Optimization of Biomass and 5-Aminolevulinic Acid Production by *Rhodobacter sphaeroides* ATCC17023 via Response Surface Methodology

Shuli Liu¹ · Guangming Zhang²,³ · Jianzheng Li³ · Xiangkun Li³ · Jie Zhang³

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**Abstract** Microbial 5-aminolevulinic acid (ALA) produced from wastewater is considered as potential renewable energy. However, many hurdles are needed to be overcome such as the regulation of key influencing factors on ALA yield. Biomass and ALA production by *Rhodobacter sphaeroides* was optimized using response surface methodology. The culturing medium was artificial volatile fatty acids wastewater. Three additives were optimized, namely succinate and glycine that are precursors of ALA biosynthesis, and D-glucose that is an inhibitor of ALA dehydratase. The optimal conditions were achieved by analyzing the response surface plots. Statistical analysis showed that succinate at 8.56 mmol/L, glycine at 5.06 mmol/L, and D-glucose at 7.82 mmol/L were the best conditions. Under these optimal conditions, the ALA yield was significantly increased.

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conditions, the highest biomass production and ALA yield of 3.55 g/L and 5.49 mg/g-biomass were achieved. Subsequent verification experiments at optimal values had the maximum biomass production of 3.41 ± 0.002 g/L and ALA yield of 5.78 ± 0.08 mg/g-biomass.

**Keywords** Rhodobacter sphaeroides · Biomass · 5-Aminolevulinic acid yield · Influencing factor · Response surface methodology · Wastewater

**Introduction**

5-Aminolevulinic acid (ALA), a significant intermediate, involves in the tetrapyrrole biosynthesis of compounds including porphyrin, heme, chlorophyll, and vitamin B12 in various organisms [1–3]. ALA has recently drawn increasing attentions as a photodynamic chemical with wide applications in medical and agricultural fields [3]. It also has been used as a plant growth stimulator or inhibitor [3]. In terms of ALA synthesis, microbial production presents prominent advantages, including simple process, low cost, and abundant raw materials [4, 5]. Among the potential microorganisms, purple non-sulfur photosynthetic bacteria (PNSB) were identified as effective and safe ALA producers [4, 6–8]. In addition, it was proved that PNSB could efficiently treat various wastewaters including dairy wastewater, soybean wastewater, olive mill wastewater, and domestic wastewater since the 1960s [9–12]. So producing biomass and ALA together with removing pollutants via PNSB wastewater treatment has attractive advantages, and it is a green biotechnology. However, the ALA yield by PNSB was still too low to be cost-effective [9, 12], and it was lower in PNSB wastewater treatment [11, 13]. Thus most attentions have focused on increasing ALA yield by various biotechnologies [14–16].

In PNSB, ALA is synthesized via C4 pathway by ALA synthetase (ALAS) [6, 13], and it is degraded by ALA dehydratase (ALAD). Succinate and glycine are the precursors to synthesize ALA while levulinic acid (LA) and D-glucose are the inhibitors of ALAD. So the addition of succinate and glycine could increase ALA production directly. D-glucose could enhance the ALA production indirectly by inhibiting ALAD activity. Thus, in order to increase the ALA yield, addition of ALA precursors (succinate, glycine) or ALAD inhibitor (D-glucose) has been widely studied [10, 17, 18]. The results reported that all these three factors had significant enhancement on biomass or ALA production of PNSB [18–21]. However, most studies examined the effects of only one factor on the biomass production or ALA yield [18, 19], few studied the combining effects of two or more factors on the biomass production or ALA yield, and little information was available on PNSB growth in wastewater treatment. A comprehensive study would thus be beneficial.

As an effective experimental design method, Central Composite Design (CCD) was applied for many research fields [22–24]. Previous study showed that volatile fatty acids (VFAs) wastewater was beneficial to PNSB growth and ALA production [25]. Hence, CCD was selected to determine the optimal conditions for both biomass and ALA yield in PNSB treating VFAs wastewater in the present study.

This study aimed to investigate the effects of succinate, glycine, and D-glucose on the biomass production and ALA yield of PNSB. The experimental results of the design by the response surface methodology (RSM) were analyzed and the optimal conditions for the simultaneous highest biomass production and ALA yield were evaluated.
Materials and Methods

Microorganism, Wastewater, and Experimental Setup

A *Rhodobacter sphaeroides* strain (the preservation number is ATCC17023) was obtained from China General Microbiological Culture Collection Center. It belongs to PNSB species and was proved effective in our previous works [26, 27]. The strain was cultured in a thermostat shaker (static, 30 °C) under light and micro aerobic conditions with PYG medium which consisted of 10 g/L polypepton, 5 g/L yeast extract, and 1 g/L glucose. The pH of PYG medium was adjusted to 6.8–7.0. The logarithmic growth phase of *R. sphaeroides* began at 36 h and the density of *R. sphaeroides* was $6.8 \times 10^8$ CFU/mL.

VFAs wastewater is a typical non-hazardous wastewater and it can provide many micro-molecule VFAs for *R. sphaeroides* growth [25]. The VFAs wastewater in this study was artificial VFAs wastewater, and its components contained yeast extract (0.5 g/L), acetic acid (3.0 mL/L), propionic acid (1.0 mL/L), butyric (0.1 mL/L), pentanoic acid (0.1 mL/L), potassium dihydrogen phosphate (0.5 g/L), ammonium chloride (0.5 g/L), and magnesium sulfate (0.2 g/L). Its characteristics were as follows: chemical oxygen demand (COD), total nitrogen (TN), and total phosphorous (TP) were 5025, 282, and 55 mg/L, respectively. All these indexes were similar to the real VFAs wastewater [25]. The initial pH was adjusted to 6.5.

Bioreactors (erlenmeyer flasks) were sterilized at 121 °C for 30 min before use. Wastewater (460 mL) and *R. sphaeroides* (40 mL) were added to each bioreactor. Light and micro aerobic conditions were provided for *R. sphaeroides* growth. The light intensity was 5000–6000 lx, and the dissolve oxygen (DO) level was 0.5–1.0 mg/L. The reaction time was 96 h in one batch test, which was determined in previous study [26].

Experimental Design and Procedure

Succinate, glycine, and D-glucose concentrations were all optimized. CCD was used including five levels and coded ($-1.682, -1, 0, 1,$ and $1.682$). For statistical calculation, the variables were coded according to Eq. (1). A total of 20 runs were carried out to optimize the parameters.

$$X_i = \frac{x_i - x_0}{\Delta x}, \quad i = 1–3$$ (1)

The optimal concentrations of three factors were achieved through Design-Expert® Software (version 8.0.5.0) in the experimental design, data analysis, quadratic model buildings, and plots. Analysis of variance (ANOVA) was performed to estimate the validity of terms. In the model optimization, the response surface at “in maximize” was selected to obtain the maximal $Y$ (biomass production) and $Z$ (ALA yield) at the same time. The corresponding optimal values ($X_1$, $X_2$, and $X_3$) were determined. According the optimal conditions, experimental runs were carried out to check the validity of the experimental models.

The regression equation for predicting the optimal point was generated from the response surface which was shown as in Eq. (2).

$$Y (\text{or } Z) = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i,j}^{3} \beta_{ij} X_i X_j$$ (2)
where $Y$ is the biomass production and $Z$ is the ALA yield; $\beta_0$ is the intercept of the off-set term; $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are the linear, quadratic, and interactive coefficients, respectively; $X_i$ and $X_j$ are the levels of the independent variables.

**Analytical Methods**

Samples collected from bioreactors were centrifuged at 9000 rpm for 10 min. The supernatant was used to test the water quality indexes (COD, TN, and TP). The collected cells were used for measuring the biomass and intracellular ALA. pH and DO were detected with pH tester and dissolved oxygen meter, respectively. COD and biomass were tested according to APHA standard methods [28]. The ALA concentration was examined according to the description [18]. The ALA yield was calculated as the ALA content of per gram biomass (mg/g-biomass).

**Results**

Water quality examination showed that PNSB could successfully treat the VFAs wastewater, and the COD, TN, and TP reduction was more than 85, 90, and 87 % in all experimental runs (data was shown in Table S1).

**The Effect of Succinate, Glycine, and D-Glucose on Biomass and ALA Yield**

Succinate and glycine were important nutrients for microbial growth, as critical precursors; they also played significant roles in ALA biosynthesis pathway [13, 19, 20]. D-glucose affected ALA production as an inhibitor of ALAS or ALAD, and it was also one favorable nutrient in microbial metabolism [18, 21]. Hence, the effects of these three factors on the biomass production and ALA yield in *R. sphaeroides* wastewater treatment were investigated. Firstly, the respective effects of three factors on the biomass production or ALA yield by single factor experiment. This aimed to determine a range of examination for the next RSM design. The results are summarized in Fig. 1.

From Fig. 1a, it was found that the biomass production reached the maximum (3.54 g/L) when the succinate concentration was 10 mmol/L. The ALA yield had a peak of 3.63 mg/g-biomass at succinate of 7.5 mmol/L, and it experienced a sharp drop from 7.5 to 12.5 mmol/L. A range of succinate concentrations of 6.0–11.0 mmol/L was selected in the RSM optimization design.

The results from Fig. 1b showed that both the biomass production and ALA yield had the maximum values of 3.41 g/L and 3.98 mg/g-biomass simultaneously at glycine of 5 mmol/L. So glycine concentration of 5 mmol/L was selected as the center point in the RSM optimization design.

As seen from Fig. 1c, the biomass production reached the maximum value of 3.56 g/L with 10 mmol/L D-glucose addition, and then it maintained an equilibrium. The ALA yield had a peak of 3.39 mg/g-biomass with 7.5 mmol/L D-glucose addition, but it declined from 7.5 to 12.5 mmol/L. Above all, a range of D-glucose concentrations of 5.0–10.0 mmol/L was selected in the RSM optimization design.
Fig. 1 Effects of succinate, glycine, and D-glucose on biomass and ALA yield: a succinate; b glycine; c D-glucose. Bar represents the standard error of means for replicates.
Procedure Optimization

After determining the ranges of succinate, glycine, and D-glucose concentrations, a total of 20 runs (Table 1) were carried out following the design to optimize the parameters. The results were shown in Table 2.

Biomass Production

The Model Fitting and Statistical Analysis

The effects of succinate, glycine, and D-glucose on biomass production were optimized through RSM approach (Table 2). Six replicates at the central points were estimated by a pure error sum of squares. A second-order polynomial Eq. (3) was given as follows:

\[
Y = 3.53553 - 0.057693X_1 - 0.025392X_2 \\
+ 0.23601X_3 - 0.077125X_1X_2 + 0.016625X_1X_3 \\
- 0.19387X_2X_3 - 0.54526X_1^2 - 0.28310X_2^2 - 0.45457X_3^2
\]  

(3)

Statistical testing of regression equation was checked by \( F \) test, and the ANOVA for the response surface quadratic model of expansion rate for biomass production was shown in Table 3. The \( F \) value of the model was 119.65 and the \( p \) value was less than 0.0001, which indicated that the model was highly significant. There was only 0.01% chance that \( F \) value could occur due to noise. The lack of fit \( F \) value was 2.45, and \( p \) value of 0.1743 implied the lack of fit was not significant relative to the pure error. The Adj \( R \)-squared was 0.9825, which indicated that there was only a 2% chance that it could not be explained by the model. Pred \( R \)-squared was in reasonable agreement with the Adj \( R \)-squared. The Adeq Precision measured the signal to noise ratio, and a ratio greater than 4 was desirable (Table 4).

ALA Yield

The Model Fitting and Statistical Analysis

The effects of succinate, glycine, and D-glucose on ALA yield were optimized through RSM approach. The designed experiments were the same to the biomass and they were shown in Table 2. A second-order polynomial Eq. (4) was given as follows:

\[
Y = 5.43553 + 0.36654X_1 + 0.43115X_2 + 0.11770X_3 + 0.081250X_1X_2 - 0.11725X_1X_3 \\
- 0.024750X_2X_3 - 1.00304X_1^2 - 0.88318X_2^2 - 0.38397X_3^2
\]  

(4)

Table 1  Level and code of variables for the central composite design

<table>
<thead>
<tr>
<th>Variable</th>
<th>Code</th>
<th>Code levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate (mmol/L)</td>
<td>( X_1 )</td>
<td>6.0 7.0 8.5 10.0 11.0</td>
</tr>
<tr>
<td>Glycine (mmol/L)</td>
<td>( X_2 )</td>
<td>4.0 4.4 5 5.6 6.0</td>
</tr>
<tr>
<td>D-glucose (mmol/L)</td>
<td>( X_3 )</td>
<td>5.0 6.0 7.5 9.0 10.0</td>
</tr>
</tbody>
</table>
The ANOVA for the response surface quadratic model of expansion rate for ALA yield was shown in Table 5. Firstly, the results showed the $F$ value of the model was 84.45 and the $p$ value was less than 0.0001, which indicated that the model was highly significant. There was only a 0.01 % chance that $F$ value could occur due to noise. The lack of fit $F$ value was 2.48, and $p$ value of 0.1708 implied the lack of fit was not significant relative to the pure error. The Adj $R$-squared was 0.9753, implying that there was only a 3 % chance that it could not be explained by the model. The Adeq Precision of 25.074 was greater than the desirable value.

Table 2  Experimental results of the central composite design for biomass and ALA yield

<table>
<thead>
<tr>
<th>Run</th>
<th>Design</th>
<th>Biomass (g/L)</th>
<th>ALA yield (mg/g-biomass)</th>
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<tr>
<td></td>
<td>$X_1$</td>
<td>$X_2$</td>
<td>$X_3$</td>
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<td>2</td>
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</tr>
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<td>12</td>
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<td>14</td>
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<td>16</td>
<td>0.00</td>
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<td>17</td>
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<td>18</td>
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<td>1.68</td>
</tr>
<tr>
<td>19</td>
<td>1.00</td>
<td>1.00</td>
<td>-1.00</td>
</tr>
<tr>
<td>20</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The ANOVA for the response surface quadratic model of expansion rate for ALA yield was shown in Table 5. Firstly, the results showed the $F$ value of the model was 84.45 and the $p$ value was less than 0.0001, which indicated that the model was highly significant. There was only a 0.01 % chance that $F$ value could occur due to noise. The lack of fit $F$ value was 2.48, and $p$ value of 0.1708 implied the lack of fit was not significant relative to the pure error. The Adj $R$-squared was 0.9753, implying that there was only a 3 % chance that it could not be explained by the model. The Adeq Precision of 25.074 was greater than the desirable value.

Table 3  Analysis of variance (ANOVA) for regression equation for biomass production

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>$F$ value</th>
<th>$p$ value (Prob &gt; $F$)</th>
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<tbody>
<tr>
<td>Model</td>
<td>8.33</td>
<td>9</td>
<td>0.93</td>
<td>119.65</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>0.077</td>
<td>10</td>
<td>7.735 E−003</td>
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<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>0.055</td>
<td>5</td>
<td>0.011</td>
<td>2.45</td>
<td>0.1743</td>
</tr>
<tr>
<td>Pure error</td>
<td>0.022</td>
<td>5</td>
<td>4.488 E−003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor total</td>
<td>8.41</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R$-squared</td>
<td>0.9908</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Adj $R$-squared</td>
<td>0.9825</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pred $R$-squared</td>
<td>0.9468</td>
<td></td>
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<tr>
<td>Adeq Precision</td>
<td>27.179</td>
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</table>
The results of regression coefficients of polynomial function for the response surface of expansion rate were shown in Table 6.

### Analysis of Response Surface

To analyze the effects of the independent variables on the biomass production, three-dimensional (3D) response surface plots were given in Fig. 2. The plots indicated effects of two factors on the response value (biomass production) while the third one was kept at “0” level. The biomass production showed an increase-then-decrease pattern with the increasing succinate or glycine concentration. Thus, a chart depicting a “roof form” was constructed, where the apex of the roof indicates the probable optimum conditions. The effects of succinate and D-glucose on the biomass production (Fig. 2b) had the similar results with the effects of succinate and glycine on the biomass production (Fig. 2a). However, it was found that the effects of glycine and D-glucose on the biomass production (Fig. 2c) were different from the results in Fig. 2a, b. Previous study showed that D-glucose could increase the microbial biomass accumulation by enhancing glycolytic pathway in aerobic respiration process [29].

3D response surface plots of ALA yields were given in Fig. 3. The effects of any two factors among three factors on ALA yield displayed similar trends. A chart “roof form” was

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### Table 4 Coefficient of response function to predicted biomass production

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient estimate</th>
<th>df</th>
<th>Standard error</th>
<th>95 % CI Low</th>
<th>95 % CI High</th>
<th>F value</th>
<th>p value (Prob &gt; F)</th>
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<tbody>
<tr>
<td>Intercept</td>
<td>3.54</td>
<td>1</td>
<td>0.036</td>
<td>3.46</td>
<td>3.62</td>
<td></td>
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<tr>
<td>$X_1$</td>
<td>$-0.058$</td>
<td>1</td>
<td>0.024</td>
<td>$-0.11$</td>
<td>$-4.667E-003$</td>
<td>5.88</td>
<td>0.0358</td>
</tr>
<tr>
<td>$X_2$</td>
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<td>1</td>
<td>0.024</td>
<td>$-0.078$</td>
<td>0.028</td>
<td>1.14</td>
<td>0.3111</td>
</tr>
<tr>
<td>$X_3$</td>
<td>0.24</td>
<td>1</td>
<td>0.024</td>
<td>0.18</td>
<td>0.29</td>
<td>98.35</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$X_{12}$</td>
<td>$-0.077$</td>
<td>1</td>
<td>0.031</td>
<td>$-0.15$</td>
<td>$-7.843E-003$</td>
<td>6.15</td>
<td>0.0325</td>
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<tr>
<td>$X_{13}$</td>
<td>0.017</td>
<td>1</td>
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<td>$-0.053$</td>
<td>0.086</td>
<td>0.29</td>
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<td>$X_{23}$</td>
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<td>$-0.26$</td>
<td>$-0.12$</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
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<td>$-0.49$</td>
<td>553.94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$X_2^2$</td>
<td>$-0.28$</td>
<td>1</td>
<td>0.023</td>
<td>$-0.33$</td>
<td>$-0.23$</td>
<td>149.32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$X_3^2$</td>
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<td>1</td>
<td>0.023</td>
<td>$-0.51$</td>
<td>$-0.40$</td>
<td>385.00</td>
<td>&lt;0.0001</td>
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### Table 5 Analysis of variance (ANOVA) for regression equation for ALA yield

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F value</th>
<th>p value (Prob &gt; F)</th>
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<td>Model</td>
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<td>3.20</td>
<td>84.45</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>0.38</td>
<td>10</td>
<td>0.038</td>
<td></td>
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<tr>
<td>Lack of fit</td>
<td>0.27</td>
<td>5</td>
<td>0.054</td>
<td>2.48</td>
<td>0.1708</td>
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<td>Pure error</td>
<td>0.11</td>
<td>5</td>
<td>0.022</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor total</td>
<td>29.21</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>R-squared</td>
<td>0.9870</td>
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<tr>
<td>Adj R-squared</td>
<td>0.9753</td>
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<tr>
<td>Pred R-squared</td>
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<td>Adeq Precision</td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>
constructed in Fig. 3a–c, respectively. The ALA yield increased with increasing succinate or glycine concentration, and then slightly decreased with increasing succinate or glycine concentration (Fig. 3a). The same trends were also obtained in other plots (Fig. 3b, c).

**Discussion**

**Improvement on Biomass Production and ALA Yield**

Regression coefficients of polynomial function for the response surface of expansion rate were shown in Table 4. It was concluded that the terms including $X_1$, $X_3$, $X_{12}$, $X_{23}$, $X_1^2$, and $X_3^2$ were significant to the response. But the following terms including $X_2$ and $X_{13}$ were not significant to the response. Succinate and D-glucose had greater effects than glycine on the biomass production in *R. sphaeroides* wastewater treatment. However, the interaction of succinate and D-glucose was not significant to the biomass production. Succinate, D-glucose, and their metabolites (succinyl-CoA, pyruvic acid or acetyl-CoA, etc.) were popular substrates or carbon source for microorganism in the aerobic metabolic pathway. They could enhance the energy metabolism and material synthetic metabolism as intermediate products by improving tricarboxylic acid cycle under aerobic condition [18, 30, 31]. Hence, succinate and D-glucose increased the biomass production. Glycine had no obvious effect on the biomass production of *R. sphaeroides* in this study. Other researcher reported that high glycine (>5 mmol/L) had a negative effect on the biomass production of *Rhodopseudomonas palustris* KG 31, but the negative effect of glycine was remitted when LA was added [25]. This showed that glycine had different effect on the biomass production under different conditions. The interaction between glycine and succinate (or D-glucose) was significant in this study.

It was found from Table 6 that the terms including $X_1$, $X_2$, $X_3$, $X_1^2$, $X_2^2$, and $X_3^2$ were significant to the response ($p$ values were less 0.05). But the following terms including $X_{12}$, $X_{23}$, and $X_{13}$ were not significant to the response. Being different from the model of biomass production, succinate, glycine, and D-glucose all had obvious effects on the ALA yield, respectively. However the interactions of three factors were slight. It was well known that succinate and glycine were irreplaceable precursors and corporated to stimulate the catalytic

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient estimate</th>
<th>df</th>
<th>Standard error</th>
<th>95 % CI Low</th>
<th>95 % CI High</th>
<th>F value</th>
<th>p value (Prob &gt; F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>5.44</td>
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<td>0.079</td>
<td>5.26</td>
<td>5.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X_1$</td>
<td>0.37</td>
<td>1</td>
<td>0.053</td>
<td>0.25</td>
<td>0.48</td>
<td>48.36</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$X_2$</td>
<td>0.43</td>
<td>1</td>
<td>0.053</td>
<td>0.31</td>
<td>0.55</td>
<td>66.91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$X_3$</td>
<td>0.12</td>
<td>1</td>
<td>0.053</td>
<td>2.595 E–004</td>
<td>0.24</td>
<td>4.99</td>
<td>0.0496</td>
</tr>
<tr>
<td>$X_{12}$</td>
<td>−0.081</td>
<td>1</td>
<td>0.069</td>
<td>−0.23</td>
<td>0.072</td>
<td>1.39</td>
<td>0.2654</td>
</tr>
<tr>
<td>$X_{13}$</td>
<td>−0.12</td>
<td>1</td>
<td>0.069</td>
<td>−0.27</td>
<td>0.036</td>
<td>2.90</td>
<td>0.1195</td>
</tr>
<tr>
<td>$X_{23}$</td>
<td>−0.025</td>
<td>1</td>
<td>0.069</td>
<td>−0.18</td>
<td>0.13</td>
<td>0.13</td>
<td>0.7268</td>
</tr>
<tr>
<td>$X_1^2$</td>
<td>−1.00</td>
<td>1</td>
<td>0.051</td>
<td>−1.12</td>
<td>−0.89</td>
<td>382.16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$X_2^2$</td>
<td>−0.88</td>
<td>1</td>
<td>0.051</td>
<td>−1.00</td>
<td>−0.77</td>
<td>296.29</td>
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<tr>
<td>$X_3^2$</td>
<td>−0.38</td>
<td>1</td>
<td>0.051</td>
<td>−0.50</td>
<td>−0.27</td>
<td>56.00</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Fig. 2 Three-dimensional response surface graphs of biomass: a succinate concentration versus glycine concentration; b succinate concentration versus D-glucose concentration; c glycine concentration versus D-glucose concentration
Fig. 3 Three-dimensional response surface graphs of ALA yield: a succinate concentration versus glycine concentration; b succinate concentration versus D-glucose concentration; c glycine concentration versus D-glucose concentration
reaction of ALAS in the ALA biosynthesis pathway [32]. And their concentrations played important roles in changing ALA yields. Fu et al. [32] found succinate and glycine were effective to improve ALA production of recombinant Escherichia coli containing R. sphaeroides hemA gene in batch fermentation. When the concentrations of succinic acid and glycine were 7.0 and 4.0 g/L, respectively, the highest ALA yield reached 4.1 g/L. Chung et al. [20] found that the highest ALA production was achieved under the optimal conditions (glycine of 15 mmol/L and succinate of 30 mmol/L). Saikeur et al. [25] reported that glycine was a limiting factor of ALA formation and had more effects on ALA yield than succinate. Liu et al. [18] demonstrated D-glucose changed ALA yield by affecting ALAS or ALAD activity.

In all the experiments, six replicates (runs 2, 5, 10, 11, 16, and 20) were set at the central points and the corresponding predicted values at the central points were the highest. So, the variables at the central points were assumed as the optimum conditions. In the comparison of run 12 vs run 11, the ALA yield slightly decreased with decreasing D-glucose when the D-glucose was less than the optimum concentration, and the same case occurred that the ALA yield slightly decreased with increasing D-glucose when the D-glucose exceeded the optimum concentration in the comparison of run 18 vs run 11. However, the ALA yield sharply decreased with increasing succinate when the succinate exceeded the optimum concentration in the comparison of run 1 vs run 11. The ALA yield decreased with decreasing succinate when the D-glucose was less than the optimum concentration in the comparison of run 14 and run 11. It was noted that the ALA yield quickly decreased with decreasing glycine when the glycine was lower than the optimum concentration in the comparison of run 15 vs run 11. Above all, succinate and glycine had more prominent effects on the ALA yield than D-glucose in this study.

**Optimization and Validation of Predictive Models**

Based on the analysis of model prediction, it was found that three factors had different effects on the biomass production and ALA yield. In the final model optimization procedure, selected criteria were as follows: succinate, glycine, and D-glucose concentrations were in ranges determination in “The Effect of Succinate, Glycine, and D-Glucose on Biomass and ALA Yield” section; the biomass production and ALA yield reached the peak. Under the criteria above, the suggested solution was as follows: succinate concentration ($X_1$) of 8.56 mmol/L, glycine concentration ($X_2$) of 5.06 mmol/L, and D-glucose concentration ($X_3$) of 7.82 mmol/L, respectively. Simultaneously, the highest biomass production and ALA yield were predicted of 3.55 g/L and 5.49 mg/g-biomass at the same optimum conditions.

To verify the reliability of the equation model used for predicting the optimum response values, the optimization study was performed with the suggested parameters. The average experimental values of biomass production and ALA yield were $3.41 \pm 0.002$ g/L and $5.78 \pm 0.08$ g/g-biomass (Table 7). The analysis showed that the experimental values were in good agreement with the predicted values.

<table>
<thead>
<tr>
<th>Optimum conditions (mmol/L)</th>
<th>Biomass (g/L)</th>
<th>ALA yield (mg/g-biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>Glycine</td>
<td>D-glucose</td>
</tr>
<tr>
<td>8.56</td>
<td>5.05</td>
<td>7.82</td>
</tr>
<tr>
<td>Microorganism</td>
<td>Conditions</td>
<td>Medium</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>---------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td><em>Chlorella regularis</em> YA-603</td>
<td>Heterotrophic culture</td>
<td>Basal medium</td>
</tr>
<tr>
<td>Recombinant <em>Escherichia coli</em></td>
<td>Fermentation</td>
<td>LB medium</td>
</tr>
<tr>
<td><em>Rhodopseudomonas palustris</em> KG31</td>
<td>Batch fermentation</td>
<td>VFAs medium</td>
</tr>
<tr>
<td>Recombinant <em>Escherichia coli</em></td>
<td>Fed-batch fermentation</td>
<td>LB medium</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em> ATCC17023</td>
<td>Batch fermentation</td>
<td>VFAs wastewater</td>
</tr>
</tbody>
</table>
agreement with the predicted ones ($p > 0.05$). This indicated that the models were adequate for the biomass production and ALA yield. Therefore, based on the RSM, enhancing biomass production and ALA yield by properly adding precursors and inhibitor in *R. sphaeroides* wastewater treatment was accurate and reliable, implicating an efficient and green biotechnology in the field of PNSB wastewater treatment reutilization.

**Comparison Between Similarly Relative Studies**

Comparison between the ALA yield of this study and other relative studies (as shown in Table 8), it can be concluded that producing ALA from *R. sphaeroides* ATCC17023 treating VFAs wastewater was considerable. In previous studies, microorganisms produced ALA in the mediums, but *R. sphaeroides* could produce ALA in wastewater treatment in this study. Moreover, the ALA yield was very satisfactory [18, 19, 21, 25]. This study represented more economical value than the relative studies from the view of economics. It was noted that valuable high *R. sphaeroides* biomass and high ALA were recycled as good materials, which was superior to other relative studies.

**Conclusions**

In this study, three key influencing factors on biomass production and ALA yield in *R. sphaeroides* wastewater treatment were optimized by RSM method, and quadratic polynomial models was obtained for the first time. The predictive models analysis suggested that succinate, glycine, and D-glucose had different effects on the biomass production and ALA yield in respective way. The model results demonstrated both biomass production and ALA yield reached the maximum values of 3.55 g/L and 5.49 mg/g-biomass under the optimal conditions (succinate at 8.56 mmol/L, glycine at 5.06 mmol/L, and D-glucose at 7.82 mmol/L), and the results were verified in subsequent experiment further. The optimal parameters were reliable and suitable for practical application biomass recycling and ALA production from PNSB wastewater treatment.

**Acknowledgments** This study was financially supported by the National Natural Science Foundation of China (51278489).

**References**
