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ORIGINAL PAPER

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β-Carotene production by *Flavobacterium multivorum* in the presence of inorganic salts and urea

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Abstract *Flavobacterium multivorum*, a non-fermenting Gram-negative bacteria, normally produces zeaxanthin (3*R*, 3′ *R*-β, β-carotene-3, 3′ diol) as its main carotenoid. The effect of supplementation of various inorganic salts and urea on the growth, total carotenoid production, and proportion of β-carotene (β, β-carotene), β-cryptoxanthin (β, β-caroten-3-ol), and zeaxanthin produced by *F. multivorum* was investigated. Urea and several salts, such as calcium chloride, ammonium chloride, lithium chloride, and sodium carbonate, improved total carotenoid production by 1.5- to 2.0-fold. Urea and sodium carbonate had an unexpectedly strong positive effect on β-carotene production at the expense of zeaxanthin formation. The effect was found to be independent of incubation time, and β-carotene represented 70% (w/w) of the total carotenoid content. The cumulative effect of urea and sodium carbonate was further studied using response surface methodology. An optimum medium was found to contain 4,000 and 4,070 mg l^{-1} urea and sodium carbonate, respectively. The maximum β-carotene level was 7.85 μg ml^{-1} culture broth, which represented 80% (w/w) of the total carotenoid produced. Optimization resulted in 77- and 88-fold improvements in the volumetric and specific β-carotene levels, respectively, accompanied by a simultaneous decrease in the zeaxanthin level as compared to the control medium. The carotenoid production profile in the optimized medium indicated that β-carotene was produced maximally during the late exponential phase at 0.41 μg ml^{-1} h^{-1}. It is possible that this organism could be an excellent commercial source of either β-carotene or zeaxanthin, depending on initial culture conditions.

Keywords *Flavobacterium multivorum* · Zeaxanthin · β-Carotene · β-Cryptoxanthin · Response surface methodology

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Introduction

Carotenoids are antioxidant micronutrients reported to protect against many illnesses. Among them, β-carotene is the most widely known carotenoid, and some, but not all, clinical trials and epidemiological studies have indicated its utility against different types of cancers [18, 30, 31]. The possible protective action of β-carotene against cancer is usually attributed to its antioxidant nature, but gene regulation effects may also be involved [5]. Along with its medicinal uses, β-carotene is used in the cosmetics, food, and feed industries for its colorant and antioxidant properties [14, 17].

Researchers and biotechnologists have successfully commercialized microbial sources of β-carotene. The alga *Dunaliella* and the fungus *Blakeslea trispora* are two prominent β-carotene producers reported in the literature [20, 29]. There are also reports of mutation programs carried out using the yeasts *Xanthophyllomyces dendrorhous* and *Rhodotorula glutinis* for hyperproduction of β-carotene [7, 16].

Among natural carotenogenic bacterial sources, *Brevibacterium* sp. [19], *Micrococcus roseus* [10], *Mycobacterium* sp. [11], and *Flavobacterium* sp. are reported to produce β-carotene as a minor product only [23]. Recent efforts to use non-carotenogenic bacteria for carotenoid production by employing recombinant DNA technology have not yet reached a commercial stage [22, 26].

Flavobacterium sp. has been widely studied for production of the xanthophyll carotenoid zeaxanthin [1, 8, 23, 32], especially since this carotenoid may be

protective against age-related macular degeneration [27]. β-Carotene along with β-cryptoxanthin are known to act as precursors in the biochemical pathway of zeaxanthin production, and thus appreciable levels of these carotenoids (~5–10%) were observed during initial growth phases of *Flavobacterium* sp. [8, 23]. Hydroxylation of β-carotene and β-cryptoxanthin ultimately leads to accumulation of zeaxanthin.

Various researchers have studied the effect of nutritional factors on carotenoid production from *Flavobacterium*. However, these nutrients are known to enhance zeaxanthin production without affecting the β-carotene level. Several inorganic salts have been reported in the literature as inhibitors of the hydroxylation process [24], and inhibitors of carotenoid biosynthetic enzymes have been used previously to study the intermediates of the biosynthetic pathway [13], but most of them also inhibit growth. If, however, they can be used in the appropriate concentrations to facilitate carotenoid production without affecting growth [6], then quantitative optimization can be achieved using statistical approaches. Response surface methodology is one such statistical method, which can be employed in microbial biotechnology to study the effect of media components that play decisive roles in the process. It can be coupled with the method of "steepest ascent" to optimize quantitative values of media supplements. It has been used recently by many researchers in assessing the role of media components in carotenoid production [33, 35].

In this study, the effect of several salts and urea on carotenoid production from *Flavobacterium multivorum* ATCC 55238 was studied, with special emphasis on β-carotene production. Selected salts and urea were optimized using the sequential approach of response surface methodology and method of steepest ascent. Carotenoid production profiles in the optimized medium were studied.

Materials and methods

Reagents and chemicals

Media ingredients and β-carotene were purchased from Sigma (St. Louis, Mo.). High performance liquid chromatography (HPLC) grade methylene chloride, methanol, and hexane were obtained from Fisher Scientific (Pittsburgh, Pa.). Synthetic zeaxanthin and β-cryptoxanthin were a gift from Hoffmann-La Roche (Basel, Switzerland). Mineral salts and urea listed in Table 1 were purchased from Amresco (Solon, Ohio).

Cultivation of the organism

F. multivorum ATCC 55238 was obtained from American Type Culture Collection (ATCC, Manassas, Va.). The culture was maintained at 4°C on solidified medium containing (g l^{-1}): glucose 35, yeast extract 20, peptone 10, $MgSO_4 \cdot 7H_2O$ 0.2, at pH 6.0. The basal liquid growth medium for shake flask studies contained (g l^{-1}): glucose 25 (autoclaved separately), yeast extract 10, peptone 10, at pH 7.0. A 5% (v/v) inoculum of *F. multivorum* ATCC 55238 in the logarithmic phase (14 h, $A_{500\ nm} = 0.8$), grown in the basal medium was used throughout the studies. All experiments were performed in 50 ml medium in 250 ml Erlenmeyer flasks and incubated at 28 ± 1°C on a rotary shaker at 250 rpm for 60 h.

Pigment extraction from bacterial cells

Culture broth (1 ml) was centrifuged at 1,800 *g* for 10 min at 4°C. The supernatant was discarded, and media components in the cell pellet were washed away by repeated suspension in sterile distilled water and

Table 1 Effect of salt and urea addition to the growth medium (2,500 mg l^{-1}) on carotenoid production by *Flavobacterium multivorum* ATCC 55238 incubated for 48 h. β-C β-Carotene, β-Cryp β-cryptoxanthin, *Zea* zeaxanthin

Run	Salt	$OD_{500\ nm}$	Carotenoids ± SD ($n = 3$, μg ml^{-1})			
			β-C	β-Cryp	Zea	Total
1	Control	8.0	0.11 ± 0.015	0.14 ± 0.07	2.85 ± 0.08	3.02
2	$CaCl_2 \cdot 6H_2O$	7.4	3.70 ± 0.15	1.37 ± 0.12	2.10 ± 0.15	7.10
3	$CuSO_4 \cdot 5H_2O$[a]	2.4	0	0.079 ± 0.04	0.48 ± 0.07	0.56
4	LiCl	5.4	1.07 ± 0.06	0.54 ± 0.08	3.4 ± 0.20	5.01
5	$MgCl_2$	3.89	0.49 ± 0.02	0.63 ± 0.02	2.24 ± 0.13	3.36
6	$MgSO_4 \cdot 7H_2O$	5.6	0.19 ± 0.01	0.57 ± 0.02	3.05 ± 0.23	3.81
7	Na_2CO_3	6.8	5.61 ± 0.19	0.93 ± 0.09	1.54 ± 0.13	8.08
8	NaCl	5.0	0.25 ± 0.01	0.56 ± 0.07	3.06 ± 0.12	3.87
9	$NaH_2PO_4 \cdot H_2O$	4.8	0.15 ± 0.01	0.32 ± 0.04	2.61 ± 0.14	3.08
10	NH_4Cl	5.6	0.52 ± 0.04	0.63 ± 0.09	3.16 ± 0.27	4.31
11	$NH_4H_2PO_4$	5.0	0.11 ± 0.01	0.36 ± 0.02	3.10 ± 0.20	3.57
12	Urea (CH_4N_2O)	7.0	4.97 ± 0.01	1.35 ± 0.08	1.21 ± 0.14	7.53
13	$ZnCl_2$[a]	2.4	0.00	0.068 ± 0.08	0.53 ± 0.06	0.59
14	$ZnSO_4 \cdot 7H_2O$[a]	2.1	0.00	0.00 ±	0.50 ± 0.05	0.50

[a]Supplemented salt was 500 mg l^{-1}

centrifugation. The cell mass was subjected to sonication using a sonic dismembrator (Fisher Scientific, model number F60) in the presence of 1 ml cold, oxygen-free methanol containing 0.01% butylated hydroxytoluene (BHT) (w/v) for 30 s (output power 5 W). Methanol extraction was repeated at least three times for complete extraction of carotenoids from the bacterial cells. The sonicated sample was centrifuged to remove the white cell pellet. The supernatant contains extracted carotenoids.

High performance liquid chromatography

The pigments in methanol were dried by vacuum evaporation in a Speedvac Plus (SC110, Savant, Farmingdale, N.Y.) and re-dissolved in 1 ml HPLC mobile phase [hexane: dichloromethane: methanol: *N*, *N*′-di-isopropylethylamine (80:19.2:0.7:0.1)]. HPLC separation was carried out at a flow rate of 1.0 ml min^{-1} on a cyano column (Microsorb 25 cm length ×4.6 mm i.d., Rainin Instrument, Woburn, Mass.). The column was maintained at room temperature, and the HPLC detector was operated at 450 nm. Peak identities were confirmed by photodiode-array spectra and by coelution with authentic standards as required. Integrated areas of the carotenoid peaks were converted to carotenoid concentrations through the use of calibration curves generated from HPLC injections of known quantities of authentic carotenoid standards. Intracellular carotenoid levels were expressed in volumetric (μ g/ml^{-1} culture broth) or specific cellular units [μg g^{-1} cell dry weight (CDW)].

Cell growth measurement

Cell growth was monitored by measurement of turbidity at 500 nm with a UV-Visible spectrophotometer (Smart Spec 3000; Bio-Rad, Richmond, Calif.). Samples were appropriately diluted (to an absorbance between 0.2 and 0.8) with double distilled water, and absorbance was measured immediately at 500 nm. For CDW estimation, the 10 ml sample was centrifuged at 1,800 *g* for 10 min and washed twice with double distilled water by suspension and centrifugation. The supernatant was discarded, and the cell pellet was then dried to constant weight in an oven at 80°C.

Effect of supplementation of salts and urea

To study the effect of salts and urea, *F. multivorum* was studied for growth and carotenoid production in liquid basal medium supplemented with urea and the salts listed in Table 1 (2,500 mg l^{-1}). The experiments were performed in quadruplicate in shake flask culture at 30°C on a rotary shaker at 250 rpm. Most of the supplements were chosen based on their role in carotenoid production in microbes available in the literature [6].

Response surface methodology

After initial experiments, urea and sodium carbonate, both of which promoted β-carotene production, were selected as independent variables in a central composite design for optimization using response surface methodology. A 2^2 first order factorial design with three center points (2,500 mg l^{-1} each) was performed in random order as listed in Table 2 [2, 9, 12, 28]. All variables had a central coded value designated as zero.

The minimum and maximum ranges of variables investigated are given in actual (1,000 and 4,000 mg l^{-1}) and coded forms (−1 and +1). The levels of the variables in coded units were X_1 and X_2 for urea and sodium carbonate, respectively. Upon completion of experiments, the average maximum volumetric production of β-carotene (μg ml^{-1}) was used to assess the response. This allowed efficient fitting and checking of the first-degree polynomial model that was used to estimate the predicted response, Y_p.

$$Y_p = \beta 0 + \beta_1 X_1 + \beta_2 X_2 \quad (1)$$

This model was chosen in the belief that the predominant local characteristics of the surface were its gradients and that the local surface could be roughly represented

Table 2 Results from first factorial design with three center points

Run	Order	$OD_{500\ nm}$	Variables (mg l^{-1})		Variables in coded units		Response yield (μg ml^{-1})		
			Urea	Na_2CO_3	X_1	X_2	Actual	Predicted	Residual
0	–	4.3	0	0	–	–	0.52	–	–
1	1	4.9	1,000	1,000	−1	−1	2.14	2.65	−0.51
2	3	5.5	4,000	1,000	+1	−1	5.56	5.43	0.13
3	5	4.8	1,000	4,000	−1	+1	5.70	5.57	0.13
4	7	7.4	4,000	4,000	+1	+1	7.85	8.35	−0.50
5	2	5.3	2,500	2,500	0	0	5.40	5.5	−0.10
6	6	5.2	2,500	2,500	0	0	5.63	5.5	0.13
7	4	5.2	2,500	2,500	0	0	5.38	5.5	−0.12

by a planar model (Eq 1) with the slope β_1 in the X_1 direction and slope β_2 in the X_2 direction; β_0 represents the intercept of the equation. Further optimization was achieved using the method of steepest ascent using a suitable step size [3, 28].

Growth and carotenoid production in optimized medium

Growth and carotenoid production by *F. multivorum* were studied using optimized medium in shake flasks (in quadruplicate) at 30°C on a rotary shaker at 250 rpm. Samples were removed periodically and analyzed for cell growth as well as carotenoid production.

Results

Effect of supplementation of salts and urea

Urea, sodium carbonate, and calcium chloride had a strong stimulatory effect on volumetric production of β-carotene and resulted in only a minor inhibition of growth (Table 1). β-Carotene represented 66, 69, and 52% (w/w) of the total carotenoid content under the influence of these three compounds, respectively. Lithium chloride supported β-carotene production, but was observed to be time dependent, as the maximum β-carotene level (44%, w/w) was observed at 24 h of growth phase, and eventually fell to 21% (w/w) at the end of fermentation (48 h). Zeaxanthin was the major carotenoid in the presence of ammonium and magnesium salts. Both these latter salts had a positive effect on carotenoid production pattern but an inhibitory effect on final growth of the bacteria. Zinc and copper salts had a negative effect on growth even at low concentrations.

Optimization by response surface methodology

The effect of urea and sodium carbonate, the two compounds most stimulatory for β-carotene production, was studied using response surface methodology. Taking 2,500 mg l^{-1} as a center point, concentrations of 1,000 and 4,000 mg l^{-1} were evaluated as shown in Table 2, which summarizes the central composite design along with the experimental and predicted responses in each individual experiment.

The least squares estimates of β_1 were calculated using the method described by Box et al. [9]. The least squares estimate of intercept β_0 is the average of all seven observations and was calculated to be 5.5. Statistical analysis indicated that sodium carbonate ($\beta_2 = 1.46$) was a little more influential than urea ($\beta_1 = 1.39$) individually. We thus obtained the fitted equation $Y_p = 5.5 + 1.39X_1 + 1.46X_2$. Experimental values displayed excellent correspondence to the predicted values.

Montgomery [28] proposed that a 2^2 full factorial design should allow the experimenter to (1) obtain an estimate or error, (2) check for interactions in the model, and (3) check for quadratic effects or curvature. All three proposed calculations are essential to explore the adequacy of the first order model.

The estimate of error using the replicates at the center of the design was 0.52. The interaction between the variables was measured by the coefficient β_{12} of the added cross product term X_1X_2 in the model. The coefficient was determined to be −0.3175. The interactive effect between these two factors indicated that the maximum estimated response was obtained at the combinations of higher coded values (+1) of both urea and sodium carbonate. The three-dimensional response surface curves were then plotted to understand the interaction of urea and sodium carbonate.

The adequacy of the straightline model was further confirmed by applying the check for quadratic (curvature) effects. The "lack of fit" statistic obtained by comparing the sum of squares to the estimate of error resulted in a very low value (0.008), indicating that there is no quadratic effect. Both the interaction and curvature checks were not significant, whereas the *F*-test for the overall regression is significant. Furthermore, the standard error of β_1 and β_2 is 0.36 $\left(\sqrt{\sigma^2/4}\right)$. Both regression coefficients β_1 and β_2 are large relative to their standard errors. Hence the model was observed to be ideal for optimization. The selected concentrations of urea and sodium carbonate did not result in the appropriate curved surface in the response surface graph. Therefore, further increases in their concentrations, along with increases in time intervals were needed for optimization.

Table 3 Results of experiments performed on the path of steepest ascent for optimization

Run	Final pH	$OD_{500\ nm}$	Variables (mg l^{-1})		Coded conditions		Response yield (μg ml^{-1})
			Urea	Na_2CO_3	X_1	X_2	
5,6,7	6.36	5.23	2,500	2,500	0	0	5.47 (average)
8	6.9	7.84	4,000	4,075	1	1.05	7.70
9	7.7	4.39	5,500	5,650	2	2.10	5.23
10	7.8	3.91	7,000	7,225	3	3.15	4.34
11	8.0	3.45	8,500	8,800	4	4.20	3.40
12	8.2	3.61	10,000	10,375	5	5.25	3.28

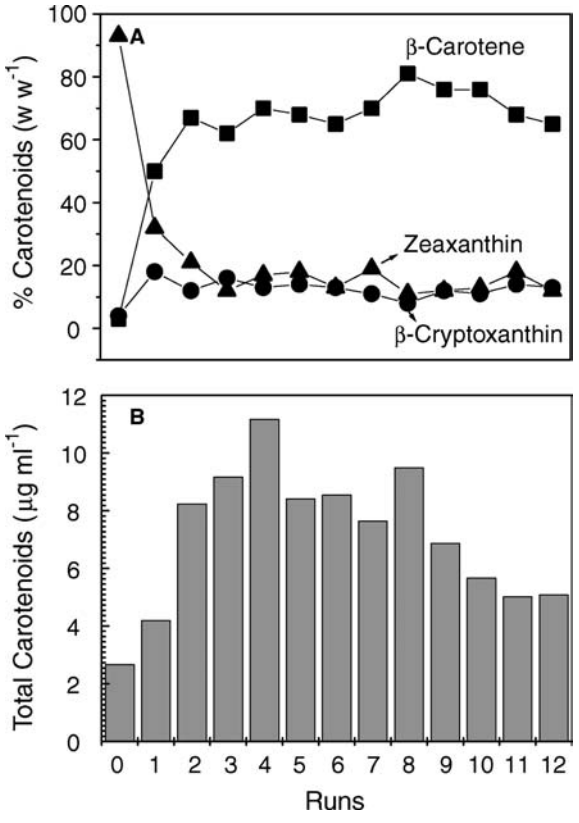

Fig. 1 Stepwise improvement in the β-carotene proportion (**a**) and content (**b**) with simultaneous decrease in zeaxanthin levels using a sequential optimization approach

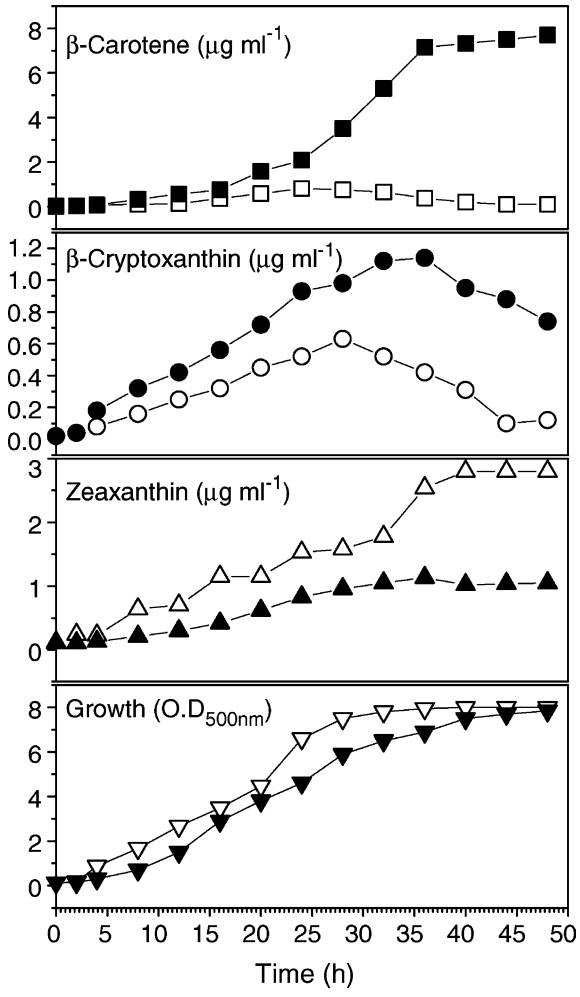

Fig. 2 Growth pattern (OD at 500 nm) and production profiles ($\mu g\ ml^{-1}$) of β-carotene, β-cryptoxanthin and zeaxanthin by *Flavobacterium multivorum* ATCC 55238 grown in basal (*open symbols*) and optimized (*closed symbols*) medium. Basal medium lacks both urea and sodium carbonate

To determine optimum values, points on the path of steepest ascent were selected by moving 1.46 units in X_2 for every 1.39 units moved in X_1 as shown in Table 3 and Fig. 1 (Experimental runs 8–12). The optimum combination was observed to contain 4,000 and 4,070.5 mg l^{-1} urea and sodium carbonate, respectively, which resulted in 7.7 $\mu g\ ml^{-1}$ β-carotene. Further increase in the quantities led to a decrease in β-carotene content and growth. This may be because of unfavorable growth conditions due to an increase in pH of the growth medium (Table 3). Ideally, a second factorial should be designed using the best combination in the steepest ascent experiment as central data points. In this case, however, the optimum combination was very close to the fourth run in the first factorial experiment of Table 2, so further experiments were unnecessary.

Carotenoid production profile

Growth and carotenoid production profiles of *F. multivorum* ATCC 55238 were studied in salt- and urea-supplemented, optimized medium and were compared with basal medium lacking these supplements (Fig. 2). The maximum specific growth rate (μ_{max}) and maximum CDW achieved with the optimum medium were 0.24 h^{-1} and 3.21 g l^{-1}, respectively, as compared to 0.36 h^{-1} and 3.28 g l^{-1} obtained in the basal medium. Supplementation of urea and sodium carbonate resulted in a slower growth rate of bacteria, but similar cell masses were ultimately achieved.

In the optimized medium, the production profile indicated that β-carotene was the major carotenoid produced throughout the fermentation (Fig. 2). The maximum production rate for β-carotene was observed between 24 and 36 h (0.41 $\mu g\ ml^{-1}\ h^{-1}$) as compared to the maximum obtained with the control medium obtained between 20 and 24 h (0.09 $\mu g\ ml^{-1}\ h^{-1}$). Zeaxanthin, on the other hand, displayed a remarkable decrease in the maximum production rate in the optimized medium (0.05 $\mu g\ ml^{-1}\ h^{-1}$) as compared to the control basal medium (0.13 $\mu g\ ml^{-1}\ h^{-1}$). β-Cryptoxanthin was produced at a similar rate in both control and optimized media.

In the basal medium, the maximum total volumetric carotenoid production was observed to be 3.02 $\mu g\ ml^{-1}$, while the specific productivity level was observed to be

921 μg g^{-1}, representing β-carotene, β-cryptoxanthin, and zeaxanthin in the proportion of 3:4:93 (w/w). In the optimized medium, total volumetric and specific carotenoid content was observed to be 9.49 μg ml^{-1} and 2,965 μg g^{-1}, respectively, and β-carotene, β-cryptoxanthin, and zeaxanthin were in the proportion of 82:7:11 (w/w). Thus, through sequential experimental optimization, β-carotene displayed 77- and 88-fold improvements in volumetric production and cellular accumulation, respectively.

Discussion

β-Carotene is an important antioxidant—provitamin A—and colorant in the market, and several microbial and non-microbial sources have been reported and exploited for potential commercialization. Among microbial sources, production of β-carotene by the alga *Dunaliella* sp. is a well-developed technology [3, 4]; however, production of carotenoids by slow-growing microalgae, particularly *Dunaliella* sp. requires maintenance of stress conditions of high salt concentration and intense light. In addition, they have a requirement for CO_2 and oxygen during day and night cycles of growth, respectively. The fungus *B. trispora* is also capable of producing high amounts of β-carotene, and detailed studies on production parameters have been reported [21, 25, 34]. However, production of carotenoids by *B. trispora* is dependent upon sexual mating of two compatible strains during fermentation, and the growth of *B. trispora* in fermenters becomes viscous and needs considerable energy input to keep the broth aerobic and well mixed. Production of β-carotene by mutants of the yeast *X. dendrorhous* and *R. glutinis* was also reported recently [7, 16], but their commercial potential is still under investigation.

Bacteria have the obvious advantage of faster growth rate over algae and fungi, but there are very few bacterial sources of β-carotene available. Johnson and Schroeder have listed a few of these β-carotene-producing bacteria, but most produce β-carotene only as a minor product [20]. *F. multivorum* ATCC 55238 is well known for the production of xanthophyll carotenoids such as zeaxanthin [8, 23] but, in this study, we report that *F. multivorum* can also be an excellent producer of β-carotene after cultural manipulation based on supplementation of urea and sodium carbonate.

Biosynthetic studies show that zeaxanthin is synthesized from β-carotene by hydroxylation of C(3) and C(3′) of the β-ionone rings via the monohydroxy intermediate β-cryptoxanthin, a process that requires molecular oxygen in a mixed-function oxidase reaction. Logically, inhibition of hydroxylase activity should lead to accumulation of β-carotene. There are literature reports of a few inhibitors, such as $CaCl_2 \cdot 6H_2O$ and solid alkaloid salts, that lead to accumulation of β-carotene [24]. However, the use of these inhibitors is limited to biochemical studies only, as most such inhibitors also severely inhibit growth.

Our initial experiments revealed that β-carotene was produced as the major carotenoid (≥70%) in the presence of urea and sodium carbonate in the growth medium. The carotenoids were analyzed by HPLC and confirmed by matching absorption spectra with authentic carotenoids. Urea and sodium carbonate were observed to stimulate β-carotene production from *Flavobacterium*. Previously, supplementation of urea has been reported to increase volumetric yield of carotenoids in *Phaffia rhodozyma* [15] and cellular levels in *R. glutinis* [7], but alterations of the biochemical pathways of xanthophyll production in the presence of urea have never been reported. Likewise, sodium carbonate is used as a nutrient source by only a few alkalophilic microorganisms, and it has never been reported to enhance carotenoid production. Urea and sodium carbonate are likely to work via inhibition of hydroxylase activity, leading to substantial inhibition of zeaxanthin formation. The interactive effects of these two supplements were further studied using response surface methodology experiments to maximize the β-carotene level without affecting the growth performance of the organism drastically. This sequential optimization approach led to a substantial increase in β-carotene proportion and content (Fig. 1), which could be significant on an industrial scale. Thus, this study opens the way for exploitation of a novel, fast-growing, bacterial source of β-carotene.

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