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ORIGINAL PAPER

Effect of magnesium ion on *crt* gene expression in improving carotenoid yield of *Rhodobacter sphaeroides*

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Abstract This study aimed at increasing carotenoid yield of *Rhodobacter sphaeroides* in wastewater treatment by adding magnesium ion (Mg^{2+}). Results showed that Mg^{2+} could improve *R. sphaeroides* biomass and carotenoid yield effectively. The highest carotenoid yield of 4.83 ± 0.14 mg/g biomass and biomass production of 3900 ± 180 mg/L were achieved at optimal Mg^{2+} concentration of 15 mmol/L. Mechanism analysis revealed that Mg^{2+} could promote carotenoid production by regulating the expressions of *crt* genes. Up-regulation of *crtBDA genes* improved carotenoid biosynthesis of *R. sphaeroides*.

Keywords Carotenoid · *Rhodobacter sphaeroides* · Magnesium ion · Gene expression · Up-regulation

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Introduction

Purple non-sulfur bacteria are (PNSB) widely spread in paddy, swamp, lakes, ocean and wastewater (Imhoff and Trüper 1989). They were used to treat various wastewaters such as olive mill wastewater, diary wastewater, soybean wastewater and domestic wastewater (Eroglu et al. 2010; Kaewsuk et al. 2010; Wu et al. 2012; Tim et al. 2014). PNSB could efficiently treat many kinds of wastewaters by degrading various pollutants; at the same time, valuable products were produced including single-cell proteins, bacteriochlorophylls, biopolymers, 5-aminolevulinic acid, carotenoids and CoQ_{10} (Kang et al. 2012; Kuo et al. 2012). Among those valuable products, carotenoids have attracted intense attention for their increasing application in the medical field (Aklujkar and Beatty 2005; Ng et al. 2011). In addition, carotenoids have also been applied as non-hazardous colorants in many fields, such as precursors of vitamin A, scavengers of active oxygen, enhancers of antibody production and functional supplements (Aksu and Eren 2005). Hence, producing carotenoids from PNSB treating non-toxic wastewater will be a green biotechnology that combines biosynthesis of valuable material and wastewater treatment.

In order to increase carotenoid yield of PNSB, many methods have been tried, including controlling and optimizing cultivation conditions, bioengineering, biochemical engineering and metabolic engineering (Chen et al. 2006; Reyes et al. 2014). As a necessary metal ion, Mg^{2+} affects PNSB growth by some important electrostatic interactions. In addition, Mg^{2+} also affects PNSB growth by regulating cellular osmotic pressure, redox processes and stabilization of intracellular macromolecules. These intracellular reactions above ensure regular PNSB growth (Hunter et al. 2008). Previous studies revealed that *Rhodopseudomonas*

faecalis growth and *Rhodobacter sphaeroides* growth could be significantly promoted by optimal Mg^{2+} concentration. Mg^{2+} plays a necessary physiological role in bacteria growth. Analysis showed that Mg^{2+} promoted photosynthetic bacteria growth by increasing pigments synthesis in the light-harvesting complexes (Liu et al. 2009; Hakobyan et al. 2012). Moreover, some studies demonstrated that Mg^{2+} of different concentrations affected the expression of distinct transport systems and interacted with ATPases in *Salmonella typhimurium* (Snavely et al. 1991; Maguire 1992; Tao et al. 1998). Another study showed that Mg^{2+} could affect carotenoid production in the protoplast fusion between *R. sphaeroides* and *Saccharomyces cerevisiae* by changing the intracellular photosynthetic reaction (Lu et al. 1999).

In carotenoid biosynthetic pathway of PNSB, *crt* genes (*crt EBICDFA*) encode different enzymes (Crt EBICDFA) which catalyze carotenoid biosynthesis. Hence, *crt* gene expression plays irreplaceable roles in carotenoid biosynthesis (Nishizaki et al. 2007; Ng et al. 2011; Takaichi 2009).

However, few studies about how Mg^{2+} affects carotenoid production of *R. sphaeroides* nor its further regulating metabolism in wastewater treatment can be found. This study aimed at examining the feasibility of Mg^{2+} addition on promoting the biomass accumulation and carotenoid production together with pollution control in *R. sphaeroides* wastewater treatment, optimizing Mg^{2+} concentration and investigating the potential mechanisms in view of the functional gene expression.

Materials and methods

Materials

A *R. sphaeroides* strain (ATCC17023) was obtained from China General Microbiological Culture Collection Center. It was cultured in a thermostat shaker (static, 30 °C) under light microaerobic conditions with PYG medium which consisted of 10 g/L polypeptone, 5 g/L yeast extract and 1 g/L glucose. The pH of PYG medium was 6.8–7.0. The logarithmic growth phase of *R. sphaeroides* began at 36 h, and the density of *R. sphaeroides* was 6.8×10^8 CFU/mL.

Sugar wastewater is a typical non-hazardous wastewater and can provide nutrients for *R. sphaeroides* growth. Artificial sugar wastewater was prepared by diluting molasses with adding ammonium chloride (0.5 g/L) and potassium dihydrogen phosphate (0.2 g/L). Its characteristics were as follows: Chemical oxygen demand (COD), total sugar, total nitrogen (TN) and total phosphorous (TP) were 5038, 4573, 153 and 34 mg/L, respectively. The initial pH was adjusted to 7.0.

Methods

Bioreactors were sterilized at 121 °C for 30 min before use. The experiment was carried out in batch fermentation. Wastewater (460 mL) and *R. sphaeroides* (40 mL) were added to the bioreactors. Light microaerobic conditions were provided for *R. sphaeroides* growth. The light intensity was 3000–6000 lux. Mg^{2+} of different concentrations (0, 1, 5, 10, 15 and 20 mmol/L) was added to the reactors.

Analysis methods

Sample collected from bioreactor was centrifuged at 9000 rpm for 10 min. The supernatant was used to test the COD, total sugar, TN and TP. The collected cells were used to measure the biomass and carotenoid. COD, biomass and TP were tested according to APHA standard methods (Clesscerl et al. 1998). Carotenoid was extracted first according to the following steps. The cells were washed twice with distilled water. The HCl-assisted extraction procedure was applied during the carotenoid extraction (Gu et al. 2008; Kuo et al. 2012). The carotenoid extractive was then dissolved in acetone and centrifuged (10,000 r/min, 20 min). The absorbance of the extractive was read by using a spectrophotometer at 480 nm, and the carotenoid yield was given by Eq. 1:

$$TC\ (\text{mg/g dry biomass}) = ADV/0.16\ \text{W} \quad (1)$$

where *TC* denotes carotenoid yield, *A* denotes the absorbance value of the diluted extractive at 480 nm, *D* denotes the dilution ratio, *V* denotes the volume of acetone, 0.16 denotes the extinction coefficient of carotenoid, and W (g) denotes the dry biomass.

Analysis of the expressions of *crt* genes

Total RNA extraction and cDNA synthesis

The total RNA extraction of *R. sphaeroides* was performed by using TRIzol reagent (Invitrogen Company) according to the manufacturer's protocol. After the purification of RNA extract with an RNeasy Mini Kit (Qiagen), total RNA was quantified at OD_{260} and OD_{280} with UV spectrophotometry (NanoDrop 2000, Thermo Scientific).

The purified RNA was used as template to generate cDNA by reverse transcription according to an AMV First Strand cDNA Synthesis Kit. The initial RT reaction system contained 1 μL total RNA, 0.2 μg random primer $p(dN)_6$ and 5 μL Rnase-free ddH_2O. They were denatured at 70 °C for 5 min and then cooled on ice for 10 s. Then reagents (4.0 μL 5 × reaction buffer, 20 nmol dNTP mix, 20 U Rnase inhibitor, 20 U AMV reverse transcriptase) were added to the reaction system. The reaction system (total

Table 1 Primers information used for RT-qPCR

Gene	Sequence		Amplicon size (bp)	Anneal temperature (°C)
	Forward primer (5′–3′)	Reverse primer(5′–3′)		
16S rRNA	CCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	196	60 60
crtE	CCTATCATCGGGCCAAGACC	CGATCTTCTCGGCATAGCGG	345	59.96 60.73
crtB	CTATTCCTTCCATGCCGCGT	GCAGATAGATCCGTCCCGAG	509	60.53 59.48
crtI	ATCGACAAGCTCGACGTTCC	CAGTAGCGCTTTTCGCTGTC	311	60.46 59.91
crtC	ACTTCCGTTTCTGGACCTGG	AATAGAAGGGCGCGTCGAG	252	59.60 59.93
crtD	GCGGCTTTATGACGCTTTCG	AGACGTTGTGATGGACGAGC	593	60.59 60.39
crtA	CCGAATCGCCGATCTACCAG	GCCCTCCCACTTACCAATCG	449	60.39 60.46
crtF	CATTCGGACGAGACGGTGAA	TGATCTCGGAAAAGCCCTGC	220	60.11 60.39

volume of 20 μL) was kept at 37 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. After the reaction, the products were stored at −20 °C for further analysis.

Primers and real-time quantitative reverse transcription PCR (RT-qPCR)

PCR primers (Table 1) were designed using Primer 5.0 and then were synthesized by Sangon Biotech.

RT-qPCR was performed with RNA extracts from samples (the growth time was 0, 12, 24, 36, 48, 60, 72 and 96 h at Mg^{2+} concentration of 0, 1, 5, 10, 15 and 20 mmol/L, respectively). Each RT-qPCR mixture (20 μL) consisted of 10 μL of SYBR Green qPCR Master Mix which consists of Taq DNA polymerase, dNTP, $MgCl_2$ and SYBR Green I dye, 1 μL forward primer, 1 μL reverse primer, 1 μL cDNA template and 7 μL Rnase-free ddH_2O. The RT-qPCR process was then carried out by an ABI Stepone Real-Time PCR System (Applied Biosystems, USA) under the following conditions: one cycle of 95 °C for 2 min and 40 cycles of 95 °C for 10 s, specific anneal temperature for 30 s and 60 °C for 40 s. Gene expression levels were assessed through Ct values. The induction ratio was calculated by method $2^{-\triangle\triangle Ct}$, and the relative transcript levels were calculated and normalized as described above (Shao et al. 2010; Li et al. 2014).

Statistical analysis

All experiments were conducted in triplicates, and the average values were reported. The significant differences were evaluated by one-way ANOVA in 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 pair-wise or individual (one-to-one) comparisons. And significant difference was considered at $p < 0.05$.

Results and discussion

Feasibility study of carotenoid production in *R. sphaeroides* wastewater treatment

Feasibility study of carotenoid production in *R. sphaeroides* wastewater treatment was set with 0 and 1 mmol/L Mg^{2+} addition. Biomass, COD removal and carotenoid yield in *R. sphaeroides* wastewater treatment were examined. In the feasibility test (Fig. 1), it was confirmed that carotenoid was produced and accumulated in *R. sphaeroides* ATCC17023 cells through the whole period. Extracellular carotenoid could hardly be detected. Therefore, in this study, only the intracellular carotenoid was tested at 96 h.

The results are shown in Fig. 1. Clearly it was feasible to achieve biomass and carotenoid production in *R. sphaeroides* wastewater treatment. In the control group without Mg^{2+}, the final biomass, COD removal and carotenoid yield were 2267 ± 109 mg/L, 72.1 ± 2.42 % and 2.49 ± 0.36 mg/g biomass, respectively. Moreover, the result in the group with 1 mmol/L Mg^{2+} showed that Mg^{2+} could improve COD removal, *R. sphaeroides* biomass accumulation and carotenoid production.

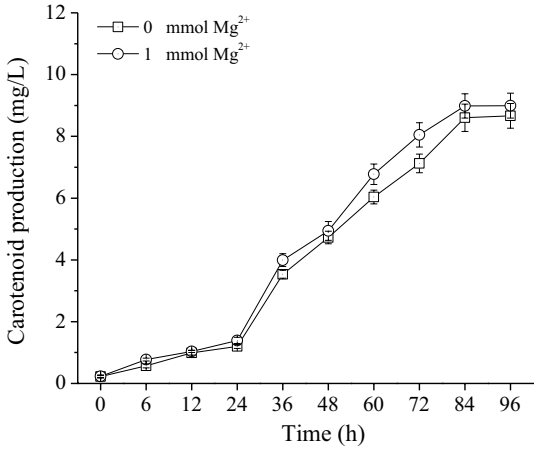

Fig. 1 Carotenoid production of the whole period in *Rhodobacter sphaeroides* wastewater treatment

Effect of Mg^{2+} concentration on biomass accumulation and carotenoid production in *R. sphaeroides* wastewater treatment

The effect of Mg^{2+} concentration on COD removal, biomass accumulation and carotenoid production was tested. The results are summarized in Figs. 2 and 3.

Effect of Mg^{2+} concentration on COD removal and biomass production

Biomass production of *R. sphaeroides* and COD removal under various Mg^{2+} concentrations indicate the efficiency of treating organic pollutants in wastewater treatment. As Fig. 2a shows, the COD removal was improved by adding Mg^{2+}. In all the experimental groups, the final COD removals were higher than that in control group after 96-h treatment. The highest and final COD removal of 91.9 ± 0.85 % was achieved with 15 mmol/L Mg^{2+} addition. It is also seen from Fig. 2a that the final COD removal increased along with increasing Mg^{2+} from 1 to 15 mmol/L. However, the COD removal displayed a decrease when Mg^{2+} concentration was increased from 15 to 20 mmol/L, but was still higher than the blank. This phenomenon showed that exceeding Mg^{2+} would slow down the enhancement on COD removal.

At the same time, Mg^{2+} addition could also increase the biomass production of *R. sphaeroides*. As Fig. 2b shows, the biomass production of *R. sphaeroides* reached a peak of 3900 ± 180 mg/L with 15 mmol/L Mg^{2+}. Mg^{2+} significantly improved PNSB growth. In previous studies, *R. sphaeroides* growth was improved by optimal 5 mmol/L Mg^{2+} (Hakobyan et al. 2012), and *Rhodopseudomonas faecalis* growth was stimulated at different levels through adding 1–15 mmol/L Mg^{2+} (Liu et al. 2009). Moreover,

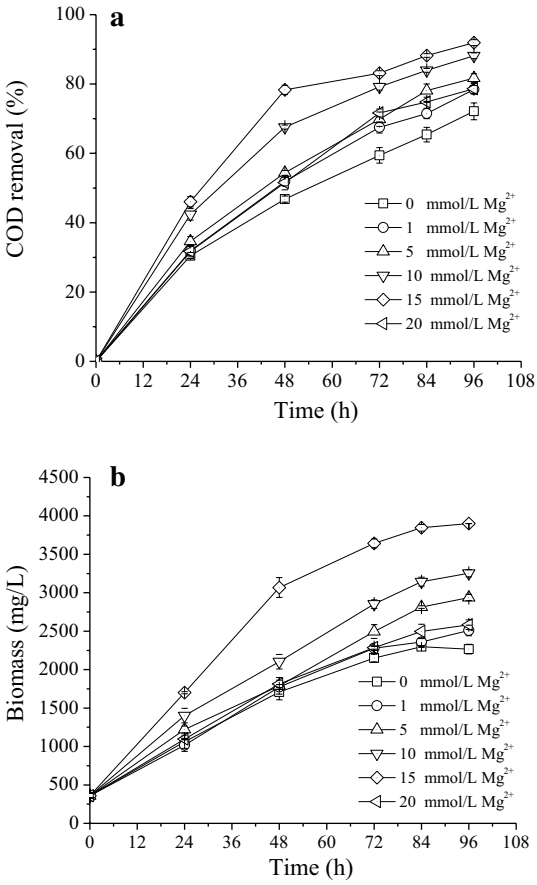

Fig. 2 Effect of Mg^{2+} concentration on biomass production and COD removal. **a** Biomass production, **b** COD removal. *Error bars* indicate the standard error on the basis of three independent experiments

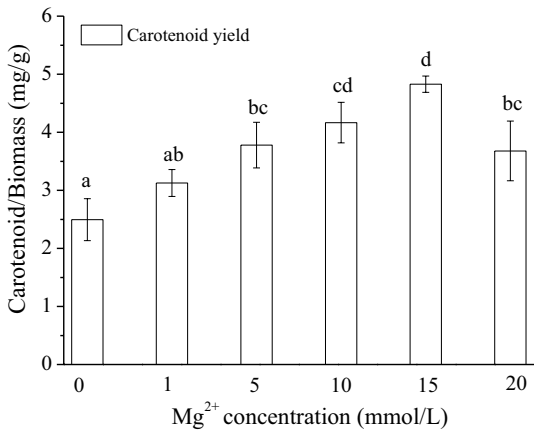

Fig. 3 Effect of Mg^{2+} concentration on carotenoid yield. *Error bars* indicate the standard error on the basis of three independent experiments

they believed that Mg^{2+} stimulated bacteria growth as one important nutrient element.

Effect of Mg^{2+} concentration on carotenoid yield

Mg^{2+} addition not only increased the biomass accumulation and COD removal in *R. sphaeroides* wastewater, but also enhanced the carotenoid yield of *R. sphaeroides* at the same time. Effects of different Mg^{2+} concentrations on carotenoid yield were investigated. Figure 3 shows that the carotenoid yields in experimental groups with 1–20 mmol/L Mg^{2+} were higher than that in the blank group. This indicated that Mg^{2+} addition of different concentrations could increase carotenoid yields at different degrees. The carotenoid yield reached the peak of 4.83 ± 0.14 mg/g biomass at 15 mmol/L Mg^{2+}. Furthermore, the results of DMRT analysis show that carotenoid yields increased differentially under varying Mg^{2+} concentrations in the following order: 15 mmol/L group ≥ 10 mmol/L group ≥ 5 mmol/L group = 20 mmol/L group ≥ 1 mmol/L group ≥ control. Hence, optimal Mg^{2+} content was at 15 mmol/L for the highest carotenoid yield.

Effect of Mg^{2+} concentration on expression of crt genes in R. sphaeroides wastewater treatment

To investigate the potential mechanism about how Mg^{2+} improves carotenoid yield, effects of Mg^{2+} on the expressions of *crt* genes in *R. sphaeroides* were explored. In the previous test (Fig. 4), it is found that *crt* gene expression displayed similar trends under different Mg^{2+} concentrations. The induction ratios of *crt* genes increased sharply in the first 60 h and then remained stable in the period of 60–72 h, and the corresponding values decreased after 72 h. So the induction ratios of *crt* genes were detected at 60 h in different groups. The results are summarized in Table 2.

As Table 2 shows, the *crtI* and *crtF* expressions under various Mg^{2+} concentrations could not be detected. The highest induction ratio (7.83 ± 0.41) of *crtE* was achieved in the blank group, and the corresponding values in the groups with 1–20 mmol/L Mg^{2+} were lower than that in blank. On the contrary, *crtB* expression reached the lowest level in blank, and the induction ratios of *crtB* increased with increasing Mg^{2+} concentrations. Further, the results of DMRT analysis showed that *crtB* expressions increased differentially under varying Mg^{2+} concentrations in the following order: 20 mmol/L group = 15 mmol/L group = 10 μmol/L group > 5 mmol/L group > 1 mmol/L group > control. At the same time, carotenoid yield was the highest in 15 mmol/L group. The possible reasons were that *crtE* expressions were inhibited by Mg^{2+} addition, but the *crtB* expressions were improved by Mg^{2+} addition. As Fig. 5 shows, *crtE* and *crtB* were important regulating genes and their transcriptional products had major influence on carotenoid biosynthesis (Nishizaki et al. 2007; Ng et al. 2011; Takaichi 2009). Up-regulating *crtE* and *crtB* would improve the initial reactions in carotenoid biosynthesis, which increased carotenoid yield. A previous study reported that the overexpression of *crtE* increased the carotenoid yield approximately by two folds when compared with the controls in *Rhodococcus erythropolis* AN12. But the overexpression of *crtB* and *crtI* did not have obvious effects on carotenoid yield in *R. erythropolis* AN12 (Brennan 2003). Expressions of *crtC* in various groups of 0–20 mmol/L Mg^{2+} did not display obvious differences. However, in *crtD* expressions, the induction ratios in the groups of 1–20 mmol/L Mg^{2+} were higher than that in control. During the sequential gene expression of carotenoid biosynthesis, *crtA* expression was later and it had important significant influence on the final carotenoid yield. As Table 2 shows, Mg^{2+} addition increased *crtA* expressions at different levels, and the highest induction ratio was 10.9 ± 0.24 with 15 mmol/L Mg^{2+}. DMRT analysis showed that *crtA* expressions increased differentially under varying Mg^{2+} concentrations in the following order: 15 mmol/L group > 20 mmol/L group ≥ 10 μmol/L group ≥ 5 mmol/L group ≥ 1 mmol/L group > control.

The regulation of crt genes based on Mg^{2+} in carotenoid biosynthetic pathway of R. sphaeroides

From Fig. 5, it was found that *crt* genes regulated the synthesis of different Crt protein products. So testing *crt* gene expression was beneficial for understanding potential metabolism at molecular level. A previous study reported that *crt*B and *crtI* gene expression had obvious effects on carotenoid biosynthesis in the *crt*B and *crtI* mutants of *R. sphaeroides* strains (Ng et al. 2011). However, *crtE* gene expression had prominent enhancement on carotenoid biosynthesis in *R. erythropolis* AN12 (Brennan 2003). The diversity might be explained that different *crt* genes are of crucial importance for effective carotenoid biosynthesis in different species. As Fig. 5 shows, carotenoid biosynthetic pathway of *R. sphaeroides* mainly included two stages: One was from diphosphate to phytoene, and the other was from phytoene to spheroidenone. This study (Table 2; Fig. 3) revealed that the expressions of latter gene (*crtB* or *crtD*, *A*) had more obvious effects on carotenoid yield than those of former genes (*crtE* or *crtC*) in sequential expression of genes. Hence, it was deduced that the enzymes from *crtBDA* gene expression might be significant regulators in carotenoid biosynthesis with Mg^{2+} addition in *R. sphaeroides*. In this way, carotenoid yield was dramatically improved, which accelerated resource utilization in *R. sphaeroides* wastewater treatment.

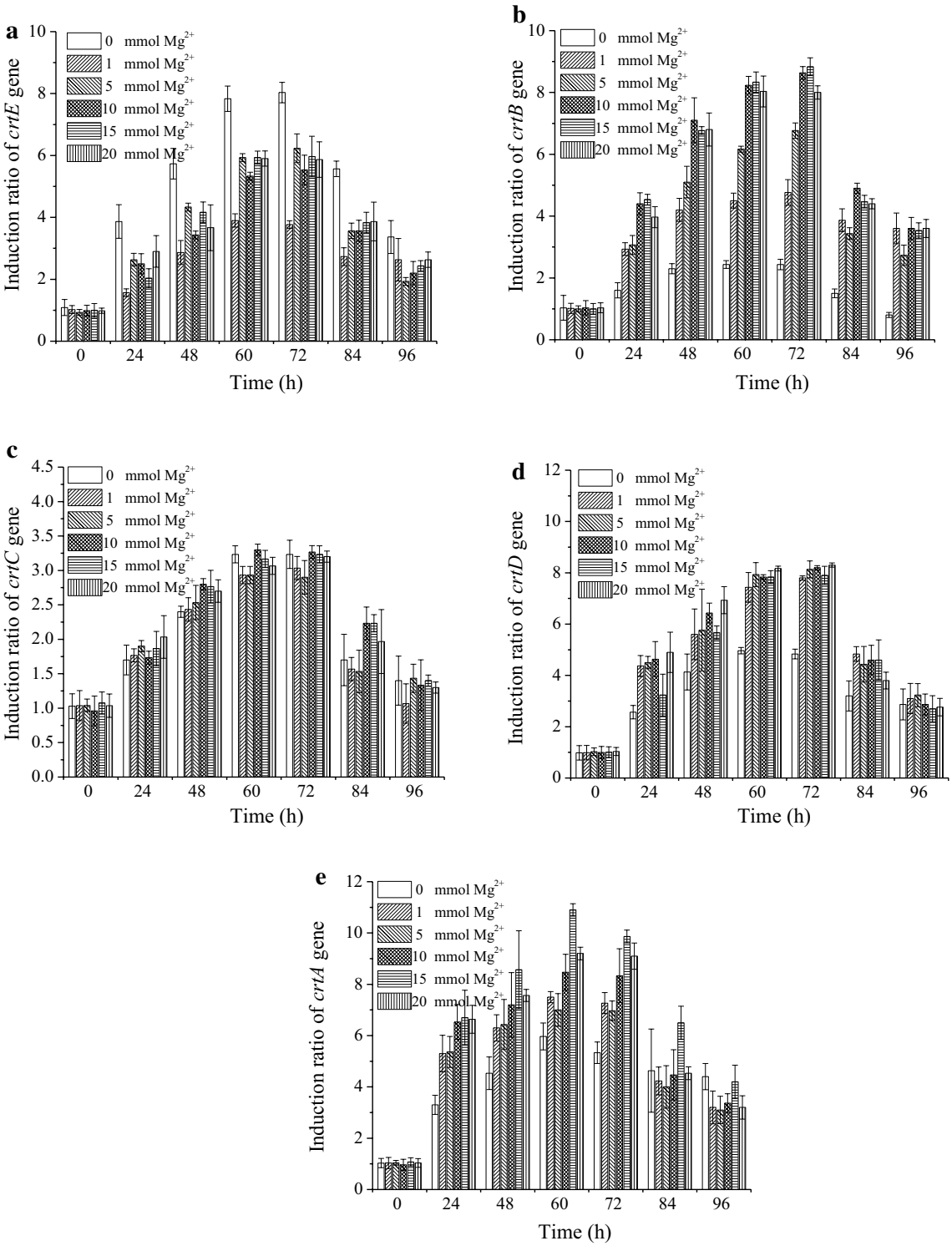

Fig. 4 Expression dynamics of *crt* genes under different Fe^{2+} concentrations in *Rhodobacter sphaeroides* wastewater treatment. **a** *crtE*, **b** *crtB*, **c** *crtC*, **d** *crtD*, **e** *crtA*. *Error bars* indicate the standard error on the basis of three independent experiments

Table 2 Expressions of *crt* genes under different Mg^{2+} concentrations at 60 h

Gene	Induction ratio (60 h) ± SD					
	0 mmol/L	1 mmol/L	5 mmol/L	10 mmol/L	15 mmol/L	20 mmol/L
crtB	7.83 ± 0.41 d	3.9 ± 0.22 a	5.93 ± 0.12 c	5.33 ± 0.12 b	5.93 ± 0.21 c	5.90 ± 0.24 c
crtE	2.43 ± 0.12 a	4.50 ± 0.24 b	6.17 ± 0.09 c	8.23 ± 0.29 d	8.33 ± 0.33 d	8.03 ± 0.50 d
crtI	–	–	–	–	–	–
crtC	3.23 ± 0.12 b	2.93 ± 0.12 a	2.93 ± 0.12 a	3.30 ± 0.08 b	3.17 ± 0.12 ab	3.07 ± 0.12 ab
crtD	4.97 ± 0.12 a	7.43 ± 0.58 b	7.93 ± 0.46 b	7.83 ± 0.09 b	7.83 ± 0.25 b	8.17 ± 0.09 b
crtA	5.96 ± 0.52 a	7.50 ± 0.22 bc	7.00 ± 0.64 b	8.47 ± 0.70 cd	10.9 ± 0.24 e	9.20 ± 0.24 d
crtF	–	–	–	–	–	–

–, Induction ratios in samples were not detected. Means sharing no common alphabet are significantly different ($p < 0.05$)

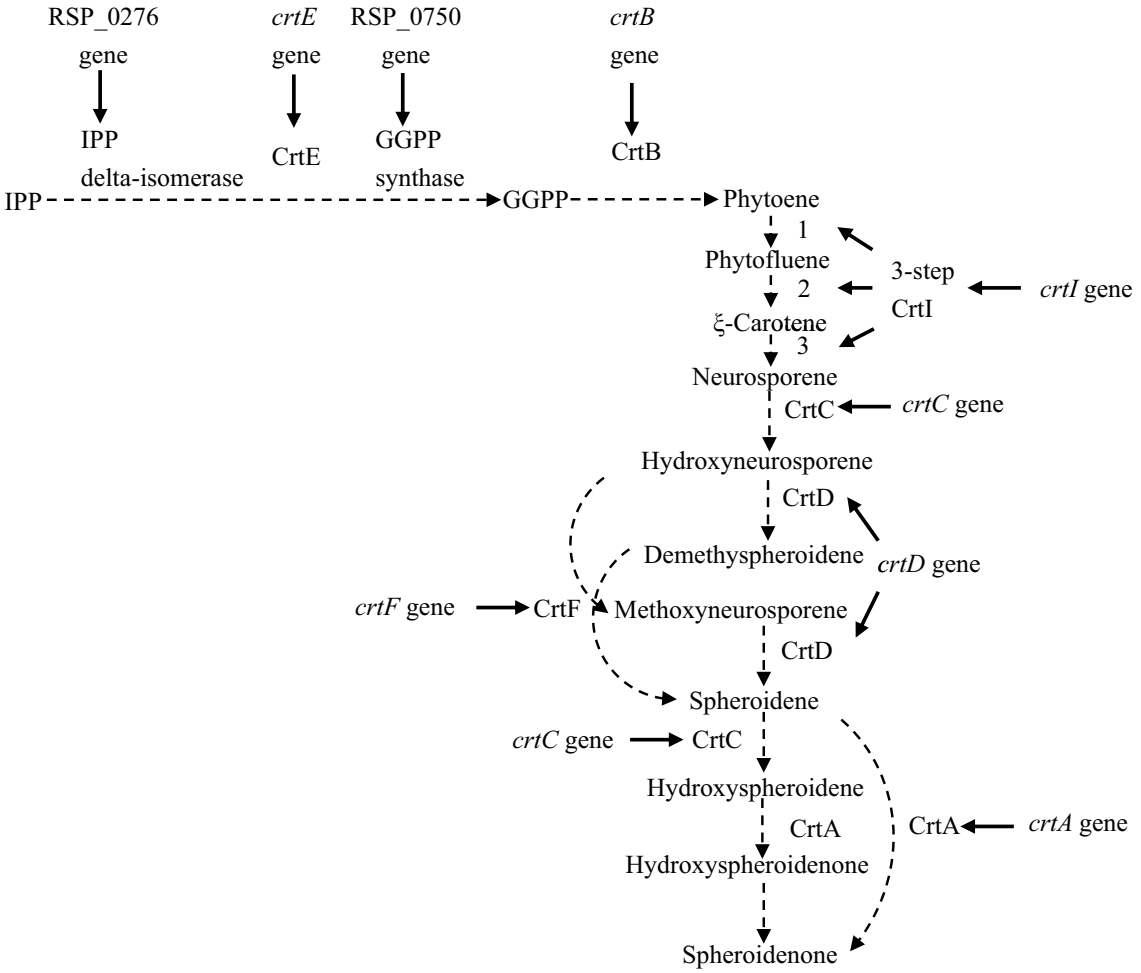

Fig. 5 Carotenoid biosynthetic pathway of *Rhodobacter sphaeroides*. This is a summary showing reactions, enzymes and regulators in the pathway based on references (Nishizaki et al. 2007; Ng et al. 2011; Takaichi 2009). Annotation: *IPP* isopentenyl pyrophosphate (diphosphate), *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate. Enzymes include (*1*) IPP delta-isomerase; (*2*) CrtE: FPP synthase; (*3*) GGPP synthase; (*4*) CrtB: phytoene synthase; (*5*) CrtI: phytoene desaturase; (*6*) CrtC: hydroxyneurosporene dehydrogenase; (*7*) CrtD: methoxyneurosporene dehydrogenase; (*8*) CrtF: hydroxyneurosporene-O-methyltransferase; (*9*) CrtA: spheroidene monooxygenase. Regulators include (*1*) RSP_0276 gene; (*2*) *crtE* gene; (*3*) RSP_0750 gene; (*4*) *crtB* gene; (*5*) *crtI* gene; (*6*) *crtC* gene; (*7*) *crtD* gene; (*8*) *crtF* gene; (*9*) *crtA* gene. *Dashed arrows* in the figure indicate the catalytic reactions by the nine enzymes above

Conclusions

This study showed that achieving valuable biomass and carotenoid from *R. sphaeroides* wastewater treatment was feasible. Optimal Mg^{2+} (15 mmol/L) could significantly improve biomass production, COD removal and carotenoid yield, respectively. Mechanism analysis showed that Mg^{2+} addition could increase carotenoid yield by regulating the expressions *of crt* genes. Genes *crtBDA* were up-regulated by optimal Mg^{2+} addition. This technology could promote the carotenoid production and improve the resource utilization in *R. sphaeroides* wastewater treatment.

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