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Optimization of Influencing Factors on Biomass Accumulation and 5-Aminolevulinic Acid (ALA) Yield in *Rhodobacter sphaeroides* Wastewater Treatment

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This study aimed to optimize four factors affecting biomass accumulation and 5-aminolevulinic acid (ALA) yield together with pollutants removal in *Rhodobacter sphaeroides* wastewater treatment. Results showed that it was feasible to produce biomass and ALA in *R. sphaeroides* wastewater treatment. Microaerobic, 1,000−3,000 lux, and pH 7.0 were optimal conditions for the highest ALA yield of 4.5 ± 0.5 mg/g-biomass. Under these conditions, COD removal and biomass production rate were 93.3 ± 0.9% and 31.8 ± 0.5 mg/l/h, respectively. In addition, trace elements Fe^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+} further improved the ALA yield, COD removal, and biomass production rate. Specifically, the highest ALA yield (12.5 ± 0.6 mg/g-biomass) was achieved with Fe^{2+} addition.

Keywords: *Rhodobacter sphaeroides*, 5-aminolevulinic acid, influencing factors, optimization, wastewater treatment

Introduction

Rhodobacter sphaeroides can utilize organic substances in wastewaters to form biomass and synthesize high-value resources, including bacteriochlorophylls, biopolymers, 5-aminolevulinic acid (ALA), carotenoids, CoQ_{10}, *etc.* [10, 13, 14]. It has shown great potential in treating various types of wastewaters [19, 32]. *R. sphaeroides* possesses dual energy metabolic pathways and photosynthetic system I [19]. Therefore, it can grow under light-anaerobic condition or dark-aerobic condition.

ALA is actively involved in the tetrapyrrole biosynthesis of compounds such as porphyrin, heme, chlorophyll, and vitamin B_{12}. It has drawn increasing interests as a photodynamic chemical, herbicide, or insecticide, which has been widely applied in medical, agricultural, and biotechnological fields [1, 12, 23, 24]. Since the 1990s, microbial production of ALA has become popular because of some prominent advantages, including simple synthesis process, low cost, and abundant synthesis materials [3, 26]. Moreover, *R. sphaeroides* has been reported to successfully produce ALA [9, 11]. Therefore, it is a green biotechnology to generate ALA by *R. sphaeroides* while treating non-hazardous wastewaters.

However, ALA production is still relatively low in *R. sphaeroides* wastewater treatment because the ALA yield is significantly affected by various factors, such as light-oxygen condition, trace element, light intensity, and pH [2, 4, 19, 27, 28, 31]. It is necessary to increase the biomass production and ALA yield by optimizing different factors in *R. sphaeroides* wastewater treatment.

Hence, this study aimed to investigate the effects of major factors on ALA yield in *R. sphaeroides* wastewater treatment, and to enhance the biomass production rate and ALA yield of *R. sphaeroides* by optimizing the operation parameters.

Materials and Methods

Materials

A photosynthetic bacteria (PSB) strain (*R. sphaeroides* ATCC17023) was obtained from China General Microbiological Culture Collection

Center (CGMCC). It was cultured in a thermostat shaker (static, 30°C) under light-microaerobic conditions with PYG medium for 36 h. The PYG medium consisted of 10 g/l polypeptone, 5 g/l yeast extract, and 1 g/l glucose. The pH of PYG medium was adjusted to 6.8–7.0. The density of *R. sphaeroides* at logarithmic growth phase was 6.8×10^8 CFU/ml. 5-Aminolevulinic acid hydrochloride was provided by Tokyo Chemical Industry (Tokyo, Japan). Trace elements used in this study were provided by Harbin TianLin Science Technologies Ltd, China.

Soybean wastewater was used in the present study, which was obtained from a local soybean milk producer. The wastewater had a chemical oxygen demand (COD), total nitrogen (TN), total phosphorous (TP), and protein concentration of 8,000–8,200, 550–600, 30–50, and 1,000–1,100 mg/l, respectively, and the initial pH was around 7.0. The concentration of trace elements in the wastewater was very low and was ignored. The wastewater was used for the experiment after filtration and autoclaving in order to avoid environmental contamination (121°C, 30 min).

Methods

Experimental set-up. The bioreactors for the experiments were 1 L glass conical flasks and were sterilized at 121°C for 30 min before use. Wastewater (460 ml) and *R. sphaeroides* (40 ml) were added to each bioreactor. The initial *R. sphaeroides* concentration was 360 mg/l. The culture temperature was 30°C under still and sterile conditions.

Three different light-oxygen conditions were set as follows: (i) Light-anaerobic condition. Light intensity was around 2,000 lux and the bioreactors were saturated with nitrogen before reaction. (ii) Light-microaerobic condition. Light intensity was around 2,000 lux. Microaerobic condition was achieved by microaeration and the dissolved oxygen (DO) concentration in the bioreactors was kept within 0.5–1.0 mg/l. This aimed to save some energy of aeration. (iii) Dark-aerobic condition. Bioreactors were covered by four layers of black cloth to avoid light. The aerobic condition was realized by aeration and the DO concentration was kept at around 2.0 mg/l [19].

In the effects of trace element experiments, Fe^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Mo(+6), B(+3), and Se(+4) were investigated. Each different trace element was individually added in the bioreactors according to the formula of No. 0259 medium in CGMCC, which contained the following ingredients (mg/l): 1.8 $FeCl_2{\cdot}4H_2O$, 0.5 $MgCl_2$, 0.25 $CoCl_2{\cdot}6H_2O$, 0.01 $NiCl_2{\cdot}6H_2O$, 0.01 $CuCl_2{\cdot}2H_2O$, 0.7 $MnCl_2{\cdot}4H_2O$, 0.1 $ZnCl_2$, 0.5 H_3BO_3, 0.03 $Na_2MoO_4{\cdot}2H_2O$, and 0.01 $Na_2SeO_3{\cdot}5H_2O$. The blank group had no trace element addition.

Light intensity gradients were set as 200–500, 500–1,000, 1,000–3,000, 3,000–6,000, and 6,000–8,000 lux. Different initial pH gradient was set as 5.0, 6.0, 7.0, 8.0, and 9.0.

Analysis Methods

Sample from the bioreactor was centrifuged at 9,000 rpm for 10 min. The supernatant was used to test the COD, TN, TP, and protein. The collected cells were used for measuring the dry biomass and ALA. The pH and DO were detected with a pH tester and dissolved oxygen meter. COD, TP, and biomass were tested according to APHA standard methods [5]. The intracellular ALA concentration was measured according to previous description [16]. The biomass production rate (mg/l/h) was calculated by Eq. (1):

$$Y_1 = W/T \quad (1)$$

where Y_1 denotes the biomass production rate, W (mg/l) denotes the dry biomass at the end of the period (96 h), and T (h) denotes the whole time period. The ALA yield (mg/g-biomass) was calculated by Eq. (2):

$$Y_2 = 1000 \times C/W \quad (2)$$

where Y_2 denotes the ALA yield, C (mg/l) denotes the ALA concentration at 96 h, and W (mg/l) denotes the dry biomass at 96 h.

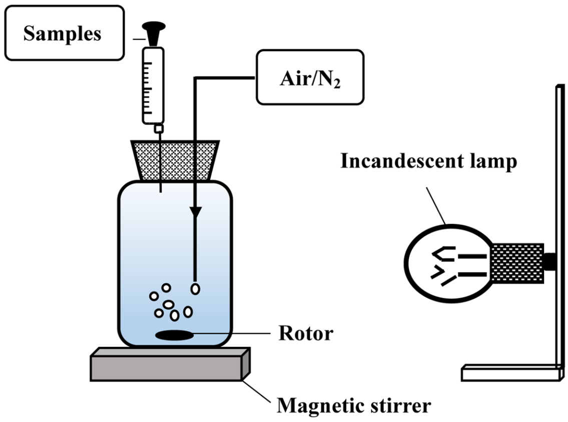

Fig. 1. Schematic drawing of the *Rhodobacter sphaeroides* wastewater treatment system.

Statistics

All values are presented as the mean ± SEM. The significant differences among multiple data were analyzed by one-way ANOVA in the Statistical Package for Social Sciences for Windows (ver. 19.0; SPSS Inc., Chicago, IL, USA). Duncan's multiple range tests (DMRT) were used for pair-wise or individual (one-to-one) comparisons. A difference of $p < 0.05$ was considered significant.

Results and Discussion

Effects of Light-Oxygen Condition on Pollutant Removal, Biomass Production Rate, and ALA Yield in *R. sphaeroides* Wastewater Treatment

R. sphaeroides, as one popular species, was selected to treat wastewater with high efficiency. In order to enhance pollutant removal, biomass growth, and ALA yield in

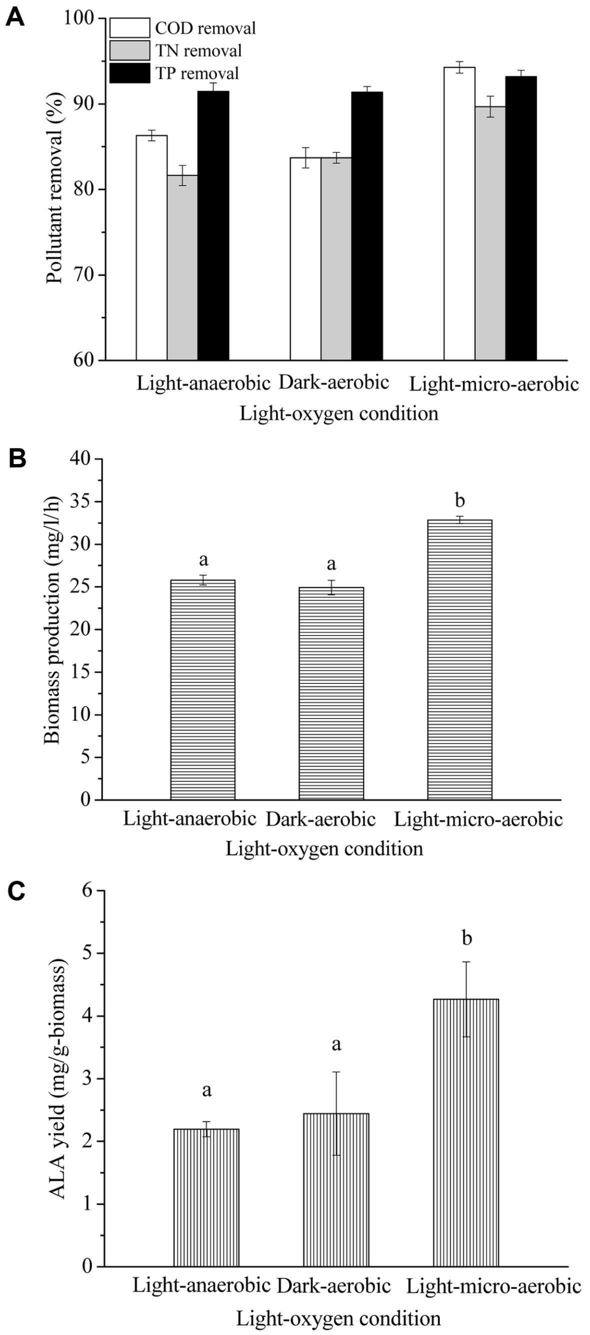

Fig. 2. Effects of light-oxygen condition on pollutant removal, biomass production rate, and ALA yield in *Rhodobacter sphaeroides* wastewater treatment.

(**A**) COD removal, TN removal, and TP removal. (**B**) Biomass production rate. (**C**) ALA yield. Bars represent the standard error of mean for replicates. Means sharing no common alphabet (above bar ± SEM) are signicantly different ($p < 0.05$).

R. sphaeroides wastewater treatment, the effects of three different light-oxygen conditions, including light-anaerobic, dark-aerobic, and light-microaerobic, were investigated. As Fig. 2A shows, the three pollutants COD, TN, and TP removals displayed a similar trend under different light-oxygen conditions. The COD, TN, and TP removals were the highest after 96 h treatment under the light-microaerobic condition. Thus, the light-microaerobic condition mostly benefits removing pollutants of *R. sphaeroides*. Light and oxygen conditions both had obvious effects on pollutant removal of *R. sphaeroides* wastewater treatment. It has been reported that different PSB species need differential light and oxygen conditions to treat a variety of wastewaters [18, 20, 22].

From Fig. 2B, it could be found that the light-microaerobic condition not only promoted pollutant removal, but also it improved the biomass accumulation of *R. sphaeroides*. The biomass production rate was 25.8 ± 0.6, 24.9 ± 0.9, and 32.8 ± 0.4 mg/l/h under light-anaerobic, dark-aerobic, and light-microaerobic conditions, respectively. As Fig. 2C showed, ALA yields were 4.3 ± 0.6, 2.2 ± 0.1, and 2.4 ± 0.7 mg/g-biomass by *R. sphaeroides*, which were higher than those in previously related studies [11, 26]. This demonstrated that *R. sphaeroides* could efficiently produce high ALA in the wastewater medium. ALA yield was higher under light-microaerobic condition than those under light-anaerobic and dark-aerobic conditions. This showed that proper light or oxygen possibly stimulated ALA biosynthesis of *R. sphaeroides*.

Above all, the favorable light-microaerobic condition was selected for the following experiment of light intensity optimization.

Effects of Light Intensity on Pollutant Removal, Biomass Production Rate, and ALA Yield in *R. sphaeroides* Wastewater Treatment

As an important factor, light intensity affected PSB growth, and it seemed to be a limiting factor because light energy was used as an energy resource in the photosynthesis process [14, 30]. As Fig. 3A showed, TN and TP removals had a similar change with COD removal under different light intensities. Representing three pollutants, the COD removals were 77.4 ± 0.9%, 82.8 ± 1.0%, 91.4 ± 0.8%, 90.4 ± 0.5%, and 82.8 ± 1.5% under light intensity of 200−500, 500−1,000, 1,000−3,000, 3,000−6,000, and 6,000−8,000 lux, respectively. The 1,000−3,000 lux light mostly improved COD removal. The COD removals were relatively high at 91.4 ± 0.8% and 90.4 ± 0.5% under 1,000−3,000 and 3,000−6,000 lux. The COD removal decreased when light intensity exceeded 6,000 lux, but it was higher than that under lower light (200−500 lux). This agreed with the previous study,

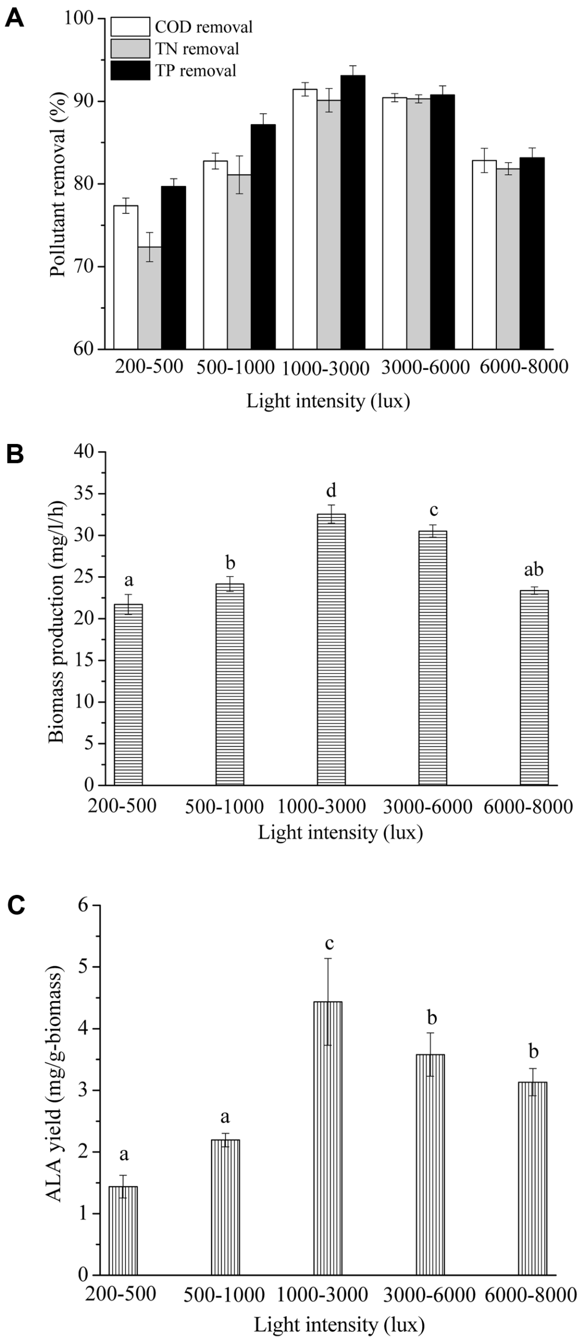

Fig. 3. Effects of light intensity on pollutant removal, biomass production rate, and ALA yield in *Rhodobacter sphaeroides* wastewater treatment.

(**A**) COD removal, TN removal, and TP removal. (**B**) Biomass production rate. (**C**) ALA yield. Bars represent the standard error of mean for replicates. Means sharing no common alphabet (above bar ± SEM) are signicantly different ($p < 0.05$).

COD removal would increase with increasing light intensity below the threshold of 5,000 lux, but COD removal decreased at higher intensity with light inhibition of PSB growth [33].

The effects of light intensity on biomass accumulation of *R. sphaeroides* are summarized in Fig. 3B. The results of DMRT analysis revealed that biomass production rates were ranked in the following order: 1,000–3,000 group > 3,000–6,000 group > 500–1,000 group ≥ 6,000–8,000 group ≥ 200–500 group. The biomass production rate reached a peak of 32.5 ± 1.09 mg/l/h at 1,000–3,000 lux. The biomass production rate increased with increasing light intensity below 3,000 lux. However, the biomass production rate fell when light intensity exceeded 3,000 lux, which showed that too high light would inhibit *R. sphaeroides* growth. A corresponding research described that the biomass of *R. sphaeroides* did not increase when the light intensity reached a threshold of more than 3,000 lux [21].

Optimal light intensity could promote *R. sphaeroides* growth and ALA production. The results are described in Fig. 3C. The ALA yields were 1.4 ± 0.2, 2.2 ± 0.1, 4.4 ± 0.7, 3.6 ± 0.4, and 3.1 ± 0.2 mg/g-biomass under 200–500, 500–1,000, 1,000–3,000, 3,000–6,000, and 6,000–8,000 lux. The results of DMRT analysis showed that ALA yields were ranked in the following order: 1,000–3,000 group > 3,000–6,000 group = 6,000–8,000 group > 500–1,000 group = 200–500 group. The highest ALA yield was achieved with 1,000–3,000 lux. The ALA yield would decrease slightly when the light intensity was higher than 3,000 lux, but it was still higher than that with low light (less than 1,000 lux). This was because too low light could not provide enough energy for ALA biosynthesis, because ALA biosynthesis is an energy-consuming process in *R. sphaeroides* [17]. Hence, the optimal light intensity was 1,000–3,000 lux for ALA yield in *R. sphaeroides* wastewater treatment.

The favorable light-microaerobic condition and light intensity of 1,000–3,000 lux were used for the following experiment of pH optimization.

Effects of pH on Pollutant Removal, Biomass Production Rate, and ALA Yield in *R. sphaeroides* Wastewater Treatment

Pollutant removal, biomass production, and ALA yield were detected under different pH after 96 h. From Fig. 4A, it was found that COD removal was 83.5 ± 0.9%, 89.3 ± 0.8%, 93.3 ± 1.0%, 92.5 ± 0.7%, and 91.2 ± 0.4% when the pH was 5.0, 6.0, 7.0, 8.0, and 9.0, respectively. The highest COD removal was achieved at pH 7.0, as acidic pH was harmful to pollutants removal, and alkaline pH will decrease pollutants removal compared with neutral pH.

Similarly, in Fig. 4B, the results of DMRT analysis revealed that biomass production was ranked in the following pH order: 7.0 group = 8.0 group > 9.0 group > 6.0 group >

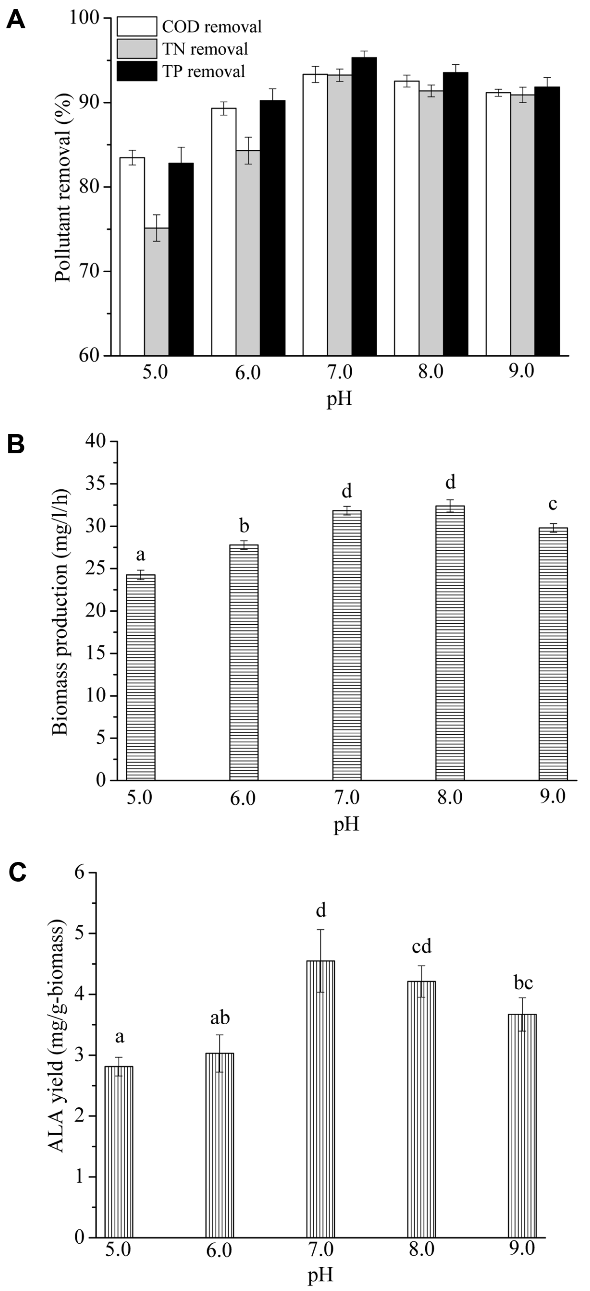

Fig. 4. Effects of pH on pollutant removal, biomass production rate, and ALA yield in *Rhodobacter sphaeroides* wastewater treatment.

(**A**) COD removal, TN removal, and TP removal. (**B**) Biomass production rate. (**C**) ALA yield. Bars represent the standard error of mean for replicates. Means sharing no common alphabet (above bar ± SEM) are signicantly different ($p < 0.05$).

5.0 group. pH 7.0 and 8.0 were relatively favorable to *R. sphaeroides* growth in this study.

The ALA yields were 2.8 ± 0.2, 3.0 ± 0.3, 4.6 ± 0.5, 4.2 ± 0.3, and 3.7 ± 0.3 mg/g-biomass under pH 5.0, 6.0, 7.0, 8.0, and 9.0. The results of DMRT analysis revealed that the ALA yields were ranked in the following pH order: 7.0 group ≥ 8.0 group ≥ 9.0 group ≥ 6.0 group > 5.0 group. The highest ALA yield was achieved at pH 7.0, which showed that a neutral pH could promote ALA biosynthesis in *R. sphaeroides* wastewater treatment. A previous study showed that ALA production by *R. sphaeroides* reached a peak value of 16 mmol/l at neutral pH 6.8–7.0 in the VFA medium [25, 28]. The effects of pH on ALA yield mainly presented in changing the activity of ALAS or ALA dehydratase (ALAD). ALAS activity was enhanced and ALAD activity was inhibited when the pH was nearly neutral (6.8 ± 0.1). However, ALAS activity was low and ALAD activity was high when the pH was 8.0. ALAS activity was low under pH 6.0–6.5 [28]. Therefore, ALA production should be carried out at a neutral or a slightly alkaline pH in this study.

The favorable light-microaerobic condition, light intensity of 1,000–3,000 lux, and pH of 7.0 were used for the following experiment of trace element optimization.

Effects of Trace Elements on Pollutant Removal, Biomass Production Rate, and ALA Yield in *R. sphaeroides* Wastewater Treatment

In order to investigate the effects of major trace elements, Fe^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Mo(+6), B(+3), and Se(+4) were added. It was found from Fig. 5A, the COD removals were 88.9 ± 0.9%, 94.1 ± 1.4%, 90.9 ± 0.4%, 90.5 ± 0.5%, 90.0 ± 0.8%, 88.9 ± 1.1%, 89.4 ± 0.6%, 89.2 ± 0.7%, 89.0 ± 1.3%, 83.9 ±1.3%, and 83.7 ± 0.6% in the blank and experimental groups. Compared with the blank, trace elements Fe^{2+}, Mg^{2+}, Ni^{2+}, and Co^{2+} improved the pollutant removal (COD, TN, and TP) at different levels. The pollutant removals in the groups with added Zn^{2+}, Mo(+6), B(+3), and Se(+4) represented no obvious difference with the blank. However, Cu^{2+} and Mn^{2+} decreased the pollutant removals.

As Fig. 5B shows, adding trace elements (Fe^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, and Mo(+6)) improved *R. sphaeroides* growth. Compared with the blank, biomass production rates were increased by 28%, 14%, 15%, 15%, 10%, and 9%, respectively. Moreover, a previous study revealed that optimal Fe^{2+} could increase the biomass production and COD removal of *R. sphaeroides* Z08 by up-regulating energy metabolism [32]. Liu *et al.* [15] showed that appropriate Ni^{2+}, Fe^{2+}, and Mg^{2+} significantly promoted cell growth of PSB *Rhodopseudomonas faecalis* RLD-53 by increasing the bioactivity (including [Ni-Fe] hydrogenase and nitrogenase, Mg-containing pigments) of bacteria. However, trace elements Cu^{2+} and Mn^{2+} have

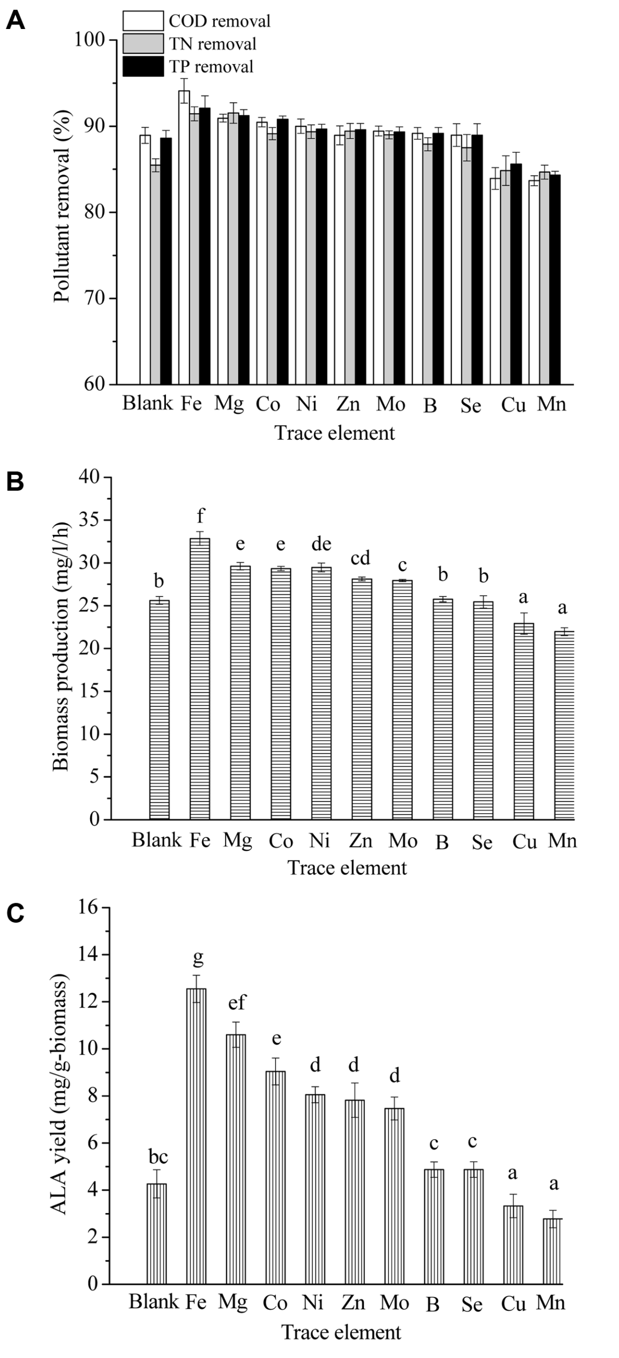

Fig. 5. Effects of trace element on pollutant removal, biomass production rate, and ALA yield in *Rhodobacter sphaeroides* wastewater treatment.

(**A**) COD removal, TN removal, and TP removal. (**B**) Biomass production rate. (**C**) ALA yield. Bars represent the standard error of mean for replicates. Means sharing no common alphabet (above bar ± SEM) are signicantly different ($p < 0.05$).

been reported to inhibit *R. sphaeroides* growth because they were possibly harmful to the bacterium [2, 8, 27]. The results of DMRT analysis showed that biomass production varied differentially in the following order: Fe^{2+} group > Mg^{2+} group = Co^{2+} group ≥ Ni^{2+} group ≥ Zn^{2+} group ≥ Mo(+6) group > B(+3) group = Se(+4) group = blank group > Cu^{2+} group = Mn^{2+} group.

COD degradation and biomass accumulation could provide proper substrates and energy sources for ALA biosynthesis by *R. sphaeroides*. From Figs. 5A and 5B, some trace elements could increase COD removal and biomass production, so ALA yields were improved by adding trace elements. As Fig. 5C demonstrated, Fe^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, and Mo(+6) significantly increased the ALA yields of *R. sphaeroides*. The results of DMRT analysis showed that ALA yields varied differentially in the following order: Fe^{2+} group > Mg^{2+} group ≥ Co^{2+} group > Ni^{2+} group = Zn^{2+} group = Mo(+6) group > B(+3) group = Se(+4) group ≥ blank group > Cu^{2+} group = Mn^{2+} group. Adding Cu^{2+} and Mn^{2+} inhibited ALA biosynthesis. Many studies showed that metal ions played significant roles in affecting the enzyme activity of ALA biosynthesis. Metal irons such as Fe^{2+}, Cu^{2+}, Ca^{2+}, and nitrate had important effects on ALAS activity [27, 31]. Metal ions Fe^{2+} and Co^{2+} regulated ALA biosynthesis by *R. sphaeroides* [23]. Zn^{2+} and Mg^{2+} were important domains of GluRS for substrate recognition [6, 7, 29]. The study demonstrated that ALA yield was increased with the addition of Fe^{2+} by increasing ALAS activity [27, 31]. Another research revealed that the ALAS activity of *Rp. palustris* KUGB306 was strongly inhibited by Pb^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}, but it was only slightly affected by Mg^{2+} and K^{+} [2]. Moreover, different metal ions had divergent effects on different species. Fe^{2+}, Mg^{2+}, Ni^{2+}, and Co^{2+} could improve COD removal, biomass production, and ALA yield in *R. sphaeroides* wastewater treatment in the present study.

This study demonstrated that it was feasible to produce biomass and ALA by *R. sphaeroides* wastewater treatment. Under the conditions of light-microaerobic, 1,000–3,000 lux, and pH 7.0, the COD removal (93.3 ± 0.9%), biomass production rate (31.8 ± 0.5 mg/l/h), and ALA yield (4.5 ± 0.5 mg/g-biomass) were the highest. Adding Fe^{2+} significantly improved the ALA yield and the highest ALA reached 12.5 ± 0.6 mg/g-biomass.

Validation Test Based on the Optimal Conditions

To verify the reliability of the one-factor-at-a-time strategy for optimizing influencing factors, a validation test was performed with the suggested parameters in the one-factor tests.

The average experimental values of biomass production

Table 1. Comparison of the one-factor test and the validation test based on optimal conditions.

Optimum conditions				Biomass production rate (mg/l/h)		ALA yield (mg/g-biomass)	
Light-oxygen condition	Light intensity	pH	Trace element	One-factor test	Validation test	One-factor test	Validation test
Light-microaerobic condition	1,000−3,000 lux	7.0	Fe^{2+}	31.8 ± 0.5	32.4 ± 0.6	12.5 ± 0.6	12.1 ± 0.5

rate and ALA yield in the validation test were 32.4 ± 0.6 mg/l/h and 12.1 ± 0.5 mg/g-biomass (Table 1). Compared with the values (31.8 ± 0.5 mg/l/h and 12.5 ± 0.6 mg/g-biomass) in the final one-factor test, the statistical analysis indicated that the values in the two groups were in good agreement with the predicted ones (p > 0.05). Therefore, enhancing biomass and ALA production using the experimental design of a one-factor test in *R. sphaeroides* wastewater treatment was accurate and reliable.

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