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Research Article

Enhancement of Carotenoids Production from *Rhodobacter sphaeroides* by Nitrosoguanidine Mutagenesis

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Abstract: To enhance carotenoids production from *Rhodobacter sphaeroides*, Nitrosoguanidine (NTG) mutagenesis was employed to construct high-carotenoids *Rb. sphaeroides* strain in the present study. Experimental results suggested that the NTG concentration and treat duration was 3 mg/mL and 15 min, respectively. One high-carotenoids *Rb. sphaeroides* strain was finally obtained and the production of carotenoids was 2 times higher than that of the production from wild type *Rb. sphaeroides*. The present study promotes to large scale production of natural carotenoids from *Rb. sphaeroides*.

Keywords: Carotenoids, mutagenesis, NTG, *Rhodobacter sphaeroides*

INTRODUCTION

Carotenoids are the most common, naturally occurring terpenoid pigments and responsible for yellow, orange and red color and synthesized in plants, algae and bacteria (Heider *et al*., 2014; Mata-Gómez *et al*., 2014). They possess a wide variety of biological functions, including anti-carcinogenic, immune-modulator and antioxidant activities (Liu *et al*., 2013; Maldonade *et al*., 2008). However, animals themselves are not able to synthesize carotenoids and require uptake of carotenoids through their diet. Carotenoids are the precursors of vitamin A and thus widely used as food supplements to modify the color of fats, cheese and drinks.

Traditionally, carotenoids are extracted from plants or produced by chemical way. Currently, microbial production of carotenoids shows increasing interest and higher safety to use. Microbial production has the advantages to the traditional methods since lower-cost substrates can be used in this approach and thus lower costs of production were gained. Owing to the apparent advantages, more and more attention has been paid to produce carotenoids by microbial method (Vachali *et al*., 2012). Literature regarding to production of carotenoids from microorganisms has been extensively reported (Liu, *et al*., 2014; Taskin and Erdal, 2011). *Rhodobacter sphaeroides* is a model purple non-sulfur photosynthetic bacterium for studying photosynthesis (Kiley and Kaplan, 1988). The bacterium itself has a complete pathway to synthesize carotenoids tightly regulated by oxygen tension and light intensity (Lang and Hunter, 1994). However, the yield of carotenoids from *Rb. sphaeroids* is relatively lower. Enhancing carotenoids production before industrial production is required. In the present study, one high-carotenoids *Rb. sphaeroides* strain was constructed through chemical mutagenesis by NTG. The yield of carotenoids was enhanced by over 100% compared to that of the wild type strain. The present study will supply an insight into increasing carotenoids production by *Rb. sphaerides* and promote the industrial scale production of carotenods.

MATERIALS AND METHODS

This study was conducted in Sichuan University of Science and Engineering at June, 2015.

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

Construction of high-carotenoids *Rb. sphaeroides* strain by NTG mutagenesis: A single colony was inoculated into a 50 mL-flask containing 40 mL of malate minimal media and grown under micro-aerobic conditions in dark at 30°C. One mL of the cell cultures was harvested when the OD_{660} had reached 0.6-0.7 and

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washed twice with normal saline. Then, the cell pellets were resuspended with 0.1 M PBS buffer (pH6.0) and then NTG solution was added to the resuspension to a final concentration of 3 mg/mL and treated for 40 min. After treatment, the cell cultures were harvested by centrifugation and washed twice with normal saline. Finally, the pellets were resuspended in 1 mL of normal saline. The cell cultures were diluted and plated on malate minimal agar media and grown at 30°C for 4 days in the dark. The survival rate *R* was estimated by $R=\frac{N2}{N1}\times 100\%$, in which N_1 is the number of bacteria without NTG treatment and N_2 is the number of bacteria treated by NTG.

The bacteria with the *R* up to 50% were considered the candidates and used for further demonstrated. For primary demonstration, all the candidates were respectively inoculated into a 50 ml-flask containing 40 ml of malate minimal media and grown under micro-aerobic conditions in the dark at 30°C until the OD_{660} had reached 0.5-0.6, respectively. Then, the precultures were, respectively incubated into a 100 ml-flask containing 80 ml of malate minimal media and grown under micro-aerobic conditions in the dark at 30°C for 48 h. Xr value was employed to primarily measure the production of carotenoids. The Xr was estimated by Xr = OD_{480}/OD_{660}, in which OD_{480} is the spectral value of carotenoids at 480 nm and OD_{660} is the spectral value of cell culture at 660 nm. Higher Xr indicates more production of carotenoids. Finally, the high-carotenoids *Rb. sphaeroides* strain was demonstrated by fermentation, extraction and quantification of carotenoids.

Production of carotenoids from *Rb. sphaeroides*: *Rb. sphaeroides* was grown in 30 °C in malate minimal media under micro-aerobic conditions (Van Niel, 1944). A single colony was inoculated into about 40 ml of malate minimal media medium in 50-mL flask and grown under micro-aerobic conditions overnight at 30°C. The pre-cultures were then incubated into 80 mL of malate minimal medium at the ratio of 1% in 100-ml flasks and grown under micro-aerobic conditions at 30°C for 48 h. Cell cultures of *Rb. sphaeroides* were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cell pellets were 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 3 M HCl for 1 h with gentle shake in the dark. The supernatant was collected by centrifugation at 10,000 rpm for 10 min and then shaken at 150 rpm for 30 min. The supernatant containing carotenoids was harvested again by centrifugation at 12,000 rpm for 10 min in the dark. Vacuum distillation and saponification reaction were used for further purification.

Determination of total carotenoids: The absorbance 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):

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

where *A* is the absorbance of diluted extract solution at 480 nm, D is the dilution ratio, V_1 is the volume of acetone and methanol mixture added, 0.16 is extinction coefficient of carotenoids, V_2 is the volume of fermentative liquid.

RESULTS AND DISCUSSION

Determination of NTG concentration and NTG treat duration: NTG is one of the most commonly used chemicals for screening (Kada *et al*., 1972). The high-carotenoids *Rb. sphaeroides* strain was constructed by NTG in the present study. The survival rate was increased, while the colony number was decreased with the increased concentration of NTG (Fig. 1). Strains

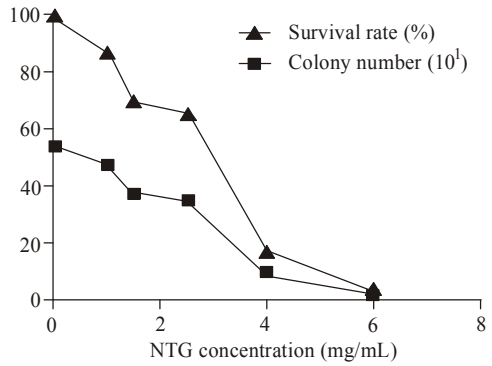

Fig. 1: Survival rate and colony number of the *Rb. sphaeroides* treated by NTG.

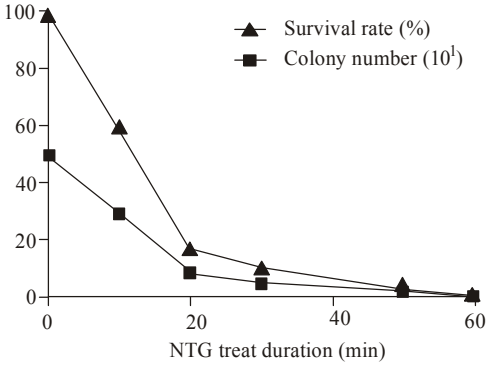

Fig. 2: Relationship between NTG treat duration and *Rb. sphaeroides* survival rate

with the survival rate of 50% were suitable for screening high-carotenoides *Rb. sphaeroides* strain and the used NTG concentration was 3 mg/ml. Subsequently, the relationship between NTG treat duration and *Rb. sphaeroides* survival rate was further measured (Fig. 2). Clearly, *Rb. sphaeroides* cells treated by 3 mg/mL NTG for 15 min were used for further screen of high-carotenoids strains.

Screen of high-carotenoids *Rb. sphaeroides* strain: Three steps were performed to screen the high-carotenoids *Rb. sphaeroides* strain. For the primary screen, Xr value was used as an indicator. Four times and totally 27 NTG treated candidates were separately measured (Fig. 3). It is obvious that Xr values for most of the candidates were enhanced, suggesting the higher concentration of carotenoids. For secondary screen, candidates of 5, 21, 72, 3, C2, E5 and F4 were further tested (Fig. 4). As expectation, carotenoids concentrations of all the candidates were significantly increased compared to that of the wild type strain. For the final demonstration, C2, 3 and E5 candidates were further measured (Fig. 5). Production of carotenoids from 3 and E5 was 2 times higher than that of production from wild type and E5 were demonstrated as the high-carotenoids *Rb. sphaeroides* strains constructed by NTG. However, the yields of carotenoids from E5 was relatively lower than that of our previous study because of the differently employed method for cell disruption.

Growth curve for the E5 strain and wild type strain: To check whether the growth rate of E5 strain and wild type strain will be affected by NTG treatment, growth curve was made by using the OD_{660} value (Fig. 6). Obviously, the mutant strain grew faster than that of wild type. The E5 mutant strain reached a stationary phase of growth after growing for 32 h and reached a decline phase after growing for 36 h. While, the wild type strain reached a stationary phase of growth after growing for 36 h. Furthermore, the wild type strain kept in stationary phase after growing for 48 h. It could be concluded that the treatment by NTG affected the growth dramatically. It agreed well with the previous report (Yang *et al*., 1994).

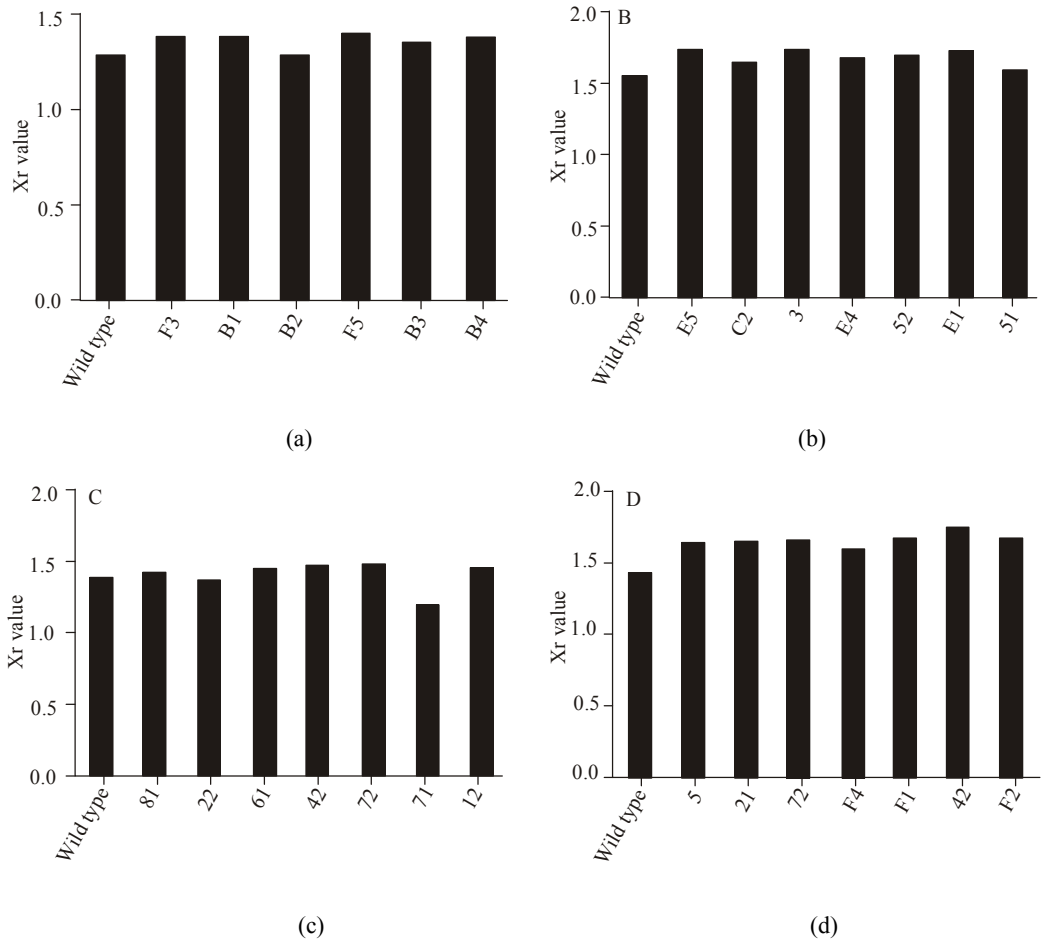

Fig. 3: Primary screen of high-carotenoids *Rb.sphaeroides* strain by using Xr value

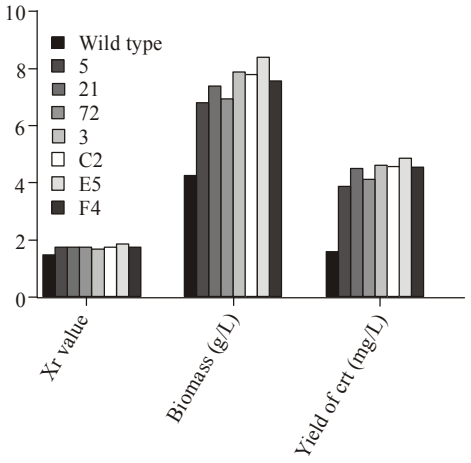

Fig. 4: Secondary screen of high-carotenoides *Rb. sphaeroides* strains

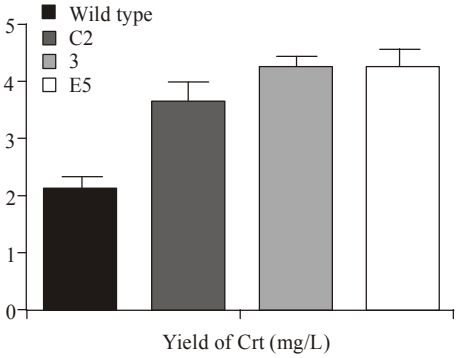

Fig. 5: Demonstration of high-carotenoids *Rb. sphaeroides* strain

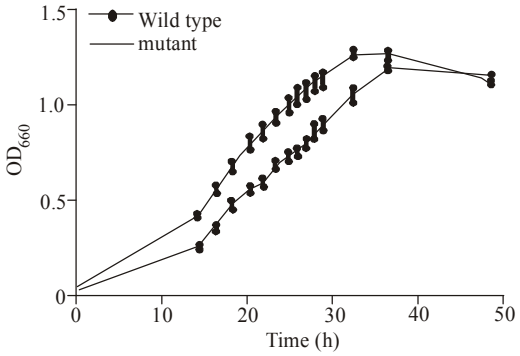

Fig. 6: Growth curve for E5 mutant and wild type strain

CONCLUSION

- A high-carotenoids *Rb. sphaeroides* stain was obtained by NTG mutagenesis. The concentration for NTG was 3 mg/mL and the treat duration was 15 min.
- The high-carotenoids *Rb. sphaeroides* stain was demonstrated by three steps including primary screen, secondary screen and final demonstration. The production of carotenoids from mutant strain was 2 times than that of the production from wild type strain.

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