Available online at www.sciencedirect.com

ScienceDirect

www.journals.elsevier.com/journal-of-environmental-sciences

JOURNAL OF ENVIRONMENTAL SCIENCES
www.jesc.ac.cn

Enhancement of *Rhodobacter sphaeroides* growth and carotenoid production through biostimulation

Shuli Liu[1], *Guangming Zhang*[1,2,*], *Xiangkun Li*[1], *Pan Wu*[1], *Jie Zhang*[1]

1. *School of Municipal and Environmental Engineering, Harbin Institute of Technology, Harbin 150090, China. Email: liushuli117288@sina.com*
2. *School of Environment and Resource, Renmin University of China, Beijing 100872, China*

ARTICLE INFO

Article history:
Received 12 October 2014
Revised 14 January 2015
Accepted 15 January 2015
Available online 14 April 2015

Keywords:
Biostimulant
Rhodobacter sphaeroides
Biomass
Carotenoid yield

ABSTRACT

Bacillus thuringiensis/cereus L2 was added as a biostimulant to enhance the biomass accumulation and carotenoid yield of *Rhodobacter sphaeroides* using wastewater as the culturing medium. Results showed that biostimulation could significantly enhance the *R. sphaeroides* biomass production and carotenoid yield. The optimal biostimulant proportion was 40 μL (about 6.4×10^5 CFU). Through the use of biostimulation, chemical oxygen demand removal, *R. sphaeroides* biomass production, carotenoid concentration, and carotenoid yield were improved by 178%, 67%, 214%, and 70%, respectively. Theoretical analysis revealed that there were two possible reasons for such increases. One was that biostimulation enhanced the *R. sphaeroides* wastewater treatment efficiency. The other was that biostimulation significantly decreased the peroxidase activity in *R. sphaeroides*. The results showed that the highest peroxidase activity dropped by 87% and the induction ratio of the *RSP_3419* gene was 3.1 with the addition of biostimulant. The enhanced carotenoid yield in *R. sphaeroides* could thus be explained by a decrease in peroxidase activity.

Introduction

Carotenoids are fat-soluble pigments that can exhibit an array of colors, such as red, yellow, orange, and purple. They are important antioxidants and active components of light-harvesting complexes in photosynthetic organisms, including bacteria, algae, and plants (Han et al., 2012; Sheehan et al., 2012). They perform different functions to protect against destructive photo-oxidation. Carotenoids have attracted intense attention because they may provide protection against cancer and heart disease (Aklujkar and Beatty, 2005; Ng et al., 2011). Furthermore, carotenoids have also been applied as non-hazardous colorants in many fields, precursors of vitamin A, scavengers of active oxygen, enhancers of antibody production, and functional supplements (Aksu and Eren, 2005; Sandmann, 2001). So it is desirable to produce more carotenoids. Currently, the production of carotenoids is mostly carried out by chemical synthetic methods. However, chemical synthetic methods have many disadvantages, including high cost and toxicity. Alternative biosynthesis by carotenoid-producing microorganisms such as *Rhodobacter sphaeroides* has attracted intensive study, because it has the advantages of relatively high carotenoid yield, negligible toxicity, and low cost (Chen et al., 2006; Kuo et al., 2012; Ng et al., 2011).

R. sphaeroides are species of photosynthetic bacteria that are non-toxic and rich in single-cell proteins. Furthermore, they contain high-value materials, including bacteriochlorophylls, biopolymers, 5-aminolevulinic acid, carotenoids, CoQ_{10}, *etc.* (Jeya et al., 2010; Kien et al., 2010; Kuo et al., 2012; Yen and Shih, 2009). *R. sphaeroides* are widely used as commercial aqua-culturing feed and chicken supplements, and they have potential application in pharmaceutical and food industries (Sasaki et al., 2005;

* *Corresponding author*. E-mail: zgm@ruc.edu.cn (Guangming Zhang).

http://dx.doi.org/10.1016/j.jes.2015.01.005

Tangprasittipap and Prasertsan, 2002). Furthermore, *R. sphaeroides*, which can utilize organic substances in wastewaters to form biomass and synthesize pigments, have shown great potential in treating various types of wastewaters (Lu et al., 2011; Wu et al., 2012). Therefore, *R. sphaeroides* biomass and carotenoids may be accumulated by using non-hazardous organic wastewater as the culturing medium. The carotenoid yields of *R. sphaeroides* have been further increased by optimizing the medium (Chen et al., 2006) or improving the carotenoid extraction conditions (Gu et al., 2008). However, these studies were all implemented in specific mediums rather than wastewater.

Various technologies have been tested to enhance the production of valuable substances in microorganisms, including optimizing the cultivation conditions, bioengineering, biochemical engineering, metabolic engineering, and biostimulant addition (Chen et al., 2006; Reyes et al., 2014). A biostimulant refers to chemicals or microorganisms that can stimulate the growth of native microorganisms or increase the secretion of intracellular materials. Biostimulant addition has achieved favorable results in various studies (Gopinath and Sims, 2011; Tyagi et al., 2011). Therefore, it is highly possible that biostimulation might significantly increase the carotenoid yield in *R. sphaeroides* while using wastewater as the culturing medium.

The purpose of this study was to increase the *R. sphaeroides* biomass and carotenoid production in wastewater treatment through biostimulation. The results might provide a potential approach for economical and sustainable *R. sphaeroides* production and carotenoid synthesis.

1. Materials and methods

1.1. *R. sphaeroides* strain and biostimulant

A *R. sphaeroides* strain (ATCC17023) was obtained from the China General Microbiological Culture Collection Center. It was cultured in a thermostatted shaker (static, 30°C) under light-micro aerobic conditions with peptone-yeast extract-glucose medium medium, which consisted of 10 g/L polypepton, 5 g/L yeast extract and 1 g/L glucose. The pH of the PYG medium was adjusted to 6.8–7.0.

The selected biostimulant was a bacterial strain that was obtained from a photosynthetic wastewater treatment process with mixed culture (Zhao and Zhang, 2014). In six isolated and purified strains, one strain was found to effectively stimulate the growth of *R. sphaeroides* and degradation of pollutants. The strain was identified as *Bacillus thuringiensis/cereus* by 16 S rDNA sequencing (accession number is KM076927 in GenBank), and it was named L2. *R. sphaeroides* and L2 were in their logarithmic growth phase when they were used for experimentation. The densities of *R. sphaeroides* and L2 were 6.8×10^8 and 1.6×10^7 CFU/mL, respectively.

1.2. Wastewater

Synthetic peptone wastewater was used, which contained 10 g/L polypepton, 5 g/L yeast extract and 1 g/L magnesium sulfate heptahydrate. The synthetic wastewater was similar to the actual soybean wastewater, which contained abundant protein nutrients (Wu et al., 2012). The main characteristics of the wastewater were as follows: pH was around 7.0, and chemical oxygen demand (COD), total nitrogen (TN), and total phosphorous (TP) were 12,502, 916, and 34 mg/L, respectively. The wastewater was used for the experiment after filtration and autoclaving in order to avoid environmental contamination (121°C, 30 min).

2. Experimental

Bioreactors were 250 mL glass flasks and were sterilized at 121°C for 30 min before use. 92 mL of wastewater and 8 mL of *R. sphaeroides* were added into each bioreactor. L2 was then added as the biostimulant. The volume of L2 was 0, 5, 10, 20, 40 and 80 μL for the control and experimental groups. The corresponding L2/*R. sphaeroides* ratio was 1/1600, 1/800, 1/400, 1/200 and 1/100 (V/V) in the five experimental groups, respectively. The dosage of biostimulant addition was selected following previous studies and the ratio was based on previous trial tests (Appendix A Fig. S1). The initial weight of *R. sphaeroides* was 360 mg/L and the L2 added was 0.075–1.2 mg/L.

The bioreactors were in static cultivation at a temperature of 30 ± 1°C under sterile conditions. The light intensity was 1500 lx, and the dissolved oxygen was kept within 0.5–1.0 mg/L (Lu et al., 2011).

To ensure the validity of experimental results, control experiments were performed. A positive control without L2 and a negative control group without L2 or *R. sphaeroides* were run in parallel to the experimental set.

2.1. Analysis methods

2.1.1. Measurement of water quality indexes

All water quality indexes were measured following APHA standard methods (Clesscerl et al., 1998). The supernatant was used for COD, TP and TN tests, and was obtained from the reaction zone after centrifugation (9000 r/min, 10 min). The cells were used for biomass, carotenoid, and peroxidase activity tests.

2.1.2. Measurement of biomass

A microscope blood counting chamber was used to calculate the number of bacteria. The total biomass (*R. sphaeroides* and L2) was measured by dry weight (103°C, overnight). The *R. sphaeroides* biomass was obtained following the method of Zhao and Zhang (2014), using an Agilent 1200 HPLC (Agilent Technologies, Inc., Santa Clara, CA, USA). Due to the constant ratio of CoQ_{10} in *R. sphaeroides*, the CoQ_{10} concentration was measured to represent *R. sphaeroides* concentration. The CoQ_{10} concentration was measured by HPLC under certain chromatographic conditions (C18 column, 10 cm × 4. 6 mm ID; mobile phase, methanol–ethanol (V/V: 9/1); flow rate: 1 mL/min; column temperature, 35°C; wavelength of UV detector, 275 nm; sample size, 15 μL; column pressure, 16.4 MPa). The *R. sphaeroides* concentration was calculated by Eq. (1):

$$\rho = 1000(Y + 37.487)/25.431 \quad (1)$$

where, ρ (mg/L) denotes *R. sphaeroides* concentration, Y denotes the peak area.

2.1.3. Extraction and determination of carotenoids

The bacteria cells in the bioreactors were harvested by centrifugation (10,000 r/min, 20 min) and were used for the carotenoid concentration test. The cells were washed twice with distilled water. The HCl-assisted extraction procedure was applied during the carotenoid extraction (Gu et al., 2008). The carotenoid extract was then dissolved in acetone and centrifuged (10,000 r/min, 20 min). The carotenoid content was measured through determining the absorbance of the acetone supernatant by a UV–Vis spectrophotometer (T6, Chuangyu Technology Inc., Shanghai, China) at 480 nm. At a proper diluted concentration, the total carotenoid yield (mg/g dry biomass) was calculated by Eq. (2):

$$TC = ADV/0.16W \quad (2)$$

where, TC (mg/g dry biomass) denotes the total carotenoid yield, *A* denotes the absorbance value of the diluted extractant at 480 nm, *D* denotes the dilution ratio, *V* denotes the volume of acetone, 0.16 denotes the extinction coefficient of the carotenoids, and *W* (g) denotes the dry biomass (Gu et al., 2008; Kuo et al., 2012).

2.1.4. Peroxidase activity assay

The bacteria cells were harvested by centrifugation (4000 r/min, 10 min) after removing the supernatant to determine the peroxidase activity. The cells were washed twice with phosphate buffer solution. The enzyme extraction was carried out by ultrasonication (JY88-II, Scientz Biotechnology Com., Ningbo, China). The cell suspension in phosphate buffer solution was subjected to ultrasonication in an ice bath according to the following conditions: 45 kHz, 280 W, 220 V, ultrasound time 2 sec, ultrasound interval 10 sec, and 60 repetitions in continuous operation as described above. The suspension, after ultrasonication, was centrifuged at 4000 r/min for 10 min, and the supernatant was the enzyme liquid. The enzyme liquid, or its diluted supernatant, was kept at 4°C for peroxidase activity detection. In the peroxidase activity detection test, the reaction mixture contained 1.68 μL guaiacol, 1.14 μL H_2O_2 (30%), and 1 ml enzyme liquid. The mixture was then adjusted to 4 mL with the addition of 0.1 mol/L phosphate buffer (pH 7.0). The absorbance was measured at 470 nm every 60 sec. One unit of enzyme was defined as the amount of enzyme that was able to catalyze the oxidation of 1 μmol of guaiacol per min (U/(g·min)) (the value of $0.01 \triangle A_{470}$ in 1 min) at 30°C.

$$\text{Peroxidase activity} = \Delta A_{470} \times V_T \times B/(W \times V_S \times 0.01 \times t). \quad (3)$$

In the preceding equation, ΔA_{470} represents the change in the absorbance value of the enzyme liquid at 470 nm, V_T represents the total volume of the extracted enzyme liquid, *B* represents the diluted value of the enzyme liquid, V_S represents the detected volume of the extracted enzyme liquid, and *t* (min) represents the reaction time (Kalin et al, 2014).

2.1.5. Analysis of RSP_3419 gene expression

The expression analysis procedure of the *RSP_3419* gene coding a putative peroxidase-related enzyme included total RNA extraction, cDNA synthesis, and real-time quantitative reverse transcription-PCR (RT-qPCR). The cells were collected after centrifugation, and RNA extraction was performed by a TRIzol reagent (Invitrogen Company, Shanghai, China) according to the manufacturer's protocol. The RNA extracts were purified with an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was then quantified at OD_{260} and OD_{280} with UV spectrophotometry (NanoDrop 2000, Thermo scientific Inc., Waltham, MA, USA). The purified RNA was then stored at −80°C for further analysis.

The cDNA synthesis was carried out according to an AMV First Strand cDNA Synthesis Kit. The RT reaction system (l μL total RNA, 0.2 μg Random Primer $p(dN)_6$ (0.2 μg), 5 μL Rnase-free ddH_2O) was denatured at 70°C for 5 min and then cooled on ice for 10 sec. Next, 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, which (total volume of 20 μL) was kept at 37°C for 5 min, 42°C for 60 min, and 70°C for 10 min.

The target gene was amplified using primers, which were designed from the *RSP_3419* gene sequence in *R. sphaeroides* ATCC17023 by Primer 5.0. The primers were as follows: the forward primer (5′–3′) was GGATATCTGGGACZTCGCCG, the reverse primer (5′–3′) was TGTCTGACAGAACGGCAACC, and the primers of the reference genes were 16S-F (5′–3′), CCTACGG GAGGCAGCAG; 16S-R (5′–3′), ATTACCGCGGCTGCTGG. Each RT-qPCR mixture (20 μL) consisted of 10 μL of SYBR Green qPCR Master Mix, which consisted 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 performed by an ABI Stepone Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: one cycle of 95°C for 2 min and 40 cycles of 95°C for 10 sec, specific anneal temperature for 30 sec and 60°C for 40 sec. Gene expression levels were assessed through threshold cycle (Ct) values. The induction ratio was calculated by the $2^{-\triangle\triangle Ct}$ method, and the relative transcript levels were calculated and normalized as described previously (Li et al., 2014; Shao et al., 2010).

All experiments above were performed in triplicate, and the reported results are the averaged values. Statistical one-way analysis of variance (ANOVA) was used to test the significance among effects of biostimulant addition on carotenoid yield, peroxidase activity and *RSP_3419* gene expression, respectively ($p < 0.05$).

3. Results and discussion

The *R. sphaeroides* biomass accounted for more than 95% of the total biomass in all experimental runs.

3.1. Effects of biostimulation on the wastewater treatment efficiency

In order to investigate the effects of biostimulation on *R. sphaeroides* wastewater treatment efficiency, changes of COD, TN and TP concentrations were detected. As Fig. 1A shows, the wastewater COD removal was only 20.3% after 96 hr in the control group. Biostimulant addition at various dosages all increased the COD removal, the optimal dosage was 40 μL, and the corresponding COD removal was 56.5%, showing a

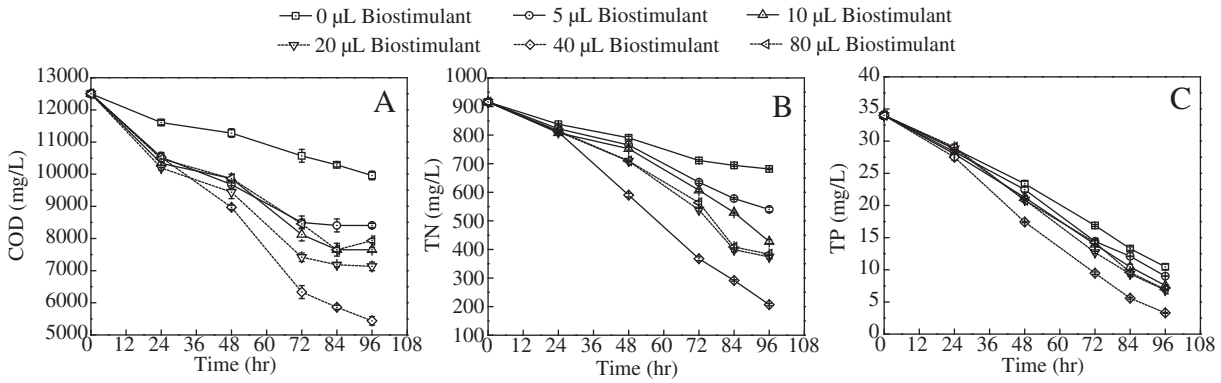

Fig. 1 – Effects of biostimulant (*Bacillus thuringiensis/cereus* L2) addition on the removals of chemical oxygen demand (COD) (A), total nitrogen (TN) (B), and total phosphorous (TP) (C) by *Rhodobacter sphaeroides*.

178% increase compared to the control. In addition, TN removal was enhanced by different biostimulant dosages. Fig. 1B indicates that the TN removals were 41.0%, 53.3%, 59.2%, 77.4% and 58.1% in the experimental groups, showing 60.3%, 108.3%, 131.1%, 202.3% and 127.1% increase compared to the control (25.6%). In Fig. 1C, it can be seen that TP removals followed a similar trend as TN; the TP removals in the experimental groups were all higher than that in the control. Moreover, TN and TP removals both reached the highest values, as COD removal did as well, when the optimal dosage was 40 μL. Clearly, biostimulant addition significantly benefitted *R. sphaeroides* wastewater treatment.

3.2. Effects of biostimulation on biomass production and intracellular carotenoid yield

Fig. 1 shows that the biostimulant improved the wastewater treatment, meaning that the increasing degradation of COD, TN and TP might provide more substrates for *R. sphaeroides* growth and intracellular carotenoid biosynthesis. To clarify the effects of biostimulation on biomass production and intracellular carotenoid yield of *R. sphaeroides*, the biomass production and carotenoid concentration were assessed. Fig. 2A shows that the optimal biostimulant dosage was still 40 μL, and the corresponding *R. sphaeroides* concentration was 3848 mg/L, showing a 67% increase over that of the control (2302 mg/L).

The carotenoid production was further investigated. Results showed that biostimulation greatly enhanced the carotenoid production, and the optimal dosage of biostimulant was 40 μL (Fig. 2B). The corresponding carotenoid concentration reached 11.8 mg/L, which was increased by 214% in comparison to the control.

Further analysis of the carotenoid yield in *R. sphaeroides* was carried out. The carotenoid yield was defined as the content of carotenoids per unit of biomass, and the unit was mg/g biomass. A high carotenoid yield (3.24 mg/g biomass) meant that the bacterial cells were suitable for carotenoid synthesis (Fig. 2C). For the control group, the final carotenoid yield was only 1.90 mg/g biomass. In the experimental groups, the carotenoid yields increased with increasing biostimulant dosage when the added biostimulant was less than 40 μL. In contrast, a decrease was shown when the added biostimulant was 80 μL.

The results above were related to the ratio of *R. sphaeroides* in total biomass. As Appendix A Fig. S1 shows, the ratio of *R. sphaeroides* in total biomass was more than 95% when the added biostimulant was not more than 80 μL, which demonstrated that *R. sphaeroides* was dominant in the wastewater treatment systems. The carotenoids in the system were only synthesized by *R. sphaeroides* since the biostimulant could not produce carotenoids. So the carotenoid yields were relatively higher in the systems containing high levels of *R. sphaeroides* (Fig. 2C). However the ratio of *R. sphaeroides* in total biomass sharply decreased to 80% when the added biostimulant was increased to 120 μL (>80 μL), which exceeded the proper range (80 μL). The ratio of *R. sphaeroides* in total biomass reached a peak when the biostimulant dosage was 40 μL in Appendix A Fig. S1. *R. sphaeroides* gradually lost its dominance when biostimulant exceeded 40 μL. Therefore, the preferred biostimulant addition was 40 μL.

3.3. Effects of biostimulation on peroxidase activity and RSP_3419 gene expression in *R. sphaeroides*

One determinant factor of intracellular carotenoid content is whether carotenoids are degraded or not (Takaichi, 2008). As pigments, carotenoids are easily degraded by various oxidases. High oxidase activity may lead to more degradation of carotenoids and thus results in a low carotenoid yield in bacterial cells. Among various oxidases, peroxidase can catalyze the hydrogen peroxide-dependent oxidation of wide variety of substrates, and it exists in most plants and microorganisms (Kalin et al., 2014). Moreover the peroxidase system is mostly coded by the *RSP_3419* gene, and this gene is widely regarded as the target gene for encoding peroxidase in *R. sphaeroides* ATCC17023. This enzyme is an antioxidant protein with alkyl hydroperoxidase activity, and it has molecular functions including acting as an antioxidant, lyase, oxidoreductase and peroxidase. So it is widely used as an important and typical oxidase. The possible interactive relationship among *R. sphaeroides*, biostimulant (*B. thuringiensis/cereus* L2), carotenoids, peroxidase and *RSP_3419* gene is summarized in Fig. 3 (Kalin et al., 2014; Ng et al., 2011; Takaichi, 2008).

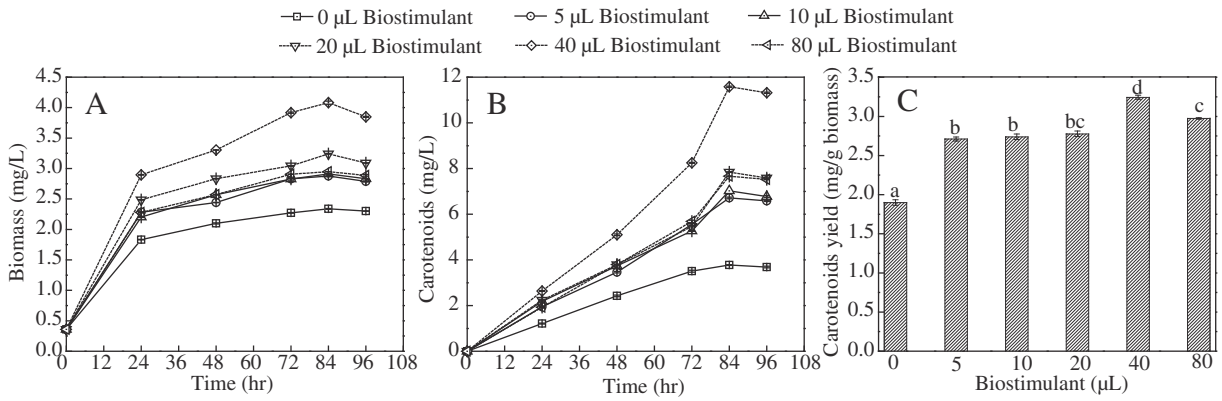

Fig. 2 – Effects of biostimulant (*Bacillus thuringiensis/cereus* L2) addition on biomass production (A) carotenoids production (B) and carotenoids yield (C) by *Rhodobacter sphaeroides*. Bar represents the standard error of means for replicates. Means sharing no common alphabet (above bar ± s.e.m.) are significantly different ($p < 0.05$).

Therefore, in order to investigate the mechanism of biostimulation effects on carotenoid yield of *R. sphaeroides*, the peroxidase activity, the *RSP_3419* gene expression change and the induction ratios of *RSP_3419* gene were evaluated under different biostimulant additions. The peroxidase activity in different systems was measured when the carotenoid yields reached their peak. As Fig. 4A shows, the peroxidase activity in the control was higher than in the experimental groups, and peroxidase activity values decreased with increasing biostimulant dosage. The control group had a peroxidase activity of 3258 U/(g·min), while the 40 μL group had a peroxidase activity of 408 U/(g·min), showing a 87% decrease. The lower the peroxidase activity was, the less carotenoids were degraded by oxidization in bacterial cells. Thus, Fig. 4A demonstrates that the addition of biostimulant enhanced the carotenoid yield by decreasing the peroxidase activity in *R. sphaeroides* so that fewer carotenoids were degraded by peroxidase.

The results (Fig. 4B) show that the induction ratios of the *RSP_3419* gene were 2.73, 3.16, 2.99, 3.09 and 2.30 in the experimental groups, while the value was defined as 1.00 in the control. The induction ratios of the *RSP_3419* gene in the experimental groups all increased compared to that of the control, and there was a significant difference between the control group and the experimental groups. The *RSP_3419* gene expressions were in the following order: 40 μL group = 10 μL group ≥ 20 μL group ≥ 5 μL group > 80 μL group > control. This demonstrated that adding biostimulant enhanced positive up-regulation of the target gene expression at the transcriptional level.

3.4. *Theoretical analysis on biostimulation increased biomass and carotenoid yield in R. sphaeroides*

In this study, added biostimulant resulted in improved wastewater treatment efficiency and high carotenoid yield. Figs. 1 and 2 demonstrate that COD, TN, TP removals were enhanced and *R. sphaeroides* biomass and carotenoid yield were obviously improved at the optimal biostimulant dosage of 40 μL. The COD removal and biomass were improved by 178% and 67% compared to the control when the biostimulant dosage was 40 μL. The carotenoid yield reached a peak of

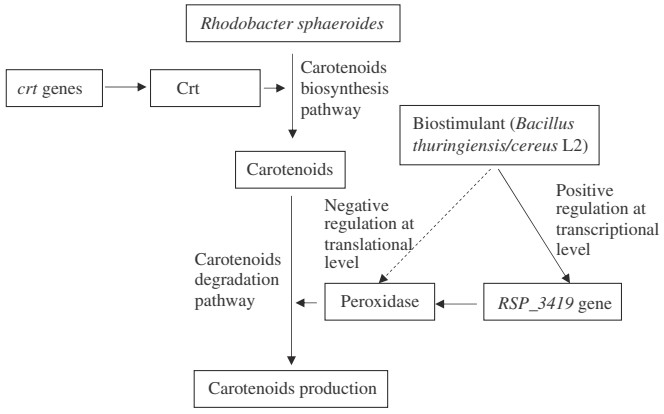

Fig. 3 – Interactive relationship among *Rhodobacter sphaeroides*, biostimulant (Bacillus thuringiensis/cereus L2), carotenoids, peroxidase and *RSP_3419* gene. Abbreviations: crt genes: crt EBICDAF genes; Crt synthases: CrtE; CrtB; CrtI; CrtC; CrtD; CrtA; CrtF. The process that the dashed line indicated was deduced by the results of the present study.
Sources: Kalin et al., 2014; Ng et al., 2011; Takaichi, 2008.

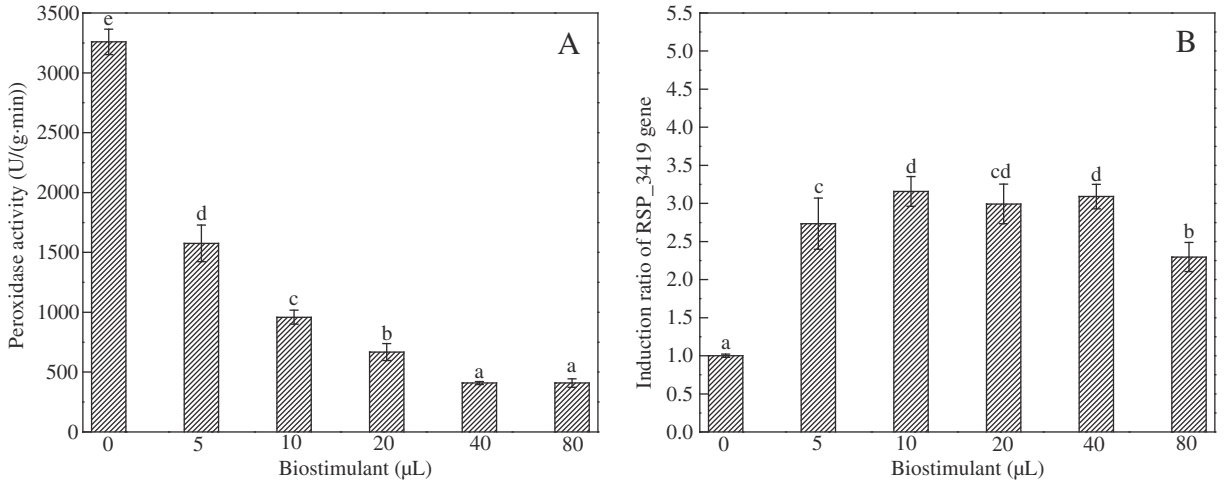

Fig. 4 – Effects of biostimulant (*Bacillus thuringiensis/cereus* L2) addition on peroxidase activity (A) and *RSP_3419* gene expression (B) by *Rhodobacter sphaeroides*. Bar represents the standard error of means for replicates. Means sharing no common alphabet (above bar ± s.e.m.) are significantly different ($p < 0.05$).

3.24 mg/g biomass and was improved by 70% compared to the control, which was comparable with previous reports (Gu et al., 2008; Kuo et al., 2012).

So far there has been no study on increasing the biomass and carotenoid yield of *R. sphaeroides* by biostimulation in wastewater treatment. However, in previous studies (El Fantroussi and Agathos, 2005; Gopinath and Sims, 2011; Li et al., 2009), the addition of a pre-adapted pure bacterial strain, a pre-adapted consortium, or the introduction of a genetically engineered bacteria all could significantly enhance the pollutant degradation rate of native microbes in the soil. In particular, adding the consortium demonstrated an effective pollutant degradation rate for optimal proportions of different microbials in the consortium (Li et al., 2009). These studies showed that the added biostimulant (bacterial strains) could enhance the native microbes' growth as well as their specific physiological functions. The optimal dosage of biostimulant addition varied in different studies.

Simultaneously, the possible mechanism by which a biostimulant increases the biomass and carotenoid yield of *R. sphaeroides* has been analyzed. Some studies revealed that in the co-culture systems, adding bacteria (biostimulant) could intensify the function of the initial bacteria by providing substrates, secreting enzymes, and changing the metabolic pathways of the initial bacteria (Ding et al., 2009; Fang et al., 2006; Kawaguchi et al., 2001; Kirschbaum et al., 2010; Miura et al., 1992; Warthmann et al., 1992). Based on previous literature and results in this study (Figs. 1 and 2), it is proposed that providing substrates may be the reason that biostimulants enhance the bacterial growth and pigment content. Miura et al. (1992) reported that *Chlamydomas* sp. MGA161 could provide more acetic acid and ethanol as electron donors for hydrogen production for the photosynthetic bacterium W-1S. Warthmann et al. (1992) demonstrated that coupling the acetate-oxidizing and the sulfur-reducing-oxidizing cycles enhanced the hydrogen production of green sulfur bacteria. Kawaguchi et al. (2001) found that the hydrogen production of *Rhodobium marinum* A-501 increased, because *Lactobacillus amylovorus* promoted amylase activity to produce lactic acid as an electron donor. THE studies OF Fang et al.(2006) and Ding et al. (2009) showed that photosynthetic bacteria syntrophically interacted with *Clostridium butyricum*, using acetate and butyrate for hydrogen production. Kirschbaum et al. (2010) found that high levels of epithelial mRNA of IL-6 and IL-8 were detectable in the mixture of periodontopathogenic bacteria and KB cells. Similarly, the biostimulant in this study could enhance the pollutant (COD, TN and TP) degradation by *R. sphaeroides* and produce more substrates for the biomass production and carotenoid biosynthesis by interspecific reaction. The biostimulant dosage in this study was very low (1/1600-1/100), so most substrates were used for *R. sphaeroides* growth and carotenoid biosynthesis.

The other possible mechanism was that biostimulant could bring about an alteration of enzyme activity or gene expression in *R. sphaeroides*, based on Fig. 4. Fig. 4A shows that the peroxidase activity was decreased by 87% at optimal biostimulant dosage. This indicated that added biostimulant could inhibit peroxidase activity to increase carotenoid yield indirectly. The more peroxidase activity was decreased, the fewer carotenoids were degraded, thus the carotenoid yield became higher.

Kirschbaum et al. (2010) found that high levels of epithelial mRNA of IL-6 and IL-8 were detectable in a mixture of periodontopathogenic bacteria and KB cells. This result indicated that the functional gene expression of the initial bacteria would change in a co-culture system. Fig. 4B shows that the induction ratios of the *RSP_3419* gene were increased by adding biostimulant, but carotenoids were degraded by peroxidase and were not directly determined by *RSP_3419* gene expression, though the *RSP_3419* gene coded peroxidase. As a result, it was found that the peroxidase activity was negatively correlated with the increasing target gene expression (transcriptional level), and it decreased with increasing biostimulant addition. This indicated that the added biostimulant probably induced negative regulation of enzymatic protein synthesis of peroxidase at the translational level, which is described by the process (dashed line) in Fig. 3.

4. Conclusions

In *R. sphaeroides* wastewater treatment, a biostimulant could successfully enhance the wastewater treatment efficiency, biomass production and carotenoids yield, which certainly improved biomass and carotenoid yield. The optimal biostimulant dosage was 40 μL, and the corresponding biomass production and carotenoid yield were 3848 mg/L and 3245 mg/g-biomass, increasing by 67% and 70%, respectively. Theoretical analysis demonstrated that the increasing carotenoid yield was caused by decreasing peroxidase activity. Such changes caused by peroxidase activity and *RSP_3419* gene expression through biostimulation have not been reported before, so more work is needed to further clarify the mechanism of the biostimulation.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 51278489).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.01.005.

REFERENCES

Aklujkar, M., Beatty, J.T., 2005. The PufX protein of *Rhodobacter capsulatus* affects the properties of bacteriochlorophyll and carotenoid pigments of light-harvesting complex 1. Arch. Biochem. Biophys. 443 (1-2), 21–32.

Aksu, Z., Eren, A.T., 2005. Carotenoids production by the yeast *Rhodotorula mucilaginosa*: use of agricultural wastes as a carbon source. Process Biochem. 40 (9), 2985–2991.

Chen, D.M., Han, Y., Gu, Z.X., 2006. Application of statistical methodology to the optimization of fermentative medium for carotenoids production by *Rhodobacter sphaeroides*. Process Biochem. 41 (8), 1773–1778.

Clesscerl, L.S., Greenberg, A.E., Eaton, A.D., 1998. Standard Methods for the Examination of Water and Wastewater. 20th ed. American Pubblic Health Association, Washington, DC.

Ding, J., Liu, B.F., Ren, N.Q., Xing, D.F., Guo, W.Q., Xu, J.F., et al., 2009. Hydrogen production from glucose by co-culture of *Clostridium butyricum* and immobilized *Rhodopseudomonas faecalis* RLD-53. Int. J. Hydrog. Energy 34 (9), 3647–3652.

El Fantroussi, S., Agathos, S.N., 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Curr. Opin. Microbiol. 8 (3), 268–275.

Fang, H.H.P., Zhu, H.G., Zhang, T., 2006. Phototrophic hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides*. Int. J. Hydrog. Energy 31 (15), 2223–2230.

Gopinath, K.R., Sims, G.K., 2011. Biostimulation for the enhanced degradation of herbicides in soil. Appl. Environ. Soil Sci. http://dx.doi.org/10.1155/2011/843450 (Article ID 843450).

Gu, Z.X., Chen, D.M., Han, Y.B., Chen, Z.G., Gu, F.R., 2008. Optimization of carotenoids extraction from *Rhodobacter sphaeroides*. LWT Food Sci. Technol. 41 (6), 1082–1088.

Han, R.-M., Zhang, J.-P., Skibsted, L.H., 2012. Reaction dynamics of flavonoids and carotenoids as antioxidants. Molecules 17 (2), 2140–2160.

Jeya, M., Moon, H.-J., Lee, J.-L., Kim, I.-W., Lee, J.-K., 2010. Current state of coenzyme Q_{10} production and its applications. Appl. Microbiol. Biotechnol. 85 (6), 1653–1663.

Kalin, R., Atasever, A., Özdemir, H., 2014. Single-step purification of peroxidase by 4-aminobenzohydrazide from Turkish blackradish and Turnip roots. Food Chem. 150, 335–340.

Kawaguchi, H., Hashimoto, K., Hirata, K., Miyamoto, K., 2001. H_2 roduction from algal biomass by a mixed culture of *Rhodobium marinum* A-501 and *Lactobacillus amylovorus*. J. Biosci. Bioeng. 91 (3), 277–282.

Kien, N.B., Kong, I.-S., Lee, M.-G., Kim, J.K., 2010. Coenzyme Q_{10} production in a 150-l reactor by a mutant strain of *Rhodobacter sphaeroides*. J. Ind. Microbiol. Biotechnol. 37 (5), 521–529.

Kirschbaum, M., Schultze-Mosgau, S., Pfister, W., Eick, S., 2010. Mixture of periodontopathogenic bacteria influences interaction with KB cells. Anaerobe 16 (4), 461–468.

Kuo, F.S., Chien, Y.H., Chen, C.J., 2012. Effects of light sources on growth and carotenoid content of photosynthetic bacteria *Rhodopseudomonas palustris*. Bioresour. Technol. 113, 315–318.

Li, X.J., Lin, X., Li, P.J., Liu, W., Wang, L., Ma, F., et al., 2009. Biodegradation of the low concentration of polycyclic aromatic hydrocarbons in soil by microbial consortium during incubation. J. Hazard. Mater. 172 (2-3), 601–605.

Li, J.M., Peng, L.J., Li, J., Qiao, Y.H., 2014. Divergent responses of functional gene expression to various nutrient conditions during microcystin-LR biodegradation by *Novosphingobium* sp. THN1 strain. Bioresour. Technol. 156, 335–341.

Lu, H.F., Zhang, G.M., Wan, T., Lu, Y.F., 2011. Influences of light and oxygen conditions on photosynthetic bacteria macromolecule degradation: different metabolic pathways. Bioresour. Technol. 102 (20), 9503–9508.

Miura, Y., Saitoh, C., Matsuoka, S., Miyamoto, K., 1992. Stably sustained hydrogen production with high molar yield through a combination of a marine green alga and a photosynthetic bacterium. Biosci. Biotechnol. Biochem. 56 (5), 751–754.

Ng, I.W., Adams, P.G., Mothersole, D.J., Vasilev, C., Martin, E.C., Lang, H.P., et al., 2011. Carotenoids are essential for normal levels of dimerisation of the RC-LH1-PufX core complex of *Rhodobacter sphaeroides*: characterisation of R-26 as *crtB* (phytoene synthase) mutant. Biochim. Biophys. Acta Bioenerg. 1807 (9), 1056–1063.

Reyes, L.H., Gomez, J.M., Kao, K.C., 2014. Improving carotenoids production in yeast via adaptive laboratory evolution. Metab. Eng. 21, 26–33.

Sandmann, G., 2001. Carotenoid biosynthesis and biotechnological application. Arch. Biochem. Biophys. 385 (1), 4–12.

Sasaki, K., Watanabe, M., Suda, Y., Ishizuka, A., Noparatnaraporn, N., 2005. Applications of photosynthetic bacteria for medical fields. J. Biosci. Bioeng. 100 (5), 481–488.

Shao, J.H., Yu, G.L., Wu, Z.X., Peng, X., Li, R.H., 2010. Responses of *Synechocystis* sp. PCC 6803 (cyanobacterium) photosystem II to pyrene stress. J. Environ. Sci. 22 (7), 1091–1095.

Sheehan, N.L., van Heeswijk, R.P., Foster, B.C., Akhtar, H., Singhal, N., Seguin, I., et al., 2012. The effect of β-carotene supplementation on the pharmacokinetics of nelfinavir and its active metabolite M8 in HIV-1-infected patients. Molecules 17 (1), 688–702.

Takaichi, S., 2008. Distribution and biosynthesis of carotenoids. In: Hunter, C.N., Daldal, F., Thurnauer, M.C., et al. (Eds.), The Purple Phototrophic Bacteria. Springer, pp. 97–117.

Tangprasittipap, A., Prasertsan, P., 2002. 5-Aminolevulinic acid from photosynthetic bacteria and its applications. Songklanakarin J. Sci. Technol. 24 (4), 717–725.

Tyagi, M., da Fonseca, M.M.R., de Carvalho, C.C., 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation 22 (2), 231–241.

Warthmann, R., Cypionka, H., Pfennig, N., 1992. Photoproduction of H_2 from acetate by syntrophic cocultures of green sulfur bacteria and sulfur-reducing bacteria. Arch. Microbiol. 157 (4), 343–348.

Wu, P., Zhang, G.M., Li, J.Z., Lu, H.F., Zhao, W., 2012. Effects of Fe^{2+} concentration on biomass accumulation and energy metabolism in photosynthetic bacteria wastewater treatment. Bioresour. Technol. 119, 55–59.

Yen, H.W., Shih, T.Y., 2009. Coenzyme Q_{10} production by *Rhodobacter sphaeroides* in stirred tank and in airlift bioreactor. Bioprocess Biosyst. Eng. 32 (6), 711–716.

Zhao, W., Zhang, G.M., 2014. Optimization of photosynthetic bacteria wastewater treatment and study of microbial species diversity. Desalin. Water Treat. 52 (28-30), 5357–5365.