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OPTIMIZATION OF *Bacillus paramycoides* FERMENTATION MEDIUM TO INCREASE THE PRODUCTION OF 5-AMINOLEVULINIC ACID IN A 10 LITER FERMENTER

Optimasi Medium Fermentasi *Bacillus paramycoides* untuk Meningkatkan Produksi 5-*Aminolevulinic Acid* pada Fermentor 10 Liter

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ABSTRACT
5-Aminolevulinic acid is an essential precursor for the biosynthesis of tetrapyrrole compounds, such as chlorophyll and heme. 5-ALA has the potential to be used as a plant growth and antioxidant activity enhancer. 5-ALA can be produced through fermentation by Bacillus paramycoides. This study aimed to optimize B. paramycoides fermentation medium to increase 5-ALA production. The optimization was carried out using response surface method (RSM) experimental design. 5-ALA production in a 10 L fermenter was conducted using an optimized medium and supplemented with MSG as a precursor and wood vinegar as an inhibitor. The results showed that the best medium composition was 27.78 g L^{-1} molasses; 9.145 g L^{-1} urea; 8.838 g L^{-1} NaCl; and 32.07 g L^{-1} glucose, resulting in 10.749 (log CFU mL^{-1}) and 255.30 µM 5-ALA. 5-ALA production trial in a 10 L fermenter produced 581.82 µM 5-ALA. Medium optimization and precursor-inhibitors addition in the fermentation increased the 5-ALA yield 3.2 times compared to before optimization.

***Keywords**: 10 L fermenter, 5-aminolevulinic acid (5-ALA),* Bacillus paramycoides*, optimization, response surface method*

ABSTRAK
5-*Aminolevulinic acid* (5-ALA) merupakan prekursor penting dalam pembentukan senyawa tetrapirol seperti klorofil dan heme. 5-ALA memiliki potensi sebagai senyawa pemacu pertumbuhan dan peningkatan kandungan antioksidan tanaman. 5-ALA dapat dihasilkan melalui fermentasi oleh *Bacillus paramycoides*. Tujuan dari penelitian ini adalah melakukan optimasi media fermentasi *B. paramycoides* untuk meningkatkan konsentrasi 5-ALA. Optimasi media fermentasi dilakukan dengan menggunakan desain eksperimen *response surface method* (RSM). Setelah itu, dilakukan uji coba produksi 5-ALA pada fermentor 10 L menggunakan media hasil optimasi RSM dan suplementasi prekursor MSG dan inhibitor asap cair. Hasil optimasi media RSM menunjukkan bahwa komposisi media terbaik adalah 27,78 g L^{-1} molase; 9,145 g L^{-1} urea; 8,838 g L^{-1} NaCl; dan 32,07 g L^{-1} glukosa dengan jumlah sel (log CFU mL^{-1}) sebesar 10,749 dan konsentrasi 5-ALA sebesar 255,30 µM. Uji coba produksi 5-ALA pada fermentor 10 L menghasilkan konsentrasi 5-ALA sebesar 581,82 µM. Optimasi media dan penambahan prekursor-inhibitor pada fermentasi dapat meningkatkan produksi 5-ALA sebanyak 3,2 kali lipat dibandingkan sebelum optimasi.

Kata Kunci: 5-a*minolevulinic acid* (5-ALA), *Bacillus paramycoides*, fermentor 10 L, optimasi, *response surface method*

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INTRODUCTION

5-Aminolevulinic acid (5-ALA) is an essential precursor for the biosynthesis of tetrapyrrole compounds, such as chlorophyll, heme, and vitamin B12 needed by organisms (Kang et al. 2017). 5-ALA has the potential to be used as livestock dietary supplements (Hendawy et al. 2020), photodynamic cancer therapy (Yi et al. 2015, Tewari and Eggleston 2018), biodegradable herbicide (Xu et al. 2018), plant growth-promoting factor (Turan et al. 2014, Helaly 2017), and antioxidant activity enhancer (Kang et al. 2014, Liu et al. 2018). 5-ALA production can be carried out by chemical synthesis methods through enzymatic conversion and biosynthesis by some organisms such as animal cells, plants, bacteria, yeast, and algae (Kang et al. 2012). Meanwhile, biosynthesis of 5-ALA using microbes has certain advantages, such as a relatively quick production process, more accessible production stages, and relatively low production costs (Nishikawa and Murooka 2001). Generally, two distinct metabolic pathways of 5-ALA biosynthesis, the Shemin or C4 and the Beale or C5 pathway, had been described (Kang et al. 2012). In this study, 5-ALA is synthesized by *Bacillus paramycoides* through the Beale or C5 pathway, in which glutamic acid is used as the precursor. Glutamic acid is converted to 5-ALA in three steps, (i) ligation of tRNA to glutamate, (ii) reduction of glutamyl-tRNA to generate glutamate 1-semialdehyde (GSA), and (iii) transamination of GSA to generate 5-ALA. Then, two molecules of 5-ALA are condensed to porphobilinogen (PBG), catalyzed by ALA dehydratase (ALAD) encoding by the *hemB* gene (Zhang et al. 2015). Thus, in the strategy for enhancing 5-ALA from *B. paramycoides*, the precursors of 5-ALA and the competitive inhibitor of ALAD are added in the fermentation. Levulinic acid (LA) and glucose are competitive inhibitors of ALAD to accumulate 5-ALA more efficiently (Kang et al. 2012).

Besides the addition of precursors and inhibitors, the fermentation medium also has a vital role in cell growth that affects the synthesis of 5-ALA (Lee et al. 2005). Supposed 5-ALA is produced on an industrial scale. In that case, cost-effective production is required, so it is necessary to optimize the composition of the fermentation medium suitable for optimal growth and 5-ALA production (Dinarvand et al. 2013). The composition of fermentation medium for lowering production cost on the industrial scale was determined in the previous study, consisting of molasses, urea, NaCl, and glucose. The conventional optimization process (one-at-a-time strategy) of carbon and nitrogen sources was carried out by optimizing variations in molasses and urea (Abou-Taleb and Galal 2018). The result showed that the optimized medium composition was 25 g L^{-1} molasses; 7 g L^{-1} urea; 10 g L^{-1} NaCl; and 30 g L^{-1} glucose (Maraziha 2020). Afterward, the production of 5-ALA using a 200 L fermenter was carried out, but the yield of 5-ALA production was still low (179 µM).

The production using a fermenter still produced a low 5-ALA yield that had a relatively low selling value compared to a high 5-ALA yield because it can produce more volume when applied to plants. Therefore, it is necessary to optimize the composition of the fermentation medium for *B. paramycoides* using an experimental design that can predict a more optimal result, which is response surface method (RSM) (Sunaryanto and Nurani 2019). Saikeur et al. (2009) reported that both 5-ALA and biomass products could be increased using RSM optimization, giving 5-ALA and biomass of 183 µM and 3.1 g L^{-1} within 54 h. RSM is a collection of mathematical and statistical techniques useful for modeling and analyzing problems where several variables influence the desired responses and aim to optimize the responses (Montgomery 2012). This research aimed to optimize *the B. paramycoides* fermentation medium in producing 5-ALA using RSM experimental design and to conduct a trial of 5-ALA production in a 10 L fermenter using the best optimization medium and supplemented with monosodium glutamate (MSG) as a precursor, and wood vinegar as an inhibitor.

MATERIALS AND METHODS

Location and time

This research was conducted at the microbiology laboratory, School of Life Sciences and Technology, Institut Teknologi Bandung from May 2021 to April 2022.

Microorganism

B. paramycoides was derived from peat soil in Burung Island, Riau, Indonesia. The isolate was then identified using 16S DNA sequencing (Farah 2020). The isolate was subcultured on a nutrient agar (NA) medium and incubated at room temperature (28 – 30ºC) for 24 hours.

Inoculum preparation

B. paramycoides on NA medium (Figure 1) was activated in a 100 mL Erlenmeyer flask containing 25 mL Luria-Bertani + glucose (LBG) medium (1% $^{w}/_{v}$ tryptone; 1% $^{w}/_{v}$ NaCl; 0,5% $^{w}/_{v}$ yeast extract; 3% $^{w}/_{v}$ glucose) and incubated at room temperature (25 – 29ºC), 130 rpm for 24 hours (Farah 2020). Then, 10 mL activated culture with 0.8 optical density (OD) at 625 nm was inoculated into a 250 mL Erlenmeyer flask containing 90 mL LBG medium and incubated as previously described.

Optimization of fermentation medium

The optimization of fermentation medium composition was carried out using response surface method (RSM) and Box-Behnken design (BBD) experiment. The four factors for statistical analysis were molasses, urea, NaCl, and glucose concentration. The upper and lower limit values of factors determined based on previous studies were input into Minitab® software (Maraziha 2020). The upper and lower limit values for each factor are shown in Table 1. Then, the 54 medium variations (Table 2) were tested by observing cell count and 5-ALA concentration responses. Afterward, the results of response data were input into the Minitab® software for analysis of response surface design, such as analysis of variance response, response surface regression, determination of factor significance, the residual plot of response, a contour plot of response, and prediction of response results from medium composition from each response optimization (cell count, 5-ALA yield, and both).

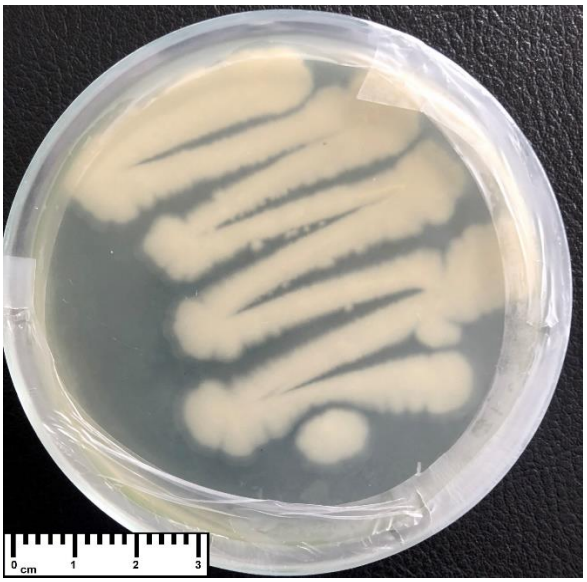


Figure 1. *Bacillus paramycoides* on nutrient agar (NA) medium

The experiment was performed by inoculating 5 mL of inoculum culture with 0.8 optical density (OD) at 625 nm to 250 mL Erlenmeyer flask containing 45 mL test medium (Table 2) and incubating the work culture at room temperature (25 – 29ºC), 130 rpm for 24 hours. The initial pH of this medium was adjusted to 6.8. *B. paramycoides* cell count and 5-ALA yield were measured after 24 hours of incubation. The response surface method was employed to answer the most suitable conditions and effect of each independent variable on cell growth and 5-ALA production. The response data obtained from the experiment (Table 2) were input into Minitab® software. Then, selecting an appropriate model type for the response was done by testing the sequential F-tests and checking the R-square, starting with a linear model and changing terms (linear, square, interaction, and quadratic). The quality of fit of the model equation was expressed by the coefficient of determination (R-squared) and adjusted-R-squared.

Using the Minitab® software, the optimal values of cell growth and 5-ALA of the experimental conditions were obtained by analyzing the response surface contour plots. Then, the response surface at optimal points was selected. Finally, the prediction of the best medium composition for optimized response (cell count, 5-ALA, and both) was operated by a response optimizer. Furthermore, the predictive

Table 1. Factors and levels used in the Box-Behnken Design

Factor	Medium ($g\ L^{-1}$)	Level		
		-1 (low)	0 (med)	1 (high)
A	Molasses	20	25	30
B	Urea	5	7.5	10
C	NaCl	5	10	15
D	Glucose	25	30	35

Table 2. Response analysis data on media optimization using Box-Behnken Design

Run	Design				Cell Count ($\log CFU/mL$)	ALA (µM)
	A	B	C	D		
1	0	-1	0	1	10.37	238.64
2	0	-1	-1	0	10.43	184.09
3	0	-1	0	-1	10.34	197.73
4	0	1	1	0	10.46	187.73
5	1	0	0	1	10.74	215.91
6	0	0	1	1	10.52	283.18
7	0	0	-1	-1	10.55	208.64
8	1	-1	0	0	10.73	215.00
9	0	0	0	0	10.69	229.55
10	-1	-1	0	0	10.42	169.55
11	0	0	0	0	10.68	229.55
12	1	0	0	-1	10.70	213.18
13	0	0	0	0	10.72	228.64
14	0	0	0	0	10.72	239.55
15	-1	-1	0	0	10.54	186.82
16	1	0	-1	0	10.56	242.27
17	0	1	0	-1	10.44	330.45
18	0	1	0	1	10.58	304.09
19	0	-1	1	0	10.23	186.82
20	-1	0	0	1	10.51	193.18
21	1	0	-1	0	10.74	133.18
22	-1	1	0	0	10.45	212.27
23	1	0	1	0	10.65	229.55
24	0	1	1	0	10.62	154.09
25	0	0	-1	-1	10.69	213.18
26	0	-1	-1	0	10.27	195.91
27	-1	0	1	0	10.43	201.36
28	0	0	-1	1	10.49	223.18
29	0	1	0	-1	10.55	286.82
30	0	-1	0	-1	10.46	194.09
31	0	0	0	0	10.65	243.18
32	-1	0	1	0	10.47	192.27
33	0	0	-1	1	10.74	239.55
34	1	1	0	0	10.71	251.36
35	0	0	1	-1	10.53	254.09
36	1	0	0	-1	10.74	123.18
37	-1	0	0	1	10.50	204.09
38	-1	0	0	-1	10.55	232.27
39	0	0	1	1	10.59	251.36
40	0	1	0	1	10.58	274.09
41	-1	0	-1	0	10.57	236.82
42	0	-1	0	1	10.29	196.82
43	0	0	1	-1	10.58	224.09
44	0	0	0	0	10.71	242.27
45	-1	1	0	0	10.25	233.18
46	-1	0	0	-1	10.57	266.82
47	0	-1	1	0	10.41	195.91
48	-1	0	-1	0	10.41	248.64
49	1	1	0	0	10.68	250.45
50	0	1	-1	0	10.48	306.82
51	1	0	0	1	10.63	141.36
52	0	1	-1	0	10.62	290.45
53	1	-1	0	0	10.75	109.55
54	1	0	1	0	10.61	256.82

medium (Table 3) was validated to ensure the predictive response value fit the test result. The validation experiment was performed by inoculating 5 mL of inoculum culture with 0.8 optical density (OD) at 625 nm to 250 mL Erlenmeyer flask containing 45 mL predictive medium (Table 3) and incubated at room temperature (25 – 29ºC), 130 rpm for 24 hours. The initial pH of this medium was adjusted to 6.8. *B. paramycoides* cell count and 5-ALA yield were measured after 24 hours of incubation.

Fermenter experiment

Fermenter (BIOF-10L, LABFREEZ, China) experiment was performed by inoculating 700 mL inoculum culture in LBG medium to 10 L fermenter containing 6.3 L of the best medium and supplemented with 0.5% $^{v}/_{v}$ MSG 0.5 M (Aiguo and Meizhi 2019). Under optimal operating conditions (Liu et al. 2017), the fermentation was carried out for 48 hours at 30ºC, initial pH of 7.1, and 130 rpm of mixer speed. DO and aeration were controlled at 40-60% and 1.5-2.5 vvm. After 24 hours of incubation, 0.1% $^{v}/_{v}$ glucose 30% and 0.5% $^{v}/_{v}$ wood vinegar (Nunkaew et al. 2018) were added and the pH was adjusted to 6.8. The fermentation was then continued for the next 24 hours at 30ºC, 110 rpm. DO and aeration were controlled at 35-50% and 1.25-1.75 vvm (Nishikawa and Murooka 2001). Bacterial growth and 5-ALA yield were measured after fermentation.

Analytical methods

B. paramycoides optical density was measured at 625 nm with a spectrophotometer (UV-1900, Shimadzu), and then correlated with *B. paramycoides* standard curve. Before measurement, culture samples were centrifuged at 8000 rpm for 5 minutes, and the supernatant was collected for 5-ALA yield determination. Then, 1 mL saline (0,9% $^{w}/_{v}$ NaCl) was added, and the cells were re-suspended. The samples were centrifuged again. The supernatant was removed, 1 mL saline was added, and the cells were re-suspended (Yap and Trau 2019). The cell count was then measured. The 5-ALA yield was determined by the colorimetric method by Mauzerall and Granick (1956). A 100 µL samples were mixed with 2 mL acetate buffer and 1% $^{v}/_{v}$

Table 3. Composition of the response optimization medium (multiple response prediction)

Response Optimization	Medium composition (g L^{-1})				Multiple Response Prediction	
	Molasses	Urea	NaCl	Glucose	Cell Count ($\log CFU/mL$)	ALA Yield (µM)
Cell growth (OD medium)	30	8.081	9.646	30.3535	10.798	-
ALA yield (ALA medium)	21.62	10	5	25	-	328.151
Both (ODALA medium)	27.78	9.145	8.838	32.07	10.72	251.3

acetylacetone. After the mixtures were boiled for 15 minutes and cooled, 3.5 mL of Ehrlich's reagent was added to each sample. The 5-ALA formed was measured at a wavelength of 553 nm.

RESULTS AND DISCUSSION

Fermentation medium optimization

The best model that could provide the best coefficient of determination for all responses is given in Table 4. The best model term for cell count and 5-ALA yield were full quadratic because their R-squared was higher than other model terms, and R-squared for cell count and 5-ALA yield were 0.6553 and 0.5437, respectively. The R-squared of the model means the soundness of the model. A higher R-squared value will give a more accurate prediction and fit the model. Both cell count and 5-ALA yield R-squared obtained in this study were not too high. It did not mean a low R-squared value was inherently poor because other factors affected the results, like the physiology and metabolic behavior of the microorganism. Moreover, if the R-squared value was low, but the result was statistically significant, the conclusion could still be drawn about the association between variable and response (Montgomery 2012). This type of information could be so valuable. The value of the adjusted R-squared suggested that the response variation of 53.16% and 37.99% for cell count and 5-ALA yield, respectively, were attributed to the independent variables and about 46.84% (cell count) and 62.01% (5-ALA yield) of the total variation could not be explained by the model.

The analysis of variance for the model full quadratic (Table 5) shows that the model significantly (p-value model of cell count and 5-ALA yield was 0.00 and 0.002, respectively) could affect the resulting response. It indicated that the model chosen could be used for predicting the value of responses. However, the p-value lack-of-fit was less than α (0.05), meaning the model did not accurately fit the data and the determination of error cannot be determined from the lack-of-fit value (Montgomery 2012). From the response surface design analysis results, the response regression equation could be used to determine and predict the response of the factor (fermentation medium). Thus, the

Table 4. Summary of statistical models for *B. paramycoides* cell count and ALA yield responses

Source (Model terms)	Standard deviation		R-squared		Adjusted R-squared		Predicted R-Squared	
	Cell count	ALA yield	Cell count	ALA yield	Cell count	ALA yield	Cell count	ALA yield
Linear	0.1181	39.766	0.3350	0.2755	0.2807	0.2164	0.2002	0.1044
Linear + square	0.0908	38.300	0.6390	0.3828	0.5749	0.2731	0.4802	0.1113
Linear + interaction	0.1246	37.440	0.3513	0.4364	0.2005	0.3054	0.0000	0.0650
Full quadratic	0.0953	35.374	0.6553	0.5437	0.5316	0.3799	0.3149	0.0918

Table 5. Analysis of variance for the full quadratic model of responses

Source	Degree of Freedom		Sum of Squares		Mean Square		F-value		P-value	
	Cell count	ALA yield	Cell count	ALA yield	Cell count	ALA yield	Cell count	ALA yield	Cell count	ALA yield
Model	14	14	0,67446	58156	0,048175	4154	5,30	3,32	0	0,002
Lack-of-Fit	10	10	0,17732	24523	0,017732	2452,3	2,90	2,93	0,012	0,012

following regression equations for cell count and 5-ALA yield were obtained. The quadratic equations for cell count and 5-ALA yield are presented.

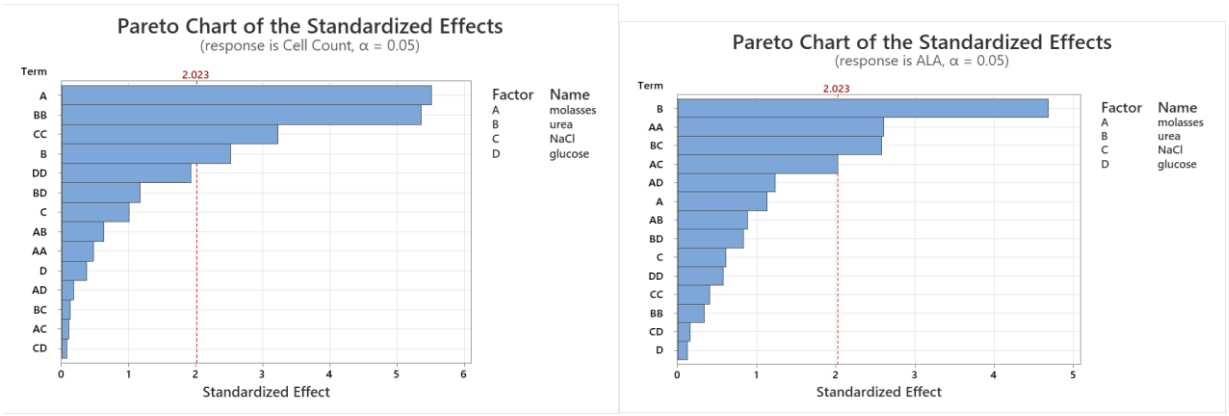
$$y_1 = 7{,}25 + 0{,}0277\,x_1 + 0{,}254\,x_2 + 0{,}0620\,x_3 + 0{,}1027\,x_4 - 0{,}00055\,{x_1}^2 - 0{,}02503\,{x_2}^2 - 0{,}00377\,{x_3}^2 - 0{,}00225\,{x_4}^2 + 0{,}00171\,x_1\,x_2 + 0{,}00015\,x_1\,x_3 + 0{,}00024\,x_1\,x_4 + 0{,}00035\,x_2\,x_3 + 0{,}00317\,x_2\,x_4 + 0{,}00010\,x_3\,x_4$$

$$y_2 = 169 + 19{,}7x_1 + 50{,}8x_2 - 5{,}6x_3 - 24{,}6x_4 - 1{,}129\,{x_1}^2 - 0{,}58\,{x_2}^2 - 0{,}177\,{x_3}^2 + 0{,}248\,{x_4}^2 + 0{,}88x_1\,x_2 + 1{,}014\,x_1\,x_3 + 0{,}614\,x_1\,x_4 - 2{,}58x_2\,x_3 - 0{,}83x_2\,x_4 + 0{,}077x_3\,x_4$$

y_1 = cell count ($\log CFU/mL$)
y_2 = 5-ALA yield (µM)
x_1 = molasses (g L^{-1})
x_2 = urea (g L^{-1})
x_3 = NaCl (g L^{-1})
x_4 = glucose (g L^{-1})

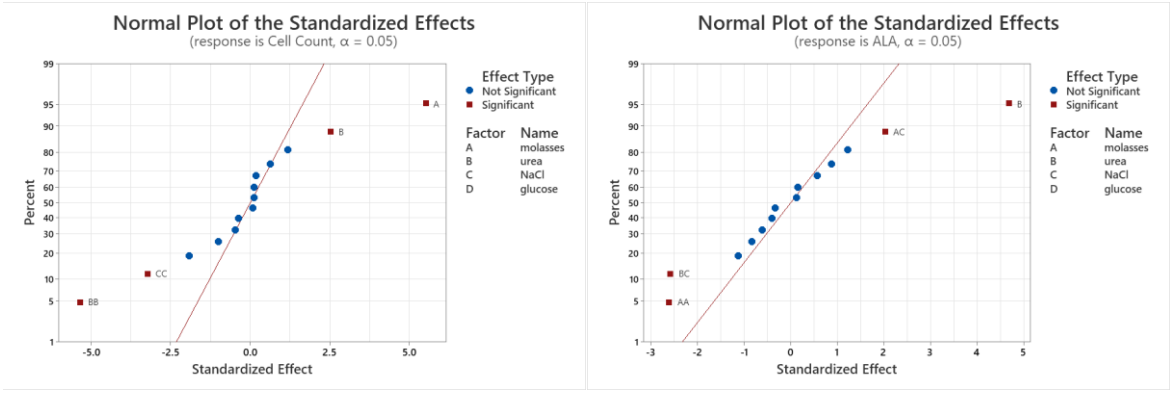
Based on the Pareto chart in Figure 2, the factors that significantly affected the cell count response (Figure 2A) were linear model A (molasses), quadratic model BB (urea), quadratic model CC (NaCl), and linear model B (urea). These factors were known to affect the cell's growth during fermentation. The factors that significantly affected the 5-ALA yield response (Figure 2B) were linear model B (urea), quadratic model AA (molasses), linear model BC (urea-NaCl), and linear model AC (molasses-NaCl). These factors were known to affect the 5-ALA production during fermentation. However, studying which model affects the cell count and 5-ALA yield positively or negatively is necessary.

From the normal plot in Figure 3, significant factors negatively or positively affect the responses. The significant factors that could have a negative effect on the cell count response (Figure 3A) were the quadratic model of NaCl (CC) and urea (BB). The significant factors that could have a positive effect on the cell count response (Figure 3A) were linear models of urea (B)



A B

Figure 2. The Pareto chart of the standardized effects: (A) cell count and (B) ALA yield



A B

Figure 3. The normal probability plot of the standardized effects: (A) cell count and (B) ALA yield

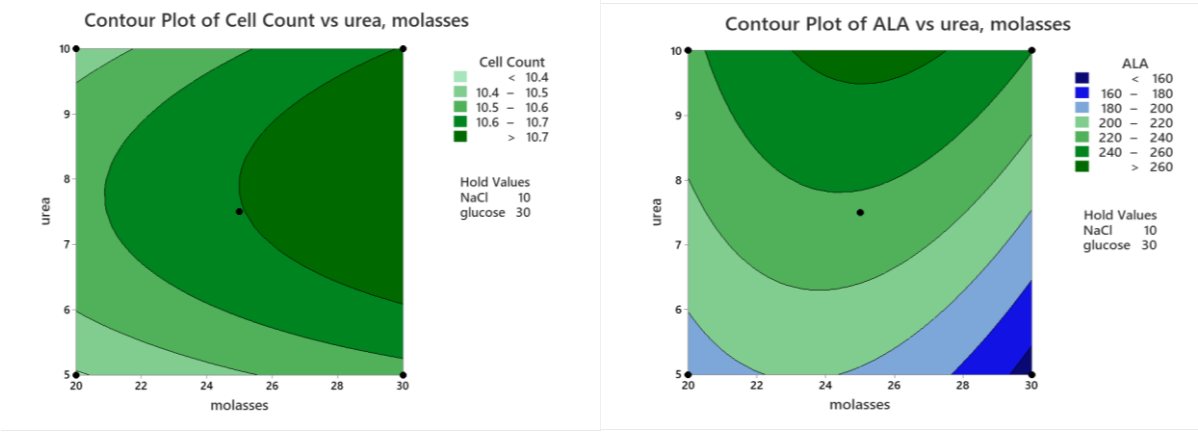
and molasses (A). The significant factors that could negatively affect the 5-ALA production were the molasses (AA) quadratic model and the urea-NaCl (BC) linear model. The significant factors that could positively affect the 5-ALA yield response were linear models of urea (B) and molasses-NaCl (AC). Lee et al. (2005) reported that the composition of fermentation medium has a key role in influencing bacterial growth. The carbon and nitrogen sources also play a role in how bacteria can grow properly and produce the desired metabolites.

In this research, the compositions of the medium used were molasses, urea, NaCl, and glucose. Molasses is a carbon source with quite complex nutrition containing sources of sucrose, glucose, fructose, amino acids, protein, minerals such as Cu, Fe, Mn, Zn, Co, Mg, K, Na, and vitamins such as riboflavin, pyridoxine, biotin, folic acid, thiamine, niacin, and choline (Puspitasari 2008). Urea is a nitrogen source that functions as an ingredient for forming amino acids and protein for the nutritional needs of microbes during the fermentation process (Prabandari et al. 2017). NaCl is a source of minerals that plays a role in maintaining osmotic pressure so that microbial cells are in optimum growth conditions (Omotoyinbo and Omotoyinbo 2016). Glucose is a monosaccharide carbon source needed in cell metabolism as a primary substrate source that can produce energy during cell growth. Moreover, glucose can also act as a carbon source for forming precursors of 5-ALA naturally in cells so that the concentration of 5-ALA can increase (Kang et al. 2012). Then, glucose can also inhibit the expression of the ALAD, which converts 5-ALA into tetrapyrrole compounds (PBG) so that more 5-ALA can be accumulated (Zhang et al. 2015).

Based on the analysis of factors that significantly affect the cell count of *B paramycoides*, molasses and urea are the compositions of the medium that can positively affect the *B. paramycoides* growth. However, if the urea concentration is too high, the growth of *B. paramycoides* will be inhibited because urea is a significant factor that negatively affects *B. paramycoides* growth. This is highly correlated with the C/N ratio, which affects the *B. paramycoides* growth. The very low and too high C/N ratio can inhibit the *B. paramycoides* growth, so proper concentrations of carbon and nitrogen sources are needed for *B. paramycoides* growth in producing 5-ALA (Syafitri et al. 2022).

Wulan et al. (2022) also reported that a low C/N ratio (high nitrogen source) could inhibit bacterial growth because an increased emission of nitrogen as ammonium inhibited bacterial growth. Then, the exceptionally high C/N ratio (low nitrogen source) caused limited nitrogen sources for producing important compounds in metabolism and cell formation. Furthermore, a high concentration carbon source could increase the acidification process in culture conditions that inhibit bacterial growth.

Then another significant factor, NaCl, is a significant factor that negatively affects the cell count if the concentration used is too high. Liu et al. (2017) reported that the optimum salinity for the *B. paramycoides* growth was 0.5% $^{w}/_{v}$ NaCl. However, it is necessary to search for the right salinity concentration so that *B. paramycoides* has optimal growth and can produce optimal 5-ALA yields.



A B

Figure 4. The contour plot shows the relationship between molasses and urea toward the fitted responses: (A) cell count and (B) ALA yield.

From the contour plot of the cell count response (Figure 4A), the relationship between molasses and urea toward the cell count response can be revealed to obtain the best cell count, which is above 10.7 $\log CFU/mL$; the factor concentration must be in the dark green area. Thus, the best cell count areas are 25-30 g L^{-1} molasses and 6-10 g L^{-1} urea. From the contour plot of the 5-ALA yield response (Figure 4B), the relationship between molasses and urea to the 5-ALA yield response can be revealed to obtain the best 5-ALA yield above 260 µM; the factor concentration must be in the dark green area. Thus, the best 5-ALA production areas are between 23-27 g L^{-1} molasses and 9.5-10 g L^{-1} urea.

After determining the response surface model, response optimization was done by maximizing the response target at the Response Optimizer menu in Minitab® software. Then, optimization medium composition variation was obtained with response prediction (Table 3). Such medium variation needed further validation to ensure the predicted response value matched the test result (Montgomery 2012). If the test results get a lower or higher value than the prediction, it can be seen what the percentage difference between the test results and predictions is. As long as the percentage difference between the test results and predictions is still below the confidence interval value (α = 5%), the test results and predictions are still considered appropriate. If the difference exceeds the confidence interval, the other medium composition variations close to the desired response value, the best 5-ALA yield, must be found (Stevens and Anderson-Cook 2019).

Based on the validation test of the optimized medium, the cell count in the medium composition optimization of cell count response (OD medium) was only 10.745 $\log CFU/mL$ (Figure 5) and has a difference of 0.49% from the predicted response to the cell count with a value of 10.798 $\log CFU/mL$ (Table 3). It means that the results of the OD medium validation composition are still in accordance with the predictions of the response optimizer analysis because the difference in the cell count is still below 5%. Then, the 5-ALA yield in the medium composition optimization of 5-ALA yield response (ALA medium) was only 221.06 µM (Figure 6) and had a difference of 32.6% from the predicted response to the concentration of 5-ALA yield with a value of 328,151 M (Table 3). It means that the results of the composition of ALA medium validation are not in accordance with the predictions of the response optimizer analysis because the difference in the concentration of 5-ALA compounds is already above 5%. However, the cell count and 5-ALA yield in the medium composition optimization of both responses (ODALA medium) matched the predicted response value. The values for cell count and 5-ALA yield in ODALA medium, respectively, were 10.749 $\log CFU/mL$ (Figure 5) and 255.3 µM (Figure 6), which had differences of 0.27% and 1.56% of the ODALA response prediction with values were 10.72 $\log CFU/mL$ and 251.3 µM (Table 3),

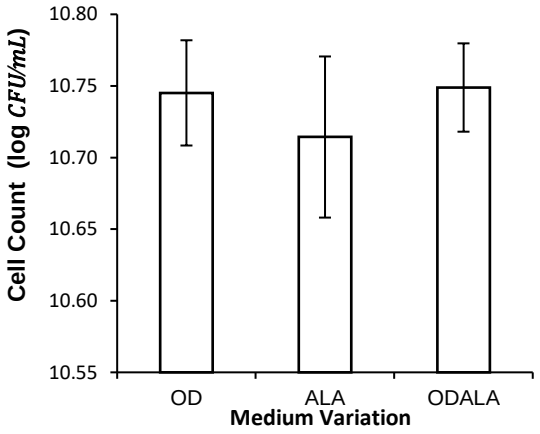


Figure 5. Comparison of the cell count at 24 hours between the predicted response optimization of cell count (OD), ALA yield (ALA), and both (ODALA)

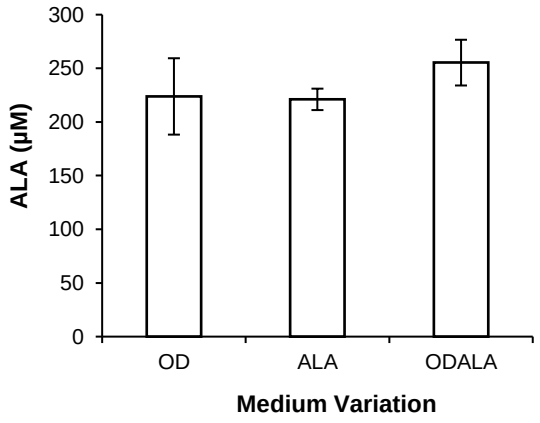


Figure 6. Comparison of the ALA yield at 24 hours between the predicted response optimization of cell count (OD), ALA yield (ALA), and both (ODALA)

respectively. As reported by Lee et al. (2005), the proper C/N ratio in the fermentation medium could produce an optimum 5-ALA yield. This indicated that the ODALA medium with 27.78 g L-1 molasses; 9.145 g L-1 urea; 8.838 g L-1 NaCl; and 32.07 g L-1 glucose was the best medium composition with the most appropriate C/N ratio in producing 5-ALA and growth of *B. paramycoides* compared to OD and ALA medium. The 5-ALA yield in the OD medium was lower than the ODALA medium because nitrogen concentration in the OD medium composition was much lower than in the ODALA medium, in which nitrogen was an important source in the formation of amino acids (Arnold et al. 2015). The cell count in the ALA medium was lower than in the ODALA medium because the carbon source in the ALA medium composition was much lower concentration than in the ODALA medium, which carbon was an important source in the cell growth that affected the production of 5-ALA (Lee et al. 2005). This experiment showed that 5-ALA could be produced well by optimizing the cell growth and the proper C/N ratio, which the ODALA medium was the best-optimized medium used in the fermenter experiment. In this research, the calculation of the C/N ratio was carried out by calculating the ratio of the total atomic weight of carbon and nitrogen contained in the ODALA medium, which was equal to 5.41:1.

Fermenter experiment

The production of 5-ALA in fermentation needs precursors of 5-ALA and enzyme inhibitors that avoid the conversion of 5-ALA into tetrapyrrole compounds. The inhibitor commonly used in the production of 5-ALA is levulinic acid (LA) which can inhibit the ALA-Dehydratase, which converts 5-ALA into porphobilinogen (Zhang et al. 2022). Nunkaew et al. (2018) reported that using wood vinegar as a substitute for LA could increase the production of 5-ALA in *Rhodopseudomonas palustris* by up to 4 times. The composition of wood vinegar consists of methanol, acetic acid, propionic acid, butyric acid, phenol, and levulinic acid. As much as 2.5% $^{v}/_{v}$ of wood vinegar contains levulinic acid compounds of 236 µM. However, the excessive use of wood vinegar (over 2.5% $^{v}/_{v}$) could inhibit cell growth and reduce the 5-ALA yield.

The microbe used in this study (*B. paramycoides*) is a bacterium with the C5 (Beale) pathway in the 5-ALA metabolic pathway. The precursor for the 5-ALA synthesis in the C5 pathway is glutamic acid, in its salt form, sodium L-glutamate monohydrate (Aiguo and Meizhi 2019). Sodium L-glutamate monohydrate analytical grade (glutamate) is expensive when used in industrial-scale production. Therefore, monosodium glutamate (MSG) was chosen to replace sodium L-glutamate monohydrate as a precursor for the 5-ALA synthesis because it is more economical and easier to obtain. If MSG is dissolved in water, MSG will dissociate into free salt and anion form glutamic acid by 78% (Siregar 2009). Thus, such glutamic acid is suitable as a precursor for the 5-ALA synthesis.

In this experiment, MSG and wood vinegar were tested on the lab scale to replace glutamate and LA. Figure 7 shows that the best 5-ALA yield was ODALA medium with MSG and wood vinegar supplementation. Replacement of precursor-inhibitor with MSG and wood vinegar could produce 298.94 µM 5-ALA which was significantly different (Tukey Test, one-way ANOVA, α=0.05) from the unoptimized medium that only produced 244.39 µM 5-ALA. Hence, ODALA medium, MSG, and wood vinegar were used in the fermenter

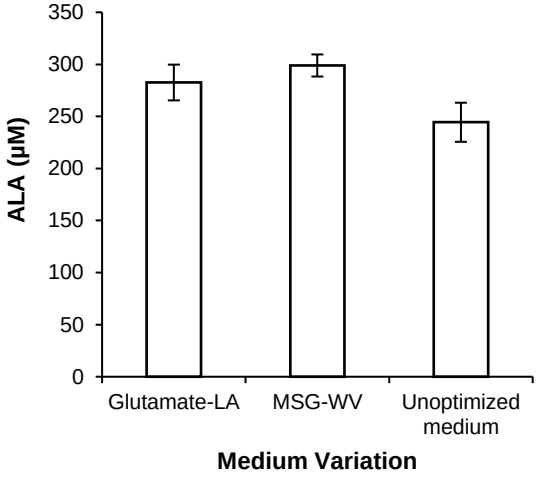


Figure 7. Comparison of the ALA yield at 48 hours between ODALA medium supplemented by glutamate as a precursor and LA as an inhibitor LA (Glutamate-LA); MSG as a precursor and wood vinegar as an inhibitor (MSG-WV); and medium before RSM optimization (unoptimized medium)

experiment to produce 5-ALA in a 10 L fermenter. According to Kang et al. (2012), the supplementation precursor and inhibitor will increase 5-ALA production. Higher 5-ALA yield will be achieved when the best precursor is supplemented at the beginning of the exponential phase and the best inhibitor is supplemented after the exponential phase ends (Choorit et al. 2011). The supplementation of the precursor at the beginning of the fermentation was intended so that the culture could produce 5-ALA during the growth phase. Meanwhile, an inhibitor was given at 24 hours so that the accumulation of 5-ALA could occur after the logarithmic growth phase (Saikeur et al. 2009).

This fermenter experiment was performed by using the best-optimized medium with a composition of 27.78 g L^{-1} molasses; 9.145 g L^{-1} urea; 8.838 g L^{-1} NaCl; and 32.07 g L^{-1} glucose. As much as 0.5% $^{v}/_{v}$ MSG 0.5 M was supplemented at the beginning of fermentation and 0.5% $^{v}/_{v}$ wood vinegar as an inhibitor and 0.1% $^{v}/_{v}$ glucose 30% were supplemented after 24 hours of fermentation. In the first 24 hours, the fermentation conditions were carried out at 130 rpm, initial pH of 7.1, aeration 1.5-2.5 vvm, 30ºC, and DO 40-60%. The DO value was maintained between 40-60% so that cell growth continued well and the production of 5-ALA could occur. According to Nishikawa and Murooka (2001), low dissolved oxygen (DO) in the culture can activate ALA synthase (*hemA*) and produce 5-ALA. After 24 hours of incubation, agitation was reduced to 110 rpm. The DO value was maintained between 35% and 50%, so the ALA synthase continued to produce 5-ALA in the stationary phase of cell growth. As reported by Yang et al. (2013), the low dissolved oxygen content can increase ALA synthase activity by 30% and produce a better 5-ALA yield. However, the very low oxygen saturation for a long duration can inhibit growth and reduce 5-ALA production.

From Figure 8, it is known that the cell count at 24 hours of fermentation was 10.74 ($\log CFU/mL$), which still fitted with the prediction, 10.72 ($\log CFU/mL$). The cell count shows that the *B. paramycoides* cell grew well in the fermenter because it had already reached the prediction. After 48 hours of fermentation, the cell count did not significantly increase, only 10.76 ($\log CFU/mL$). These results indicated the growth after supplementation of wood vinegar and glucose did not occur exponentially. The limiting factor like carrying capacity was one of the factors that indicated the cell growth was already in the stationary phase. Then, the decrease in DO was another factor affecting the fermenter's cell growth (Yang et al. 2013). Furthermore, the addition of wood vinegar could affect cell growth because other various compounds in wood vinegar might affect cell growth (Nunkaew et al. 2018). Thus, the processes that occurred in the fermenter could focus on the accumulation of 5-ALA.

From Figure 9, it is known that the 5-ALA yield at 24 hours of fermentation was 423.18 µM which was relatively higher than the prediction at the same time. This might be because the production of 5-ALA using a fermenter could achieve optimal fermentation conditions, like optimal initial pH and aeration, for cell growth and 5-ALA synthesis. Then, not only the use of the best fermentation medium

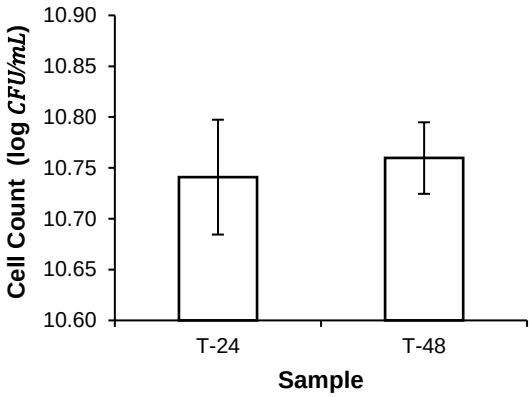


Figure 8. Comparison of the cell count between samples at 24 hours and 48 hours

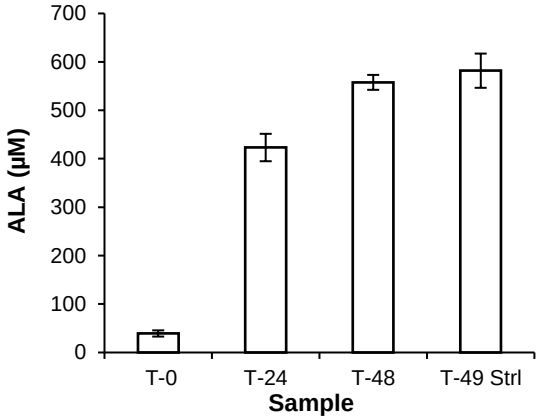


Figure 9. Comparison of the ALA yield between samples at the beginning, 24 hours, 48 hours, and 49 hours (after sterilization)

suitable for optimal cell growth and 5-ALA synthesis but also the supplementation of MSG was one of the factors why the 5-ALA yield at 24 hours was relatively high. After supplementation of wood vinegar and glucose and the addition of incubation time for 24 hours, the concentration of 5-ALA was 557,73 µM at 48 hours and 581,82 µM after sterilization. It indicated that wood vinegar could accumulate 5-ALA production by inhibiting ALA-Dehydratase, according to Nunkaew et al. (2018), who reported that wood vinegar could be used as an inhibitor to increase the synthesis of 5-ALA. There was a slight increase in the concentration of 5-ALA after sterilization. This might be because there was 5-ALA still trapped in the cells and released after the sterilization process. From this fermenter experiment which used the best fermentation medium (ODALA medium), MSG as a precursor, and wood vinegar as an inhibitor, an increase of 401.82 µM or 3.2-times compared to 5-ALA production in a 200 L fermenter was achieved.

This research was expected to be used optimally, especially in the study and application of bioprocess technology for the production of 5-ALA effectively and efficiently on an industrial scale. Therefore, ALA with various benefits such as plant biostimulators, bioherbicides, cosmetic drugs, and health supplements, can later be used by the wider community, especially in Indonesia.

CONCLUSION

The best composition of *B. paramycoides* fermentation medium in producing 5-ALA was 27.78 g L^{-1} molasses; 9.145 g L^{-1} urea; 8.838 g L^{-1} NaCl; and 32.07 g L^{-1} glucose. The best medium composition was suitable for *B. paramycoides* cell growth and 5-ALA synthesis. With the supplementation of MSG as a precursor and wood vinegar as an inhibitor, the fermentation could achieve high 5-ALA production up to 581,82 µM. Medium optimization using Box-Behnken design and supplementation with MSG and wood vinegar could increase the 5-ALA yield 3.2 times compared to that before optimization.

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