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Influence of Precursors and Inhibitor on the Production of Extracellular 5-Aminolevulinic Acid and Biomass by *Rhodopseudomonas palustris* KG31

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5-Aminolevulinic acid (ALA) and the biomass of photosynthetic bacteria, *Rhodopseudomonas palustris* KG31, have very high potential for development and exploitation as bioherbicide and biofertilizer respectively. In this work, the effects of two precursors and an inhibitor of aminolevulinic dehydratase (ALAD) added to the VFA culture medium on the production of ALA and biomass were investigated. The experimental runs were carried out according to a Box-Behnken design. The precursors were added to the medium at the beginning of cultivation, while the inhibitor was added after 24 h. Statistical analysis indicated that levulinic acid (LA) has a positive effect on ALA production while glycine has a negative effect on biomass production. In order to enhance both ALA and biomass products, the most suitable medium was VFA medium supplemented with 3.0 mM glycine and 10 mM LA, giving ALA and biomass of 182.91 μM and 3.1 gDCW/l within 54 h.

Key words: 5-aminolevulinic acid; Box-Behnken design; response surface methodology

ALA is an intermediate product found during the biosynthesis of tetrapyrroles such as heme, chlorophyll, and vitamin B12. Generally, two distinct metabolic pathways of ALA synthesis, the Shemin or C_4 and the Beale or C_5 pathways have been described.[1)] In prokaryotes, such as purple nonsulfur photosynthetic bacteria, usually ALA is synthesized *via* the C_4 pathway in which succinyl-CoA and glycine are used as the precursors, and aminolevulinic synthase (ALAS) catalyzes these two precursors to ALA. Next, two molecules of ALA are condensed to porphobilinogen (PBG) by the action of ALAD.[2,3)] Thus, in the strategy for enhancing ALA from the bacteria, the precursors of ALAS and the competitive inhibitor of ALAD, for example LA and glucose are added.[4)]

Among ALA-producing microorganisms, photosynthetic bacteria have received much attention. Besides the production of ALA, biomass, and pigments, the bacteria are also able to fix atmospheric nitrogen. These properties are suitable for application of the culture broth containing cells and their products in the agricultural field. Biomass of photosynthetic bacteria has been used as a source of protein with high amounts of essential amino acids *e.g.*, methionine and lysine,[5,6)] and also as a feed supplement for producing low-cholesterol chicken eggs.[7)] ALA can be applied as a plant growth-regulator and as a weed growth inhibitor,[1)] in which a concentration range of 0.06–0.6 mM demonstrated plant regulating activities[8)] whereas 4 mM ALA could be used to control weed (*Trifolium repens*) growth.[9)] Application of ALA to date palm revealed that the fruit chlorophyll content at the Khalal stage was significantly increased.[10)]

Despite intensive study of the optimal conditions for ALA production by adding precursor and inhibitor to the medium, most of these works have been carried out by traditionally optimization process (the one-at-a-time strategy) and were not focused on biomass formation. Hence in this study, the Box-Behnken design was applied, whereby the interactions among the factors were included. A photosynthetic bacterium strain, KG31, was selected for this study based on our previous finding that the strain assimilated volatile fatty acids (acetic acid, propionic acid, butyric acid, and valeric acid), and grew at wide pH (4.5–8.0) and temperature ranges (30–42 °C). Thus, these properties might be useful for the production of ALA and biomass from wastewater containing volatile fatty acids.

In this work, the influences of succinate, glycine, and LA on ALA and biomass products of the photosynthetic bacterium *R. palustris* KG31 were evaluated. The results of the design were analyzed and suitable conditions for ALA and biomass production were selected after evaluation of the data by the response surface method (RSM).

Materials and Methods

Media and culture conditions. R. palustris KG31 was isolated from soil collected from a paddy field in Nakhon Si Thammarat Province, Thailand, and identified using morphological, biochemical, and

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Abbreviations: ALA, 5-aminolevulinic acid; ALAD, aminolevulinic dehydratase; ALAS, aminolevulinic synthase; ANOVA, analysis of variance; DCW, dry cell weight; df, degree of freedom; LA, levulinic acid; MS, mean square; PBG, porphobilinogen; RSM, response surface method; SS, sum of squares

Table 1. Level and Code of Variables Chosen for the Box-Behnken Design

Variable	Coded	Coded levels (mM)		
		−1	0	1
Succinate	X_1	0	5	10
Glycine	X_2	0	5	10
LA	X_3	0	5	10

physiological properties and 16S rDNA sequencing by Pichit Chodok, a master student of Biotechnology Program, School of Agricultural Technology, Walailak University, Thailand.

The GM medium was used to maintain the bacterial cells and to prepare a starter culture. The medium contained 2.0 g/l of yeast extract, 2.7 g/l of malate, 3.7 g/l of monosodium glutamate, 0.8 g/l of $(NH_4)_2SO_4 \cdot 7H_2O$, 0.5 g/l of KH_2PO_4, 0.5 g/l of K_2HPO_4, 0.2 g/l of $MgSO_4 \cdot 7H_2O$, 0.053 g/l of $CaCl_2 \cdot 2H_2O$, 1.2×10^{-3} g/l of $MnSO_4 \cdot 7H_2O$, 1.0×10^{-3} g/l of thiamine-HCl, 1.0×10^{-3} g/l of nicotinic acid, and 1.0×10^{-5} g/l of biotin.[11] The initial pH of this medium was adjusted to 6.5 with 6 M NaOH.

The VFA medium, used as a test medium, had a composition similar to the GM medium, except that the yeast extract was 0.5 g/l, and 1.8 ml/l acetic acid, 1.0 ml/l butyric acid, and 0.2 ml/l propionic acid were added instead of malate.[12] The initial pH of this medium was adjusted to 7.0 with 6 M NaOH.

Starter cultures were prepared by cultivating *R. palustris* KG31 in 375-ml flat bottles containing 350-ml the GM medium, and incubated under the static condition with light intensity of 5,500 lux at 35 ± 1 °C for 48 h. Then the cells were harvested by centrifugation at 12,000 rpm for 15 min (RC 5 plus, Sorvell, CT, USA). To prepare the inoculums, the cell pellets were washed twice and re-adjusted to a cell concentration (OD_{660}) of 1.0 with the VFA medium. The batch fermentation was performed by inoculating 10% of a starter culture to a 375-ml flat bottle containing 350-ml of VFA medium plus succinate, glycine, and LA, as described below. Then the vessels were incubated as previously described.

Experimental design. Succinate and glycine are the precursors for ALA production, and LA is a competitive inhibitor of ALAD.[13] These chemical agents were added to the VFA medium at three levels (low, medium, and high), and coded (−1, 0 and +1) (Table 1), according to the Box-Behnken design.[14] In this study, the design consisted of triplicate at the center point and the points as shown in Table 2. A total of 15 experimental runs were carried out, the precursors were added to the VFA medium at time 0 h; then the inhibitor was added after 24 h the cultivation. The experiments were carried out in duplicate. The actual results are shown in Table 2.

The optimal concentrations of the succinate, glycine, and LA were optimized by the response surface method (Design-Expert Software, Stat-Ease, Minneapolis, USA). The three variables (succinate, glycine, and LA) selected for the statistical analysis were designed X_1, X_2, and X_3 respectively. As for the predicted responses, Y stands for ALA yield whereas Z stands for biomass yield. First, the data were entered into the Design-Expert Software. This software contains sequential techniques that were used for evaluation of the effect of the variables on the response. Secondly, selecting an appropriate type of model for the response was done by testing the sequential F-tests, starting with a linear model and adding terms (quadratic, and higher if appropriate). Finally, the F-statistic was calculated for each model, and the highest order model with significant terms was chosen. Tests for significant sequential models, model equation, and model terms were performed by employing analysis of variance (ANOVA). The quality of fit of the model equation was expressed by the coefficient of determination (R^2) and adjusted R^2. The software was used for regression analysis of the experimental data and also to plot the response surface graphs. The optimal values of the ALA and biomass yields of the experimental conditions were obtained by analyzing the response surface contour plots. Then the response surface at in-range was selected to achieve the maximal values of ALA and biomass. Finally, experimental runs were performed to check the validity of the selected experiments.

Table 2. Box-Benkhen Design with the Actual and Predicted Values of ALA Production by *R. palustris* KG31

Run no.	Design			ALA at 48 h (μM)	
	X_1	X_2	X_3	Actual	Predicted
1	10	5	0	9.67	0.76
2	10	0	5	38.32	42.18
3	0	5	10	134.47	118.40
4	5	10	10	64.00	112.81
5	10	10	5	47.30	53.99
6	0	10	5	118.75	76.97
7	5	0	0	10.33	6.34
8	10	5	10	119.54	95.42
9	5	5	5	56.20	59.58
10	0	0	5	38.24	65.16
11	5	5	5	61.61	59.58
12	0	5	0	15.28	23.74
13	5	10	0	10.28	18.15
14	5	0	10	106.20	101.00
15	5	5	5	63.49	59.58

The data in the table under "Actual" represent means of duplicate experiments.

The linear model for predicting the optimal point was expressed according to the equation:

$$Y \text{ (or } Z) = b_1X_1 + b_2X_2 + b_3X_3 \quad (1)$$

The quadratic second order polynomial equation was as follows:

$$Y \text{ (or } Z) = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (2)$$

The coefficient, b_0 is the free or off-set term called the intercept. The terms b_1, b_2, and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are cross-product coefficients, and b_{11}, b_{22}, and b_{33} are the quadratic coefficients.

The difference between means was evaluated by Duncan's Multiple Range Test and $P < 0.05$ was considered as significant.

Analytical methods. Biomass was measured as turbidity using a spectrophotometer (UV-1601, Schimadzu, Kyoto, Japan) at a wavelength of 660 nm and correlated with the dry cell weight (DCW) measured by drying the cells at 105 °C for 12 h. Extracellular ALA was determined by the colorimetric method,[15] with the following modification: The cells were centrifuged at 10,000 rpm for 20 min at 4 °C. Then 1-ml of the clear supernatants was mixed with 2-ml of 1 M sodium acetate buffer, pH 4.7, and 50-μl of acetyl acetone. After the mixtures were boiled for 15 min and cooled, 3.5-ml of modified Ehrlich's reagent was added to each sample. The ALA formed was measured at a wavelength of 553 nm. The residual volatile fatty acids were determined with HPLC (Waters, MA, USA) with reflective index detector (Waters, MA, USA). The HPLC condition was as follows: column, metaCarb H plus column (Varian, CA, USA); temperature, 68 °C; mobile phase, 8.5 mM H_2SO_4; and flow rate, 0.4 ml/min. The concentrations of residual volatile fatty acids were calculated from the peak areas. The pH value was measured using a pH meter (CyberScan pH 510, Ayer Rajah Crecent, Singapore).

Results and Discussion

ALA production

Biochemical data showed that the bacterium utilized various types of organic carbon such as acetic acid, propionic acid, butyric acid, glycine, and succinate (data not shown). Acetic acid, propionic acid, and butyric acid were supplied as sources of carbon and electron donor while glycine and succinate were added as precursors to increase the ALA yield.

The ALA yields of the 15 experimental runs are given in Table 2. Cells growing in the medium supplemented with 5 mM glycine and 10 mM LA (run no. 3) produced

the highest ALA yield, at least 8 to 13 fold higher than cells growing in the medium without added LA (runs nos. 1, 7, 12, and 13). This indicates that ALA was greatly enhanced after the addition of LA. It should be noted that at 24 h, before the addition of LA, the pH value of all the experimental runs was 6.2–7.6; after addition, LA, the pH value dropped about 0.5 units. The pH value of experimental runs nos. 3, 6, 8, and 14 containing ALA in range of 106.20–134.47 μM was 6.6–7.6, while the pH value of experimental run nos. 1, 7, 12, and 13 (9.67–15.28 μM ALA) was 8.1–8.4. Possibly not only LA but also the pH value caused a positive effect in enhancing the ALA yield. As reported by Lee *et al.*, ALAD was inhibited under slight acidic environments.[3)]

This study aimed to investigate the combined effects of the selected variables (succinate, glycine, and LA) to the responses (ALA and biomass), to find the best models to represent ALA and biomass production and the optimal concentrations of the selected variables to the responses. Hence the response surface method was used. The RSM is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response.[16)] Box *et al.* mentioned that the RSM has been used to answer questions: (1) How is a particular response affected by a given set of input variables over some specified region of interest? (2) Which of the inputs gives a product simultaneously satisfying the desired specifications. And (3), what values of the inputs yield a maximum for a specific response, and what is the response surface like close to this maximum.[17)] In order to answer, a suitable approximation for the true functional relationship between the response and the set of variables should be found. Normally, that relationship is explained by the selected model (linear, quadratic, cubic, or so on).

For ALA production, an ANOVA table for the sequential model sum of squares showed that the linear model is the highest order model with significance terms (*Prob > F is 0.0010*) whereas the quadratic and cubic models are the *Prob > F* of 0.7012 and 0.0130 respectively (data not shown). Thus the linear model represented by regression Eq. (3) for ALA production by *R. palustris* KG31 was given as follows:

$$Y\,(\mu M) = 17.83 - (2.298X_1) + (1.181X_2) + (9.466X_3) \quad (3)$$

where the variables take their coded values, and represent ALA yield (Y) as a function of succinate (X_1), glycine (X_2), and LA (X_3).

Table 3 shows the ANOVA for response surface linear model. The ANOVA of linear model of Eq. (3) clearly demonstrates that the model was significant, as can be seen from the low probability value (P value = *0.0010*). The soundness of the model can be checked by the determination coefficient, R^2, and the adjusted R^2. The value of adjusted R^2 suggested that the total variation of 69.61% for the ALA was to be attributed to the independent variables and about 31.39% of the total variation could not be explained by the model. The coefficient estimates of Eq. (3) and the corresponding P-value are shown in Table 4. The P-value of variable b_3 was 0.0001. This result indicates a big effect of LA.

Table 3. Analysis of Variance (ANOVA) for the Fitted Linear Model for Optimization of ALA Production

Source	df	SS	MS	*P*-Value
Model	3	19256.86	6418.95	0.0010
Residual	11	6039.47	549.04	
Lack of fit	9	6010.82	667.87	0.0212
Pure error	2	28.56	14.32	
Corrected total	13	25296.33		

$R^2 = 0.7613$, adjusted $R^2 = 0.6961$

Table 4. Coefficient of the Response Function to Predicted ALA Formation

Variable	Coefficient	Standard error	*F*-Value	*P*-Value
b_0	59.58	6.05	11.69	0.0010
b_1	−11.49	8.28	1.92	0.1930
b_2	5.91	8.28	0.51	0.4908
b_3	47.33	8.28	32.64	0.0001

The predicted ALA yield was obtained by solving regression Eq. (3). The analysis showed that the maximum ALA yield was 118.40 μM (run no. 3) (Table 2). Equation (3) indicated that in order to enhance the ALA yield, glycine and LA should be supplied to the medium. This also showed that another precursor, succinate, was available for incorporation with added glycine, leading to ALA formation by the action of ALAS.[18)]

It has long been known that the precursors for ALA formation *via* the C_4 pathway are succinate and glycine. This work demonstrated that glycine produced by *R. palustris* KG31 was a limiting factor as compared with succinate. In order to improve the ALA yield, glycine should be added to the culture medium. However, in this experiment, the effect of glycine was not as big as LA. As reported by Nishikawa *et al.*, *R. sphaeroides* CR-286 produced 1.5 mM ALA in the presence of 50 mM glucose, 60 mM glycine, 15 mM LA, and 1.0% (w/v) yeast extract.[19)] Fu *et al.* reported that high initial concentrations of glycine and succinate did not improve the ALA yield produced from recombinant *E. coli* containing the *R. sphaeroides hemA* gene.[20)] In batch fermentation, the addition of glucose and glycine was effective to improve ALA production.[18)] But Chung *et al.* found that the optimal condition for ALA production of recombinant *E. coli* harboring *hemA* from *Bradyrhizobium japanicum* was 15 mM glycine and 30 mM succinic acid.[4)] Qin *et al.* found that the highest ALA yield of recombinant *E. coli* harboring *hemA* from *Agrobacterium radiobacter* was achieved in a medium containing glycine, succinic acid, and glucose, and by adding LA at the end of the log phase.[2)]

Biomass production

R. palustris biomass was reported to be used as sources of biofertilizer and Single Cell Protein.[21)] Moreover, the bacteria are able to fix molecular nitrogen.[22)] Accordingly, the influence of added succinate, glycine, and LA on the biomass yield was evaluated.

Table 5 shows the biomass yield of 15 experimental runs. The data obtained were analyzed as mentioned above. The highest biomass was obtained in run no. 14 (2.6 gDCW/l), while runs nos. 4, 5, and 13 gave low biomass content as compared to the others. The F tests

Table 5. Box-Benkehn Design with the Actual and Predicted Values of Biomass for *R. palustris* KG31

Run no.	Design			Biomass at 48 h (gDCW/l)	
	X_1	X_2	X_3	Actual	Predicted
1	10	5	0	1.2	1.1
2	10	0	5	2.6	2.8
3	0	5	10	1.3	1.5
4	5	10	10	0.4	0.5
5	10	10	5	0.8	0.8
6	0	10	5	1.7	1.5
7	5	0	0	1.6	1.5
8	10	5	10	1.8	1.8
9	5	5	5	2.2	2.3
10	0	0	5	2.3	2.3
11	5	5	5	2.3	2.3
12	0	5	0	1.4	1.4
13	5	10	0	0.5	0.7
14	5	0	10	2.6	2.5
15	5	5	5	2.2	2.3

The data in the table under "Actual" represent means of duplicate experiments.

Table 6. Analysis of Variance (ANOVA) for the Fitted Quadratic Model for Optimization of Biomass

Source	df	SS	MS	*P*-Value
Model	9	7.12	0.79	0.0041
Residual	5	0.26	0.05	
Lack of fit	3	0.25	0.08	0.0639
Pure error	2	0.01	0.01	
Corrected total	14	7.38		

$R^2 = 0.9641$, adjusted $R^2 = 0.8996$

are performed as mentioned above. Thus the following regression equation for biomass yield (Z) was obtained. The quadratic Eq. (4) is presented.

$$\begin{aligned} Z\,(\text{gDCW/l}) = {} & (1.278) + (0.08X_1) + (0.085X_2) \\ & + (0.341X_3) - (0.012X_1X_2) \\ & + (0.006X_1X_3) - (0.011X_2X_3) \\ & - (0.006{X_1}^2) - (0.011{X_2}^2) \\ & - (0.028{X_3}^2) \end{aligned} \quad (4)$$

where Z (biomass, gDCW/l) is the predicted response variable; $X_1 - X_3$ = the actual values of the independent variables, *viz.*, succinate, glycine, and LA respectively.

A statistical analysis of the process parameters along with the biomass yields as response values is shown in Table 5. The optimal biomass of the predicted value was 2.8 gDCW/l (run no. 2). Run no. 14 achieved a predicted value of the biomass of 2.5 gDCW/l. The statistical significance of Eq. (4) was checked by the F-test, and the ANOVA for the response surface quadratic model is summarized in Table 6. The ANOVA of the quadratic model of the equation demonstrated that the model was significant, as can be seen from the low probability values (P value = 0.0041). The value of adjusted R^2 suggested that the total variation of 89.96% for the biomass is to be attributed to the independent variables and about 10.04% of the total variation cannot be explained by the model.

Table 7. Coefficient of the Response Function to Predicted Biomass Formation

Variable	Coefficient	Standard error	*F*-Value	*P*-Value
b_0	2.26	0.13	14.94	0.0041
b_1	−0.03	0.08	0.14	0.7274
b_2	−0.71	0.08	76.71	0.0003
b_3	0.18	0.08	4.76	0.0809
b_{12}	−0.30	0.12	6.80	0.0478
b_{13}	0.16	0.12	1.82	0.2357
b_{23}	−0.28	0.12	5.92	0.0591
b_{11}	−0.14	0.12	1.42	0.2874
b_{22}	−0.28	0.12	5.37	0.0683
b_{33}	−0.70	0.12	34.42	0.0020

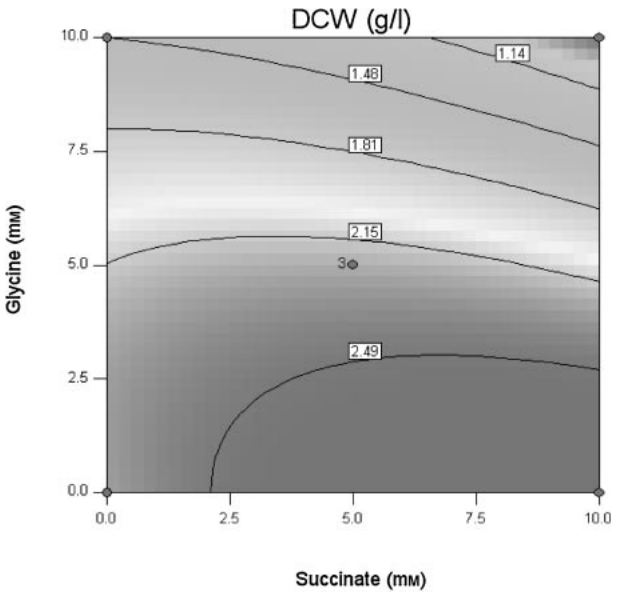

Fig. 1. Contour Plot of the Interactions of Succinate and Glycine in Biomass Yield.

The coefficient estimates of Eq. (4) and the corresponding P-value are shown in Table 7. The data imply that glycine (b_2) and an interaction of glycine and succinate (b_{12}) were significant. The P-value of variable b_2 was 0.0003. The P-value of variable b_{12} was 0.0478. These results clearly indicate a big effect of glycine. A contour curve was drawn by fixing the LA concentration at 5 mM, so that the interaction between succinate and glycine on the biomass yield during cultivation of the bacterium for 48 h was obtained (Fig. 1). Figure 1 implies that if succinate was not added or was added to 10 mM, supplementation of glycine >5 mM would result in a decrease in biomass contents. In order to receive about 2.5 gDCW/l, about 3–6 mM succinate should be added.

Hence, growth and ALA production did not depend on the same factor. LA had the highest effect on ALA formation. In the case of glycine, it also had influence on the ALA yield. Moreover, the addition of succinate together with glycine resulted in a decrease in biomass production. Besides, LA lowered the pH value of the medium and it can also be used as a carbon source for the bacterium (data not shown).

An attempt to add these precursors at 12, 24, and 36 h of cultivation time (supplementation of LA at 24 h) showed that adding times did not increase the ALA

Table 8. Concentrations of Succinate, Glycine, and LA Used in the Verification Test

Vessel	Concentration (mM)		
	Succinate	Glycine	LA
Run A	0	3	10
Run 3	0	5	10
Run 14	5	0	10
Control	0	0	0

concentration significantly. On the other hand, the addition of LA at 12, 24, and 36 h (supplementation of the precursors at 0 h) had an effect on ALA formation (data not shown). Time 24 h, at which the cells growth entered an early log phase, was the best for adding LA.

Verification the models

As mentioned above, after cultivation of the bacterium for 48 h, the data of runs nos. 3 and 14 gave the highest ALA and biomass yields respectively (Table 2 and Table 5). The application of RSM and statistical analysis clearly confirmed that different factors influenced ALA and biomass production. In order to enhance both products, response surface method analysis was performed. The results showed that the most suitable medium for ALA and biomass production was VFA medium supplementation with 3.0 mM glycine and 10 mM LA (Table 8).

Figure 2a shows the levels of ALA along with the incubation times. The ALA profiles increased with increasing incubation time, especially in run no. 3 and run A. At 48 h, the yield of ALA was 8.06, 164.24, 115.00, and 180.21 μM in the control, runs nos. 3 and 14 and run A respectively. The predicted ALA yield in runs nos. 3 and 14 and run A was 118.40, 101.00, and 116.30 μM respectively. This experimental finding is in agreement with the model's prediction. Duncan's Multiple Range Test was used to evaluate these results, and it was found that the amount of ALA produced in the control experiment was significantly different ($p < 0.05$) from the other runs. At 54 h, the ALA yields of run no. 3 (194.70 μM) and run A (182.96 μM) were significantly different ($p < 0.05$) from those of run no. 14 (101.79 μM). Thus, the results of run no. 3 and run A indicated that succinate was not a limited precursor while glycine and LA were needed for ALA production by the cells. Also this indicated a major effect on LA toward ALA formation. It should be noted that in the course of the experiments, the ALA contents of run no. 3 and run A were not significant different. This may be because the optimization experiment was focused on receiving both ALA and biomass yields. Figure 2a also shows that the levels of ALA were drastically decreased after 60 h. Moat and Foster stated that succinate is formed by reducing malate to fumarate and then, to the succinate in the Krebs cycle under anaerobic conditions.[23)]

ALA production by this bacterium was relatively low as compared with those previously reported. However, the utilization of volatile fatty acids which often create an odor problem and the utilization of light energy are of the best points for economic wastewater treatment with ALA production. It should be emphasized that the ALA content obtained in this study was enough to be used to promote plant growth.[8)]

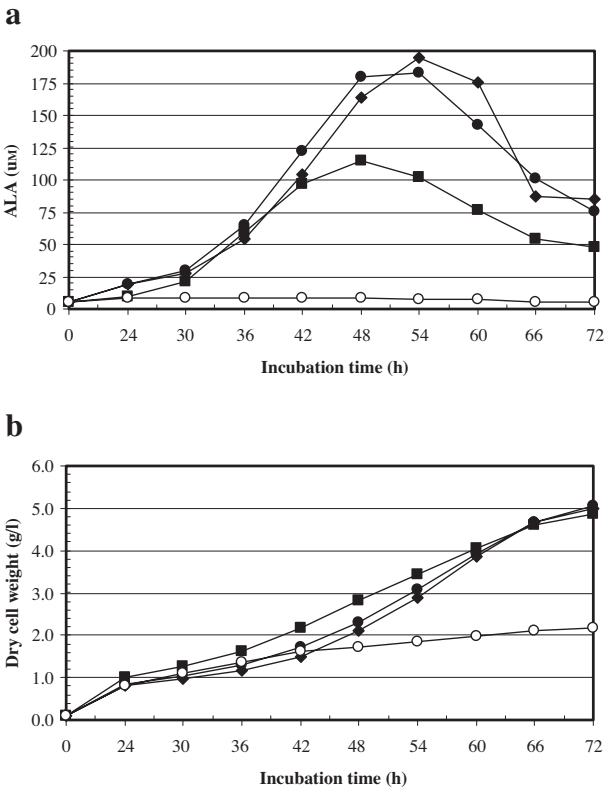

Fig. 2. Time Profiles of ALA Yields (a) and Biomass Yields (b) of Run A (●), Run no. 3 (◆) Run no. 14 (■) and the Control (○).

The growth of the bacterium under the optimal conditions is shown in Fig. 2b. In run no. 14, growth of the bacterial cell rapidly developed as compared with run no. 3 and run A. At 48 h, the biomass yield in the control, runs nos. 3 and 14, and run A was 1.7, 2.1, and 2.8 and 2.3 gDCW/l respectively (Fig. 2b). However, the predicted yield of biomass was 1.5, 2.5, and 1.7 gDCW/l, for runs nos. 3 and 4 and run A respectively. At 54 h, the biomass of runs nos. 3 and 14, and run A was 2.9, 3.4, and 3.1 gDCW/l respectively. At 60 h, the biomass was in a range of 3.8–4.1 gDCW/l. The biomass yields obtaining at 48 and 54 h under the experiments of all runs and control were not significantly different ($p < 0.05$).

The volatile fatty acid profiles of run A rapidly decreased as compared with runs nos. 3 and 14. At 48 h, acetic acid was 0.88, 1.23, and 0.81 g/l in runs nos. 3 and 14, and run A respectively; butyric acid was 0.71, 0.72, and 0.46 g/l in runs nos. 3 and 14, and run A respectively (Fig. 3). After 18 h, propionic acid was completely exhausted (data not shown). Normally, the profiles of acetic acid were decreased along the cultivation time but at the end of cultivation (72 h) the levels of acetic acid of run no. 14 and run A were slightly increased (Fig. 3a). This is because acetic acid was formed during the assimilation of propionic and butyric acids by the bacterium (data not shown). Figure 3 shows that the volatile fatty acids contained in run no. 14 were consumed lower than run no. 3 and run A. This may be because the bacterium preferred to utilize succinate as a carbon source instead of volatile fatty acids. This result agrees with the studies of Qin *et al.*, who reported that succinate serves as a carbon source for cell growth of the bacterium.[2)]

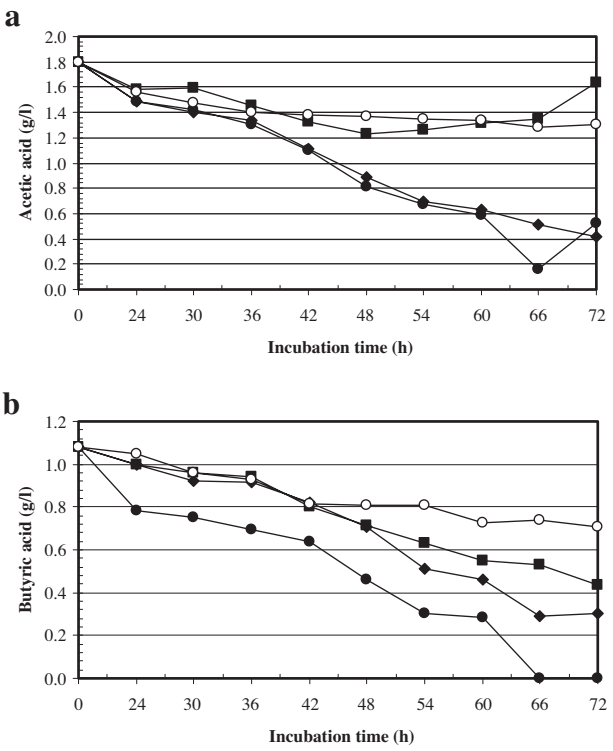

Fig. 3. Time Profiles of Residual Acetic Acid (a) and Residual Butyric Acid (b) of Run A (●), Run no. 3 (◆), Run no. 14 (■), and the Control (○).

It should be noted that while the bacterium grew under the control conditions, both ALA (at 48 h = 8.06 μM) and biomass formations (at 48 h = 1.7 gDCW/l) were lowest. The profiles of volatile fatty acid consumption were also low. Apart from the reasons mentioned above, these phenomena may be explained by the fact that the pH value of the culture broth was alkaline.

The results obtained from this design experiment led us to predict the medium composition to be used for ALA and biomass productions. The advantage of this statistical technique, RSM, led us to build an appropriate model, evaluating the effects of relevant variables and searching for optimum conditions for the production of products and reducing the number of experiments, whereas traditionally the optimization process in which a single factor is varied while all other factors are kept fixed at a specific set of conditions is laborious, time consuming, and incapable of reaching the true optimum due to ignoring the interaction among variables.

Conclusion

This experiment demonstrated the effects of succinate, glycine, and LA on ALA and biomass yields to be produced by *R. palustris* KG31 in VFA medium under illumination conditions. Application of the RSM and statistical analysis led us to find the optimal ALA and biomass yields in response to the affecting factors, glycine and LA. Thus, the ALA of 183.0 μM and the biomass of 3.1 gDCW/l were achieved within 54 h, cultivation time in VFA medium supplemented with 3.0 mM glycine and 10 mM LA.

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