Expression analysis of *Lycopene* β-cyclase gene in *Dunaliella* sp. M22

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**ABSTRACT**

*Dunaliella* is a halophile green microalga. It accumulates massive amounts of β-carotene recognized as additives in food and cosmetic industry due to their antioxidant, colorant, provitamin and therapeutic properties. The algae are commercially cultivated in Australia and Israel except Thailand. In this study, growth, β-carotene content and *Lcyβ* gene encoding lycopene β-cyclase enzyme in β-carotene biosynthesis pathway were studied. The highest β-carotene content was obtained from culture containing 2.5 M NaCl with 3.65 pg cell⁻¹. High salinity could increase β-carotene content whereas reduced growth of the algae population. The expression analysis revealed that an increase of salinity concentration could elevate *Lcyβ* transcript level. The result indicated that *Lcyβ* is one of the salt-inducible genes.

Key words: β-carotene, *Lcyβ* gene, *Dunaliella*, microalgae.

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INTRODUCTION

*Dunaliella* is a green microalga that belongs to the phylum Chlorophyta. *D. salina* and *D. bardawil* are known to store large amounts of carotenoids and intracellular β-carotene more than 10% of cell dry weight (Borowitzka, 1988). It massively accumulates β-carotene under extreme environmental conditions (Lamers et al., 2008), such as high light intensity, high salinity, extreme temperatures and nutrient depletion. In nature, there are over 600 known carotenoids. Carotenoids are an essential group of natural pigments found in all photosynthetic organisms (plants, algae and cyanobacteria). Carotenoids have antioxidants properties and attracted thoughtfulness as potential agents in chemoprevention of cancers, particularly β-carotene which one molecule of β-carotene is found to neutralize up to 1000 molecules of free radical oxygen (Foote et al., 1970). One of the most important for any organism is β-carotene, which has a provitamin A activity (Heldt and Heldt, 2005). Carotenoids are composed from the 5-carbon compound isopentenyl diphosphate (IPP), pass through the dimethylallyl diphosphate (DMAPP), geranylgeranyl pyrophosphate (GGPP) to produce phytoene, lycopene and β-carotene, respectively. Lycopene β-cyclase is one of an important key enzyme in the production of β-carotene which was encoded by *Lcyβ* gene. It catalyzes β-cyclization that creates one β-ionone ring at each end of the lycopene molecule to produce β-carotene (Hao et al., 2012). Moreover, the regulation of β-carotene biosynthesis in *Dunaliella* remains to be elucidated especially in the salt soil isolated strain. The study reported the expression of *lycopene β-cyclase* gene in *Dunaliella* sp. M22 isolated from the salt soil sample collected from Northeastern part of Thailand.

MATERIALS AND METHODS

1. Growth and β-carotene accumulation of *Dunaliella* sp. in photobioreactor

The green algae *Dunaliella* sp. was isolated from salt soil collected from Chaiyaphum in the Northeastern part of Thailand. The *Dunaliella* sp. M22 strain was screened by changing of green to orange color in remodified Johnson's agar medium (Borowitzka, 1988) containing 1.5 M NaCl, 3.05 mM MgSO₄·7H₂O, 9.389 mM KCl, 0.392 mM Ca(NO₃)₂, 88.18 μM KH₂PO₄, 85.7 μM H₂BO₃, 0.809 (NH₄)₆Mo₇O₂₄·4H₂O, 0.240 μM CuSO₄·5H₂O, 0.126 μM CoCl₂·6H₂O, 0.733 μM ZnCl₂, 0.909 μM MnCl₂·4H₂O, 11.903 mM NaHCO₃, 141 μM Na₂EDTA and 2.2 μM FeCl₃. Culture were kept under continuous cool daylight (200 μmol m⁻² s⁻¹) at 25±2 °C with air/CO₂ (ratio 95/5) at the flow rate of 30 ml/min. The cell samples were mixed with 1 μl of 3% formalin and counted using a 0.1 mm deep counting Neubauer chamber.
2. β-Carotene determination

Ten ml of the samples were collected and washed to remove the traces of adhering salts by centrifugation. The β-carotene was extracted by adding 2-3 ml acetone (80%) and stored in dark at 4°C for a week. The amounts of β-carotene was quantified photometrically using the extinction coefficient ε_{453nm} = 0.233 l mg⁻¹ cm⁻¹ according to Goodwin (1980). Carotenoid content was calibrated against values obtained for β-carotene (Sigma, USA) samples dis-solved in 80% (v/v) acetone.

3. Characterization of Lcyβ gene

The Dunaliella sp. M22 cells were grown in remodified Johnson's medium (Borowitzka, 1988) containing 1.0 and 1.5 M NaCl by centrifugation. NaCl concentration was elevated from 1.0 M to 2.5 M NaCl and 1.5 M to 2.5 M NaCl after exponential phase. The cells were extracted the total RNA for expression analysis at 0hr, 4hr, 1d and 3d. The total RNA samples were DNase treated to remove residual genomic DNA. Then, RNA concentrations were quantified and adjusted to 1.0 μg μl⁻¹ by using Nano Drop Spectrophotometer ND-100.

The cDNAs were generated using SuperScript® III First-Strand Synthesis System (Invitrogen, USA). RT reaction was performed at 50°C for 50 min and terminated by heat inactivation at 85°C for 5 min. To determine relative levels of Lcyβ expression, primer sequences and PCR amplification conditions were performed as followed: Lcyβ 5’-GTACCAAGGCGGTACGGCAT-3’ and 5’- CTTAACCATCCTGTGACG-3’ (one cycle of 95°C, 120 s; 29 cycles of 95°C, 30 s; 61°C, 30 s; 68°C, 30 s; and one cycle of 68°C, 10 min). Lcyβ gene specific primers were designed from accession number KM016906. An α-tubulin was used as an internal control using primer — namely; Atub (5’- GTACTGCTGAGCAGCGCA -3’ and 5’- TGCTCCAGCAGGGAGTGGA -3’). The cycle number was determined at exponential amplification stage as the following conditions: one cycle of 95°C, 120 s; 25 cycles of 95°C, 30 s; 63°C, 30 s; 68°C, 30 s; and one cycle of 68°C, 10 min. Then, semi-quantitative RT-PCR was used to amplify cDNAs from 12 samples (4 concentrations of NaCl and 3 growth phases). The PCR products were separated by electrophoresis in a 1% agarose gel containing 0.5 mg l⁻¹ ethidium bromide and visualized using Gel DOC 2000 (Biorad, USA).

RESULTS AND DISCUSSION

1. Growth

Growth of all cultures rapidly reached an exponential phase within 3 days (Figure 1). Maximum cell density occurred in 1.0 M NaCl (2.03±40.37×10⁷ cell ml⁻¹) with specific growth rate of 2.27 and the lowest cell density was 8.49±1.41×10⁵ cell ml⁻¹ in 3.5 M NaCl with specific growth rate of 0.89. These results are in agree with the result of (Borowitzka et al., 1990; Marín et al., 1998) that the higher salinity affect the cell density and growth rate.
Growth of *Dunaliella* sp. M22 grown in remodified Johnson’s medium containing different salinities (1.0, 1.5, 2.5 and 3.5 M NaCl) under continuous light intensity of 80 μmol photon m\(^{-2}\)s\(^{-1}\) at 25 ± 2 °C and sparged with 5% CO\(_2\). Arrows indicate day when cultures were harvested for the estimation of β-carotene and mRNA transcript levels. Plotted data are the averages ± SD of three replicates.

2. **β-carotene determination**

At the exponential phase, the highest β-carotene content was found in the culture containing 2.5 M NaCl, followed by 3.5, 1.5 and 1.0 M with 1.55, 1.49, 0.86 and 0.33 pg cell\(^{-1}\), respectively (Figure 2). The study clearly revealed that elevation of salinity could increase β-carotene content. These factors could also retard cell division but increase the β-carotene-to-chlorophyll ratio (Ben-Amotz et al., 1982).

![Figure 1](image1.png)  
**Figure 1** Growth of *Dunaliella* sp. M22 grown in remodified Johnson’s medium containing different salinities (1.0, 1.5, 2.5 and 3.5 M NaCl) under continuous light intensity of 80 μmol photon m\(^{-2}\)s\(^{-1}\) at 25 ± 2 °C and sparged with 5% CO\(_2\). Arrows indicate day when cultures were harvested for the estimation of β-carotene and mRNA transcript levels. Plotted data are the averages ± SD of three replicates.

![Figure 2](image2.png)  
**Figure 2** The effect of salinity on carotene accumulation in *Dunaliella* sp. M22. The culture grown at remodified Johnson’s medium containing 1.0 M NaCl were transferred to fresh remodified Johnson’s medium with 1.0, 1.5, 2.5 and 3.5 M NaCl. β-carotene accumulation per cell was determined at 2 days of cultivation.
3. Characterization of \textit{Lcy\textbeta} gene

During exponential phase, the high \textit{Lcy\textbeta} transcript level was found in the culture grown in 1.5, 2.5 and 3.5 M NaCl, while lower \textit{Lcy\textbeta} transcript level was occurred in 1.0 M NaCl (Figure 3). These expression patterns were correlated with \textit{\beta}-carotene content (Figure 2), indicating that \textit{Lcy\textbeta} is directly involved in \textit{\beta}-carotene biosynthesis. Similar result was reported by Ramos et al. (2008) who found that the mRNA levels of \textit{Lcy\textbeta} gene increased when \textit{D. salina} were submitted to abiotic stress conditions such as salinity and high light intensity.

![Expression patterns of \textit{Lcy\textbeta} at different growth periods](image)

**Figure 3** Expression patterns of \textit{Lcy\textbeta} at different growth periods (exponential phase, early senescence phase and late senescence phase) of \textit{Dunaliella} sp. M22 was grown in 1.0, 1.5, 2.5 and 3.5 M NaCl were identified using the intensity of RT-PCR product. \textit{\alpha}-tubulin was used as an internal control.

**CONCLUSION**

Different salt concentrations had effects on algae density and \textit{\beta}-carotene content in \textit{Dunaliella} sp. M22. Higher salinity increased \textit{\beta}-carotene accumulation and up-regulates the expression of \textit{Lcy\textbeta}, but retarded growth of the population.

**ACKNOWLEDGEMENTS**

The authors would like to thank the National Research Council of Thailand (NRCT) for the financial support to carry out this research work.

**REFERENCES**


