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RESEARCH ARTICLE

ISOLATION, IDENTIFICATION AND PHYSIOLOGICAL CHARACTERISTICS OF HIGH CAROTENOIDS YIELD *RHODOPSEUDOMONAS FAECALIS* PSB-B

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ABSTRACT

Carotenoid is a natural antioxidant and rich in the photosynthetic bacteria. We described here the isolation of strain PSB-B, which has the ability of high produce with carotenoids, from Fenhe River in China. Based on phenotypic, physiological, biochemical and phylogenetic (16S rDNA) studies, strain PSB-B could be classified as a strain of *R. faecalis*. The condition of fermentation carotenoids from strain PSB-B was optimized of temperature, inoculum concentration and light intensity 3 aspects by response surface methodology. The results showed that the optimal conditions were 30.88 °C, 2172 lux and 9.66 % inoculum concentration. Under these conditions, the practical yield of carotenoids was 16.93 mg/L.

INTRODUCTION

Carotenoids are colourful compounds possessing yellow, orange and red pigments. They show important biologic activities associated with antioxidant properties, such as strengthening the immune system, decreasing the risk of degenerative illnesses, reducing the risk of cardiovascular disease, and preventing macular degeneration and cataracts [1]. Carotenoids are naturally occurring tetraterpenes found in various fruits, vegetables, plants, algae and bacteria. Currently, chemical synthesis, extraction from plants, animals and microorganism fermentation are the commonly methods of carotenoids production. However, most of the carotenoids sold in the market are derived from chemical synthesis and cannot meet consumers' desire for natural carotenoids [2].

Using the way of microbial fermentation production of carotenoids can not only avoid the dangerous chemical synthetic pigments caused by carcinogenic, teratogenic, but overcome the pigment extracted from plants which were affected by the seasons and the raw material. Microbial fermentation production of carotenoids received wide attention of experts both at home and abroad. To improve carotenoids production by microbial, researchers adopt different way, such as the selection of strain and mutant [3, 4] with higher yield caroteniods, the optimization of the many parameters of carbon to nitrogen ratio (C/N), light intensity, and so on. So far, researchers have found that the production of carotenoids microorganisms have *Chlorella zofingiensis* [5], *Phaffia rhodozyma* [6, 7] of the yeasts, *Rhodospirillum rubrum* [8, 9], and *Rhodobacter sphaeroides* [10] of the photosynthetic bacteria.

At present, the scholars' study of carotenoids production mainly focused on yeast and mould, some of which have been put into commercial production, while the study of carotenoids from photosynthetic bacteria mainly focused on the effect of the pigment and its synthesis route [3, 11-13].

Photosynthetic bacteria are photosynthetic Gram-negative prokaryotes that convert light energy into chemical energy by the process of anoxygenic photosynthesis. They contain photosynthetic pigments, bacteriochlorophyll (Bchl) and carotenoids, and can grow autotrophically with CO_2 as the sole carbon source. This energy is ultimately transferred via Bchl to the photosynthetic reaction center and carotenoids act as photooxidative protectors against the damaging combination of oxygen, light and photosensitizing Bchl molecules [14, 15]. Carotenoids serve as accessory pigments in light-harvesting complexes by increasing the absorption cross-section of photosystems for the absorption of radiant energy in photosynthetic bacteria.

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This study isolated *R. faecalis* PSB-B with a high capability of carotenoids production from the sludge of Fenhe River. The effects of temperature, illumination intensity, and the inoculum concentration on carotenoids yields were investigated by using RSM in order to determine the optimal conditions for the higher carotenoids yields. This will provide an important step in the development of more successful strategies aiming at exploiting more beneficial microbe resources for carotenoids.

MATERIALS AND METHODS

Isolation of photosynthetic bacteria

The bottom silt samples were collected from Fenhe River in Shanxi Province of China. Photosynthetic bacteria were enriched by inoculating with the samples 300ml sterile plastic bottles that had been completely filled with YP medium, which contained Yeast extract 3 g, Peptone 3 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.3 g and the initial pH value was adjusted to 6.8-7.2. Anaerobic conditions were established by tight up the sealed vials. The vials were incubated in 30 °C 3000 Lux illumination incubator. After 15 days of incubation, the color of cultures varied dark red. Bacteria from each culture were streak-purified on YP agar plates that were then incubated in (CO_2 Incubator) anaerobic jars with a CO_2 generator (ESCO, America) placed in front of a tungsten lamp. One isolated colony from each plate was cultured in YP agar. The capability of carotenoids production was further evaluated.

Morphological, biochemical and spectral characteristics

Visual observations of both morphological and microscopic characteristics using the light microscope were conducted. Gram reactions were determined according to standard microbiological procedures [16]. Cell size was observed by scanning electron microscope (S570, HITACHI, Japan).

The carbon sources including glucose, mannitol, mannose, sodium pyruvate, Xylose, fructose and tartrate were sterilized by filtration (pore size 0.22 μm, Jinteng Company, China) and added aseptically to the autoclaved-based medium at final concentration of 0.2% (w/v). The strain was incubated for 24 h at 35 °C 2000 lux and subcultured in the same medium three times. Basal medium was used as the negative control. All experiments were done in triplicate.

Formation of H_2S, utilization of citrate, gelatin liquefaction tests were performed according to standard microbiological procedures [16].

16S rDNA amplification and sequence analysis

Used a commercial kit (SK8255, Sangon Biotech), genomic DNA was extracted. A pair of universal primers was prepared according to previously described methods [17], upstream primer (formerly named 7F), 5'-CAG AGT TTG ATC CTG GCT-3'; downstream primer (named 1540R), 5'-AGG AGG TGA TCC AGC CGC A-3'.

Almost 1400 bp of 16S rDNA product was amplified from all nucleic acid samples by PCR. The PCR reaction mixtures (30 μl) contained 0.5 μl of template, 1 μl of primers, 2.5 μl of 10×Buffer. PCR MasterMix (KP201-01, Tiangen Biotech CO., LED, China), and the volume was adjusted to 25 μl with deionized water. 2×Pfu PCR MasterMix was composed of 0.1 U Pfu polymerase, 500 μM dNTP each, 50 mM Tris–HCl (pH 8.7), 20mM KCl, 4 mM $MgCl_2$. The PCR was performed in the PTC-100^{TM} for 4 min, followed by 30 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, followed by a final extension performed at 72 °C for 10 min. Electrophoresis analysis was performed by loading 10 μl of PCR-amplified DNA product onto 1.5% (w/v) agrose gel containing ethidium bromide (EtBr) of 0.5 μg/ml final concentration. Gels were electrophoresed at a constant voltage of 150 V for 20 min before gels were scanned digitally with an Alpha Imager system (IS2200, Alpha Innotech, USA). PCR products were purified with the Qiaquick DNA gel purification kit (SK8131, Sangon Biotech) according to the manufacturer's instructions. DNA sequences were determined by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd (China). The closest matches to the partial 16S rDNA sequence were identified based on the results of a BLAST search of the EzBioCloud data-base. The phylogenetic tree was constructed using the neighbor-joining method with the MEGA5 software. Bootstrap analysis for 1000 replicates was performed to estimate the confidence of the tree topologies [18].

Optimization of fermentation conditions

The experimental results were analyzed by response surface methodology (RSM) using the software Design-Expert 8.0.6. Calculations were done at 95% of confidence level. In optimization of cultivation, the combination effect of incubation temperature as X_1, illumination intensity as X_2 and inoculum concentration as X_3 was studied using RSM. In order to optimize the incubation conditions and investigate effects of above independent variables on the yield of carotenoids, a central-composite rotary design with the variables at five levels (Table 1) was used in the experiments. Table 2 presents the design matrix for the experiment and the regression model proposed for response was given below:

$$Y = {}_0 + \sum_{i=1}^{3} {}_i X_i + \sum_{i=1}^{3} {}_{ii} X_i^2 + \sum_{i=1}^{3} {}_{ij} X_i X_j \qquad (1)$$

where β_0 is the value of the fixed response at the central point of the experiment which is the point (0,0,0); β_i, β_{ii}, and β_{ij} are the linear, quadratic and cross-products coefficients, respectively. While demonstrating the significant effects 3-dimensional fitted surfaces were drawn.

Table 1 Independent variable values and their corresponding levels

Coded	Temperature (°C)	Illumination intensity (lux)	Inoculum Concentration (%)
1.682	38.409	3681.79	18.409
1	35	3000	15
0	30	2000	10
-1	25	1000	5
-1.682	21.591	318.207	1.59104

Determination of carotenoids

Cells were incubated at 30 °C under 60 W tungsten lamp illumination of 2000 lux 3 days. Thalli are attained from the fermented liquid which was centrifuged by 3000 r/min for 10 minutes. Cells were washed free of medium with distilled water. Acetone solvent was added in thalli. Ultrasonic power and duration were set at 390 W and 6 min. After ultrasonic treatment in ultrasonic crasher for 6 min in order to break the cells of PSB-B, the flask was kept in water bath of 20 °C. Then make the centrifugal treatment with the mixture in the speed of 3000 r/min for 10 minutes. The supernatant is the pigment crude extract liquid.

Total carotenoids content was determined at 480 nm using a spectrophotometer (722SJinghua, China) following the recommendation of [19]

$$\text{Caroteniods yield (mg/L)} = ADV_1/0.16V_2 \qquad (2)$$

where A is the absorbance value of diluted extraction at 480 nm, D is the dilution rate, V_1 is the volume of acetone, 0.16 is the extinction coefficient of carotenoids, and V_2 is the volume of fermentation liquor.

Statistical analysis

Software SPSS 13.0 (SPSS Inc., USA) was used for statistical analysis. Nonparametric Test was at the 1 % level and ANOVA was at the 5 % level.

RESULTS AND DISCUSSION

Isolation and identification of the photosynthetic bacteria

Of eleven isolates of producing carotenoids obtained from the sludge of Fenhe River in China, every strains showed different carotenoid-producing activity, only strain PSB-B exhibited stronger carotenoid-producing activity than others (Fig. 1). Strain PSB-B was selected for the further studies. Colony diameter was about 0.6-0.8 mm. The color of colony in anaerobic environment was dark red, but colorless under the aerobic. The microscopic studies revealed that strain PSB-B was Gram-negative, straight to slightly curved rods, size 0.6-0.8*1.2-2.0 μm (Fig. 2). The species could grows phototrophic in the light under anaerobic conditions, but it also had the capable of growing in the dark under microaerophilic or anaerobic conditions, depending on the carbon source. The biological test showed that strain PSB-B can utilize sodium pyruvate, xylose, not utilize glucose, mannitol, mannose, fructose and tartrate. The following reactions were negative: gelatin liquefaction, formation of H_2S.

The scale of Bacteriochlorophyll (Bchl) and carotenoids is a major reason that affects the certain color of photosynthetic bacteria. The wavelength of maximum absorption (λ max) and the shape of the spectrum (spectral fine structure) are characteristic of the chromophore. The ultraviolet and visible spectrum is the first diagnostic tool for the identification of Bchl and carotenoids. The conjugated double-bond system of carotenoids constitutes the light-absorbing chromophore that gives carotenoids their attractive color and provides the visible absorption spectrum that serves as a basis for their identification and quantification. Most carotenoids absorb maximally at three wavelengths, resulting in three-peak spectra. Therefore, the shape of the spectrum (spectral fine structure) is a characteristic of the photosynthetic bacteria. In the 200-900nm wavelength range, the characteristic absorption peak of strain PSB-B was 380, 465, 495, 525, 805, 850 nm. Results indicated the presence of carotenoids of normal spirilloxanthin series and bacteriochlorophyll a. The spectral characteristic showed that strain PSB-B was most closely allied to *R. faecalis* RLD-53 [20]. However, recent calculations have shown that the wavelength of absorption by any given bound Bchl a molecule is determined by its specific micro-environment and is affected by both pigment-pigment and pigment-protein interactions [21]. In order to identify the types of the carotenoids, we need use some analytical methods, such as TLC, HPLC-MS, Infrared spectroscopy and so on.

We used a 16S rDNA gene sequence-based strategy to identify isolate PSB-B. Full-length (approximately 1.4 kb fragment) 16S rDNA gene was PCR amplified (Fig. 3). The complete 16S rDNA of strain PSB-B (1373 bp) was sequenced and is available at GenBank under accession number KM272172. A BLAST search of EzBioCloud database showed PSB-B most resembled *R. faecalis* strain gc^T (99.93 % similarity). The physiological and morphological characteristic shows that the strain PSB-B was most closely allied to *R. faecalis*. A phylogenetic tree (Fig. 4) was constructed based on an alignment of 1335 bp of 16S rDNA sequences.

Optimization of incubation conditions of carotenoids.

Light intensity is a very important factor in photosynthetic bacteria growth since photosynthetic bacteria convert light energy into chemical energy through anaerobic photosynthesis [22]. Light provides the energy supporting photosynthetic bacteria growth and controlled carotenoids synthesis and photosynthesis in photosynthetic membrane vesicles. Under anaerobic conditions, the number of photosynthetic membrane vesicles produced varied inversely with the incident light intensity [23]. Therefore, the impact of light intensity on the photo-pigments synthesis in photosynthetic organisms varied greatly. Zhou Q *et al.* indicated that 8000 lux was an optimal light intensity for carotenoids production, but 2000 lux was an optimal intensity for biomass production [22]. The high energy cost for light irradiation is one of those problems which have adverse effect of carotenoids production, because that well bacterial growth and carotenoids production require an intensive light energy containing specific light wavelength depending on Bchl [24]. Therefore, appropriate light intensity for commercial production of carotenoids has an important significance.

Irrespective of the type of fermentation, whether it is solid-state fermentation or liquid-state fermentation, inoculum concentration affects the yield of carotenoids. When inoculum concentration was too high, carotenoids production and the growth of cell were little, it was probably because high inoculum concentration contributed to lower light penetrability and resulted in lower light intensity for the growth of bacteria. In addition, the temperature was also the main factor that affected the production of carotenoids. Different strains have

different optimum temperatures for carotenoids production. Therefore, appropriate temperature for commercial production of carotenoids has an important significance. Optimal light intensity, inoculum concentration and temperature should have a high light conversion efficiency to optimize the productivity and make photosynthetic bacteria cultivation economically sustainable. Response surface methodology generates mathematical models that precisely represent the overall process of carotenoids fermentation from strain PSB-B. For cost-saving purpose, the final condition would be considered optimum if the operation parameters were as low as possible [25].

The graphical representations of the regression Eq. (3), called the response surfaces (3-D) presented in Fig. 5 was obtained using Design-Expert 8.0.6. Twenty experimental points run randomly according to the experiment planning (Table 2). The yield of carotenoids ranged from 7.53mg/L to 16.98mg/L.

The predicted model can be described by

$$Y = -65.06151 + 4.53153 * X_1 + 0.014587 * X_2 - 0.79299 * X_3 - 9.75815*10^5 * X_1 * X_2 + 0.044856 * X_1 * X_3 - 6.17815*10^5 * X_2 * X_3 - 0.076947 * X_1^2 - 2.52648 * 10^5 * X_2^2 - 0.023734 * X_3^2 \quad (3)$$

with R^2= 81.19%. By applying ANOVA for the mode (Eq. (3)), the established model was found to be significant ($P = 0.0201 < 0.05$) and it could be used to predict the carotenoids yield. ANOVA also demonstrated that the model adequately represents the real relationship between the parameters, giving a high coefficient of determination.

Table 2 Experimental planning (central-composite rotary design)

run	X_1	X_2	X_3	Response (the yield of carotenoids)
1	25	1000	5	9.78
2	35	1000	5	9.87
3	25	3000	5	12.78
4	35	3000	5	10.75
5	25	1000	15	7.53
6	35	1000	15	11.94
7	25	3000	15	9.13
8	35	3000	15	11.75
9	21.5	2000	10	11.23
10	38.5	2000	10	15.03
11	30	318	10	9.22
12	30	3680	10	13.65
13	30	2000	1.59	16.97
14	30	2000	18.41	16.81
15	30	2000	10	16.45
16	30	2000	10	16.97
17	30	2000	10	16.81
18	30	2000	10	16.98
19	30	2000	10	15.98

The P-value was used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller was the value of P, the more significant was the corresponding coefficient [18, 26]. The lack of fit is an indication of the failure for a model representing the experimental data at which points are not included in the regression or variations in the models cannot be accounted for random error [27]. According to the model, all the terms including quadratic terms of temperature (X_1^2), illumination intensity (X_2^2) were significant. The other term coefficients were not significant.

The optimal conditions were given by RSM as following: temperature, 30.88 °C; illumination intensity, 2172 lux; inoculum concentration, 9.66 %. Under these conditions, the practical yield of carotenoids was 16.93 mg/L, which was close to the predicted yield calculated according to the regression model presented in Eq. (3).

The correlative surface response graphs are shown in Fig. 5. They provided a visual interpretation of interactions between two tested variables and the relationships between responses and experiment levels of each variable. Different shapes of the contour plots indicated different interactions between the variables. Elliptical contours were obtained when there was a perfect interaction between the independent variables while circular contour plots indicated otherwise [28, 29].

As shown in Fig. 5 (A), where the carotenoids fermentation yield was given as temperature and illumination intensity at fixed inoculum concentration (0 level). The carotenoids fermentation yield increase with increase of temperature from 25.0 to 30.88°C. But beyond 30.88 °C, the carotenoids fermentation yield decreased with increasing temperature when illumination intensity was set. Similarly, when the temperature was set, the carotenoids fermentation yield was also found to increase rapidly with increase of illumination intensity from 1000 to 2172 lux, and then there was a gentle decrease when the illumination intensity continued to increase.

What Fig. 5 (B) showed was that the influence of change of temperature and inoculum concentration on the carotenoids fermentation yield when illumination intensity was set as 0 level. In the range of temperature and inoculum concentration, there was a maximum of the carotenoids fermentation yield. When temperature was set as a constant, the carotenoids fermentation yield increased with increase of inoculum concentration from 5 to 9.66 %, but beyond 9.66 %, the carotenoids fermentation yield decreased with increasing inoculum concentration. What is more, when inoculum

Table 3 Comparison of microbial carotenids production using different waste substrates as carbon source

Microorganism	Molecule	Culture medium	Carotenoids production
Rhodopseudomonas sp.(bacteria) [21]	Carotenoids	RCVBN medium	1.455 mg/L
Dietzia natronolimnaea (bacteria) [30]	Canthaxanthin	Whey	3.29 g/L
Rhodobacter sphaeroides (bacteria) [10]	Carotenoids	Basal medium	17.245 mg/L
Blakeslea trispora (fungus) [31]	β-carotene	Corn steep liquor	20 g/L
Sporobolomyces roseus (yeast) [32]	β-carotene	Sodium succinate	2.58 g/L
Phaffia rhodozyma (yeast) [33]	Astaxanthin	Cassava residues	8.6 g/L
Sporobolomyces ruberrimus (yeast) [34]	Torularhodine	Technical glycerol	30 g/L
Rhodopseudomonas faecalis. PSB-B	Carotenoids	YP medium	16.93 mg/L

concentration was lower, the maximum of carotenoids fermentation yield was reached at a higher temperature while a higher temperature indicated otherwise. On the other hand, the carotenoids fermentation yield varied with the change of temperature presents a similar rule.

Fig. 5 (C) showed the 3-D contour plot at varying illumination intensity and inoculum concentration at fixed temperature (0 level). It indicated that carotenoids fermentation yield increased when the illumination intensity extended from 1000 to 2172 lux, and then began to decrease. At a fixed illumination intensity, the variety of carotenoids fermentation yield changed with inoculum concentration similarly as illumination intensity.

Comparison of carotenoids production capability with the literature

Microbial fermentation of carotenoids production has more advantage than the methods of chemical synthetic and extracted from plants. But the ability of carotenoids fermentation is different among various kinds of microbe. Table 3 lists fermentation carotenoids yield on several common microbe. The ability of fermentation carotenoids on yeast and mold is higher than bacteria. Carotenoids yield was compared PSB-B with the other photosynthetic bacteria strains in the literature. The carotenoids production of strain PSB-B is 16.93 mg/L, only next to *Rhodobacter sphaeroides* [10].

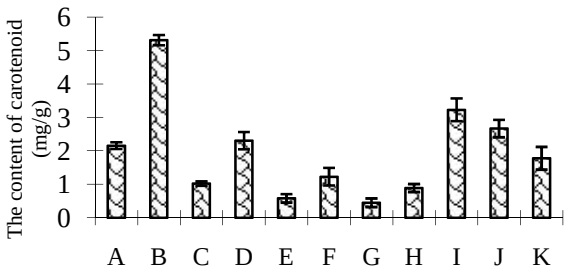

Figure 1 The determination of carotenoids content from eleven strains isolate

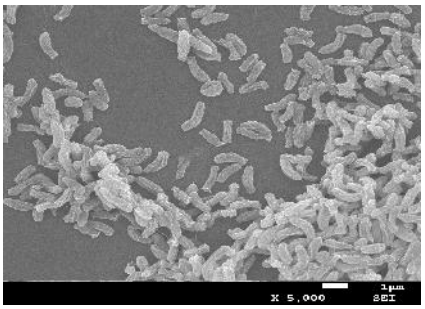

Figure 2 Morphological characteristic of strain PSB-B

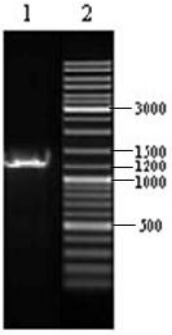

Figure 3 The electrophoretogram shows amplification of strain PSB-B 16S rDNA by a pair of universal primer 7F and 1540R. Line 1: DNA Marker; Line 2: isolate PSB-B

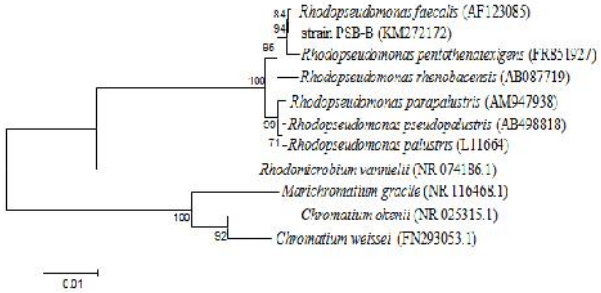

Figure 4 Phylogenctic tree shows the 16S rDNA relationships of strain PSB-B with other members of photosynthetic bacteria. The tree was constructed by MEGA 5 by the neighbor-joining method with bootstrap values calculated from 1000 trees.

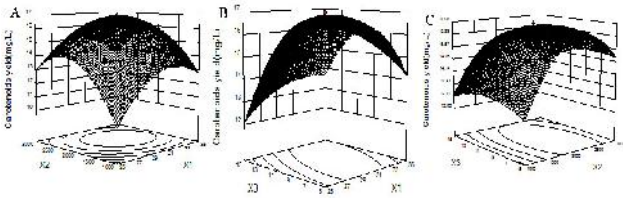

Figure 5 The 3-D plots showing the effects of variables (X_1: temperature, X_2: illumination intensity, X_3: inoculum concentration) on carotenoids yield. A: The interaction of temperature and illumination intensity; B: The interaction of temperature and inoculum concentration; C: The interaction of inoculum concentration and illumination intensity.

CONCLUSION

This study showed that *R. faecalis* PSB-B has the potential for carotenoids production at high efficiency under anaerobic light conditions. Optimal conditions of carotenoids production as follows: temperature 30.88 °C, illumination intensity 2172 lux, inoculum concentration 9.66 %. Under these conditions, the practical yield of carotenoids was 16.93 mg/L. Compared with carotenoids yield from other PSB strains, carotenoids extraction from strain PSB-B is of commercial value.

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