Effects of metal ions on biomass and 5-aminolevulinic acid production in *Rhodopseudomonas palustris* wastewater treatment

Shuli Liu, Guangming Zhang, Jianzheng Li, Xiangkun Li and Jie Zhang

**ABSTRACT**

This work investigated the effects of eight metal ions on *Rhodopseudomonas palustris* growth and 5-aminolevulinic acid (ALA) yield in wastewater treatment. Results show that metal ions (Mg²⁺ of 15 mmol/L, Fe²⁺ of 400 μmol/L, Co²⁺ of 4 μmol/L, Ni²⁺ of 8 μmol/L and Zn²⁺ of 4 μmol/L) could effectively improve the chemical oxygen demand (COD) removal, *Rp. palustris* biomass and ALA yield. The highest ALA yield of 13.1 mg/g-biomass was achieved with Fe²⁺ of 400 μmol/L. ALA yields were differentially increased under different metal ions in the following order: Fe²⁺ group > Mg²⁺ group > Ca²⁺ group = Ni²⁺ group > Zn²⁺ group = Mn²⁺ group > control. Cu²⁺ and Mn²⁺ inhibited *Rp. palustris* growth and ALA production. Mechanism analysis revealed that metal ions changed ALA yields by influencing the activities of ALA synthetase and ALA dehydratase.

**Key words** | 5-aminolevulinic acid yield, ALA dehydratase, ALA synthetase, metal ion, *Rhodopseudomonas palustris*

**INTRODUCTION**

Purple non-sulfur bacteria (PNSB), used to treat various wastewaters, have received increasing attention since the 1960s. They could effectively remove the pollutants from different wastewaters including dairy wastewater, soybean wastewater, olive mill wastewater, and domestic wastewater (*Kaewsuk et al. 2010*; *Eroglu et al. 2011*; *Wu et al. 2012*; *Tim et al. 2014*). Simultaneously, the accumulated PNSB biomass contains a variety of valuable materials, including single cell proteins, bacteriochlorophylls, biopolymers, 5-aminolevulinic acid (ALA), carotenoids, and CoQ₁₀ (*Kang et al. 2012*; *Kuo et al. 2012*). Among those materials, ALA attracted special attention because it is an important photo-dynamic chemical involved in the biosynthesis of tetrapyrrole compounds, and it is widely applied in both medical and agricultural fields (*Sasaki et al. 2002, 2005*).

ALA production largely depends on different influencing factors, including light-oxygen conditions, pH, carbon and nitrogen sources, temperature, and metal ions (*Choi et al. 2004*; *Chung et al. 2005*; *Liu et al. 2014*). Trace metal ions, such as iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), cobalt (Co), copper (Cu), zinc (Zn) and nickel (Ni), play essential roles in regulating osmotic pressure and redox processes and stabilizing macromolecules through electrostatic interactions. Most of them are transition metals with strong abilities to form complex compounds (*Hunter et al. 2008*). In previous studies, Fe²⁺, Mg²⁺, Ca²⁺ and Ni²⁺ have been proved to improve PNSB growth of *Rhodobacter sphaeroides* and *Rhodopseudomonas faecalis* (*Liu et al. 2009*; *Hakobyan et al. 2012*; *Wu et al. 2012*). In addition, some metal ions were activators to various enzymes. Previous work revealed that metal ions (Fe²⁺, Cu²⁺ and Ca²⁺) had important effects on the activity of ALA synthetase (ALAS) (*Sasaki et al. 1989*; *Tangprasittipap et al. 2007*). Fe²⁺ and Co²⁺ were important elements for regulating tetrapyrrole biosynthesis of *R. sphaeroides* (*Sasi-kala et al. 1994*).

This study was aimed at investigating the effects of eight different metal ions on *Rp. palustris* growth and ALA yield in wastewater treatment; optimizing the metal ion concentration to increase the ALA yield; investigating the potential mechanism on influencing ALA yield by changing ALAS and ALA dehydratase (ALAD) activities.

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MATERIALS AND METHODS

Materials

An *Rp. palustris* strain (ACCC10649) used in this study was obtained from Agricultural Culture Collection of China. It was cultivated with 0317 medium (detailed information can be found at www.accc.org.cn/show.asp) under light-anaerobic conditions in a serum bottle (120 rpm, 30 °C). The medium consisted of 3 g/L yeast extract, 0.5 g/L magnesium sulfate heptahydrate, 3 g/L peptone, 0.3 g/L calcium chloride. pH was adjusted to 7.0. The inoculated bacteria in logarithmic growth phase were used for experiments, and the density was $7.5 \times 10^8$ colony forming units (CFU)/mL.

Synthetic wastewater was selected in this study. It comprised peptone (2 g/L), sodium acetate (2 g/L), yeast extract (1.5 g/L) and potassium dihydrogen phosphate (0.5 g/L). The characteristics were as follows: chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP) were 3,425, 232 and 85 mg/L, respectively. pH was adjusted to around 7.0. The wastewater was used for the experiments after filtration and autoclaving to avoid environmental contamination (121 °C, 30 min).

Methods

Experimental

The bioreactors for the experiments were glass conical flasks of 1 L volume, and each reaction volume was 500 mL, as shown in Figure 1. They were sterilized at 121 °C for 30 min before use. Synthetic wastewater (460 mL) and *Rp. palustris* (40 mL) were added to each bioreactor. The initial concentration of *Rp. palustris* was 550 mg/L.

Light and oxygen conditions were as follows: light intensity was 3,000–6,000 lux; anaerobic conditions were maintained by continuous nitrogen inflow from nitrogen cylinder to bioreactor. The cultivating temperature was 30 °C.

Metal ions addition

Different metal ions were individually added to bioreactors according to the trace elements formula of No. 0259 medium in China General Microbiological Culture Collection Center. The formula contained the following ingredients (mg/L): 1.8 FeCl$_2$·4H$_2$O, 0.5 MgCl$_2$, 0.25 CoCl$_2$·6H$_2$O, 0.01 NiCl$_2$·6H$_2$O, 0.01 CuCl$_2$·2H$_2$O, 0.7 MnCl$_2$·4H$_2$O, 0.1 ZnCl$_2$, 0.5 H$_2$BO$_3$, 0.03 Na$_2$MoO$_4$·2H$_2$O, and 0.01 Na$_2$SeO$_3$·5H$_2$O. In this study, 100, 200, 400 and 500 μmol/L for FeCl$_2$, 5, 10, 15 and 20 mmol/L for MgCl$_2$, and 2, 4, 8 and 12 μmol/L for CoCl$_2$, NiCl$_2$, ZnCl$_2$, Na$_2$MoO$_4$, CuCl$_2$, and MnCl$_2$ were added into the reaction systems, respectively. No metal ion was added in the blank group.

Analysis methods

Each sample collected from a bioreactor was centrifuged at 9,000 rpm for 10 min. The supernatant was used to test COD, TN and TP. The collected cells were used to measure the biomass and intracellular ALA. pH and dissolved oxygen were detected by a pH tester and dissolved oxygen meter, respectively. COD and biomass were tested according to American Public Health Association *Standard Methods* (Clesserl et al. 1998). The COD detection method was potassium dichromate oxidation spectrophotometric method. Biomass was measured by dry weight (105 °C, overnight). The intracellular ALA concentration was examined according to the description (Liu et al. 2010). The ALA yield (mg/g-biomass) was calculated by Equation (1):

$$Y = \frac{C}{W}$$

where $Y$ denotes the ALA yield, $C$ (mg/L) denotes the ALA concentration at 96 h, $W$ (g/L) denotes the dry biomass at 96 h.

The assay for ALAS and ALAD activities were measured by the method described (Lin et al. 2009). The reaction containing ALAS extract was performed with acetylacetone addition for 15 min at 100 °C. After the reaction, the mixture was cooled down to room temperature.
and mixed with modified Ehrlich’s reagent for 30 min, then the absorbance at 554 nm was measured. One unit of ALAS activity was defined as the amount of the enzyme needed to produce 1 nmol of ALA in 1 min. The assay for ALAD activity involved a total volume of 500 μL, consisting of 50 mmol/L of potassium phosphate buffer (pH = 7.5), 50 mmol/L of ZnCl₂, 1 mmol/L of MgCl₂, and 5 mmol/L of ALA. The reaction (37 °C, 10 min) was stopped by 20% trichloroacetic acid. The next procedures were the same as the assay for ALAS activity (15 min reaction with acetylacetone at 100 °C, cooled down, 30 min reaction with modified Ehrlich’s reagent, and then measured at 554 nm absorbance). One unit of ALAD activity was defined as the amount of the enzyme needed to produce 1 nmol of porphobilinogen (PBG) in 1 h.

Statistical analysis

In order to ensure data accuracy, parallel experiments were carried out and all results were represented as mean ± standard deviation. Significant differences between the treatments were evaluated by one-way analysis of variance with the Statistical Package for Social Sciences for Windows (version 19.0; SPSS Inc., Chicago, IL, USA). Duncan’s multiple range tests (DMRT) were used for pairwise or individual (one-to-one) comparisons. Significant difference was considered at \( P < 0.05 \).

RESULTS AND DISCUSSION

Optimization of metal ions on COD removal, biomass production and ALA yield in *Rp. palustris* wastewater treatment

The first step was to determine the optimal dosages of various metal ions. Metal ions at different concentrations were added to the wastewater, and the COD removal, biomass production and ALA yield at different levels. The optimal dosage of each metal ion was 400 μmol/L for Fe²⁺, 15 mmol/L for Mg²⁺, 4 μmol/L for Co²⁺, 8 μmol/L for Ni²⁺, 4 μmol/L for Zn²⁺, and 4 μmol/L for Mo²⁺, respectively. Metal ions of Cu²⁺ and Mn²⁺ decreased the COD removal, biomass production and

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>COD removal (%)</th>
<th>Biomass (g/L)</th>
<th>ALA yield (mg/g-biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank for Fe²⁺</td>
<td>88.46 ± 0.69a</td>
<td>2.44 ± 0.07a</td>
<td>5.42 ± 0.66ab</td>
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<td>Fe²⁺ (100 μmol/L)</td>
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<td>2.58 ± 0.08b</td>
<td>7.91 ± 0.54bc</td>
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<td>Fe²⁺ (200 μmol/L)</td>
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<td>8.99 ± 0.42c</td>
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<td>Fe²⁺ (400 μmol/L)</td>
<td>91.17 ± 1.09b</td>
<td>2.85 ± 0.05c</td>
<td>13.1 ± 0.68d</td>
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<tr>
<td>Fe²⁺ (500 μmol/L)</td>
<td>88.60 ± 0.73a</td>
<td>2.80 ± 0.03c</td>
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<td>88.46 ± 0.69a</td>
<td>2.44 ± 0.07a</td>
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<tr>
<td>Mg²⁺ (5 mmol/L)</td>
<td>89.37 ± 0.41ab</td>
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<td>Mg²⁺ (10 mmol/L)</td>
<td>90.93 ± 1.02b</td>
<td>2.91 ± 0.06c</td>
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<td>Mg²⁺ (15 mmol/L)</td>
<td>93.93 ± 0.53c</td>
<td>3.16 ± 0.06c</td>
<td>10.56 ± 0.46c</td>
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<tr>
<td>Mg²⁺ (20 mmol/L)</td>
<td>90.43 ± 0.78b</td>
<td>2.94 ± 0.06c</td>
<td>7.61 ± 0.52b</td>
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<tr>
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<tr>
<td>Co²⁺ (2 μmol/L)</td>
<td>88.87 ± 0.76a</td>
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<tr>
<td>Co²⁺ (4 μmol/L)</td>
<td>89.73 ± 0.45a</td>
<td>2.72 ± 0.10b</td>
<td>8.35 ± 0.23b</td>
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<td>Co²⁺ (8 μmol/L)</td>
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<td>1.64 ± 0.10b</td>
<td>8.08 ± 0.51b</td>
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<tr>
<td>Co²⁺ (12 μmol/L)</td>
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<td>2.71 ± 0.09b</td>
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<td>Ni²⁺ (4 μmol/L)</td>
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<td>Ni²⁺ (8 μmol/L)</td>
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<td>9.12 ± 0.56b</td>
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<td>Ni²⁺ (12 μmol/L)</td>
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<td>Zn²⁺ (2 μmol/L)</td>
<td>89.37 ± 0.45ab</td>
<td>2.63 ± 0.05b</td>
<td>5.43 ± 0.36b</td>
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<td>Zn²⁺ (4 μmol/L)</td>
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<td>Zn²⁺ (8 μmol/L)</td>
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<td>2.75 ± 0.06b</td>
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<td>Zn²⁺ (12 μmol/L)</td>
<td>89.17 ± 0.58b</td>
<td>2.72 ± 0.10b</td>
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<td>2.44 ± 0.07a</td>
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<tr>
<td>Mo²⁺ (2 μmol/L)</td>
<td>88.43 ± 0.74a</td>
<td>2.62 ± 0.06b</td>
<td>5.62 ± 0.87a</td>
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<td>Mo²⁺ (4 μmol/L)</td>
<td>88.77 ± 0.25a</td>
<td>2.72 ± 0.05b</td>
<td>6.11 ± 0.11a</td>
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<td>Mo²⁺ (8 μmol/L)</td>
<td>88.43 ± 0.68a</td>
<td>2.64 ± 0.05b</td>
<td>5.97 ± 0.48b</td>
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<tr>
<td>Mo²⁺ (12 μmol/L)</td>
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<td>2.59 ± 0.06b</td>
<td>5.92 ± 0.42b</td>
</tr>
<tr>
<td>Blank for Cu²⁺</td>
<td>88.46 ± 0.69c</td>
<td>2.44 ± 0.07b</td>
<td>5.42 ± 0.66b</td>
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<tr>
<td>Cu²⁺ (2 μmol/L)</td>
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<td>2.21 ± 0.09a</td>
<td>3.77 ± 0.38a</td>
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<tr>
<td>Cu²⁺ (4 μmol/L)</td>
<td>83.67 ± 0.79ab</td>
<td>2.32 ± 0.04ab</td>
<td>3.68 ± 0.43ab</td>
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<tr>
<td>Cu²⁺ (8 μmol/L)</td>
<td>82.37 ± 0.66ab</td>
<td>2.24 ± 0.06a</td>
<td>3.67 ± 0.31a</td>
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<td>Cu²⁺ (12 μmol/L)</td>
<td>81.63 ± 0.57a</td>
<td>2.21 ± 0.07a</td>
<td>2.96 ± 0.59a</td>
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<td>Blank for Mn²⁺</td>
<td>88.46 ± 0.69a</td>
<td>2.44 ± 0.07b</td>
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<td>Mn²⁺ (2 μmol/L)</td>
<td>83.80 ± 0.33b</td>
<td>2.12 ± 0.07a</td>
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<td>Mn²⁺ (4 μmol/L)</td>
<td>82.67 ± 0.61ab</td>
<td>2.10 ± 0.06a</td>
<td>3.10 ± 0.06ab</td>
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<tr>
<td>Mn²⁺ (8 μmol/L)</td>
<td>82.13 ± 0.62b</td>
<td>2.11 ± 0.06a</td>
<td>2.77 ± 0.53ab</td>
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<tr>
<td>Mn²⁺ (12 μmol/L)</td>
<td>81.63 ± 1.17a</td>
<td>2.06 ± 0.07a</td>
<td>2.72 ± 0.50a</td>
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</tbody>
</table>

*Significantly different, \( P < 0.05 \).*
ALA yield. The less Cu^{2+} or Mn^{2+} was added, the weaker the inhibition on ALA yield.

Effects of different metal ions on COD removal, biomass production and ALA yield in *Rp. palustris* wastewater treatment

Based on the results in Table 1, the optimal dosage of each metal ion was selected in the following experiments.

The biomass production and COD removal

Figure 2(a) shows that, compared with the blank group, metal ions such as Fe^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Mo^{2+} increased the COD removals in *Rp. palustris* wastewater treatment. The COD removals were 91.2, 93.9, 89.7, 90.7, 90.0, 88.4, 88.6, 83.8, and 83.8%, respectively. Metal ion addition was a simple method, and this method could improve the pollutant degradation efficiency in *Rp. palustris* wastewater treatment from the results above.

With the same trend, Figure 2(b) shows the biomass production reached the peak of 3.16 g/L with 15 mmol/L for Mg^{2+}. Clearly, Fe^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Mo^{2+} stimulated *Rp. palustris* growth, and the biomass displayed an increase of 16.6, 29.5, 11.5, 10.9, 12.4 and 11.2%, respectively. Analysis results of DMRT show that the biomass increased differentially under different metal ions in the following order: Mg^{2+} group > Fe^{2+} group = Co^{2+} group = Ni^{2+} group = Zn^{2+} group = Mo^{2+} group > control. In addition, Cu^{2+} and Mn^{2+} inhibited *Rp. palustris* growth. The possible reason is that Cu^{2+} and Mn^{2+} are toxic to bacteria to some extent. Wu *et al.* (2015) reported that the optimal Fe^{2+} content (20 mg/L) could significantly increase the biomass production and COD removal in *R. sphaeroides* Z08 wastewater treatment. Through further analysis, Wu *et al.* (2015) found that Fe^{2+} improved the *R. sphaeroides* growth and COD removal by enhancing the energy metabolism pathway of *R. sphaeroides*. Likewise, previous studies showed that Fe^{2+}, Mg^{2+}, Ca^{2+} and Ni^{2+} ions promoted the growth of PNSB including *R. sphaeroides* and *Rp. faecalis* (Liu *et al.* 2009; Hakobyan *et al.* 2012). On the one hand, some metal ions were essential elements for cell growth. On the other hand, it was proved that Mg^{2+} and Ni^{2+} could enhance *R. sphaeroides* growth by synthesizing more pigment complexes. In addition, Hakobyan *et al.* (2012) reported that Mg^{2+} also affected *R. sphaeroides* growth by regulating the pH of the medium. In this study, Fe^{2+} and Mg^{2+} both significantly increased *Rp. palustris* growth and COD removal.

The ALA production

Metal ions improved the biomass and COD removal, and the potential of ALA production in *Rp. palustris* wastewater treatment under different metal ions was examined. Figure 2(c) shows the ALA yields in the groups with
metal ions (Fe²⁺, Mg²⁺, Co²⁺ and Ni²⁺) were improved to some extent. Compared with the blank group, the ALA yields were increased by 142.5, 95.1, 54.1 and 68.5%, respectively. Metal ions Zn²⁺ and Mo²⁺ had no obvious effect on the ALA yield. The ALA yields in the groups with Cu²⁺ and Mn²⁺ were lower than that of the blank. Moreover, it was proved that Rp. palustris growth was inhibited by Cu²⁺ and Mn²⁺ (Figure 2(b)). So Cu²⁺ and Mn²⁺ were possibly toxic to Rp. palustris cells or ALAS activity in the ALA biosynthesis pathway. A previous study reported that Cu²⁺ had critical effects on ALAS activity (Sasaki et al. 1989). Further, the analysis results of DMRT showed that ALAS activities varied differentially under different metal ions in the following order: Fe²⁺ group > Mg²⁺ group > Co²⁺ group > Ni²⁺ group > Zn²⁺ group > Mo²⁺ group > control > Cu²⁺ group > Mn²⁺ group. Hence, the addition of Fe²⁺ could dramatically improve ALA production.

Effects of metal ions on ALAS and ALAD activities

Previous studies showed that many metal ions (Fe²⁺, Mg²⁺ and Ni²⁺) were used as PNSB growth medium components. Metal ions (Mo²⁺, Mg²⁺ and Ni²⁺) were critical elements in various enzymes such as hydrogenase and nitrogenase. They were also activators to some enzymes (Wang & Wan 2008; Liu et al. 2009; Eroglu et al. 2011). Studies also revealed that some metal ions (Fe²⁺, Cu²⁺ and Co²⁺) had important effects on ALAS activity and they could regulate the ALA biosynthesis process (Sasaki et al. 1989; Sasikala et al. 1994; Tangprasittipap et al. 2007). ALAS and ALAD are key enzymes in the ALA biosynthesis pathway. In this pathway, ALA is formed by ALAS catalysis, and ALAS activity has a positive effect on ALA production. On the contrary, ALAD can catalyze conversion of two molecules of ALA into one molecule of PBG, resulting in a decrease of ALA production (Liu et al. 2014). Hence, in order to investigate the effects of metal ions on the key enzymes in ALA biosynthesis, ALAS and ALAD activities with different metal ion additions were examined. The results are shown in Figure 3.

Figure 3(a) shows that metal ions of Fe²⁺, Mg²⁺, Co²⁺, Ni²⁺ and Zn²⁺ increased ALAS activities, and further improved the ALA yield. This agreed with the results in Figure 2(c) where those metal ions increased the ALA yield. DMRT analysis showed that ALAS activities varied differentially under metal ions in the following order: Fe²⁺ group > Mg²⁺ group > Co²⁺ group > Ni²⁺ group > Zn²⁺ group. Choi et al. (2004) demonstrated that the ALAS activity of Rp. palustris KUGB306 was strongly inhibited by Fe²⁺, Co²⁺ and Zn²⁺ at 1 mmol/L, but it was only slightly affected by Mg²⁺. In this study, metal ions of different concentrations such as 400 μmol/L for Fe²⁺, 15 μmol/L for Mg²⁺, 4 μmol/L for Co²⁺, 8 μmol/L for Ni²⁺ and 4 μmol/L for Zn²⁺ increased the ALAS activities of Rp. palustris strain ACCC10649. This finding showed that different metal ions had different effects on ALAS of different species. Moreover, different species displayed differential responses to metal ions of specific concentration.

As another key enzyme in ALA biosynthesis pathway, ALAD can catalyze the degradation of ALA; therefore, the inhibition of the activity of ALAD is desirable for higher ALA yield (Liu et al. 2014). Figure 3(b) shows that Mg²⁺, Co²⁺, Zn²⁺ and Mn²⁺ increased the ALAD activities, which consequently increased ALA degradation. The possible reason was that these metal ions might be activators.
to the enzymes. A previous study reported that Mg\(^{2+}\), K\(^{+}\) and Zn\(^{2+}\) maintained the catalytic activity of ALAD (Sasi-kala et al. 1994). It is worth underlining that Mn\(^{2+}\) also increased ALAS activity, but low ALA yield was achieved (Figure 2(c)). The reason was that Mn\(^{2+}\) promoted ALAD activity to degrade more ALA.

In addition, the highest ALA yield was achieved with 400 \(\mu\)mol/L of Fe\(^{2+}\). From Figure 3(a) and 3(b), it is found that there were two possible reasons. On the one hand, Fe\(^{2+}\) increased ALA yield by increasing ALAS activity. One the other hand, Fe\(^{2+}\) also decreased ALA degradation by inhibiting ALAD activity. A similar study reveals that 30 \(\mu\)mol/L for Fe\(^{2+}\) enhanced ALA production by increasing ALAS activity (Tang-prasittipap et al. 2007). Hence, proper Fe\(^{2+}\) addition would increase ALA yield by changing ALAS and ALAD activities.

Taking into account that too much metal ion in the effluent could be a new pollution, we detected the concentrations of residual metal ions in the effluent with a Plasma-Atomic Emission Spectrometry System (Otima 5300 DV ICP, Perkin-Elmer Inc., Boston, USA). The results in Table 2 show that metal ion additions in this study could meet a common sewage discharge standard (GB 18918-2002) by the Chinese Government, so it was accepted to add proper metal ions.

### CONCLUSIONS AND PERSPECTIVES

This study showed that metal ion addition could improve the biomass and ALA production in \(Rp.\ palustris\) wastewater treatment. Optimal dosages of metal ions were 400 \(\mu\)mol/L for Fe\(^{2+}\), 15 mmol/L for Mg\(^{2+}\), 4 \(\mu\)mol/L for Cu\(^{2+}\), 8 \(\mu\)mol/L for Ni\(^{2+}\) and 4 \(\mu\)mol/L for Zn\(^{2+}\), respectively. Four hundred micromoles per liter for Fe\(^{2+}\) led to the highest ALA yield of 13.1 mg/g-biomass. The maximum COD removal and biomass production were achieved at 15 mmol/L for Mg\(^{2+}\). Mechanism analysis showed that different metal ions had different influencing mechanisms on ALA yield by regulating ALAS or ALAD activities.

Based on the results above, proper metal ion addition improving biomass and ALA production will be a simple and green biotechnology in the field of wastewater treatment. Producing valuable ALA might be applied in other fields. This biotechnology will promote wastewater treatment recycling, and it also will provide some guidance for ALA production in agriculture, medical or other fields.

### ACKNOWLEDGEMENT

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