

Effects of light intensity and the remaining nitrate concentration on the beta-carotene accumulation of a wild *Dunaliella salina* strain isolated from the saline soil

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Abstract

An isolated *Dunaliella salina* strain from northern Thailand was cultured in modified Johnson's medium in column photobioreactor. The beta-carotene accumulation mainly depended on the quantities of cells entering into carotenogenesis condition that was significantly enhanced by high started KNO₃ concentration. Low remaining nitrate concentration in the culture of each cell (RNCC) was suitable for algae to accumulate beta-carotene. Following the cultivation time extended, RNCC of all cultures decreased and tended to the same level (10-20 pg/cell) although the biomass or beta-carotene content in the culture was higher in high started KNO₃ concentration. High light intensity restrained the growth especially in low KNO₃ concentration but improved beta-carotene accumulation and RNCC. The highest biomass and beta-carotene dry weight (DW) were 2.25 g L⁻¹ and 79.2 mg g⁻¹ DW respectively. Above results indicated that increasing the biomass and as early as possible to strengthen the stress on each cell was important to improve the final beta-carotene yield.

Introduction

Beta-carotene is a carotenoid, one of groups of plant pigments known to have antioxidant and other effects. This is a substance in plants that's quickly converted into vitamin A inside the body. Beta-carotene is often thought of as a form of vitamin A itself. Having normal levels of vitamin A is important for good vision, strong immunity, and general health.¹⁻³ The most important natural beta-carotene resource come from the cultivation of the unicellular

biflagellate marine green microalga *Dunaliella* spp.⁴ *Dunaliella salina* (*D. salina*) is an ideal species in algal production because of its extremely adaptability to various adversity stress.^{5,6}

Present studies have confirmed that beta-carotene yields can be improved under stress conditions such as high irradiance, high temperatures, high salt concentration and/or nutrient deficiency *etc.*^{4,7} However, one of problems was that increasing stresses could decrease the biomass although the beta-carotene content per cell is significantly improved.^{4,6,8} Therefore, the main optimization or research targets of beta-carotene production are finding a balance between the maximum biomass and beta-carotene yield.⁷⁻⁹ KNO₃ is one of the main factors to directly affect the biomass and beta-carotene accumulation. Low nitrate concentration is suitable for beta-carotene production but opposite for biomass accumulation. However, the living habits of each algae species or strain are different that resulted the biomass or beta-carotene yields is different under the same KNO₃ concentration.

In order to obtain the maximum beta-carotene yield, developing the photo-bioreactors or two-phase cultivation were often mentioned and developed in algal production.^{2,10} These methods mainly focused on the improvement of stress conditions, *e.g.* modifying the nutrition concentration or components of culture medium, increasing the illuminated surface, adjusting temperature, salinity or pH *etc.* These methods provided a lot useful proposes in algal production. However, conclusions of these methods were not very in agreement, *e.g.* some studies pointed that 10 mM, 5 mM or 2 mM even 1 mM nitrate were suitable for algae growth.^{4,7,11-13} Hence, the relations among the remaining nitrate concentration in the culture medium, cell quantities and beta-carotene accumulation were studied to explain these differences in this research.

Moreover, *D. salina* strain KU XI in this research was isolated from saline soil in Chaiyaphum, Thailand, where ~17% area was covered by saline-alkali soil and suitable for commercial algal production.⁹ Hence, aims of this research were also to study the growth, beta-carotene accumulation and cell morphology of this isolated wild strain under different way of adding KNO₃, with the hypothesis that improving the stress on each cell could enhance the total beta-carotene yields. Meanwhile, results of this study could also be used for future commercial algal production in northern Thailand.

Materials and Methods

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Key words: Biomass; beta-carotene; *Dunaliella salina*; light stress; nitrate limitation.

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Contributions: ZW developed the original idea and the protocol, abstracted and analyzed data, wrote the manuscript; CM provided the methods and instruments; NJ and PD provided the idea, experimental materials, equipment, manuscript correction and final support; RA and WA attended the experiment and reviewed the manuscript.

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D. salina strain KU XI was obtained from bioscience lab of Faculty of Science, Kasetsart University, Thailand and cultured in modified Johnson's medium in column photobioreactor at 30 with a working volume of 250 mL⁹. The salinity (NaCl concentration) was 2.5 M. Initial pH and cell density was controlled at 7.5 and approx. 70×10⁴ mL⁻¹. Bioreactors were aerated with compressed air containing 5% CO₂ (v/v) at approx. 0.01 vvm (volume of air per volume of medium per minute) and irradiated continuously from two sides with cool white fluorescence lamps giving 54 μmol m⁻² s⁻¹ and 200 μmol m⁻² s⁻¹ respectively¹⁴.

KNO₃ concentration was set at 0.1, 0.2, 0.3, 0.5, 0.75 and 1 g l⁻¹ respectively as 6 treatments with 4 repetitions of each. Two groups were designed, namely non-fixed and fixed KNO₃. In the first group, different KNO₃ concentrations were one-time added at the first day and the started cell density was approx. 70×10⁴ mL⁻¹; In

the second group, algae were preliminarily cultured in the modified Johnson's medium with 1.5 M NaCl and 1 g KNO₃ l⁻¹ for 7 days and the biomass was collected by centrifugation (4000 rpm, 5 min). The biomass was uniformly added into each treatment and finally the started cultivation cell density was approx. 8×10⁶ mL⁻¹. KNO₃ was multiple supplemented to the medium every two days for maintaining the same nitrate concentration as the first day.

Modified alkaline potassium persulfate digestion-UV spectrophotometric method (Chinese national standard, GB 11894-89) was used to check nitrate concentration. 10 ml algae solution was diluted 20 times or higher then filtered (0.45 μm). 1 mL HCl was added into 10 ml filtered solution and then fixed this sample to 50 ml with deionized water. The optical density (OD) of this diluted sample was checked by HP8453 ultraviolet spectrophotometer manufactured by Hewlett-Packard Company (Paolo Alto, CA, USA) and calculated the OD value difference (OD₂₂₀ - 2 × OD₂₇₅). Nitrate concentration could be directly calculated by the standard nitrate curve which was determined by a series of known KNO₃ concentration and their OD value difference (OD₂₂₀ - 2 × OD₂₇₅). Finally, the remaining nitrate concentration in the culture of each cell (RNCC) was calculated:

$RNCC = C_n / N \times 10^6$, where the C_n was concentration of nitrate in the culture (μg mL⁻¹) and N was cell density (cells mL⁻¹), the unit of RNCC was pg/cell.

The biomass was harvested after 15 days by centrifugation and dried in oven at 60°C for three days. Cell densities was checked by daily counting in a hemacytometer and the raw data was processed with the method of sigmoid curve fitting by the software of Table Curve 2D v5.01 for calculating relative growth rate (RGR) and specific growth rate (μ):

$$RGR = \ln(N_2/N_1) / (t_2 - t_1);$$

$\mu = \ln 2 / t_d$, where N₂ or N₁ was the cell density at the check day and the first day; t₂, t₁ and t_d was the check day, first day and doubling time respectively. Beta-carotene was extracted by 95% acetone and its extraction solution was analyzed and checked by Waters e2695 HPLC with Waters RP18 Column 5 μm, manufactured by Waters Company, Milford, USA. In the mobile phase, solvent A was ethyl acetate and solvent B was acetonitrile and water (9:1, v/v). The flow rate was 1 mL min⁻¹. The solvent programming was that: 0-16 min, 0-60% solvent A; 16-30 min, 60% solvent A; 30-35 min, 100% solvent A.^{2,15} Finally, beta-carotene content was converted by dry biomass weight (mg g⁻¹ DW). All the data analysis was processed by the software of Microsoft Excel 2010 and SPSS 16.0 with the method of ANOVA and t-Test.

Results and Discussion

Biomass and beta-carotene content under different light intensity and KNO₃ concentration

Increasing KNO₃ concentration could significantly improve the beta-carotene content and the growth under different light intensity, however, their specific growth rate did not show a corresponding tendency (Table 1 and Figure 1). High light intensity was suitable for beta-carotene accumulation but opposite for biomass accumulation. The beta-carotene content (mg L⁻¹) in high light intensity was not significant higher than in low light intensity (t=1.81, P=0.45). Moreover, the beta-carotene contents (pg/cell, mg L⁻¹) in high KNO₃ concentration were both higher than in low KNO₃ concentration (Table 1 and Figure 1), however, the beta-carotene content (mg L⁻¹) under the low and high light intensity was not significant difference when KNO₃ concentration higher than 0.75 g L⁻¹ (F=5.98, P=0.19 and F=5.98, P=0.16 respectively). This result could be related with the stress of nitrate limitation on each cell or required more time to accumulate beta-carotene. Above results showed that the final biomass or beta-carotene content was mainly affected by the started nitrate concentration.

Previous studies mentioned that 10 mM, 5 mM, 2 mM and 1 mM nitrate were suitable for algae growth.^{4,7,11-13} Prevalent views that high nutrition or low stress intensity was suitable for growth and opposite for beta-carotene accumulation.^{12,16,17} However, results of this study were not very consistent with these results and showed that the beta-carotene contents (pg/cell, mg L⁻¹) were both improved when increasing the KNO₃ from 0.1 g L⁻¹ to 0.75 g L⁻¹ (Table 1). Results of this study also showed that growth of this *D.salina* strain was enhanced when KNO₃ greater than 0.4 g L⁻¹. Obviously, here arises a question that what concentration of nitrate was suitable for growth or beta-carotene accumulation? The nutrition in medium would be rapidly consumed when the algae reproduced massively. Above mentioned nitrate concentrations were all the started nitrate concentration. Also, growth and nutrition consumption in different environment or algae species were different. Hence, especially in the late cultivation stage, the remaining nutrition would be more effective on the growth or beta-carotene accumulation.

Nitrogen resource was a vital factor for *D. salina* growth or beta-carotene production. Algae would die if absent nitrogen resource long time.^{8,18} Only the living cells under the long period of nitrate limitation could synthesis large amount of carotenoids for maintain-

ing the photosynthesis and metabolism.¹⁹ Therefore, some algae cells in the low started nitrate concentration could not exist long enough to accumulate beta-carotene in the late stage and died or the new reproductive cells not yet to produce beta-carotene. Hence, the beta-carotene contents (pg/cell, mg L⁻¹) in the culture of low started nitrate concentration were lower than in high started KNO₃ concentration. Moreover, it also indicated that quantities of living cells especially those cells entered into carotenogenesis conditions in the late stage would be vital for final beta-carotene yield.

Biomass and beta-carotene content under different light intensity and fixed concentration of KNO₃

Low KNO₃ concentration significantly limited the growth and beta-carotene accumulation (Figure 2 and Table 2). However, beta-carotene content (pg/cell) and its dry weight were higher in low KNO₃ concentration (Table 2). Cell densities under high light intensity was lower, whereas the beta-carotene dry weight was higher than under the low light intensity although their difference were not significant (t=1.81, P=0.36). Moreover, the beta-carotene content (mg L⁻¹) in the way of multiple supplementing KNO₃ was significantly lower than the way of one-time adding KNO₃ (Figure 1 and Figure 2). Above results showed that the final biomass or beta-carotene content was mainly affected by the KNO₃ concentration.

Carotenoids have been recognized to play essential roles in light harvesting for photosynthesis. Carotenoids could also prevent the injury to the cell from over high light intensity. Hence, light was considered an important factor to affect algae growth and beta-carotene accumulation.²⁰ Light compensation point and saturated point of algae was 30 μmol m⁻² s⁻¹ and 600 μmol m⁻² s⁻¹ respectively and Ben-Amotz *et al.* found that increasing light intensity in this range was less effective for biomass accumulation but high effects for beta-carotene accumulation.²¹ Results of this study were consistent with this view (Figure 1 and Figure 2) although the beta-carotene content (mg L⁻¹) under high and low light intensity was not significant difference (t=2.23, P=0.97).

Algae growing and beta-carotene accumulation obviously required different environment or nutrition levels. Generally, low stress intensity would promote the biomass accumulation but high stress intensity would improve beta-carotene accumulation. Hence, two-phase cultivation was an effective way to improve the beta-carotene yields.^{2,8,19,22}

The beta-carotene content (pg/cell) or dry weight was increased when the KNO₃ concentration decreased (Table 2); however, the beta-carotene content (mg L⁻¹) was improved when

the KNO_3 concentration increased (Figure 2). According to the methods of two-phase cultivation, the maximum biomass obtained at the low stress conditions, and then the biomass was quickly transferred to high stress intensity environment, such as low nitrate concentration, high light intensity, high salinity and high temperature etc.^{8,19} However, a fact that cell density was rapidly decreased when the biomass was transferred into the medium containing low KNO_3 (Figure 2). This result further reflected that nitrate deprivation would significantly restrain algae growth or low

nitrate would seriously affect the survival ratio of algae.^{8,18,19,23} Hence, high quantities of living cells in high nitrate concentration accumulated higher beta-carotene content although the accumulation of beta-carotene in each cell was lower.

It should be noticed that the total beta-carotene yields by multiple supplementing nitrate was lower than by one-time giving nitrate. This could be related with the lack of enough nitrate limitation stress on each cell. Borowitzka *et al.* considered that wild *D salina* can live better than the other organisms

because of long-term adaptability to the environment, meanwhile, the stress from the environment such as high salinity, nutrition deprivation and high light intensity *etc.* could limit the cell division. Following the stress time extended, some *Dunaliella* cells conducted into the carotenogenesis condition and started to accumulate beta-carotene.^{8,19,22,24,25} During this period, the cell morphology was also changed, from ovoid to round or cell size increased, etc. Obviously, the more cells entered into the carotenogenesis conditions, the more accumulation of beta-carotene.

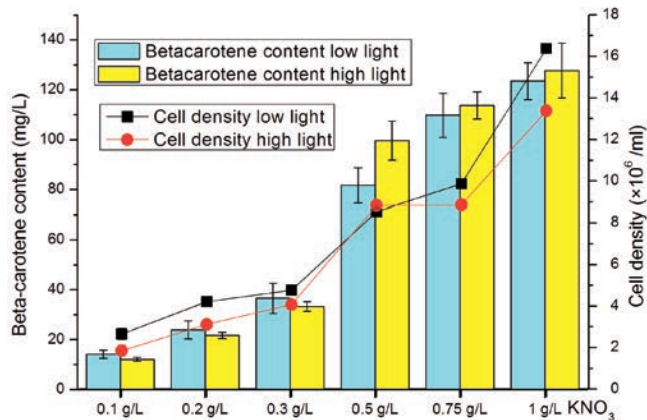


Figure 1. The cell density and beta-carotene content under different light intensity and KNO_3 concentration. KNO_3 was one-time added at the first day; all values were collected at 15th cultivation day.

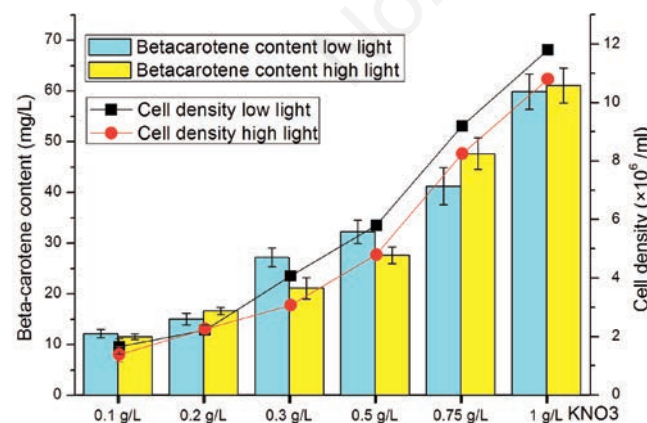


Figure 2. The cell density and beta-carotene content under different light intensity and KNO_3 concentration. KNO_3 was multiple supplemented every two days for maintaining the same concentration as the first day; all values were collected at 15th cultivation day.

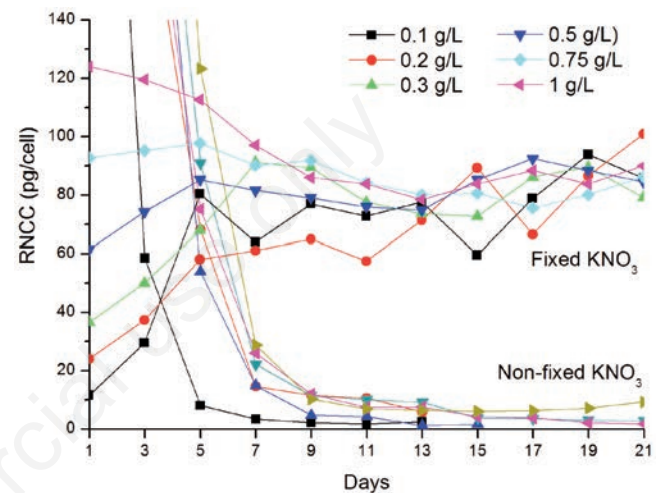


Figure 3. Remaining nitrate concentration in the culture of each cell under low light intensity. Fixed KNO_3 , multiple supplementing KNO_3 to the culture every two days for maintaining the same concentration as the first day; Non-fixed KNO_3 , one-time adding KNO_3 at the first day.

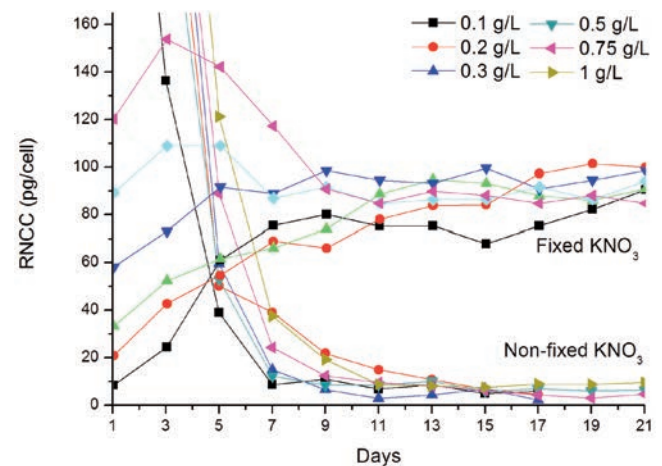


Figure 4. Remaining nitrate concentration in the culture of each cell under high light intensity. Fixed KNO_3 , multiple supplementing KNO_3 to the culture every two days for maintaining the same concentration as the first day; Non-fixed KNO_3 , one-time adding KNO_3 at the first day.

Remaining nitrate concentration in the culture of each cell under low light intensity

Following the cultivation days extended, the cell was rapidly growing and KNO_3 was quickly consumed that promote the RNCC rapidly decreased; meanwhile, RNCC of all cultures dramatically decreased and kept a stable and lowest level after 5-7 days (Figure 3). This result reflected that cell densities and the nutrition in the medium had formed a balance, which was based on the nutrition space of each cell. Long-term low RNCC was suitable to induce the algae cell into carotenogenesis conditions. According to the results shown in Figure 1 and Figure 2, beta-carotene content (mg L^{-1}) was higher in Non-fixed- KNO_3 group; it could be related with the low RNCC in this group. This result could be also explained by the algae adaptability to the environment.

Nitrate limitation is known to inhibit cell division, therefore the cells divide at a slow rate and produce more beta-carotene to protect the cells to prevent possible injury from radiation or maintain the photosynthesis.^{19,23} In addition, some algae died because of their low nitrate stress adaptability or natural senescence; hence, the way of one-time adding KNO_3 provided a relative stable nitrate limitation environment and high started nitrate concentration remained more living cells in the late stage entering into carotenogenesis con-

dition to accumulate more beta-carotene.²² In the way of multiple supplementing KNO_3 , the RNCC was decreased, however their cell division was not significantly inhibited same as one-time adding KNO_3 ; these new reproductive cells and the nitrate concentration formed a balance, but this balance could not promote more cells entering into carotenogenesis condition. Hence, beta-carotene content (mg L^{-1}) in the way of multiple supplementing KNO_3 was lower. Moreover, observing the algae cell could also reflect the relation between nitrate limitation and cell morphology variation.

Cells of *Dunaliella* are generally ovoid, 4-15 μm wide, and 6-25 μm long, but depending on stages of growth or development and environmental conditions.⁹ Cell of all cultures by one-time giving KNO_3 showed an ovoid shape and the long diameter was 6-8 μm before 10th day; most cells changed to a round shape and diameters increased 1-3 μm during the second week and this tendency was significant when KNO_3 arrange from 0.3- 0.75 g l^{-1} . After three weeks, most of cells diameters increased 2-4 μm apart from cultures that the KNO_3 was 0.1, 0.2 and 1 g L^{-1} . However, this variation was not significant in the culture by multiple supplementing KNO_3 although a part of cells shape or size in the low KNO_3 concentration was changed. Whatever conditions, this variation was closed related with RNCC. Obviously, low RNCC could be more suitable to induce more beta-carotene accumulation.

Remaining nitrate concentration in the culture of each cell under high light intensity

The variation of RNCC under high light intensity was similar as previous results. RNCC of all cultures dramatically decreased and kept a stable and lowest level after 5-7 days (Figure 4). The value of RNCC was calculated based on the nitrate concentration and cell density, in addition, effects of light intensity on cell growth was not significant as shown in Figure 1 and Figure 2; hence, the variation tendency of RNCC between high and low light intensity was not big difference especially in the way of multiple supplementing KNO_3 . However, their values still had a few differences. In the way of one-time adding KNO_3 , RNCC of all cultures under high light intensity dramatically decreased below 20 pg/cell after one week and 10 pg/cell after two weeks; correspondingly, RNCC of all cultures under low light intensity was almost below 10 pg/cell after one week. This result showed that high light intensity could improve the nitrate limitation to start to accumulate beta-carotene early. It was related with the proposed function of beta-carotene accumulation in *Dunaliella* as a protection of the cell against injury by high intensity irradiation. The higher the intensity to which the algae are exposed, the higher is their beta-carotene.²¹

Table 1. Cell growth and beta-carotene content under different KNO_3 concentration and light intensity.

KNO_3 (g L^{-1})	Specific growth rate (μ)		Biomass (g/L)		Beta-carotene dry weight (mg/g)		Beta-carotene content per cell (pg/cell)	
	L-light	H-light	L-light	H-light	L-light	H-light	L-light	H-light
0.1	0.60±0.03 ^{ab}	0.40±0.03 ^a	0.29±0.03 ^a	0.21±0.04 ^a	48.5±5.8 ^a	56.8±3.7 ^a	5.4±0.9 ^a	6.6±0.7 ^a
0.2	0.60±0.04 ^a	0.61±0.04 ^b	0.50±0.03 ^b	0.38±0.03 ^b	48.4±7.3 ^a	57.2±3.3 ^a	5.5±0.9 ^a	7.0±0.4 ^a
0.3	0.68±0.06 ^b	0.63±0.03 ^{bc}	0.65±0.03 ^c	0.58±0.04 ^c	56.7±9.3 ^{ab}	57.3±3.3 ^a	7.7±0.8 ^b	8.2±0.3 ^b
0.5	0.68±0.03 ^b	0.64±0.04 ^{bc}	1.26±0.09 ^d	1.39±0.03 ^d	64.8±4.0 ^{bc}	71.5±5.6 ^b	9.6±0.5 ^c	11.4±0.5 ^d
0.75	0.62±0.03 ^{ab}	0.73±0.03 ^d	1.53±0.04 ^e	1.48±0.04 ^e	71.6±5.8 ^c	76.7±3.7 ^b	11.1±0.8 ^d	12.8±0.6 ^e
1	0.63±0.04 ^a	0.68±0.03 ^{cd}	2.25±0.07 ^f	2.17±0.04 ^f	54.9±3.3 ^a	58.6±5.1 ^a	7.6±0.6 ^b	9.6±0.7 ^c

KNO_3 was one-time added and all data were collected at 15th day; L-light, low light intensity; H-light, high light intensity; values having a common letter are not significantly difference at P=5%.

Table 2. Cell growth and beta-carotene content under different light intensity and KNO_3 concentration.

KNO_3 (g L^{-1})	Relative growth rate (μ)		Biomass (g/L)		Beta-carotene dry weight (mg/g)		Beta-carotene content per cell (pg/cell)	
	L-light	H-light	L-light	H-light	L-light	H-light	L-light	H-light
0.1	-0.11	-0.13	0.17±0.02 ^a	0.15±0.02 ^a	73.3±4.8 ^d	79.2±3.5 ^d	7.34±0.46 ^c	8.36±0.44 ^c
0.2	-0.09	-0.09	0.24±0.03 ^a	0.26±0.03 ^a	63.1±4.8 ^c	64.1±2.7 ^c	6.75±0.53 ^b	7.25±0.63 ^b
0.3	-0.05	-0.07	0.51±0.04 ^b	0.47±0.12 ^b	53.2±3.6 ^b	51.2±4.5 ^b	6.68±0.44 ^b	6.88±0.29 ^b
0.5	-0.02	-0.04	0.80±0.03 ^c	0.65±0.06 ^c	40.5±2.9 ^a	43.7±2.5 ^a	5.56±0.18 ^a	5.76±0.2 ^a
0.75	0.01	0.002	1.05±0.14 ^d	1.06±0.15 ^d	39.6±3.5 ^a	46.7±2.9 ^{ab}	5.73±0.29 ^a	5.78±0.31 ^a
1	0.03	0.02	1.38±0.13 ^e	1.37±0.02 ^e	43.5±2.5 ^a	44.7±2.1 ^a	5.54±0.19 ^a	5.64±0.3 ^a

KNO_3 was multiple supplemented and all data were collected at 15th day; Relative growth rate (RGR), the growth under the constant KNO_3 concentration did not show a sigmoid curve, hence, RGR used here to replace the specific growth rate, also, the data analysis here was omitted; L-light, low light intensity; H-light, high light intensity; values having a common letter are not significantly difference at P=5%.

Results of this study showed that the final beta-carotene content was related with the started nitrate concentration. Therefore, the higher started nitrate concentration for this *D.salina* strain, the higher beta-carotene yields obtained. However, it was not practical for the commercial algae cultivation. High input of nitrate required more costs and long cultivation time to produce beta-carotene. As results had shown in Figure 1 and Figure 2, the beta-carotene content did not show a significant difference between 0.75 and 1 g l⁻¹ at 15th day, therefore, 0.5 or 0.75 g l⁻¹ would be suitable for this strain to produce beta-carotene. In addition, improving light intensity could improve the beta-carotene accumulation although the light intensity that over light compensation point (about 600 μmol m⁻² s⁻¹) was not test in this study because of the experimental conditions limited.

Conclusions

The final beta-carotene content related with the started nitrate concentration. High started KNO₃ concentration could promote high cell quantities and beta-carotene content. Its essence was that high started nitrate concentration could remain more cells in the late stage entering into the carotenogenesis condition to accumulate more beta-carotene. Nitrate limitation could be reflected by the value of RNCC. Low RNCC (below approx. 20 pg/cell) was an advantage for beta-carotene accumulation and the low RNCC level was irrelevant with started KNO₃ concentration. This means whatever low or high started KNO₃ concentration, it could be consumed following the algae growing and culture time extended and finally the RNCC would tend to the same level. However, the started nitrate concentration could not be too high or too low otherwise the algae required long time to accumulate beta-carotene or the beta-carotene yields was lower because of low biomass. High light intensity could limit the growth especially in low KNO₃ concentration but improve the beta-carotene accumulation and RNCC. Results of this study advised that increasing the biomass and as early to strengthen the stress on each cell was important to improve the beta-carotene yield.

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