Advance Journal of Food Science and Technology 11(12): 800-804, 2016
DOI:10.19026/ajfst.11.2795
ISSN: 2042-4868; e-ISSN: 2042-4876

Submitted: September 3, 2015 Accepted: September 22, 2015 Published: August 25, 2016

Research Article

Rapid Production of Natural Carotenoids from *Rhodobacter sphaeroides*

[1, 2]Xin Nie, [1]Changjiang Xiao, [1]Li Zhang, [1]Yang Zhao, [1]Xingbo Liu, [1]Juan Wang, [1]Yi Tang, [1]Yi Liu, [1]Zhiping Zhao and [3]Yu Pan
[1]School of Chemistry and Pharmaceutical Engineering, Sichuan University of Science and Engineering, [2]Liquor Making Biological Technology and Application of Key Laboratory of Sichuan Province, Zigong 643000,
[3]College of Horticulture and Landscape Agriculture, Southwest University, Beibei 400700, China

Abstract: *Rhodobacter sphaeroides* is abundant in natural carotenoids. However, fermentation under light growth conditions for carotenoids biosynthesis is time consuming. In the present study, fermentation for 36 h under micro-aerobic growth conditions was employed to rapidly produce carotenoids from *Rb. sphaeroides*. Orthogonal experiments suggested that the optimal fermentation process was: temperature 33°C, inoculation amount 8%, fermentation duration 36 h. The yield of total carotenoids was 11.542 mg/L. Real-time PCR indicated that expression levels for *crtA*, *crtD*, *crtE* and *crtI* at the time fermentation for 36 h were higher than that of fermentation for 48 h. TLC analysis showed that at least 6 well separated bands were observed. Temperature, pH and light tolerance tests indicated that the total carotenoids were stable. 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay suggested that the total carotenoids exhibited high anti-oxidant activity and the IC_{50} value of DPPH radical scavenging test was about 8.175 μg/mL. The present study will promote to produce natural carotenoids from microorganism in large-scale.

Keywords: Anti-oxidant activity, carotenoids, extraction, fermentation, *Rhodobacter sphaeroides*

INTRODUCTION

Carotenoids are the precursors for vitamin A and play very important roles in preventing human disease including cardiovascular diseases, cancer and other chronic diseases (Bonifacio *et al*., 2012). Carotenoids exhibit high anti-oxidant potential and thus make it be of particular significance to human health (Fiedor and Burda, 2014). Furthermore, carotenoids are widely used as food nutrition (Maiani *et al*., 2009). Because of the important applications in human health and food industry, more and more attention has been paid to carotenoids. Currently, more than 700 carotenoids have been reported, of which about 50 are presented in a typical human diet. Of these 50, about 20 carotenoids have been identified in human blood and tissues (Khachik, 2006). The scientific findings promote the rapid growth of carotenoids market. The carotenoids market in 2010 was about 1.2 billion USD and it will be 1.4 billion USD in 2018 with the considerable increase.

Rb. sphaeroides is abundant in carotenoids and could be an ideal organism to produce natural carotenoids. Metabolism pathway and regulation of carotenoid biosynthesis in *Rb. sphaeroides* has been well-studied (Lang and Hunter, 1994). However, fermentation under light growth conditions for carotenoids biosynthesis in *Rb. sphaeroides* is time consuming. In the present study, we employed fermentation under micro-aerobic growth conditions to produce carotenoids from *Rb. sphaeroides* very rapidly.

MATERIALS AND METHODS

Materials: All the chemicals used in medium, extraction and analysis of carotenoids are analytical pure.

Rapid biosynthesis of carotenoids by fermentation under micro-aerobic growth conditions: A single colony was inoculated in about 20 mL of M22+ medium (Hunter and Turner, 1988) in 100-mL flask and vigorously grown overnight at 30°C. The pre-cultures were then inoculated into 200 mL of M22+ medium at the ratio of 8% in 500-mL flasks and vigorously shaken at 30°C. Cell cultures were shifted from aerobic conditions to micro-aerobic conditions at an OD_{660} of 0.5-0.8 and incubated for 36-72 h under dark conditions. Micro-aerobic growth conditions were performed by incubating 400 mL of culture in 500-mL flask under gentle agitation at 150 rpm under dark conditions.

Corresponding Author: Zhiping Zhao, School of Chemistry and Pharmaceutical Engineering, Sichuan University of Science and Engineering, Zigong 643000, China, Tel.: 86-813-5505606

Table 1: Orthogonal experiment for the optimization of carotenoids

	Inoculation amount (%)	Time (h)	Temperature (°C)	Production (mg/L)
1	4	36	27	2.3610
2	6	48	27	6.8930
3	8	60	27	7.0480
4	8	48	30	6.7810
5	4	60	30	7.1330
6	6	36	30	6.7690
7	6	60	33	8.3380
8	8	36	33	11.542
9	4	48	33	7.8390

Table 2: Primer sequence for real-time PCR

Primer	Sequence (5'-3')
crtA-F	GAATCGCCGATCTACCAG
crtA-R	GCCGATCTTGAAGACCAC
crtD-F	GGCCGATCACATCGTCTT
crtD-R	TCGGCGCAGATGTAGAGT
crtE-F	AAGCGGCTGAAGGACATC
crtE-R	GATCTTCTCGGCATAGCG
crtI-F	AGCTTCCGCATGAACACC
crtI-R	ATCCGCATTCGAGACCAC

Optimization of fermentation process conditions: Orthogonal experiment was employed in fermentation time, temperature and inoculation amount. Parameters were listed in Table 1.

Extraction of total carotenoids from *Rb. sphaeroides*: Cell cultures of *Rb. sphaeroides* in fermentative medium were collected by centrifugation at 10,000 rpm for 10 min at 4°C. The cell pellet was washed once with distilled water. The precipitate was subsequently resuspended in acetone and methanol mixture (acetone: methanol mixture = 7:2, v/v) at the ratio of 1:40. Then, the cells were broken by ultrasonic cooled with ice, 300 W, work for 3 s and stop for 5 s, continued for 30 min in the dark. The supernatant was collected by centrifugation at 10,000 rpm for 10 min. The supernatant was shaken at 150 rpm for 30 min and centrifuged at 12,000 rpm for 10 min in the dark to obtain the supernatant containing carotenoids. Vacuum distillation and saponification reaction were used for further purification.

Determination of total carotenoids: The absorbance value of total carotenoids extracted from *Rb. sphaeroides* was evaluated by UV-vis spectrophotometer at 480 nm after suitable dilution. The total carotenoids yield (mg/L culture liquid) was calculated on the basis of culture broth volume according to the following formula (Chen *et al*., 2006):

$$\text{carotenoids yield}\,(\text{mg/L}) = \frac{\text{ADV}_1}{0.16\text{V}_2}$$

where,

A = The absorbance of diluted extract solution at 480 nm

D = The dilution ratio

V_1 = The volume of acetone and methanol mixture added, 0.16 is extinction coefficient of carotenoids

V_2 = The volume of fermentative liquid.

Real-time PCR analysis: Primers used for analyzing the expression of carotenoids biosynthesis pathway structural genes levels were listed in Table 2. Real-time PCR was performed according to manufacturers' instructions of one-step RT-PCR kit (Qiagen) with a final concentration of 4 ng/μl total RNA.

TLC assay of total carotenoids: Fraction of the total carotenoids from *Rb. sphaeroides* was performed by TLC. Silica gel G was employed as an adsorbent. Mobile phage (hexane: methanol: acetone = 70:29:1, v/v/v) were used for the separation of total carotenoids and separation was performed under dark conditions:

$$R_f = \frac{\text{Distance traveled by the substance}}{\text{Distance traveled by the solvent}}$$

Stability assay of the total carotenoids: For the effects of temperature on the stability of total carotenoids assay, a suitable mount of total carotenoids powder was dissolved in n-butyl alcohol. Then, 5 mL of the resuspension was transferred into 10-mL color comparison tube and kept in water broth at 30, 50, 70 and 90°C, respectively for 0 h and 3 h. The fatality rate of total carotenoid was calculated according to the following formula:

$$\text{fatality rate}\,(\%) = [\frac{A_0 - A_i}{A_0}] \times 100$$

where A_0 is the value of OD_{480} for control, A_i is the value of OD_{480} for treated sample.

For the effects of pH on the stability of total carotenoid assay, a suitable mount of total carotenoids powder was dissolved in methanol. Then, 5 mL of the resuspension was transferred into 10-mL color comparison tube. 5 mL of 0.1, 0.5, 1, 2 and 4%, respectively HCl and KOH was added to the tube, respectively. Mixed well and reacted at room temperature for 2 h under dark conditions. The fatality rate of total carotenoids was calculated according to the above described formula.

For the effects of light intensity on the stability of total carotenoids assay, a suitable mount of total carotenoids powder were dissolved in methanol. Then, 5 mL of the resuspension was transferred into 10-mL color comparison tube and treated under dark conditions, indoor scattered light and 2000 Lux light for 8 h. The fatality rate of total carotenoids was calculated according to the above described formula.

Anti-oxidant activity using DPPH assay: DPPH free radical assay was carried out to measure the free radical scavenging activity by the method of Brand-Williams *et al*. (1995). Radical scavenging activity was measured by the following formula:

$$\text{Scavenging } (\%) = [\frac{A_1 - (A_2 - A_3)}{A_1}] \times 100$$

where, A_1 is the absorbance of the DPPH solution, A_2 is the absorbance of sample and DPPH after treatment and A_3 is the absorbance of sample without DPPH. Total carotenoids concentration providing 50% scavenging (IC_{50}) was calculated from the graph plotted between scavenging percentage and total carotenoids concentration.

RESULTS AND DISCUSSION

Rapid fermentation of carotenoids in *Rb. sphaeroides*: The orthogonal experiments results were listed in Table 1. Obviously, temperature of 33°C, inoculation amount of 8% and fermentation time of 36 h were considered to be the optimum fermentation conditions. The yield of carotenoids reached was 11.542 mg/L. One of the major advantages in the present study is that the fermentation time is significantly reduced. 36 h is considered as the optimum fermentation time.

Biosynthesis of carotenoids is tightly regulated by *crt* operon, including 8 genes at least. Expression levels of the four important structural genes *crtA*, *crtD*, *crtE* and *crtI*, respectively encoding spheroidene monooxygenase, methoxyneurosporene dehydrogenase, geranylgeranyl pyrophosphate synthetase and phytoene dehydrogenase, were measured by real-time PCR (Fig. 1). Obviously, the four structural genes were up-regulated. Expression levels of *crtA*, *crtD* and *crtI* were up-reguated by more than 1 fold, which agreed well with the high production of carotenoids at 36 h.

TLC assay of total carotenoids: The total carotenoids were analyzed by absorbance ranging from 300 nm to 900 nm (Fig. 2). Spectral absorptions were observed at ~360, ~480, ~570 and 760 nm, respectively indicating the existence of the BChl without saponification treatment. However, the spectral absorbance of total carotenoids treated by saponification was only observed at ~480 nm, suggesting the complete removal of BChl in the total carotenoids extraction (Toomey and McGraw, 2007). TLC chromatography analysis was

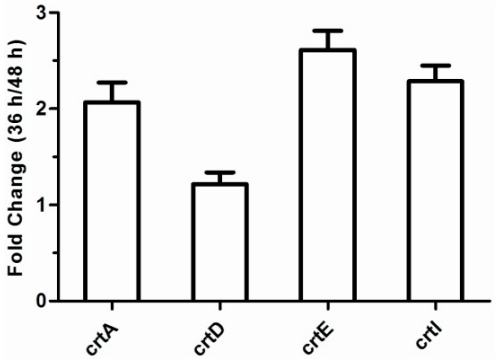

Fig. 1: Real-time PCR analysis of carotenoids biosynthesis pathway structural genes *crtA*, *crtD*, *crtE* and *crtI* expression levels at 36 h and 48 h

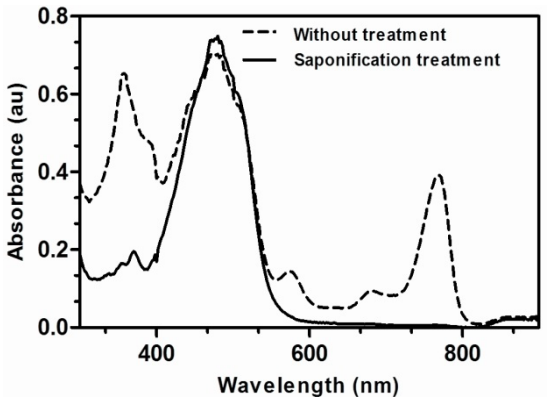

Fig. 2: Spectral absorption of total carotenoids with and without saponification treatment

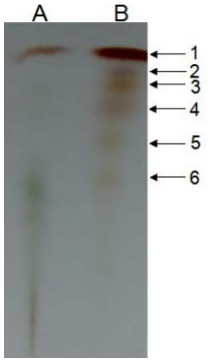

Fig. 3: TLC chromatography analysis of total carotenoids. A represents total carotenoids without saponification, while, B represents total carotenoids with saponification treatment

shown in Fig. 3, suggested the presence of the total carotenoids. A indicated the total carotenoids without saponification and B indicated the total carotenoids treated by saponification. Clearly, green bands were observed in line A in the thin layer plate, indicating the existence of BChl. In line B, there were at least 6 bands well separated with different colors. The R_f values of bands observed in line B are listed in Table 3. Based on the experimental observations, it could be concluded

Table 3: R_f values of bands observed in line B of TLC chromatography

	Color of band	R_f value
1	Orange-red	0.96
2	Purple	0.92
3	Yellow-orange	0.88
4	Pink	0.83
5	Light yellow	0.77
6	Light yellow	0.67

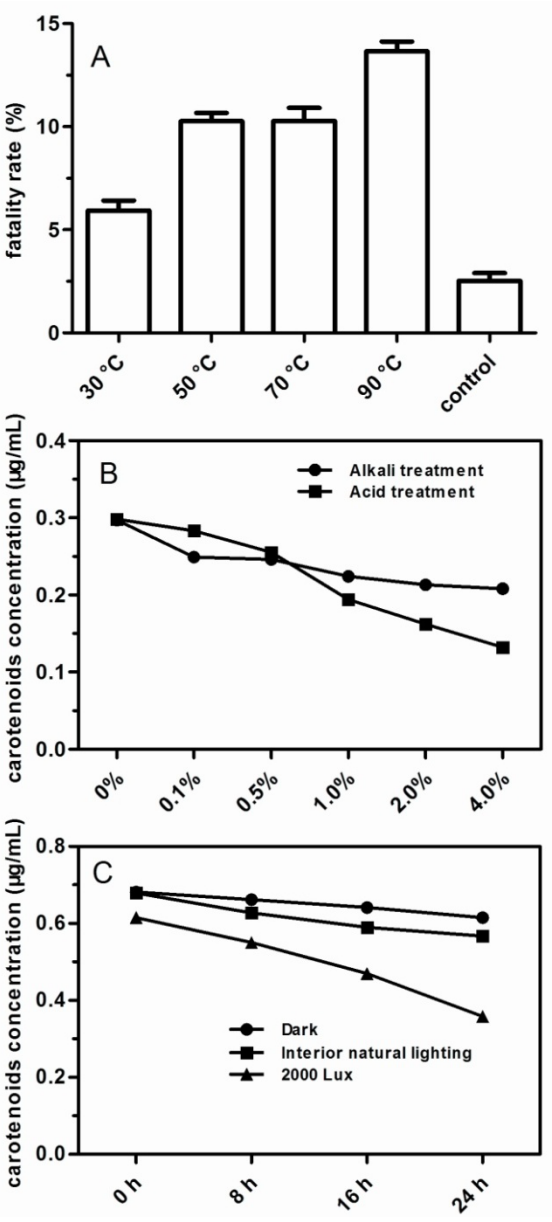

Fig. 4: Stability test of the total carotenoids. A, B and C indicate temperature, pH and light intensities on the stability of the total carotenoids

that total carotenoids were isolated from *Rb. sphaeroides.*

Stability assay of the extracted total carotenoids: Experimental results for the effects of temperature on the stability of the total carotenoids were described in Fig. 4A. Clearly, it affected the stability of total carotenoids slightly. The fatality rate of each sample was: 30°C, 6.25%; 50°C, 10.42%; 70°C, 10.83%; 90°C, 13.61%. While, the control treated at room temperature was much more stable. The effects of pH on the stability of isolated carotenoids were shown in Fig. 4B. Obviously, the amount of the total carotenoids was decreased with the increase of the concentrations of alkali and acid. However, the concentration of the total carotenoids changed slightly. The total carotenoids were more stable in dark than in 2000 Lux and indoor light, as revealed in Fig. 4C. Strangely, the indoor light affected the total carotenoids stability more dramatically than that of the 2000 Lux light. However, according to the experimental results, the total carotenoids were stable.

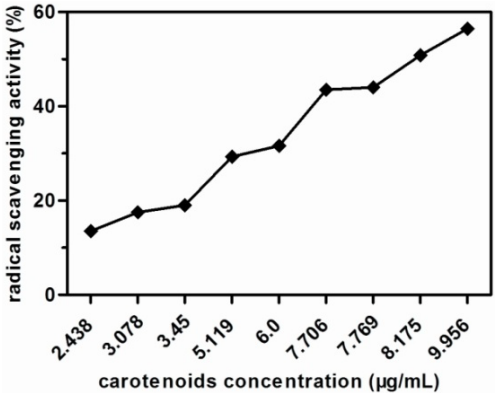

Fig. 5: Radical scavenging activity (%) of the total carotenoids

Antioxidant assay: DPPH radical scavenging activity was measured, as revealed in Fig. 5. It was obvious that the radical scavenging activity increased with the increase of total carotenoids concentration. The radical scavenging activity for the total carotenoids with the concentration of 2.438, 3.078, 3.45, 5.119, 6, 7.969, 8.175L and 9.956μg/mL was 13.51%, 17.55%, 19.05%, 29.33%, 31.64%, 44%, 50.85% and 56.47%, respectively. Clearly, IC_{50} value of DPPH radical scavenging test for the total carotenoids was approximately 8.175μg/mL. It is better than carotenoids extracted from plants and some bacteria (Faith-Anthony *et al*., 2014; Sindhu *et al*., 2010).

CONCLUSION

In the present study, we employed the *Rb. sphaeroides* to very rapidly produce carotenoids by fermentation under micro-aerobic growth conditions:

- Orthogonal experiments suggested that the optimal fermentation process is: temperature 33°C, inoculation amount 8%, fermentation time 36 h. The yield of total carotenoids was 11.542mg/L.

- The extracted total carotenoids were stable to temperature, pH and light.
- The total carotenoids exhibited high anti-oxidant activity and the IC_{50} value of DPPH radical scavenging test was about 8.175 μg/mL.

ACKNOWLEDGMENT

This study was supported by Research Project of Sichuan University of Science and Engineering (2011RC12, 2014KY02), Scientific Research Foundation of the Education Department of Sichuan Province (15ZA0222), Research Project of Liquor Making Biological Technology and Application of Key Laboratory of Sichuan Province (NJ201306, NJ201512) and Sichuan Province Undergraduate Training Programs for Innovation and Entrepreneurship (201410622021).

REFERENCES

Bonifacio, A., R. Guidetti, T. Altiero, V. Sergo and L. Rebecchi, 2012. Nature, source and function of pigments in tardigrades: *In vivo* raman imaging of carotenoids in *Echiniscus blumi*. PLoS One, 7(11): e50162.

Brand-Williams, W., M.E. Cuvelier and C. Berset, 1995. Use of a free radical method to evaluate antioxidant activity [J]. LWT-Food Sci. Technol., 28(1): 25-30.

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

Faith-Anthony, A.O., N. Ibrahim and A. Hamzah, 2014. Clinical potentials of bacteriocarotenoids: Rhodopin and β-carotene from phototrophic *Rhodopseudomonas palustris*. ISOR J. Dent. Med. Sci., 13(12): 52-58.

Fiedor, J. and K. Burda, 2014. Potential role of carotenoids as antioxidants in human health and disease. Nutrients, 6(2): 466-488.

Hunter, C.N. and G. Turner, 1988. Transfer of genes coding for apoproteins of reaction centre and light-harvesting LH1 complexes to *Rhodobacter sphaeroides*. J. Gen. Microbiol., 134: 1471-1480.

Khachik, F., 2006. Distribution and metabolism of dietary carotenoids in humans as a criterion for development of nutrional supplements. Pure Appl. Chem., 50(8): 1551-1557.

Lang, H.P. and C.N. Hunter, 1994. The relationship between carotenoid biosynthesis and the assembly of the light-harvesting LH2 complex in *Rhodobacter sphaeroides*. Biochem. J., 298(Pt1): 197-205.

Maiani, G., M.J. Castón, G. Catasta, E. Toti, I.G. Cambrodón, A. Bysted, F. Granado-Lorencio, B. Olmedilla-Alonso, P. Knuthsen, M. Valoti, V. Böhm, E. Mayer-Miebach, D. Behsnilian and U. Schlemmer, 2009. Carotenoids: Actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. Mol. Nutr. Food Res., 53(Suppl. 2): S194-S218.

Sindhu, E.R., K.C. Preethi and R. Kuttan, 2010. Antioxidant activity of carotenoid lutein *in vitro* and *in vivo*. Indian J. Exp. Biol., 48(8): 843-848.

Toomey, M.B. and K.J. McGraw, 2007. Modified saponification and HPLC methods for analyzing carotenoids from the retina of quail: implications for its use as a nonprimate model species. Invest. Ophth. Vis. Sci., 48(9): 3976-3982.