops, autoclave, oven, vortex, analytical balance, 2mL capacity microtube, centrifuge, UV-Vis spectrophotometer, pH meter, hotplate stirrer, cuvette, incubator shaker, Laminary Air Flow, incubator, and heatblock.

The basic material used in this research is broiler chicken feathers (*Gallus domesticus*), *Bacillus haynesii* BK1H isolate, liquid Lysogeni Broth (LB), skimmed milk, distilled water, bacto agar, sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), potassium hydrogen phosphate (K_2HPO_4), Tris-HCl, casein (Sigma Aldrich), trichloroacetic acid, sodium carbonate (Na_2CO_3), citric acid ($C_6H_8O_7$), sodium citrate ($Na_3C_6H_5O_7$), folin ciocalteu, ethylenediaminetetraacetate (EDTA) solution, and phenyl methyl sulfonyl fluoride (PMSF) solution.

2.2 Procedure

2.2.1 Bacterial Culture Regeneration

Bacterial regeneration was carried out aseptically using Skim Milk Agar (SMA) medium. *Bacillus haynesii* BK1H pure isolate taken from glycerol stock was inoculated in sterile SMA media using quadrant scratch technique. The culture was then incubated at 37°C for 24 hours until the bacterial colonies grew and produced a clear zone [3].

2.2.2 Preparation of Liquid Preculture Media

Bacillus haynesii BK1H isolate was inoculated on liquid Lysogeni Broth (LB) media and incubated in an incubator shaker at 37°C and aeration speed of 100 rpm for one night (± 15 hours) until the media became cloudy. The liquid culture is then used as a preculture to make starter, as much as 1% of the liquid culture is inoculated on the starter preculture media. The preculture media was then incubated in an incubator shaker at 37°C and an aeration speed of 100 rpm (± 4-5 hours) until the Optical Density (OD) reached 0.4-0.5 [4].

2.2.3 Preparation of Sub-merge Fermentation Media

Chicken feathers as much as 1% added 100 mL of salt solution. Furthermore, the fermentation media was autoclaved at 121°C for 15 minutes. 1% *Bacillus haynesii* BK1H starter was added to each fermentation medium, then incubated in an incubator shaker at 37°C and 100 rpm for 3 days.

2.2.4 Tyrosine Standard Curve

2 mL of tyrosine solution with concentrations of 0 (blank), 10, 30, 50, 70, 90, and 110 ppm, added 5 mL of 0.5 M Na_2CO_3 and 1 mL of folin ciocalteu (1:1). The solution was incubated in a dark room for 30 minutes. Sample absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 660 nm.

2.2.5 Protease Activity Assay

1 mL of the supernatant was added to 2.5 mL of 0.5 M Na_2CO_3 solution and 0.5 mL of folin ciocalteu solution (1:1). The solution was incubated for 30 minutes in a dark room and then its absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 660 nm. Enzyme activity calculations were carried out using equation 1 [4].

$$Enzyme\ Activity\ \left(\frac{U}{mL}\right) = \frac{Tyrosine\ concentration\ (ppm)\ x\ (Substrate\ volume + enzyme\ volume)(mL)}{Enzyme\ volume\ (mL)\ x\ Incubation\ time\ (minutes)} \quad (1)$$

2.2.6 Protease Type Determination

Identification of the type of protease was carried out using EDTA and PMSF solutions at concentrations of 5 mM and 10 mM. 200µL of the protease extract was added to 35µL of EDTA or PMSF solution, then homogenized and allowed to stand for 10 minutes. Then 0.5 mL of 1% (w/v) casein was added to the sample, and incubated for 5 minutes in a water bath shaker at 37°C and 100 rpm. 1 mL of 10% (w/v) TCA solution was added to the sample and allowed to stand for 15 minutes. The solution was centrifuged (21°C, 10.000, 10 minutes) and then the same procedure was carried out to test the protease activity. While a solution with a concentration of 10 mM was prepared with the same treatment but with the addition of 70µL of EDTA or PMSF solution.

2.3 One Factor at a Time (OFAT)

2.3.1 Temperature

150µL of protease extract was added to 0.5 mL of 1% (w/v) casein (dissolved in 50 mM Tris-HCl pH 8), then homogenized and incubated for 5 minutes in heatblock at various temperatures (37°C-72°C). 1 mL of 10% (w/v) TCA solution was added to the sample and allowed to stand for 15 minutes. The solution was centrifuged at 21°C and 10.000 RPM for 10 minutes, then the same procedure was carried out to test the protease activity.

2.3.2 pH

150μL of protease extract was added to 0.5 mL of casein which was dissolved in a buffer with various pH. Casein solution pH 6 was prepared by dissolving 1% (w/v) casein in 50 mM citrate buffer; casein solutions pH 7 and pH 8 were prepared by dissolving 1% (w/v) casein in 50 mM Tris-HCl buffer; while casein solutions pH 9 and pH 10 were prepared by dissolving 1% (w/v) casein in 50 mM glycine-NaOH buffer. The mixture was homogenized and incubated for 5 minutes in a heatblock (37°C). 1 mL of 10% (w/v) TCA solution was added to the sample and allowed to stand for 15 minutes. The solution was centrifuged at 21°C and 10.000 rpm for 10 minutes, then the same procedure was carried out to test the protease activity.

2.3.3 Divalent Metal Cations

150 μL of protease extract was added with various metal solutions (Ca^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+}), at concentrations of 0.5 mM and 1 mM. The mixture was homogenized and incubated for 5 minutes in a heatblock (37°C). 1 mL of 10% (w/v) TCA solution was added to the sample and allowed to stand for 15 minutes. The solution was centrifuged at 21°C and 10.000 rpm for 10 minutes, then the same procedure was carried out to test the protease activity.

2.3.4 Plackett Burman Design (PBD)

Based on the results of factor selection by OFAT, two values were selected that produced the lowest protease activity and the highest protease activity as Low Level (-1) and High Level (+1) of each factor analyzed [5]. Then these values were inputted into the Minitab 20 software, and tested in 12 sets of experiments (triplicate) to determine the significance of each factor on protease activity. Furthermore, the protease activity of each experimental set was analyzed by Analysis of Factors (ANOVA). The PBD experimental design and result can be seen in Table 6.

2.3.5 Response Surface Methodology-Box Behnken Design (RSM-BBD)

The experimental design using RSM was made with Minitab 20 software. There were 13 experimental sets for RSM-CCD (triplicate), the results of the protease activity of each experimental set were analyzed by ANOVA to determine the optimum value of each factor on protease activity. The RSM-CCD experimental design and result can be seen in **Table 9**.

3 RESULT AND DISCUSSION

3.1 Bacterial Culture Regeneration

Fig. 1. Skim Milk Agar Media (SMA) (a) and (b) BK1H regeneration results in SMA medium

Based on preliminary research, BK1H bacteria has been isolated from Bledug Kuwu mud [3]. The genotype test results stated that BK1H had a high percentage of similarity to the 16S rRNA gene of *Bacillus haynesii* [3]. In this study, BK1H bacterial cells were used to produce extracellular proteases. Proteases with optimal activity can be produced by fresh cell cultures, so before carrying out the optimization process it is necessary to regenerate the bacterial cells. Figure 1(a) is Skim Milk Agar (SMA) medium which is cloudy white in color because it contains casein which is emulsified in water. Meanwhile, Figure 1(b) is the result of BK1H regeneration in SMA media. BK1H produces extracellular proteases that are used to hydrolyze casein in skim milk, producing amino acids and short polypeptides that dissolve in water so that the white emulsion around the bacterial colonies turns into clear zones.

3.2 Protease Fermentation Using Sub-merge Fermentation (SmF) Method

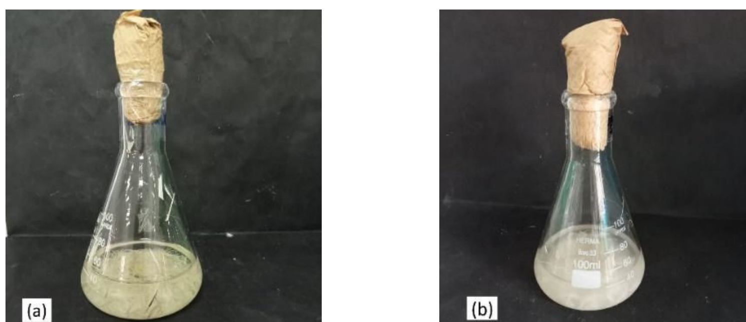

Fig. 2. Fermentation Media with Chicken Feather Substrate (a) Before Fermentation, and (b) after fermentation

The SmF method was chosen because the high amount of water and the intensity of the media shaking in the shaker helped the bacterial pili adhere to the surface of the substrate [4]. Chicken feathers are used as a source of nutrition for bacterial growth due to their abundant availability in nature and the potential for high amino acid content, the proximate test results show that as much as 82% of the crude protein extract is contained in chicken feathers [6]. In addition, the fermentation media also contains a salt solution to stabilize DNA and bacterial cell walls so that they can survive and degrade chicken feathers properly.
Based on Figure 2(b), the production medium after fermentation is cloudy white because the chicken feathers are degraded by the protease synthesized by *Bacillus haynesii* BK1H. Extracellular proteases break peptide bonds and disulfide bridges in keratin to produce polypeptides and amino acids. Furthermore, amino acids are reabsorbed by bacteria as nutrients for the process of growth and reproduction.

3.3 Protease Type Determination

Table 1. Results of adding inhibitors to the crude extract of protease

Sample	Protease Activity (U/mL) ± Standard Deviation
Controls (Without Inhibitors)	240.469 ± 0.000
EDTA 0.5 mM	192.131 ± 0.584
EDTA 1.0 mM	68.524 ± 0.894
PMSF 0.5 mM	116.121 ±0.584
PMSF 1.0 mM	47.031 ± 6.258

Based on Table 1 the addition of EDTA and PMSF can cause a decrease in protease activity. EDTA inhibited protease by 20.10% (0.5 mM) and 71.50% (1.0 mM), EDTA as a chelating agent can bind metal cofactors in the active site which functions to stabilize enzymes, causing a drastic decrease in enzyme activity [7]. While PMSF inhibited protease by 51.71% (0.5 mM) and 80.44% (1 mM), PMSF can interact with the -OH group of serine, this effect inhibits product formation because PMSF does not have a functional group that can be involved in enzyme catalytic process. These results are consistent with the research by Riesmi, et al have also reported that keratinase activity, which is a type of protease enzyme, can decrease after adding EDTA and PMSF [8]. Based on the identification results, it can be concluded that the types of protease from *Bacillus haynesii* BK1H are metalloprotease and serine protease.

3.4 One Factor at a Time (OFAT)

3.4.1 Temperature

Table 2. The effect of temperature on protease activity

Temperature (°C)	Protease Activity (U/mL) ± Standard Deviation
37	28.779 ± 1.145
42	36.469 ± 4.579
47	59.540 ± 6.869
52	110.945 ± 2.290
57	133.207 ± 0.572
62	130.576 ± 10.017
67	87.469 ± 3.435
72	11.779 ± 2.290

Based on Table 2, optimal protease activity occurs at a temperature of 57°C (133.207 ± 0.572 U/mL). The source of the protease comes from *Bacillus haynesii* BK1H which was isolated from the hot mud of Bledug Kuwu, so there is a possibility that the enzyme is still active at high temperatures. At the optimum temperature, there is an increase in kinetic energy so that the frequency of effective collisions is more intense, the impact is the more effective formation of the enzyme-substrate complex so that high enzyme activity is obtained.

The decrease in protease activity occurred at temperatures >57°C. If the temperature continues to increase, the increased kinetic and vibrational energy will put pressure on the bonds, resulting in breaking of the bonds which function to hold the enzyme molecule in order to have the proper structure [9]. This causes the tertiary structure of the enzyme to change, the active site is permanently damaged, and the shape is no longer complementary to the substrate.

3.4.2 pH

Based on Table 3, minimum protease activity occurs at pH 6 (45.374 ± 6.869 U/mL), at low pH (acidic conditions) the $-COO^-$ group will bind hydrogen ions, so that the negative charge is lost and the ability to form salt bridges will be reduced [10]. While optimal protease activity occurs at pH 8 (125.921 ± 2.862 U/mL), at optimum pH the proton donor and acceptor groups on the catalytic side of the enzyme are at the appropriate ionization level resulting in high protease activity. A decrease in activity occurs at pH> 8, at high pH (alkaline conditions) the $-NH3^+$ group will release hydrogen ions so that their ability to form ionic interactions is lost

[11]. This causes changes in the 3D structure of the enzyme, so that the ability to form bonds between substrates is reduced.

Table 3. The effect of pH on protease activity

pH	Protease Activity (U/mL) ± Standard Deviation
6	45.374 ± 6.869
7	79.779 ± 7.155
8	125.921 ± 2.862
9	89.695 ± 6.583
10	77.957 ± 6.010

3.4.3 Divalent Metal Cations

Table 4. The effect of divalent metal cations on protease activity

Sample (mM)	Protease Activity (U/mL) ± Standard Deviation
Controls (Without metals)	63.183 ± 1.145
Ca^{2+} 0.5	70.268 ± 5.842
Ca^{2+} 1.0	94.231 ± 1.490
Co^{2+} 0.5	74.398 ± 8.763
Co^{2+} 1.0	98.446 ± 14.006
Mn^{2+} 0.5	72.540 ± 4.966
Mn^{2+} 1.0	62.835 ± 12.515
Zn^{2+} 0.5	70.681 ± 11.684
Zn^{2+} 1.0	83.696 ± 18.178

In this study, several common types of metals were used, such as Ca^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} with concentrations of 0.5 mM and 1.0 mM. Based on Table 4, all metals can increase protease activity at either one or both concentrations, but with a very low percentage increase. This is possible because the active site of the enzyme has bound the cofactor during fermentation and is in a saturated state, so that the addition of cations during the activity test does not have much effect on the protease activity [12]. The addition of 1.0 mM Ca^{2+} showed the highest activity with a relatively lower standard deviation (94.231 ± 1.490 U/mL), so Ca^{2+} was chosen for Plackett-Burman Design (PBD) analysis.

3.4.4 Plackett Burman Design (PBD)

Table 5. Low Level (-) and High Level (+) in PBD

Factor	Unit	*Low Level* (-1)	*High Level* (+1)
Temperature	°C	37	57
pH	-	6	8
$CaCl_2$	mM	0.5	1.0

PBD is a factorial design to determine the significance of each factor. PBD uses a 2-level multivariate approach, namely high level (+) and low level (-). Table 5 shows the 2 levels used in PBD. while Table 6 shows the design of the PBD experiment in this experiment, there were 12 runs with 3 repetitions to obtain protease activity.

Table 6. PBD Experiment Result

Run	Temperature (°C)	pH	Ca^{2+} (mM)	Respons (U/mL)
1	57	6	1.0	144.030 ± 4.263
2	57	8	0.5	96.981 ± 8.316
3	37	8	1.0	19.006 ± 8.460
4	57	6	1.0	123.521 ± 2.632
5	57	8	0.5	98.909 ± 8.479
6	57	8	1.0	98.515 ± 10.445
7	37	8	1.0	16.618 ± 8.443
8	37	6	1.0	57.216 ± 5.235
9	37	6	0.5	49.200 ± 4.313
10	57	6	0.5	153.161 ± 3.155
11	37	8	0.5	22.623 ± 8.151
12	37	6	0.5	69.303 ± 2.687

ANOVA (Analysis of Variance) is used to test the average difference between all the factors tested. In PBD, ANOVA is used to analyze the significance of temperature, pH, and metal cations on protease activity. ANOVA has 2 hypotheses, namely H_0 (operational hypothesis) and Ha (alternative hypothesis). H_0 means that there is no average difference between all the test factors, H_0 is proven by a $p>0.05$ value. Whereas H_a means that there is a significant difference between the averages of all test factors, H_a is evidenced by a p-value <0.05.

Table 7. ANOVA in PBD

Source	F-Value	P-Value
Model	**106.160**	0.000
Linear	106.160	0.000
Temperature	252.570	**0.000**
pH	64.840	**0.000**
$CaCl_2$	1.070	**0.332**
Lack-of-Fit	0.470	**0.762**

Based on Table 7. P-value of temperature and pH showed results of less than 0.05. This indicates that the H_a hypothesis is proven, meaning that at the 5% significance level all factors have a significant effect on protease activity. While the F-value is the average variation in the sample [13], the model's F-value is 106.16 indicating that the model is significant. Meanwhile, the lack-of-fit or deviation of the inaccuracy of the model in this study was 0.762 ($p >0.05$) meaning that the model used did not have significant deviations so that it could predict results accurately.

3.4.5 Response Surface Methodology-Central Composite Design (RSM-CCD)

Response Surface Methodology (RSM) is an optimization method consisting of factorial design and regression analysis to analyze significant factors and select optimum experimental conditions to maximize the response. There are 5 levels used in Response Surface

Methodology-Central Composite Design (RSM-CCD), namely -α, -1, 0, +1, and +α. All values are taken from One Factor at a Time (OFAT) results. Table 8 shows the 5 levels in the RSM-CCD, while Table 9 represents the experimental design of the RSM-CCD in this study.

Table 8. Low Level (-) and High Level (+) in RSM-BBD

Factor	Unit	-α	-1	0	+1	+α
Temperature	°C	42.857	47	57	67	71.142
pH	-	5.171	6	8	10	10.828

Table 9. RSM-CCD Experiment Result

Run	Temperature (°C)	pH	Response (U/mL)
1	47	6	35.525 ± 2.695
2	67	6	12.588 ± 14.004
3	47	10	65.342 ± 5.675
4	67	10	147.779 ± 16.845
5	42.857	8	17.445 ± 2.103
6	71.142	8	13.667 ± 12.239
7	57	5.171	8.945 ± 4.907
8	57	10.828	123.763 ± 5.613
9	57	8	95.025 ± 5.729
10	57	8	83.961 ± 11.536
11	57	8	134.287 ± 6.920
12	57	8	98.937 ± 1.236
13	57	8	85.504 ± 5.204

Table 10. ANOVA in RSM-BBD

Source	F-Value	P-Value
Model	10.640	0.004
Linear	14.230	0.003
Temperature	0.760	0.413
pH	27.700	0.001
Cuadratic	9.510	0.010
Temperature*Temperature	18.520	0.004
pH*pH	1.580	0.249
2-Way Interaction	5.740	0.048
Temperature*pH	5.740	0.048
Lack-of-Fit	1.360	0.374

Based on Table 10. The P-value of several factors such as pH (linear equation), temperature*temperature (quadratic equation), and the interaction between temperature*pH showed results of less than 0.05. At the 5% significance level, these factors proved to have a significant effect on protease activity. The model's F-value is 10.640 indicating that the model

is significant. While the Lack-of-fit 0.374 (p> 0.05) indicates that the model used does not have significant deviations so that it can predict accurate results.

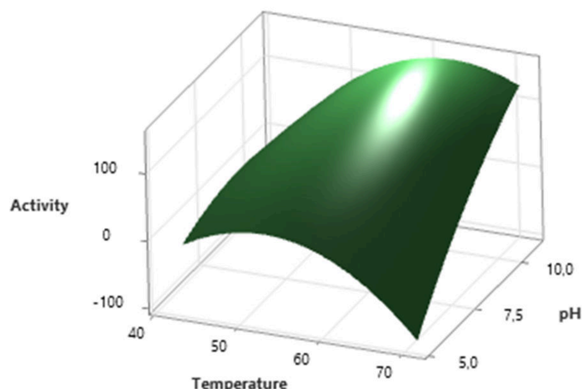

Fig. 3. Surface Plot in RSM-BBD

Based on **Figure 3**, the optimum temperature for protease activity from *Bacillus haynesii* BK1H has a temperature range of 50°C-60°C, because basically the source of the protease comes from the hot mud of Bledug Kuwu so there is a possibility that the enzyme is still active at high temperatures. Based on **Figure 3**, the pH is still experiencing an increase in activity, so in further research it is recommended that the pH range be widened.

Table 11. Confirmation of RSM-CCD with experiment

Factor		**Protease Activity (U/mL)**	
Temperature (°C)	pH	RSM Prediction	Actual Result
63.100	10.820	149.910	157.630 ± 16.242

Based on **Table 11**, RSM-CCD suggested experimental conditions of 63.100 and DpH 10.820, and to obtain protease activity of 149.910 U/mL. To determine the accuracy of the RSM optimization results, the RSM response predictions must be verified with experimental data. Under the same experimental conditions, an activity of 157.630 ± 16.242 U/mL was obtained. This value has an accuracy of 95.102% from the RSM-CCD predicted value.

4 Conclusion

The results of the PBD screening showed that temperature and pH had a p-value <0.05 or significant, while the RSM-CCD suggested conditions of 63.100°C and pH 10.820, a predicted activity of 149.910 U/mL, while the results actually has an activity of 157.630 ± 16.242 U/mL. This value has an accuracy of 95.102% of the prediction.

References

[1] A. Razzaq *et al.*, no. JUN **7** pp. 1–20 (2019)
[2] futuremarketinsights.com, “Protease Market Outlook (2023 to 2033),” *futuremarketinsights.com*, (2023)
[3] N. Faridah and S. Suharti, *AIP Conf. Proc* **2353** (2021)
[4] S. Suharti, H. N. Rozaq, A. Qisti, and M. Alvionita, MD24 in Sub-merge and Solid State Fermentation **19**, pp. 460–470 (2023)

[5] A. P. Karlapudi, S. Krupanidhi, R. R. E., I. M., N. B. Md., and V. T.C., *Beni-Suef Univ. J. Basic Appl. Sci.*, (2018)
[6] T. Tesfaye, B. Sithole, D. Ramjugernath, and T. Mokhothu, *Sustain. Chem. Pharm.*,. **9**, no. May, pp. 27–34, (2018)
[7] J. Polaina and A. P. MacCabe, *Industrial enzymes: Structure, function and applications*. (2007)
[8] S. Suharti, M. T. Riesmi, A. Hidayati, U. F. Zuhriyah, S. Wonorahardjo, and E. Susanti, *Pertanika J. Trop. Agric. Sci.*, **41**, no. 3, pp. 1449–1461, (2018)
[9] N. H, "The Effect of Temperature on Enzyme Reactions," (2023)
[10] J. C. & D. Harvey, "The Effect of pH on Enzyme Kinetics," Chemistry Libretext, (2023)
[11] D. L. N. and M. M. Cox, *Lehninger Principles of Biochemistry*. W.H. Freeman and Company, (2005)
[12] S. M. Smith, "Metal Reconstitution of Particulate Methane Monooxygenase and Heterologous Expression of the pmoB Subunit," (2012)
[13] Minitab, "Analysis of variance table for Fit Regression Model," (2023)