THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Genetic Engineering)

DEGREE

Genetic Engineering | Interdisciplinary Graduate Program

FIELD | **PROGRAM**

TITLE: Sequencing, Characterization and Expression Analysis of *Lycopene β-cyclase* Gene in *Dunaliella* sp.

NAME: Mr. Thanawat Boontip

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Niran Juntawong, Dr.rer.nat.)

THESIS CO-ADVISOR

(Associate Professor Prasart Kermanee, Ph.D.)

THESIS CO-ADVISOR

(Assistant Professor Chotika Yokthongwattana, Dr.Sc.)

GRADUATE COMMITTEE CHAIRMAN

(Assistant Professor Siriwan Prapong, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

SEQUENCING, CHARACTERIZATION AND EXPRESSION ANALYSIS OF *LYCOPENE β-CYCLASE* GENE IN *DUNALIELLA* SP.

THANAWAT BOONTIP

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Genetic Engineering)
Graduate School, Kasetsart University
2014

Thanawat Boontip 2014: Sequencing, Characterization and Expression Analysis of *Lycopene β-cyclase* Gene in *Dunaliella* sp.. Master of Science (Genetic Engineering), Major Field: Genetic Engineering, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Niran Juntawong, Dr.rer.nat. 102 pages.

Currently, β-carotene is used as food coloring agent, cosmetics and drugs. It is found in all photosynthetic organisms including green microalgae *Dunaliella* with massive amounts of β-carotene accumulation. Lycopene β-cyclase (Lcyβ) is an enzyme which is responsible for β-carotene biosynthesis in photosynthetic organisms. To approach a large-scale β-carotene production, information about suitable condition for growth and β-carotene accumulation relating to *Lcyβ* expression is important. The aim of this study is to report full-length sequence and expression of *Lcyβ* in *Dunaliella* sp. M22 under different salt concentrations. The full-length sequence of *Lcyβ* cDNA contains 193 nucleotides of 5′ untranslated region (UTR), 569 nucleotides of 3′ UTR and 1392 bp open reading frame (ORF) encoding for 463 amino acid protein. Phylogenetic analysis revealed that the *Dunaliella* sp. M22 *Lcyβ* grouped with the *Dunaliella salina Lcyβ* gene. It showed an overall amino acid identity of 77%. In this study, high salinity reduced growth of the algae population whereas β-carotene content in the cell was increased. The expression analysis revealed that an increase of salinity concentration at exponential phase could elevate *Lcyβ* transcript level and β-carotene content, but the *Lcyβ* transcript level at senescence phase was not correlated with salinity concentration. The *Lcyβ* transcript levels at senescence phase might be regulated by other factors, in particular by nutrient starvation.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I would like to express my deep gratitude and indebtedness to my respected research advisor, Associate Professor Dr. Niran Juntawong for his scholastic guidance, kind supervision, constant encouragement and valuable advice during the entire period of study.

I also gratefully acknowledge to my committee members, Associate Professor Dr. Prasart Kermanee from Department of Botany and Assistant Professor Dr. Chotika Yokthongwattana from Department of Biochemistry, Faculty of science, Kasetsart University for their valuable guidance and encouragement throughout my research. Thanks are extended to Mr. Ramaraj Sathasivam and Mr. Piyawat Pongpadung my nice friends in Bioscience Program for great supports.

Finally, I am grateful to my family for their moral support and to relatives for their love and encouragement for pursuing my higher studies.

Thanawat Boontip

December 2014

TABLE OF CONTENTS

Page

TABLE OF CONTENTS	i
LIST OF TABLES	ii
LIST OF FIGURES	iii
LIST OF ABBREVIATIONS	x
INTRODUCTION	1
OBJECTIVES	3
LITERATURE REVIEW	4
MATERIALS AND METHODS	22
Materials	22
Methods	26
RESULTS	40
DISCUSSIONS	67
CONCLUSIONS	74
LITERATURE CITED	75
APPENDIX	84
CURRICULUM VITATE	102

LIST OF TABLES

Table		Page
1	Function of carotenoids in natural.	5
2	Maximum absorption in light petroleum of some well-known carotenoids.	9
3	The quantity of β-carotene used in different fat-based food products.	10
4	Instrument list for this study.	22
5	Chemical list for culturing *Dunaliella sp.*	23
6	Chemical list for molecular biology.	24
7	Primers for the amplification of *Lcyβ* gene.	25
8	Oligonucleotide forward and reverse primers of *Lcyβ* gene for the PCR amplification of cDNA. An α tubulin gene (*Atub)* primers were used for the PCR amplification as an internal control.	38
9	Optimized PCR conditions for the cDNA amplification of *Lcyβ* and *Atub* gene.	39
10	Sequence similarity result of partial *Lcyβ* fragment isolated from *Dunaliella* sp. M22 with other species from NCBI database.	52

Appendix Table

1	The recipe of modified Johnson's medium and remodified Johnson's medium.	85
2	Effect of NaCl concentration (1.0, 1.5, 2.5 and 3.5 M) on *Dunaliella* sp. M22 growth in flask containing modified Johnson's medium.	89
3	Effect of NaCl concentration (1.0, 1.5, 2.5 and 3.5 M) on *Dunaliella* sp. M22 growth in flask containing remodified Johnson's medium.	89
4	Effect of NaCl concentration (1.0, 1.5, 2.5 and 3.5 M) on *Dunaliella* sp. M22 growth in remodified Johnson's medium in photobioreactor.	90

LIST OF FIGURES

Figure		Page
1	The structure of isoprene and beta-ionone ring a) Isoprene structure; 1 and 4 depicted carbon atom position b) Types of beta-ionone ring.	7
2	Structure of β-carotene and its common isomers a) all-trans form b) 15-cis form c) 13-cis form.	8
3	β-carotene quench oxidizing free radical pathway; Equation 1, electron was transferred from free radical to β-carotene. Equation 2, β-carotene captured free radical molecule into the polyene chains directly.	12
4	β-carotene scavenge oxidizing free radical pathway; Left, electron transferring yielding cation radicals $Car^{\bullet+}$. Right, direct addition yielding free radical adducts which are stabilized by delocalization on the polyene chain.	12
5	The isopentenyl pyrophosphate synthesis in the plastids proceeds *via* 2-methyl erythriol 4-phosphate pathway (MEP).	16
6	Carotenoid biosynthesis. The phytoene synthase (Pds) catalyzes the head-to-head addition of two molecules of geranylgeranyl diphosphate to phytoene. The later is converted by desaturases with neurosporene as the intermediate to lycopene. β-carotene is formed by cyclization. Finally, zeaxanthin is formed by additional hydroxylation.	17
7	Production of β-carotene by the isolated strain on modified Johnson's medium agar plate containing 1.5 M NaCl. The *Dunaliella* sp. strain was isolated from salt soil sample (A). The β-carotene producing strains were shown in red circles (B).	40
8	*Dunaliella* sp. strains M02, M04, M07, M11 and M22 were cultured in liquid 1.5 M NaCl modified Johnson's medium (A), the M22 strain turned orange after 2 months (B).	41

LIST OF FIGURES (Continued)

Figure		Page
9	Growth of *Dunaliella* sp. M22 strain in modified Johnson's medium containing different NaCl concentrations in 250 ml Erlenmeyer flask under continuous illuminated cool daylight (80 μmol photon $m^{-2}s^{-1}$) at 25±2 ˚C. Plotted data are the averages ± SD of three replicates.	42
10	Growth of *Dunaliella* sp. M22 strain in remodified Johnson's medium containing different NaCl concentrations. The strain was grown in 250 ml Erlenmeyer flask under continuous cool daylight (80 μmol photon $m^{-2}s^{-1}$) at 25±2 ˚C. Plotted data are the averages ± SD of three replicates.	43
11	Accumulation of β-carotene by *Dunaliella* sp. M22 cultured in remodified Johnson's medium supplemented with 1.0, 1.5, 2.5 and 3.5 M NaCl concentrations. The strain was grown under continuous light intensity of 80 μmol photon $m^{-2}s^{-1}$ at 25±2 ˚C. Plotted data are the averages ± SD of three replicates.	44
12	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). The cultures were grown under continuous light intensity of 80 μmol photon $m^{-2}s^{-1}$ at 25 ± 2°C and the cultures were spurged with 5% CO_2. Arrows indicate day (I, II, III) when cultures were harvested for the estimation of β-carotene and mRNA transcript levels. Plotted data are the averages ± SD of three replicates.	45

LIST OF FIGURES (Continued)

Figure		Page
13	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 different stages of growth A) Period I B) Period II C) Period III and β-carotene accumulation per milliliter D) Period I E) Period II F) Period III.	47
14	Salt stress accelerated the biosynthesis of orange pigment in *Dunaliella* sp. M22. The NaCl concentrations were 1.0, 1.5, 2.5 and 3.5 M from left to right tube. The initial cell density in each tube was 5.0×10^5cells ml^{-1}. All cultures were illuminated under 200 µmol photons $m^{-2}s^{-1}$ at 25±2°C and were sparged continuously with filtered-sterile air and 5% CO_2.	49
15	Screening of cloned PCR products using colony PCR (universal primers M13F and M13R), the expected size of 0.5 kb was amplified using the colony 5^{th} as template.	50
16	Partial sequence of *lycopene β-cyclase* gene (*Lcyβ*) amplified from *Dunaliella* sp. M22 using LcyβWF and LcyβNR degenerated primers.	51
17	Graphical view of nucleotide sequence alignment of partial *Lcyβ* (458 bp) using BLASTN (2.2.29) program from NCBI.	52
18	3′ RACE PCR of *lycopene β-cyclase* gene, Lane 1: negative control 2: 3′ RACE fragment was amplified using LcyβNF and Q_0 primers; Lane 3: 3′ nested PCR was carried out using Lcyβ33F and Q_1 primers.	55

LIST OF FIGURES (Continued)

Figure		Page
19	5′ RACE PCR of *lycopene β-cyclase* gene, Lane 1: negative control; Lane 2: 5′ RACE fragment was amplified using LcyβNR and Q_0 primers; Lane 3: 5′ nested PCR was performed by Lcyβ124R and Q_1 primers.	56
20	Full-length nucleotide sequences with 2,154 bp in size of *lycopene β-cyclase* gene isolated from *Dunaliella* sp. M22. The sequence was submitted to the NCBI with the accession number KM016906.	57
21	Nucleotide and deduced amino acid sequence of full-length *lycopene β-cyclase* gene, start codon (ATG) and stop codon (TGA) are underlined (length 463 aa).	58
22	Phylogenetic analysis of the deduced amino acid sequences of *lycopene β-cyclase* from *Dunaliella* sp. M22 with other *lycopene β-cyclase* sequences from NCBI. The tree was constructed using MEGA version 5.2 software and conducted by the neighbor joining method. Bootstrap values were calculated from 1000 replicates. The GenBank accession numbers are indicated in brackets.	59
23	Total RNA was extracted from exponential phase, early senescence phase and late senescence phase. *Dunaliella* sp. M22 cultured in remodified Johnson's medium containing 1.0, 1.5, 2.5 and 3.5 M NaCl concentrations.	61
24	RT-PCR product of *Atub* and 18S rRNA gene amplified with different $MgCl_2$ concentrations of 1, 2, 3, 4 and 5 mM.	62
25	Lane 1-5: RT-PCR product of *Lcyβ* gene amplified with 1.0, 2.0, 3.0, 4.0 and 5.0 mM $MgCl_2$ concentrations at annealing temperature of 63℃; Lane 6-11: Different annealing temperatures were carried out to amplify the *Lcyβ* gene at 50, 54, 60, 63, 65 and 66.5℃ with 1.5 mM $MgCl_2$.	63

LIST OF FIGURES (Continued)

Figure		Page
26	The different cycle numbers were carried out to amplify the *Atub* (15, 19, 22, 25, 29 and 33 cycles) and *Lcyβ* gene (19, 22, 25, 29, 33 and 37 cycles).	63
27	The cDNA normalization of *Atub* gene from cell grown in 1.0, 1.5, 2.5 and 3.5 M NaCl remodified Johnson medium in 3 periods of growth (exponential, early and late senescence phases).	64
28	The cDNA normalization of *Atub* gene from cell grown in the culture shifted from 1.0 M to 2.5 M and 1.5 M to 2.5 M NaCl.	64
29	Expression patterns of *Lcyβ* at different growth period (exponential phase, early senescence phase and late senescence phase) of *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.	65
30	Expression of *Lcyβ* mRNA levels in hyper-saline shock treated *Dunaliella* sp. M22 cells. The culture was shifted from 1.0 to 2.5 M and 1.5 to 2.5 M NaCl at senescence phase. The RNA samples were collected at 0 h, 4 h, 1 day and 3 days after the salinity shock.	66

Appendix Figure

1	Illustration showed the relationship between cell density and β-carotene content under different salinity A) 1.0 M B) 1.5 M C) 2.5 M D) 3.5 M. The cell was grown in modified Johnson medium under continuous illumination (80 μmol $m^{-2}s^{-1}$). Plotted data are the averages ± SD of three replicates.	86

LIST OF FIGURES (Continued)

Appendix Figure		Page
2	Illustration showed *Dunaliella* sp. M22 in culture containing A) 1.5 M and B) 2.5 M NaCl remodified Johnson's medium at late senescence phase under continuous illumination (200 μmol $m^{-2}s^{-1}$) and flow air with 5% CO_2 under light microscope (1000x).	88
3	Amplification of partial *Lcyβ* gene with LcyβWF and LcyβNR primer.	91
4	The multiple aligned sequences were used as template from design primer.	91
5	3′ end *Lcyβ* transformed clone was detected by colony PCR using M13F and M13R primer.	93
6	5′ end *Lcyβ* transformed clone was detected by colony PCR using M13F and M13R primer.	93
7	5′ Race of *lycopene β-cyclase* gene sequence of *Dunaliella* sp. M22 strains.	94
8	3′ Race of *lycopene β-cyclase* gene sequence of *Dunaliella* sp. M22 strains.	95
9	Amplification of full-length *Lcyβ* gene using LcyβfullFW and LcyβfullRW.	96
10	Graphical view of amino sequences alignment of Lcyβ (463 amino acids) using BLASTP (2.2.29) program from NCBI.	97
11	Results amino acid alignment of lycopene β-cyclase by CLUSTAL 2.1 multiple sequence alignment.	97

LIST OF FIGURES (Continued)

Appendix Figure		Page
12	An illustration of the difference of using new RACE and classic RACE. a) In classic RACE, early termination in the reverse transcription step results in polyadenylation of less than full-length first-strand cDNAs, all of which can be amplified using PCR to generate less than full-length cDNA 5′ ends. (*) cDNA ends created that will be amplified in the subsequent PCR reaction. b) In new RACE, less than full-length cDNAs are also created but are not terminated by the anchor sequence; hence, they cannot be amplified in the subsequent PCR reaction.	100
13	Schematic representation of classic RACE. At each step, the diagram is simplified to illustrate only how the new product formed during the previous step is utilized. (GSP1) Gene-specific primer 1; (GSP2) gene-specific primer 2; (GSP-RT) gene-specific primer used for reverse transcription. a) Amplification of 3′ partial cDNA ends; b) amplification of 5′ partial cDNA ends; c) schematic representation of the primers used in classic RACE. The 52-nucleotide Q_T primer (5' Q_O-Q_I-TTTT 3′) contains a 17-nucleotide oligo(dT) sequence at the 3′ end followed by a 35-nucleotide sequence encoding HindIII, SstI, and XhoI recognition sites.	101

LIST OF ABBREVIATIONS

aa	=	Amino acid
bp	=	Base pairs
C	=	Carbon
$Ca(NO_3)_2$	=	Calcium nitrate
Car	=	β-carotene
cDNA	=	Complementary deoxyribonucleic acid
chl	=	Chlorophyll
CoA	=	Coenzyme A
$CoCl_2$	=	Cobalt (II) chloride
$CuSO_4$	=	Copper (II) sulfate
cyt	=	Cytochrome
D	=	*Dunaliella*
Da	=	Daltons
dATP	=	Deoxyadenosine triphosphate
DEPC	=	Dietyl pyrocarbonate
DMADP	=	Dimethylallyl diphosphate
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphat
dNTP	=	Deoxyribonucleotide
DOXP	=	1-deoxy-D-xylulose 5-phosphate
DPME	=	4-diphosphocytidyl-2-C-methyl-D-erythritol
DTT	=	Dithiothreitol
Dxr	=	1-deoxy-d-xylulose 5-phosphate synthase
Dxs	=	Isoprenoid synthase
E	=	*Escherichia*
EDTA	=	Ethylenediaminetetraacetic acid
EtBr	=	Ethidium bromide
$FeCl_3$	=	Iron(III) chloride

LIST OF ABBREVIATIONS (Continued)

Fig	=	Figure
FPP	=	Farnesyl diphosphate
×g	=	Earth's gravitational
G3P	=	Glyceraldehyde-3-phosphate
GGPP	=	Geranylgeranyl diphosphate
GGPS	=	Geranylgeranyl diphosphate synthase
GSP-F	=	Forward gene specific primer
GSP-R	=	Reverse gene specific primer
GSPs	=	Gene specific primers
h	=	Hour
H	=	Hydrogen
H_3BO_3	=	Boric acid
HMBPP	=	4-hydroxy-2-methylbut-2-enylpyrophosphate
HMG CoA	=	β-hydroxy-β-methylglutaryl coenzyme A
IPP	=	Isopentyl diphosphate
kb	=	Kilobase pair
KCl	=	Potassium chloride
kDa	=	Kilodalton
KH_2PO_4	=	Monopotassium phosphate
l	=	Liter
LB	=	Luria-Bertani medium
Lcyβ	=	*Lycopene β-cyclase*
M	=	Molar
MEcDP	=	4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate
MEP	=	2-C-methyl-D-erythritol 4-phosphate
$MgCl_2$	=	Magnesium chloride
$MgSO_4$	=	Magnesium sulfate
min	=	Minute

LIST OF ABBREVIATIONS (Continued)

ml	=	Mililitre
mM	=	Millimolar
mm	=	Millimetre
$MnCl_2$	=	Manganese (II) chloride
mRNA	=	Masseger ribonucleic acid
MVA	=	Mevalonate pathway
Na	=	Sodium
NaCl	=	Sodium chloride
$NaHCO_3$	=	Sodium bicarbonate
NaOH	=	Sodium hydroxide
NCBI	=	The National Center for Biotechnology Information
ng	=	Nanogram
$(NH_4)_6Mo_7O_{24}$	=	Ammonium molybdate hydrate
NK	=	Natural killer cell
nm	=	Nanometre
no	=	Number
^oC	=	Degree Celsius
ORF	=	Open Reading Frame
PCR	=	Polymerase Chain Reaction
Pds	=	*Phytoene desaturase*
pg	=	Picogram
PP	=	Pyrophosphate
Psy	=	*Phytoene synthase*
qPCR	=	Quantitative Polymerase Chain Reaction
RACE	=	Rapid Amplification cDNA Ends
RNA	=	Ribonucleic acid
Rnase	=	Ribonuclease
rpm	=	Revolutions per minute
RT	=	Reverse transcriptase

LIST OF ABBREVIATIONS (Continued)

RT-PCR	=	Reverse Transcriptase Polymerase Chain Reaction
sec	=	Second
sp	=	Species
TAE	=	Tris-acetate-EDTA
TBE	=	Tris-Borate-EDTA
TE	=	Tris-EDTA
Temp	=	Temperature
TNF-α	=	Tumor necrosis factor α
TPP	=	Thiamine pyrophosphate
U	=	Unit
ZDS	=	ξ-carotene desaturase
ZnCl2	=	Zinc chloride
µg	=	Microgram
µl	=	Microlitre
µM	=	Micromolar

SEQUENCING, CHARACTERIZATION AND EXPRESSION ANALYSIS OF *LYCOPENE β-CYCLASE* GENE IN *DUNALIELLA* SP.

INTRODUCTION

Carotenoids are natural compounds found in yellow and green vegetables and fruits. β-carotene is one of carotenoids. It plays a crucial role on protection of photo-oxidation of chlorophyll. It is used as a potential antioxidant which can prevent the uncontrolled formation of activated oxygen species and free radicals. One molecule of β-carotene is found to neutralize up to 1000 molecules of free radical oxygen (Foote *et al.*, 1970). It has broadly beneficial properties and used in drug, cosmetic, food industries (Fazeli *et al.*, 2006).

Dunaliella is the main natural source for β-carotene. It accumulates massive amounts of β-carotene under high light intensity and growth-limiting conditions (Oren, 2005). It can be morphologically distinguished by the lack of a rigid cell wall (Ben-Amotz *et al.*, 1987). *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; Raja *et al.*, 2008). Commercial production of β-carotene from *D. salina* faced a problem with cultivation because β-carotene accumulation is enhanced by physiological stress, but its accumulation is high when growth rate is low (Marín *et al.*, 1998). Isolation, identification and optimization of culture media for high production of natural β-carotene is a great importance (Fazeli *et al.*, 2006).

There were many studies which tried to understand and modify the β-carotene biosynthesis pathway. 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). Most results indicated that *Lcyβ* gene is directly correspondent to high β-carotene accumulation which could be regulated by either stress condition or using genetic engineering (Ramos *et al.*, 2008; Ye *et al.*, 2008; Zhu

et al., 2003). However, the regulation of β-carotene biosynthesis in *Dunaliella* remains to be elucidated especially in the salt soil isolated strain. The aim of this study is to sequenced, characterize and express *lycopene β-cyclase* gene in *Dunaliella* sp. M22 isolated from the salt soil sample collected from Northeastern part of Thailand.

OBJECTIVES

1. To screen the *Dunaliella* sp. from salt soil collected from Chaiyaphum province

2. To study the effect of salinity on growth and β-carotene accumulation of *Dunaliella* sp. M22.

3. To sequence the *lycopene β-cyclase* gene in *Dunaliella* sp. M22.

4. To characterize the *lycopene β-cyclase* gene expression in *Dunaliella* sp. M22.

LITERATURE REVIEW

1. *Dunaliella sp.*

Dunaliella is a microalga that belongs to the phylum Chlorophyta and family Polyblepharidaceae. This photosynthetic microorganism produces many value-added compounds for applications in food, animal feed, cosmetics and feedstock. When exposed to specific extreme environmental conditions; such as high light intensity, high salinity, extreme temperatures and nutrient depletion, *Dunaliella* massively accumulates β-carotene with high photosynthetic efficiency (Lamers *et al.*, 2008). This led to the large-scale application of *D. salina* for commercial production of natural β-carotene (Del Campo *et al.*, 2007; Lamers *et al.*, 2008). In comparison to other microalga, *D. salina* has the following advantages: a) Disruption of cells is much easier than other algae because of its wall-less nature, b) Continuous culture in a laboratory is easy and the growth rate is relatively high, c) Resistance to various environmental conditions of *D. salina* is higher than other algae (Pick, 2004).

Carotenoids are liposoluble pigments which are responsible for the yellow, orange, and red colors of several fruits, vegetable, fungi, bacteria as well as animals. Carotenoids have not been only exploited industrially for a long time as natural pigments and provitamin A, it also has proven to offer protection against macular degeneration, UV-induced skin damage, some age-related degenerative diseases and other biological functions such as membrane constituents, photosynthetic pigments, electron transport carriers, growth substances and plant hormones (Heldt and Heldt, 2005). These carotenoids have many different functions that are shown in Table 1.

The antioxidant property is a well-known function of carotenoids, which protects the cell from damaging molecule so-called free radicals through a process known as oxidation. In addition, other unexpected biological functions of carotenoids related to gene regulation or functional communication which could provide additional health benefits, such as tumor suppressing activity (Tibaduiza *et al.*, 2002).

Table 1 Function of carotenoids in nature

Precursor	**Example**	**Function**
C_5: Dimethylallyl-PP	Isoprene	Protection of the photosynthetic apparatus against heat
Isopentenyl-PP	Side chain of cytokinin	Growth regulator
C_{10}: Geranyl-PP	Pinene, Linalool	Defense substance attractant
C_{15}: Farnesyl-PP	Capsidiol	Phytoalexin
C_{20}: Geranylgeranyl-PP	Gibberellin	Plant hormone
C_{30}: 2 Farnesyl-PP	Cholesterol, Sitosterol	Membrane constituents
C_{40}: 2 Geranylgeranyl-PP	Carotenoids	Photosynthetic pigments
n Geranylgeranyl-PP or *n* Farnesyl-PP	Prenylated proteins	Regulation of cell growth
	Prenylation of plastoquinone, Chlorophyll, cyt *a*	Membrane solubility of photosynthetic ubiquinone, pigments and electron transport carriers
	Dolichols	Glucosyl carrier

Source: Heldt and Heldt (2005)

In plant, β-carotene is mostly biosynthesized for an important component involved in photosynthesis. It contributes to trap light energy and subsequently transfers the energy to chlorophyll (Cuttriss *et al.*, 2011).

In nature, there are over 600 known carotenoids. One of the most important for any organism is β-carotene, which widely distributes in nature, i.e. general coloration of many different organisms (Shariati and Hadi, 2011). β-carotene also has

a provitamin A activity catalyzed by β-carotene 15,15'-monooxygenase in various tissues that converted β-carotene into retinol, a form of vitamin A (Heldt and Heldt, 2005).

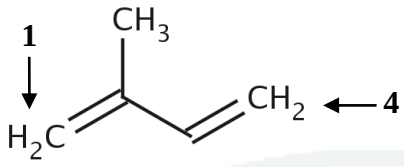
2. β-carotene

2.1 Structure and properties

β-carotene is an organic compound which is classified as a C_{40} unsaturated hydrocarbon specifically called as isoprenoid. It is a derivative of carotenoids. It has aliphatic and aliphatic-alicyclic structure. Molecular formula of β-carotene is $C_{40}H_{56}$ and its molecular weight is 536.9 Da (Shariati and Hadi, 2011). β-carotene structure comprises with 8 of isoprene, which is linked to another isoprene. Linking between two isoprene molecules could occur in three ways, such as 1-1 links, 1-4 links and 4-4 links (Figure 1) . The types of linking cause several forms of β-carotene structure. It can dissolve in carbon disulphite, benzene, chloroform, methylene chloride and hexane. It is moderately soluble in ether, petroleum ether, and oils. It is very sparingly soluble in methanol and ethanol and practically insoluble in water, acid and alkaline (Budavari, 1996).

Both ends of β-carotene comprise of two retinyl groups (β-ionone ring) that have provitamin A activity (Wang *et al.*, 2007) (Figure 1). Individual carotenes are named by the specific end groups. The end groups and their prefixes are shown in figure 1b. All retinoids derive from this compound and they maintain the characteristics of β-ionone ring. Different end groups or β-ionone ring modifications characterize the various retinoids (Cuttriss *et al.*, 2011).

a)



b)

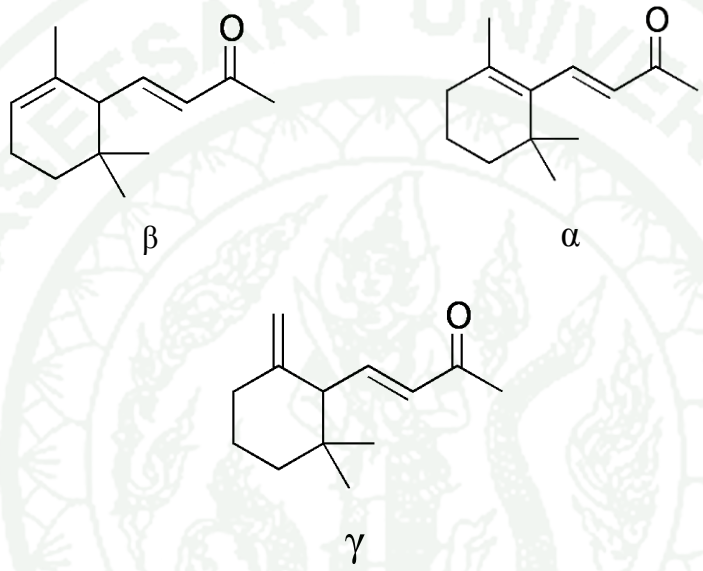


Figure 1 The structure of isoprene and beta-ionone ring

a) Isoprene structure; 1 and 4 depicted carbon atom position

b) Types of ionone ring

Source: Havaux (1998)

A characteristic of maximum light absorption is easily available for identifying β-carotene from carotenoids. The absorption spectrum of a carotenoid is a consequence of the conjugated polyene system presenting in the molecule and of various additional structural features. The increase in wavelength of the position of the absorption maxima is due to additional conjugated double bonds in the parent carotene or a ring. For example, β-carotene has more double bonds in a ring than alpha-carotene resulting in maximum absorption of β-carotene at a higher wavelength than alpha-carotene (Goodwin, 1980).

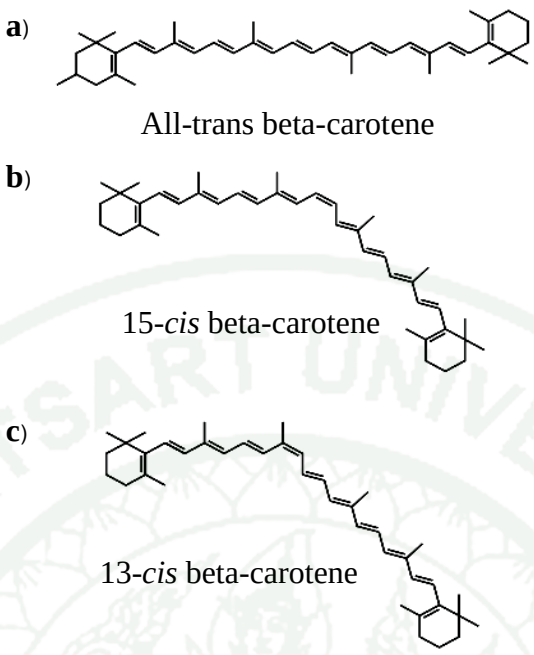


Figure 2 Structure of β-carotene and its common isomers
a) all-trans form
b) 15-cis form
c) 13-cis form

Source: (Kopsell and Kopsell, 2010)

β-carotene is one of the most active provitamin A because its tran and cis form can be cleaved to form two all-trans-retinal molecules, which are reduced to form all-trans-retinol (vitamin A) (Figure 2). The absorption spectrum of β-carotene is measured and has a marked effect on the position of the maxima and on the molecular absorbance of the compound **(**Table 2).

Table 2 Maximum absorption in light petroleum of some well-known carotenoids

Pigments	**Maximum absorption spectrum (nm)**		
β-carotene	427	449	447
α-carotene	422	444	473
β-carotene-5,6-epoxide	423	447	478
β-carotene-5,8-epoxide	409	428	450
9-cis-β-carotene	425	443	471
γ-carotene	435	461	491

Source: Goodwin (1980)

2.2 Application of β-carotene

Nowadays, β-carotene is increasingly interested because many products, such as food, pharmaceutical and cosmetic products contain β-carotene. β-carotene is widely used as food colorants. It is legally permitted in foods for most countries around the world because of its nontoxic effect. β-carotene in oil solution is used in fat based foods. It appears yellow and red at 1-10 mg l^{-1} and at 30-50 mg l^{-1} of β-carotene, respectively. The quantity of β-carotene used in different food products are shown in Table 3. β-carotene in water miscible dispersion is yellow-orange. It is used in water based food products.

Table 3 The quantity of β-carotene used in different fat-based food products

Food products	mg β-carotene/0.5 kg product
Butter	1-4
Cheese	2-8
Ice cream	2-5
Margarine	3-4
Salad oil	3-6
Frying oil	6-15
Oil spray for popcorn	50-200

Source: Bauernfeind (1981)

Pharmaceutical, cosmetic products and medicinal preparations have been appropriately colored and flavored to have a considerable psychological impact. Thus, color should look attractive to patients. In addition, different colors of pharmaceutical products are useful to distinguish one product from the others. β-carotene is satisfied as coloring agent for sugar-coated tablet and sugar syrup in orange-red shade color. It showed no color change after several months at elevated temperature or 3 months of exposure to direct light. The tablet color with β-carotene can be stored at room temperature for more than 3 years (Bauernfeind, 1981).

Carotenoids are used in fat based cosmetics or cosmetics that contain fat as a vehicle, such as in liquid emulsions and creams. The main point of interest is the use of carotenoids to replace synthetic lipstick colors. The shade of color depends on the kinds and amount of carotenoid (Bauernfeind, 1981).

2.3 Function of β-carotene in biology

2.3.1 Antioxidant

Free radicals and oxidative stress have been recognized as important factors in the biology of ageing and degenerative diseases. Free radicals are extremely reactive molecules that have unpaired electrons, a configuration that makes them likely to take part in a chemical reaction. Free radicals often associate with cell damage, mutations, and even malignancies (Krinsky, 1998). In human, free radicals are produced in human body by metabolic processes. Singlet oxygen is one of the most reactive radicals that are formed in human. Excited singlet oxygen molecules have harmful oxidation effects on epidermal protein, lipids and DNA (Darvin *et al.*, 2005).

Biological antioxidants are natural molecules which prevent the uncontrolled formation of free radicals and activated oxygen species or inhibit their reactions with biological structures. The destruction of the most free radicals and activated oxygen species relies on the oxidation of endogenous antioxidants, mainly scavenging and reducing molecules. The chemical structure of β-carotene makes it an efficient *in vitro* neutralizer of singlet oxygen ($^1O_2$), and to a lesser extent, an effective agent in reducing lipid peroxidation (Kopsell and Kopsell, 2010).

The first pathway of quenching free radical relies on electron transfer and produces a cation radical (Figure 3, Equation 1) (Chaudière and Ferrari-Iliou, 1999). $^1O_2$ is usually formed through photochemical reactions involving the absorption of light by a sensitizer molecule, which converts it to the singlet state sensitizer. A singlet state sensitizer molecule is converted to triplet state sensitizer through intersystem crossing process. The triplet state sensitizer can react with ground state oxygen, which exists in a singlet state oxygen ($X^{\bullet}$). $X^{\bullet}$ is a highly reactive species, capable of oxidizing nucleic acids, various amino acids in proteins and unsaturated fatty acids. In plants, β-carotene (CAR) is the most effective quenchers which attain excess energy from $X^{\bullet}$ (Krinsky, 1998).

$Car + X^{\bullet} \rightarrow Car^{\bullet} + X$ (Equation 1)

$Car + X^{\bullet} \rightarrow Car(X)^{\bullet}$ (Equation 2)

When Car = β-carotene

$X^{\bullet}$ = Oxygen radical

$Car^{\bullet}$ = β-carotene radical

$Car(X)^{\bullet}$ = Radical addition to the polyenic chain

Figure 3 β-carotene quench oxidizing free radical pathway; Equation 1, electron was transferred from free radical to β-carotene. Equation 2, β-carotene captured free radical molecule into the polyene chains directly.

The second pathway relies on a direct free radical addition to the polyene chain (Figure 3, Equation 2). This suggests that carbon-centered adducts free radical in reaction can add oxygen and propagate a chain reaction as shown in Figure 4 (Burton and Ingold, 1984).

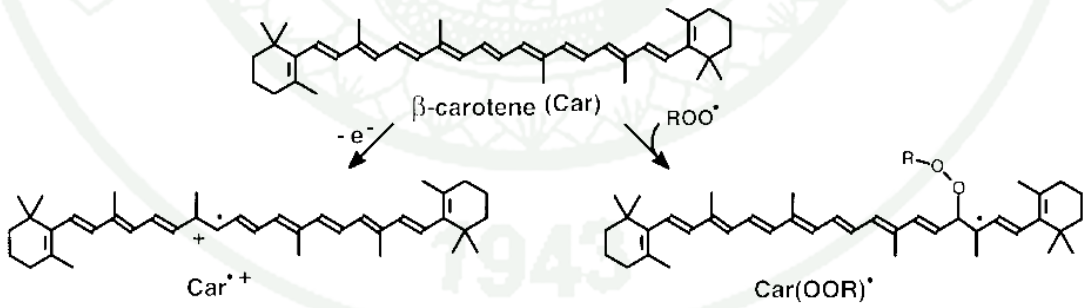


Figure 4 β-carotene scavenge oxidizing free radical pathway; Left, electron transferring yielding cation radicals $Car^{\bullet +}$. Right, direct addition yielding free radical adducts which are stabilized by delocalization on the polyene chain.

Source: Chaudière and Ferrari-Iliou (1999)

Tsuchihashi *et al.* (1995) reported that β-carotene acts as a moderate radical scavenging antioxidant, especially in the lipophilic domain of the membranes at low oxygen concentration. Moreover, it may also be possible that β-carotene and α-tocopherol act cooperatively as an antioxidant in the membranes and lipoproteins.

2.3.2 Photoprotective potential of carotenoids

In photosynthetic organisms such as plants, carotenoids are known to have a functional role because of the capability of these compounds to transfer energy in photosynthesis and in photoprotection (Krinsky, 1993). This function serves to protect cells and tissues from cellular damage related to certain photochemical reactions such as light-induced photooxidation of chlorophyll and other molecules (Rock, 1997). In higher plants and algae, photosynthesis involves two systems, photosystem I and photosystem II. Chlorophyll a and b take a role in photosystem I and photosystem II, respectively. The energy transfers from β-carotene to the chlorophyll of photosystem I is very efficient. The energy from light harvesting role transfers singlet energy to chlorophyll as in photosynthetic bacteria (Goodwin, 1980).

2.3.3 Protective effects against mutagenesis and genotoxicity

Astorg (1997) revealed protective effects against mutagenesis and genotoxicity function of carotenoids in diverse organisms. β-carotene and other carotenoids, including non-provitamin A carotenoids have anti-initiating action of carcinogenesis in animal models.

Cell proliferation is a key aspect of the promotion and progression stages of carcinogenesis but it is also involved in the initiation stage because a dividing cell is much more susceptible than a quiescent one to genotoxic damage. Carotenoids have been found to inhibit cell growth of various types of cultured cells, especially tumor cell lines (Krinsky, 1993).

2.3.4 Enhancement of the immune system

In animals, β-carotene and other carotenoids enhance the proliferative response of T and B lymphocytes to mitogens. Carotenoids increase the activity of natural killer (NK) cells. They increase both the number and the activity of cytotoxic T-cells. Carotenoids are also increase macrophage tumor-killing activity and stimulate the secretion of tumor necrosis factor α (TNF-α) (Astorg, 1997; Schwartz *et al.*, 1990). These effects are clearly involved in the action of carotenoids on the progression stage of carcinogenesis, by suppressing tumor growth, killing tumor cells and lowering tumor burden. β-carotene is also able to limit the decline of immune functions in elderly people (Astorg, 1997; Bendich, 1996).

3. Biosynthesis of β-carotene

Biosynthesis of carotenoids contains additional modifications of the end groups, the polyene chain, the methyl groups, or molecular rearrangements that contribute to the tremendous structural diversity of carotenoids. All carotenoids are derived from isoprenoid or terpenoid pathway. Condensation of one molecule of dimethylallyl diphosphate (DMADP) and three molecules of isopentyl diphosphate (IPP) produces the geranylgeranyl diphosphate (GGDP) that forms one-half of all C_{40} carotenoids (Hirschberg, 2001).

The metabolic pathway for β-carotene biosynthesis from glyceraldehyde-3-phosphate (G3P) is common in plants and other organisms, via following formations of isopentenyl pyrophosphate (IPP), phytoene, lycopene, and β-carotene. Two major pathways are in control for the biosynthesis of IPP in plants and algae. The pathways are mevalonate pathway (MVA) and non-mevalonate pathway (MEP) which recognize as 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Ye *et al.*, 2008) (Figure 4), respectively. In mevalonate pathway occurring in cytosol, two molecules of acetyl CoA initially react

to produce acetoacetyl CoA and then with another acetyl CoA yielding β-hydroxy-β-methylglutaryl CoA (HMG CoA).

In plants, carotenogenic enzymes involved in the carotenoid biosynthesis are imported into plastids by transit peptides when they are synthesized as larger precursors in the nucleus (Zhu *et al.*, 2003). It begins with glyceraldehyde-3-phosphate (G3P) and pyruvate via 1-deoxy-D-xylulose 5-phosphate (DOXP) , 2C-methyl-D-erythritol 4-cyclodiphosphate (MEP) , 4-diphosphocytidyl-2-C-methyl-D-erythritol (DPME), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcDP) and 4-hydroxy-2-methylbut-2-enylpyrophosphate (HMBPP), respectively (Ye *et al.*, 2008) (Figure 5).

The synthesis of IPP follows different pathways in cytosol. First, pyruvate is decarboxylated by isomerase through thiamine pyrophosphate (TPP) and interacted with G3P to produce DOXP. After isomerization and reduction by NADPH, MEP is synthesized. The MEP is then activated by reacting with CTP to produce DPME. Two further reduction steps, followed by dehydration and phosphorylation, finally produce IPP (Figure 5). The MEP-synthase pathway for isoprenoids is present in bacteria, algae, and plants but not in animals. A large part of plant isoprenoids, including the hemiterpene isoprene, monoterpenes like pinene and limonene, diterpenes (e.g., phytol chains, gibberellin, and abietic acid as an oleoresin constituent) as well as tetraterpenes (carotenoids) are synthesized via the MEP synthase pathway located in the plastids. In addition, this pathway synthesizes the side chains of chlorophyll and plastoquinone. (Heldt and Heldt, 2005). Moreover, the earlier pathway is mainly responsible for the biosynthesis of sesquiterpenes (C_{15}), diterpenes (C_{20}), phytyl (C_{20}), triterpenes (C_{30}) and including carotenoids (C_{40}) such as tocopherols, lycopene and β-carotene, respectively (Ye *et al.*, 2008).

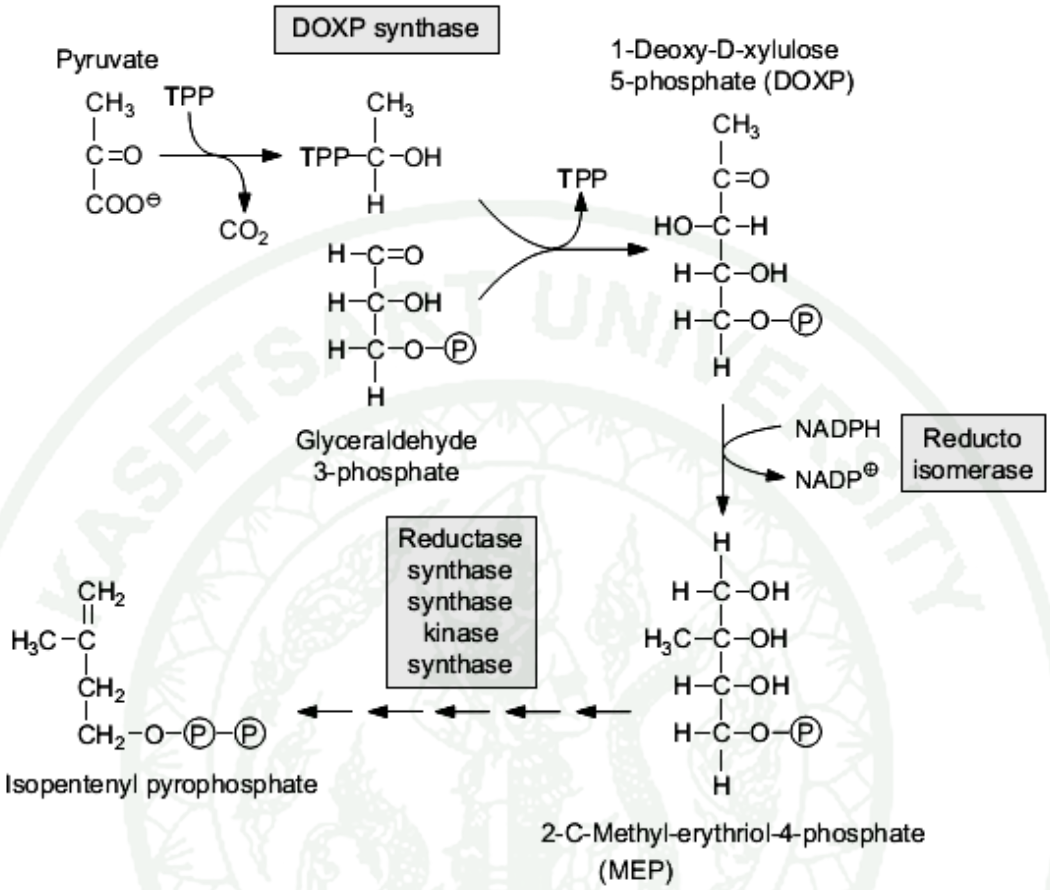


Figure 5 The isopentenyl pyrophosphate synthesis in the plastids proceeds *via* 2-methyl erythriol 4-phosphate pathway (MEP).

Source: Heldt and Heldt (2005)

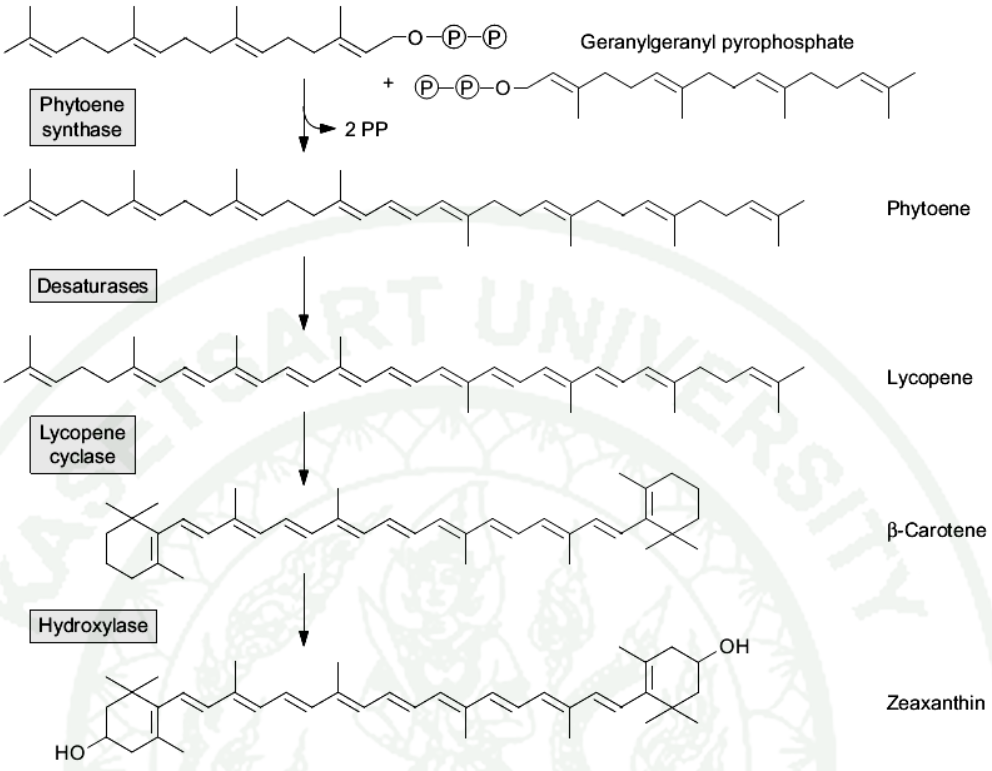


Figure 6 Carotenoid biosynthesis. The phytoene synthase (Pds) catalyzes the head-to-head addition of two molecules of geranylgeranyl diphosphate to phytoene. The later is converted by desaturases with neurosporene as the intermediate to lycopene. β-carotene is formed by cyclization. Finally, zeaxanthin is formed by additional hydroxylation.

Source: Heldt and Heldt (2005)

The first step of the carotenoid pathway is the formation of phytoene, which is the C_{40} of plant carotenoid pigments, from two molecules of GGPP. GGPP is formed by the serial addition of three molecules of IPP to one molecule of dimethylallyl pyrophosphate (DMAPP). These reactions are catalyzed by the GGPP synthase (GGPS). Phytoene synthase catalyzes the condensation of two molecules of GGPP to form phytoene. Phytoene is then converted to lycopene through two desaturation reactions catalyzed by phytoene desaturase and ξ-carotene desaturase. Lycopene can be

transformed into β-carotene by the action of lycopene β-cyclase which catalases a two-step reaction that creates one β-ionone ring at each end of lycopene (Figure 6).

4. β-carotene accumulation in response to stress condition

The rate and amount of β-carotene accumulation in *Dunaliella* sp. is determined by the conditions under which it is cultured. Under standard laboratory cultivation conditions, little β-carotene is synthesized and the algae appear green color. However, when the light intensity and salinity is increased much beyond the intensity required for normal growth, and when the rate of growth is limited, β-carotene is accumulated to the highest levels (Ben-Amotz *et al.*, 1988).

Rad *et al.* (2011) isolated *Dunaliella* sp. from hypersaline Urmia Lake water. *Dunaliella* sp. was treated in modified Johnson's medium containing different salinities (1, 2 and 3 M NaCl). The β-carotene content in culture containing 1, 2 and 3 M NaCl increasingly accumulated during the incubation period of 30 days. At the end of the experiments, the maximum cell content and the highest β-carotene content were obtained at 1 and 3 M NaCl concentrations, as 1.68 x 10^6 cell ml^{-1} and 8.94 pg $cell^{-1}$, respectively.

Ak *et al.* (2008) studied *Dunaliella viridis* Teodoresco isolated from Camalti salt works in batch system. They also grew algae in different NaCl concentrations (1, 2 and 3 M), different temperatures (25 and 28 ℃) and different light intensities (50 and 75 µmol photons $m^{-2}s^{-1}$). The amount of total carotenoid was found in culture containing 3 M NaCl at 25 ℃ and 50 µmol photons $m^{-2}s^{-1}$ light intensity. Their result revealed that the carotenoid accumulation was strongly dependent on salinity, temperature and light intensity.

Marín *et al.* (1998) studied the effect of nitrate and NaCl concentrations on growth and pigment under low light intensity in batch system. The highest carotenoid concentration was found in culture containing 1.5 M NaCl and low concentration of nitrate 212 µM at 35 days cultivation. Their results showed that microalga was

particularly sensitive to decrease of nitrate concentration, which promotes carotenoid accumulation within cell.

5. Genetic regulation of carotenoid biosynthesis

The metabolic pathways can be regulated at different hierarchical levels, for instance, at a level of transcription, translation or enzyme activity. Genes encoded enzymes in the carotenoid biosynthesis pathway have been cloned and well analyzed in various organisms, such as bacteria, cyanobacteria, fungi, algae and plants. The regulation of carotenoid biosynthesis involves either the expression of a formerly silent enzyme or the modification of key pathway enzymes.

In the carotenoid biosynthetic pathway, phytoene synthase (*Psy*), phytoene desaturase (*Pds*) and lycopene cyclase (*Lcyβ*), are the three essential enzymes responsible for the biosynthesis of both acyclic and cyclic carotenoids. Tuan *et al.* (2011) studied a relationship between carotenoid content and an expression of *Psy* and *Pds* genes in different organs and stages of fruit ripening in bitter melon *(M. charantia)*. Their results showed that the expression of both gene were mostly higher in the flowers. The expression levels of *Psy* and *Pds* decreased during the mid-stage of fruit ripening but increased in the fully ripe fruit. Salvini *et al.* (2005) also showed similar report in *H. annuus L.* that *Psy* was highly expressed in cotyledons, young and mature leaves. The *Psy* transcript levels were influenced by leaf expansion suggesting that the expression of the *Psy* gene is regulated during the process of leaf development. Phytoene synthase can catalyze the condensations of two-geranylgeranyl diphosphate (GGDP) to produce a C_{40} phytoene. Moreover, Romero *et al.* (2011) studied the function of *Pds* gene by virus-induced gene silencing (VIGS) techniques in tomato (*S. lycopersicum L.*) . They attempted to silence the *Pds* gene through the VIGS method in detached tomato fruits. After 16 days of *Pds* gene silencing, approximately 75% of the tomatoes showed *Pds* silencing symptoms. The yellow phenotype observed in *Pds*-silenced fruit was mainly due to the lack of the red-colored lycopene. The biochemical changes observed in *Pds*-silenced detached tomatoes suggested that carotenoid might be affected by the silencing of this gene. In

addition, the similar expression patterns of *Psy* and *Pds* gene during fruit maturation and leaf development may regulate the transcription of carotenoid biosynthesis genes. Thus, *Psy* and *Pds* genes feasibly regulate the flux of β, β-carotenoid branch of carotenoid biosynthesis (Tuan *et al.*, 2011).

Lycopene β-cyclase mainly plays a key role in the biosynthesis of β-carotene. Many studies have indicated that β-carotene compound is biosynthesized from lycopene by β-cyclization at both terminals. This reaction was catalyzed by lycopene β-cyclase. It catalyzes a two-step reaction that creates one β-ionone ring at each end of the lycopene molecule to produce β-carotene (Hao *et al.*, 2012).

D'Ambrosio *et al.* (2004) transformed tomato *Lcyβ* cDNA under the control of the *CaMV35S* promoter in tomato (*L. esculentum M.*) by *Agrobacterium*-mediated transformation. In the fruits of transgenic tomato, the lycopene is completely converted to β-carotene up to two folds. Their report also showed that in the wild type the *Lcyβ* gene is early downregulated from the breaker stage while *Lcyβ* transcript is very abundant during the whole ripening process in transgenic tomato. However, the use of a constitutive promoter possibly supports β-carotene biosynthesis in transgenic organisms. Rodriguez-Saiz *et al.* (2007) recently found an expression of the β-carotene biosynthetic genes *crtE, crtY, crtI,* and *crtB* from *Pantoea agglomerans* in halophilic bacteria *Halomonas elongata*. The transgenic *Halomonas elongata* was suggested as an alternative source of carotenoids under halophilic growth conditions (2% NaCl) with the highest production (560 μg g^{-1} dry weight). On the contrary, no β-carotene was detected in 15% NaCl. However, Rodriguez-Saiz *et al.* (2007) revealed that the use of genes from a non-halophilic species (*Pantoea agglomerans*) may lead to the reduction of carotenoid production under high salinity. Zhu *et al.* (2003) indicated that an alteration of *lycopene β-cyclase* and *lycopene ε-cyclase* transcript levels well correspond with a diversion of metabolic flow into the β-branch, leading to the higher proportions of β-carotene and derivatives.

Ramos *et al.* (2008) also studied *Lcyβ* transcript levels in microalgae *D. salina* under abiotic stress conditions, such as high light, nitrogen depletion, and high

salinity. *D. salina* cells pre-adapted to the culture containing 1.5 M NaCl. Northern blot analysis revealed that maximum transcript levels and β-carotene content were found in cells submitted to nutrient depletion medium containing 3.0 M NaCl after 36 h. This result indicated an important role of the combination between nutrient depletion and salinity shock on *Lcyβ* expression. Moreover, light intensity also induced an increase in *Lcyβ* transcript levels and β-carotene content. The highest *Lcyβ* transcript level was obtained in the cells, which were submitted to high (500 μmol $m^{-2}s^{-1}$) or low (45 μmol $m^{-2}s^{-1}$) light intensity, respectively. Furthermore, high light intensity coupled with nutrient deficiency increased the highest accumulation of carotenoid and supported the role of β-carotene on light harvesting and photoprotection (Ben-Amotz *et al.*, 1987).

MATERIALS AND METHODS

Materials

1. Instruments

Table 4 Instrument list for this study

Instruments	Company
Centurion Scientific K280R	Centurion Scientific Limited
DNA SUB CELL™	Bio-Rad Laboratories
Gel DOC 2000	Bio-Rad Laboratories
GeneAmp PCR System 2400	Perkin Elmer
Hybridization Water Bath	Stovall Life Science
Mettler AT201	Mettler Toledo
Mettler PM400	Mettler Toledo
MiniStar-AT 85~245	Tomos Life Science Group
Mini-Transilluminator	Bio-Rad Laboratories
Nano Drop Spectrophotometer ND-100	Nano Drop
Nanofuge	Hoefer Scientific Instruments
Optic Ivymen® System constant temperature incubator shaker	Comecta S.A.
pH/mV Meter UB-10	Denver Instrument
Power PAC300	Bio-Rad Laboratories
Px2 Thermal Cycler	Thermo Electron Corporation

2. Chemicals

Table 5 Chemical list for culturing of *Dunaliella sp.*

Chemicals	**Company**
Sodium chloride	Ajax FineChem Pty
Magnesium sulfate	Fisher Scientific
Potassium chloride	MERCK
Calcium nitrate	Ajax FineChem Pty
Monopotassium phosphate	Ajax FineChem Pty
Boric acid	MERCK
Ammonium molybdate hydrate	Fisher Scientific
Copper (II) sulfate	MERCK
Cobalt (II) chloride	May&Baker
Zinc chloride	Ajax FineChem Pty
Manganese (II) chloride tetrahydrate	Ajax FineChem Pty
Sodium bicarbonate	Ajax FineChem Pty
Ethylenediaminetetraacetic acid	Ajax FineChem Pty
Iron(III) chloride	Ajax FineChem Pty
Sodium hydroxide	Ajax FineChem Pty

Table 6 Chemical list for molecular biology

Chemicals	**Company**
Glacial acetic acid	LAB-SCAN
Agar No.2 bacteriological	Lab M Limited
Agarose ultrapure	Omnipur
Boric acid	MERCK
Calcium chloride dihydrate	Ajax FineChem Pty
Chloroform	LAB-SCAN LTD
DEPC	Bio Basic INC
D-glucose	Carlo Erba
Ethanol	LAB-SCAN
Ethidium bromide	Invitrogen
Glycerol	MERCK
Isopropyl alcohol	LAB-SCAN
Magnesium chloride	Ajax FineChem Pty
Phenol: Chloroform: Isoamyl alcohol (25: 24: 1)	USB
Potassium dihydrogen phosphate	Ajax FineChem Pty
Sodium chloride	Ajax FineChem Pty
Tris (hydroxymethyl) aminomethane	Amresco
Trizol reagent	Invitrogen
Tryptone	Lab M Limited
Yeast extract	Biobasic INC

3. Primer

Table 7 Primers for the amplification of *Lcyβ* gene

Primer name	Sequence 5′-3′
Q_T	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC$(dT)_{17}$
Q_O	CCAGTGAGCAGAGTGACG
Q_I	GAGGACTCGAGCTCAAGC
LcyβWF	CCTCTCAGATGGGCSCACCRTGYAWG
LcyβNR	ACAGCTGGGCDAGYCACCAHGKAVGT
LcyβNF	CCTCTCAGATGGGCRCACCAWKGMAAG
Lcyβ124R	CCCYCCAGTCCATGAASARCAT
LcyβfullFW	GATGTCTCGGCAGTCAGACA
LcyβfullRW	CCTCGTGGCAATGGGTAAGC
Lcyβ33F	GTACCAAGGCGCGTACGGCAT
Lcyβ33R	CCTAACCACTTCATGCGAGC
18S_F1	TGCAGTTAAAAAGCTCGTAGTTG
18S_R1	GAACATCTAAGGGCATCACAGAC

4. Kit

- GeneJET Purification Kit (Thermo Fisher Scientific, USA)
- cDNA Synthesis with SuperScript® III RT (Invitrogen, USA)
- RBC TA Cloning Vector Kit (RBC Bioscience, Korea)
- RBC HiYeildt™ Gel/PCR DNA Fragment Extraction Kit (RBC Bioscience, Korea)
- RBC HiYeildt™ Plasmid Mini Kit (RBC Bioscience, Korea)
- HIT Competent Cells (RBC Bioscience, Korea)
- Terminal Transferase (New England Biolabs, USA)
- Long PCR Enzyme Mix (Thermo Fisher Scientific, USA)
- DreamTaq DNA Polymerase (Thermo Fisher Scientific, USA)
- DNAse I (Invitrogen, USA)

Methods

1. Screening of *Dunaliella* sp.

The green alga *Dunaliella sp.* was isolated from salt soil sample collected from Chaiyaphum province in the northeastern part of Thailand. The mixed salt soil sample (10 g) was added with 30 ml of modified liquid Johnson's medium containing 2.5 M NaCl and incubated for ~3 weeks under continuous illumination cool daylight (60 µmol $m^{-2}s^{-1}$) at 25±2 °C (Borowitzka, 1988). After that, the culture was streaked on 1.5 M NaCl modified Johnson's agar medium. The single colony was sub-cultured several times on the agar medium for screening pure strains. Finally, each colony was picked and cultured in 1.5 M NaCl modified Johnson's liquid medium for next experiments.

2. Growth and β-carotene accumulation of *Dunaliella* sp. in flask culture

Effect of different NaCl concentrations on growth of *Dunaliella* sp. in modified Johnson's medium (Borowitzka, 1988) and in remodified Johnson's medium from the original recipe were compared (Appendix Table 1). Fifty milliliters of starter cells were transferred into 500 ml Erlenmeyer flasks containing 250 ml of 1.0, 1.5, 2.5 and 3.5 M NaCl in both media at a final cell density of approximately 2.5×10^5 cells ml^{-1} and were kept under continuous cool daylight (80 µmol $m^{-2}s^{-1}$) at 25±2 °C for 11 days. The cell samples were collected daily, mixed with 1 µl of 3% formalin and counted under light microscope using haemacytometer. The specific growth rate (µ), doubling per time (k) and doubling time (t_d) of microalga were calculated according to the Equation 1, 2 and 3, respectively:

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \qquad \text{(Equation 1)}$$

$$k = \mu/0.6931 \qquad \text{(Equation 2)}$$

$$t_d = 0.6931/\mu \qquad \text{(Equation 3)}$$

where N_2 and N_1 represented the cell density values at the time t_2 and t_1, respectively.

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

Fifty milliliters of starter cells were transferred into modified Johnson's medium containing 1.0, 1.5, 2.5 and 3.5 M NaCl at final cell density of 7.5×10^5 cells ml^{-1} and kept under continuous cool daylight (200 μmol $m^{-2}s^{-1}$) at 25±2 °C with air/CO_2 (ratio 95/5) at the flow rate of 30 ml/min. The cell samples were collected, mixed with 1 μl of 3% formalin and counted under light microscope using haemacytometer.

4. Carotenoid extraction and determination

Ten milliliters of the culture were harvested and centrifuged at 4000 ×g for 10 min. The pellet was washed 2-3 times with distilled water to remove traces of adhering salts. Then, 2-3 ml of acetone (80%) was added for extracting carotenoid pigment. The mixture was centrifuged for 5 min and the supernatant was collected three times until the cell debris becomes colorless. All fractions of supernatants were pooled and adjusted to a final volume of 10 ml. β-carotene content was analyzed using a UV-8453 spectrophotometer at 450 nm wavelength. The β-carotene content was calculated from a standard curve equation of authentic β-carotene (Sigma, USA) dissolved in 80% (v/v) acetone. The β-carotene amount was calculated by Goodwin (1980) equation as follow :

$$\text{β-carotene (mg ml}^{-1}) = (A_{450} \times V \times f \times 10)/2500$$

Where, A_{450} = Absorbance at 450 nm

V = Volume of the extract, and

f = Dilution factor

5. RNA Extraction

The alga cells were collected at the late senescence phase of growth in medium containing 2.5 M NaCl by centrifugation at 3000×g for 10 min. The supernatant was removed and the cell pellet was kept. One ml of TRIzol reagent (Invitrogen, USA) was added to the sample for cell lyses. Then, the sample was incubated at room temperature for 10 min to allow complete dissociation of nucleoprotein complexes. The supernatant was collected by centrifugation at 12,000×g at 4 °C for 10 min and transferred to a fresh Eppendorf tube. Two hundred microliters of chloroform were added, and the mixture was vigorously shaken for 15 sec and kept at room temperature for 3 min. Then, the sample was centrifuged at 12,000×g at 4 °C for 15 min. The upper aqueous phase was collected and transferred to a fresh Eppendorf tube. The RNA was precipitated from the aqueous phase by gently mixing with 500 µl of isopropanol, then incubated at 25ºC for 10 min, and centrifuged at 12,000×g at 4 °C for 10 min. The RNA pellet was washed twice with 0.5 ml of 75% ethanol. The ethanol was removed from the RNA pellet and the pellet was air-dried. The RNA pellet was resuspended in 20 µl RNase-free water and stored at -20 °C.

The concentration and quality of RNA were determined by measuring the absorbance at 260 nm with the UV-8453 spectrophotometer and gel electrophoresis. For long-term storage, RNA was then stored at -80 °C until further used. The RNA concentration of each sample was estimated by the following equation:

Concentration of RNA sample = A_{260} × dilution factor × 40 µg ml^{-1}

Purity of RNA was determined by the ratio of the reading at 260 nm and 280 nm (A_{260}/A_{280}). The ratio of A_{260}/A_{280} ranging from 1.8 to 2.0 indicates good quality of the RNA.

DNase digestion reaction was performed in 50 µl of the reaction mixture consisting of 5 µg of total RNA, 5 µl of RQ1 RNase-Free DNase 10X Reaction

Buffer, 5 µl of RQ1 RNase-Free DNase and final volume was adjusted to 50 µl by nuclease-free water. The reaction mixture was incubated at 37 °C for 1 h. Fifty microliters of Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added to the reaction mixture. The reaction mixture was gently mixed, and centrifuged at 12,000×g for 2 min. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube. Fifty microliters of chloroform were added to the tube and gently mixed, and then centrifuged at 12,000×g for 2 min. The clear upper phase was transferred to a fresh tube filled with 125 µl of absolute ethanol, incubated at -20 °C for 30 min and centrifuged at 12,000×g for 10 min. The RNA pellet was washed twice by adding 500 µl of 75% ethanol. The RNA pellet was air-dried, resuspended in 20 µl of RNase-free water and then stored at -80 °C until further used.

6. Sequencing of the *lycopene β-cyclase* gene

6.1 Reverse Transcription

The first-strand cDNA synthesis reaction was performed in 10 µl comprising 2 µg of total RNA, 1 µl of 50 µM Oligo $(dT)_{20}$, and 1 µl of 10 mM dNTP. Total volume was adjusted to 10 µl by RNase-free water. The reaction mixture was incubated at 60 °C for 5 min, placed on ice for 1 min. The RNA mixture was mixed with 10 µl of cDNA Synthesis Mix (Invitrogen, USA) containing 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT™ (40 U/µl), 1 µl of SuperScript®III RT (200 U/µl). The reaction mixture was incubated at 50 °C for 50 min on DNA Thermal Cycler (Perkin Elmer Cetus, USA). The cDNA synthesis reaction was terminated at 85 °C for 5 min, and then chilled on ice. One microliter of RNaseH was added to the cDNA mixture, incubated at 37 °C for 20 min and stored at -20 °C.

6.2 Amplification of *lycopene β-cyclase* gene by Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed to amplify the *lycopene β-cyclase* gene fragment. Two degenerated primers were designed based on conserve regions of the *lycopene β-cyclase* gene of the organisms in databases, i.e. ADX41685.1 (*Dunaliella sp*. ABRIINW-G4) , AAO64977.1 (*Haematococcus pluvialis*), AAX54906.1 (*Chlamydomonas reinhardtii*), XP_002957303.1 (*Volvox carteri f.* nagariensis), EIE23905.1 (*Coccomyxa subellipsoidea* C-169), Q43415.1 (*Capsicum annuum*), XP_002531498.1 (*Ricinus communis*), XP_002884784.1 (*Arabidopsis lyrata* subsp. lyrata), NP_001234226.1 (*Solanum lycopersicum*), ABF69944.1 (*Citrus sinensis*), ABP00806.1 (*Ostreococcus lucimarinus* CCE9901), CAL56827.1 (*Ostreococcus tauri*) , ACO66866.1 (*Micromonas* sp. RCC299) , EGB09881.1 (*Aureococcus anophagefferens*) and EFN58632.1 (*Chlorella variabilis*).

All amino sequences were used to identify conserve regions by ClustalW2 (Larkin *et al.*, 2007). The conserved amino acid sequence was calculated and translated to nucleotide sequence to predict degenerate primers by CODEHOP (Rose *et al.*, 2003). The degenerate primers were tested once by Oligocalc (www.basic.northwestern.edu/biotools) and FastPCR software (PrimerDigital Ltd., Finland). Finally, LcyβWF and LcyβNR primers were designed as follows:

Forward primer LcyβWF: CCTCTCAGATGGGCSCACCRTGYAWG
Reverse primer LcyβNR: ACAGCTGGGCDAGYCACCAHGKAVGT

The PCR was performed in a final volume of 25 µl containing 1.0 µl cDNA template, 2.5 µl 10X Long PCR buffer with 15 mM $MgCl_2$, 2.5 µl dNTP Mix (2 mM each), 1.0 µl LcyβWF primer (10 µM), 1.0 µl LcyβNR primer (10 µM), 1.0 µl Long PCR Enzyme Mix (5U/µl) (Thermo Fisher Scientific, USA) and 15.0 µl ddH_2O. The thermal cycling program started with preheating at 94 °C for 3 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 30 s and 68 °C for 30 s. The reaction was terminated with a final extension at 68ºC for 10 min. PCR product was

identified by gel electrophoresis and the size of the amplified fragment was compared to 1 kb DNA ladder (Thermo scientific, USA)

6.3 Agarose gel electrophoresis

The DNA sample was mixed with 6X loading dye (10 mM Tris-HCl pH 7.6, 0.1% w/v Bromophenol blue, 60% glycerol and 60 mM EDTA), loaded on 1.5% agarose gel in 0.5X TBE buffer. The gel was electrophoresed at 110 volt for 30 min, stained by 0.2 µg l^{-1} ethidium bromide. The DNA was visualized by using a UV transilluminator (Biorad, USA).

6.4 Gel extraction

The DNA band was eluted using the GeneJET purification kit (Thermo scientific, USA) according to manufacturer's protocol; Binding buffer was added to the cut gel (1:1 of volume) and incubated at 50 °C for 5 min until the gel was entirely dissolved. The solution was transferred to a purification column and centrifuged at 12,000×g for 10 sec. Seven hundred microliters of washing buffer were add to the column and centrifuged once at 12,000×g for 2 min. Finally, 30 µl elution buffer were added to column and centrifuged for 1 min to collect DNA.

6.5 Ligation

The ligation of the PCR fragment was performed in a final volume of 25 µl containing 2.0 µl purified PCR product, 2.5 µl 10X Dream PCR buffer, 1.0 µl dATP (0.1 mM) and 0.15 µl Dream *Taq* Polymerase (5U/µl). Total volume was adjusted to 25 µl with nuclease free-water and the mixture was incubated at 72 °C for 30 min. For ligation reaction, the PCR product was ligated into linearized cloning vector (RBC Bioscience, Korea), overhanging 3´ deoxythymidine (T) residues was designed for allowing PCR inserted.

Ligation reaction was performed on ice containing 1 ul Ligation buffer A, 1 ul Ligation buffer B, 2 ul RBC TA cloning vector, 5 ul PCR product and 1 ul T4 DNA ligase (3U/µl). Total volume was adjusted to 10 µl by nuclease free-water. The ligation reaction mixture was incubated at 22 °C for 30 min and kept at 4 °C overnight.

6.6 Plasmid transformation into competent cells

HIT Competent Cells™ DH5α were thawed on ice. Ten microliters of the ligation reaction mixture were added, swirled and placed on ice for 30 min. The competent cells were heat-shocked at 42 °C for exactly 30 sec and immediately kept on ice. One milliliter of LB medium was added and agitated on a rotary shaker at 200 rpm for 2 h at 37 °C. Then, 200 µl of transformed cells were spread on LB agar medium containing 100 µg ml^{-1} ampicillin and incubated at 37 °C. The transformed colony appeared on the LB agar medium within 16 h after incubation. The white colony was sub-cultured and stored at 4 °C.

6.7 Plasmid extraction from competent cells

The plasmid was extracted using HiYield Gel/PCR Fragment Extraction Kit (RBC Bioscience, Korea) according to the manufacturer's protocol. The transformed cells were grown overnight in 5 ml LB broth containing 100 µg ml^{-1} ampicillin in a shaking incubator at 37 °C for 16 h. The cells were harvested by centrifugation at 8000×g for 1 min and then resuspended in 100 µl of cell resuspension buffer. Two hundred microliters of lysis solution were added to the cell suspension, the tube was gently inverted and kept on ice for 5 min. The lysate was neutralized by adding 150 µl of neutralization buffer and kept on ice for 5 min. The plasmid DNA was precipitated with 1/2 volume of isopropanol and centrifuged at 10000×g for 10 min. The plasmid DNA was rinsed with 70% ethanol, air dried and dissolved in 100 µl of TE buffer (Sambrook and Russell, 2001). The plasmid DNA was kept at –20 °C for sequencing or for further analysis.

6.8 Sequence analysis

The plasmid DNA was sent out and sequenced at Macrogen Company (Korea). The sequence of cloning vector at Multi Cloning Site was removed from the raw sequence using DNA BASER Sequence Assembler 4.1 (Heracle BioSoft S.R.L., Romania). The sequence of *Lcyβ* gene was aligned by BlastN tool with other *Lcyβ* gene sequences from the NCBI database. The similar nucleotide sequences were multiple aligned by Clustalw tool to identify the conserved region and the gene specific primers were designed by the CODEHOP (Rose *et al.*, 2003) to amplify the *lycopene β-cyclase* gene.

7. Sequencing 5′ and 3′ end of *lycopene β-cyclase* gene

7.1 Sequencing the 5′-end of *lycopene β-cyclase* gene

7.1.1 Reverse Transcription

The first-strand cDNA synthesis reaction was performed in 10 µl reaction mixture comprising 2 µg of total RNA, 1 µl of 100 µM gene specific primer (LcyβNR), and 1 µl of 10 mM dNTP. Total volume was adjusted to 10 µl by RNase free-water. The reaction was incubated at 60 °C for 5 min and placed on ice for 1 minute. The RNA mixture was mixed with 10 µl of cDNA Synthesis Mix (Invitrogen, USA) containing 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT™ (40 U/µl), 1 µl of SuperScript®III RT (200 U/µl). The reaction mixture was incubated at 50 °C for 50 min and followed by terminating at 85 °C for 5 min on PX2 Thermal Cycler (Thermo Electron Corporation, USA), and then chilled on ice. One microliter of RNaseH was added to the cDNA mixture, incubated at 37 °C for 20 min, and stored at -20 °C.

7.1.2 cDNA precipitation

To remove the primer and remaining chemicals, 20 µl of cDNA were added by 2 µl of 3 M sodium acetate and 25 µl of 95% ethanol. The mixture was centrifuged at 18,000×g for 30 min, supernatant was discarded and the pellet was washed twice with 70% ethanol and air dried at room temperature. The pellet was dissolved in 20 µl of TE buffer.

7.1.3 Appending polyA tails

For tailing cDNA, the reaction mixture was performed in 20 µl reaction mixture containing 4.0 µl 5X Terminal Deoxynucleotidyl Transferase buffer, 0.6 µg cDNA fragments, 1.0 µl dATP (3 mM) and 1.5 µl Terminal Deoxynucleotidyl Transferase (20 U/µl) (New England Biolabs, USA). Total volume was adjusted to 20 µl by nuclease-free water and incubated at 37 °C for 10 min. The reaction was inactivated by heating at 70 °C for 10 min.

7.1.4 Adding OligodT-Adaptor

The A-tail added cDNA was extended with Q_t (Oligo(dT)-Adaptor) by using *Taq* DNA polymerase. The reaction was performed in 50 µl reaction mixture containing 2.0 µl A-tail added cDNA template, 5.0 µl 10X Long PCR buffer, 1.0 µl dNTP Mix (10 mM each), 1.0 µl Q_t primer (10 µM), 2.0 µl DMSO , 3.0 µl $MgCl_2$ (10 mM) and 0.25 µl Long PCR Enzyme Mix (5U/µl). The final volume was adjusted to 50 µl and the reaction was performed on the PX2 thermal cycler (Thermo Electron Corporation, USA) a following program: 95 °C for 5 min; 50 °C for 2 min; and 68 °C for 40 min. After that, the mixture was purified as described in method 7.1.2

7.1.5 5′ end amplification

The purified mixture was used as a template for 5′ RACE PCR. The amplification reaction was performed in to 50 µl reaction mixture containing 2.0

µl cDNA Template, 5.0 µl 10X Long PCR buffer, 1.0 µl dNTP Mix (10 mM each), 1.0 µl Lcyβ33R Primer (10 µM), 1.0 µl Q_0 Race Adaptor primer (10 µM) , 2.0 µl DMSO, 3.0 µl $MgCl_2$ (25 mM) and 0.25 µl Long PCR Enzyme Mix (5U/µl). Total volume was adjusted to 50 µl. The thermal cycling program was used as follows: one cycle of 94 °C for 3 min; 35 cycles of 94 °C for 30 s; 55 °C for 20 s; 68 °C for 2 min; and one cycle of 68 °C for 10 min. Size of the PCR product was compared with 1 kb DNA ladder (Thermo scientific, USA). Then, the largest size of DNA fragement was cut from agarose gel and purified using HiYield Gel/PCR Fragment Extraction Kit (RBC Bioscience, Korea) according to the manufacturer's protocol.

The nested PCR was carried out in 50 µl. The reaction mixture contained 2.0 µl 5′ end cDNA Template, 5.0 µl 10X Long PCR buffer, 1.0 µl dNTP Mix (10 mM each), 1.0 µl Lcyβ124R Primer (10 µM), 1.0 µl Q_I nested Adaptor primer (10 µM), 2.0 µl DMSO, 3.0 µl $MgCl_2$ (25 mM) and 0.25 µl Long PCR Enzyme Mix (5U/µl). The sample was adjusted to 50 µl final volume and reaction was performed on the thermal cycler with the following program: one cycle of 94 °C for 3 min; 15 cycles of 94 °C for 30 s, 50 °C for 20 s, 68 °C for 2 min; 25 cycles of 94 °C for 30 s, 47 °C for 20 s, 68 °C for 2 min; and one cycle of 68 °C for 10 min. PCR product was identified by gel electrophoresis. DNA band was cut, purified using HiYield Gel/PCR Fragment Extraction Kit (RBC Bioscience, Korea) and sent out to sequence at Macrogen Company (Korea).

7.2 Sequencing 3′-end of *lycopene β-cyclase* gene

7.2.1 Reverse transcription

The first-strand cDNA synthesis reaction was performed with Q_t primer as described in the 5′-end procedure.

7.2.2 3′ end amplification

The cDNA was used as a template for the amplification of 3′ end with Q_0 RACE Adaptor primer. The reaction mixture contained 2.0 µl cDNA Template, 5.0 µl 10X Long PCR buffer, 1.0 µl dNTP Mix (10 mM each) , 1.0 µl LcyβNF Primer (10 µM), 1.0 µl Q_0 Race Adaptor primer (10 µM), 2.0 µl DMSO, 3.0 µl $MgCl_2$ (25 mM), and 0.25 µl Long PCR Enzyme Mix (5U/µl). The sample was adjusted to 50 µl final volume and performed as following program: one cycle of 94 °C for 3 min; 10 cycles of 94 °C for 30 s, 57 °C for 20 s, 68 °C for 2 min; 25 cycles of 94 °C for 30 s, 54 °C for 20 s, 68 °C for 2 min; and one cycle of 68 °C for 10 min. The PCR product was identified by gel electrophoresis. DNA band was cut and purified using HiYield Gel/PCR Fragment Extraction Kit (RBC Bioscience, Korea).

The purified fragment was used as a template for nested PCR. The reaction contained 2.0 µl 3′ end cDNA template, 5.0 µl 10X Long PCR buffer, 1.0 µl dNTP Mix (10 mM each), 1.0 µl Lcyβ33F Primer (10 µM) , 1.0 µl Q_I nested Adaptor primer (10 µM), 2.0 µl DMSO, 3.0 µl $MgCl_2$ (25 mM) and 0.25 µl Long PCR Enzyme Mix (5U/µl). The sample was adjusted to 50 µl final volume and performed on the thermal cycler by the following program: one cycle of 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 50 °C for 20 s, 68 °C for 2 min; and one cycle of 68 °C for 10 min. The PCR product was identified by gel electrophoresis. DNA band was cut, purified using HiYield Gel/PCR Fragment Extraction Kit (RBC Bioscience, Korea) and sent out to sequence at Macrogen Company (Korea).

To amplifying full-length *Lcyβ* gene, PCR was performed in a final volume of 25 µl containing 1.0 µl cDNA template, 2.5 µl 10X PCR buffer, 0.5 µl of 50 mM $MgCl_2$,0.5 µl dNTP Mix (10 mM each), 1.0 µl LcyβfullFW primer (10 µM), 1.0 µl LcyβfullRW primer (10 µM), 1.0 µl recombinant *Taq* DNA Polymerase (5U/µl) (Invitrogen, USA) and 15.0 µl ddH_2O. The thermal cycling program started with preheating at 94 °C for 5 min, followed by 40 cycles of 94 °C for 50 s, 55 °C for 40 s and 72 °C for 3 min. The reaction was terminated with a final extension at 72 ºC

for 10 min. PCR product was identified by gel electrophoresis and the size of the amplified fragment was compared to 1 kb DNA ladder (Thermo scientific, USA)

8. Phylogenetic tree analysis

The nucleotide and amino acid sequences were aligned with the corresponding sequences in NCBI database using the Basic Local Alignment Search Tool, BLAST v2.2.29 (Altschul *et al.*, 1990). The amino acid sequences were multiple aligned by ClustalW2 (Larkin *et al.*, 2007). The phylogenetic relationships were calculated using Molecular Evolutionary Genetics Analysis, MEGA software 5 (Tamura *et al.*, 2011). The deduced amino acid sequences were constructed and compared by the neighbor-joining method (Saitou and Nei, 1987). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

9. *Lcyβ* gene expression study

Two types of experiment were carried out to study the *Lcyβ* gene expression. Firstly, *Dunaliella* sp. M22 was inoculated to 500 ml of liquid remodified Johnson medium containing 1.5 M NaCl and kept under continuous cool daylight (200 μmol $m^{-2}s^{-1}$) at 25±2 °C until the strain reached to exponential phase. Then, the cells were transferred to fresh remodified Johnson medium containing different initial NaCl concentrations of 1.0, 1.5, 2.5 and 3.5 M at final cell density of 7.5×10^5 cells ml^{-1} and cultured using parameters as described in method 3. During experiment, the cells were collected approximately 5×10^5 cells at 3 periods; exponential phase (period I), early stage of senescence phase (period II) and late stage of senescence phase (period III) for determining *Lcyβ* gene expression pattern during growth.

Secondly, the *Dunaliella* sp. M22 was cultured in cylinder tube containing 200 ml of 1.0 and 1.5 M NaCl modified Johnson's medium until growth reached to exponential phase. Hundred milliliters of exponential phase culture from the 1.0 and 1.5 M NaCl was mixed with 100 ml of fresh modified Johnson's medium containing

4.0 and 3.5 M NaCl, respectively (final concentration reached to 2.5 M). The cells were cultured using parameter as mentioned above in Method 3. The cells were collected approximately 5×10^5 cells for extracting total RNA at 4 h, 1 day and 3 days.

Total RNA was extracted by TRIzol reagent and residual genomic DNA was removed by DNAse (Invitrogen, USA) according to manufacturer's protocol. Then, the RNA concentration was quantified and adjusted to 0.3 μg μl^{-1}. The first-strand cDNA synthesis reaction was performed as mentioned above in Method 6.1. *Lcyβ* transcript levels were investigated using semi-quantitative RT-PCR. To determine relative levels of *Lcyβ* expression, the α-tubulin was used as an internal control. The cDNA amount was normalized by amplification of α-tubulin gene. The PCR amplification conditions were optimized. The suitable cycle number was determined at the exponential amplification stage and primer sequences are listed in Table 8 and Table 9, respectively.

Table 8 Oligonucleotide forward and reverse primers of *Lcyβ* gene for the PCR amplification of cDNA. An α tubulin gene (*Atub)* primers were used for the PCR amplification as an internal control.

Gene	Sequence	Expected size
Lcyβ	Forward: 5′- GTACCAAGGCGCGTACGGCAT -3′	264 bp
	Reverse: 5′- CCTAACCACTTCATGCGAGC -3′	
Atub	Forward: 5′- GTACTGCCTGGAGCACGGCA -3′	522 bp
	Reverse: 5′- TGCTCCAGCAGGGAGTGGGT -3′	

Table 9 Optimized PCR conditions for the cDNA amplification of *Lcyβ* and *Atub* gene.

Gene	Denaturing Temperature/ Time	Annealing Temperature/ Time	Extension Temperature/ Time	Cycles
Lcyβ	95 ºC/ 20 sec	63 ºC/ 20 sec	68 ºC/ 30 sec	29
Atub	95 ºC/ 20 sec	63 ºC/ 20 sec	68 ºC/ 30 sec	25

PCR reactions were separated by electrophoresis on a 1.2% agarose gel containing 0.5 mg l^{-1} ethidium bromide. Densitometry analysis was performed using Gel DOC 2000 and ImageJ2x software (Rawak Software, Inc., USA).

RESULTS

1. Screening of β-carotene producing *Dunaliella* sp. strain

The *Dunaliella* sp. was screened from salt soil sample by adding 2.5 M NaCl modified Johnson's liquid medium, subsequently streaked on modified Johnson's agar plate containing 1.5 M NaCl. Several hundred colonies were obtained. The colony that has an ability to produce β-carotene initially turned orange after 2 months (Figure 7).

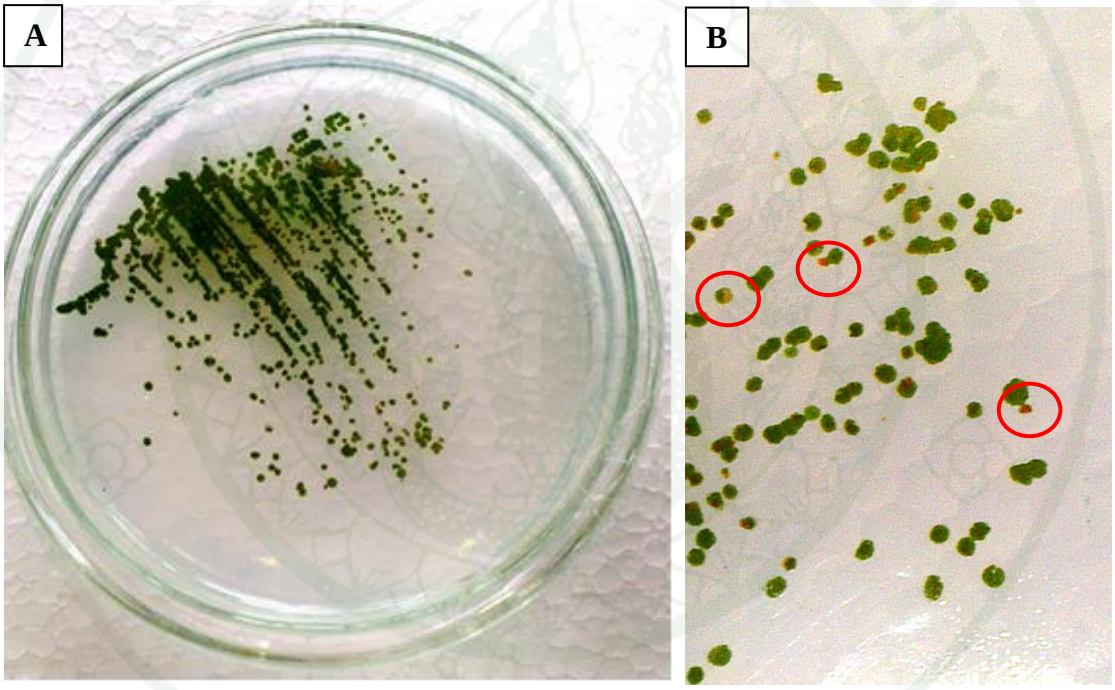


Figure 7 Production of β-carotene by the isolated strain on modified Johnson's medium agar plate containing 1.5 M NaCl. The *Dunaliella* sp. strain was isolated from salt soil sample (A). The β-carotene produc*ing* strains were shown in red circles (B).

Five orange colonies, — namely M02, M04, M07, M11 and M22 were selected and inoculated into liquid modified Johnson's medium containing 1.5 M NaCl (Figure 8). The β-carotene production ability was observed from changing of green to orange color. The highest β-carotene accumulation was observed in M22,

followed by M11, M13, M07 and M02 *Dunaliella* sp. strains. The M22 strain was used for further study.

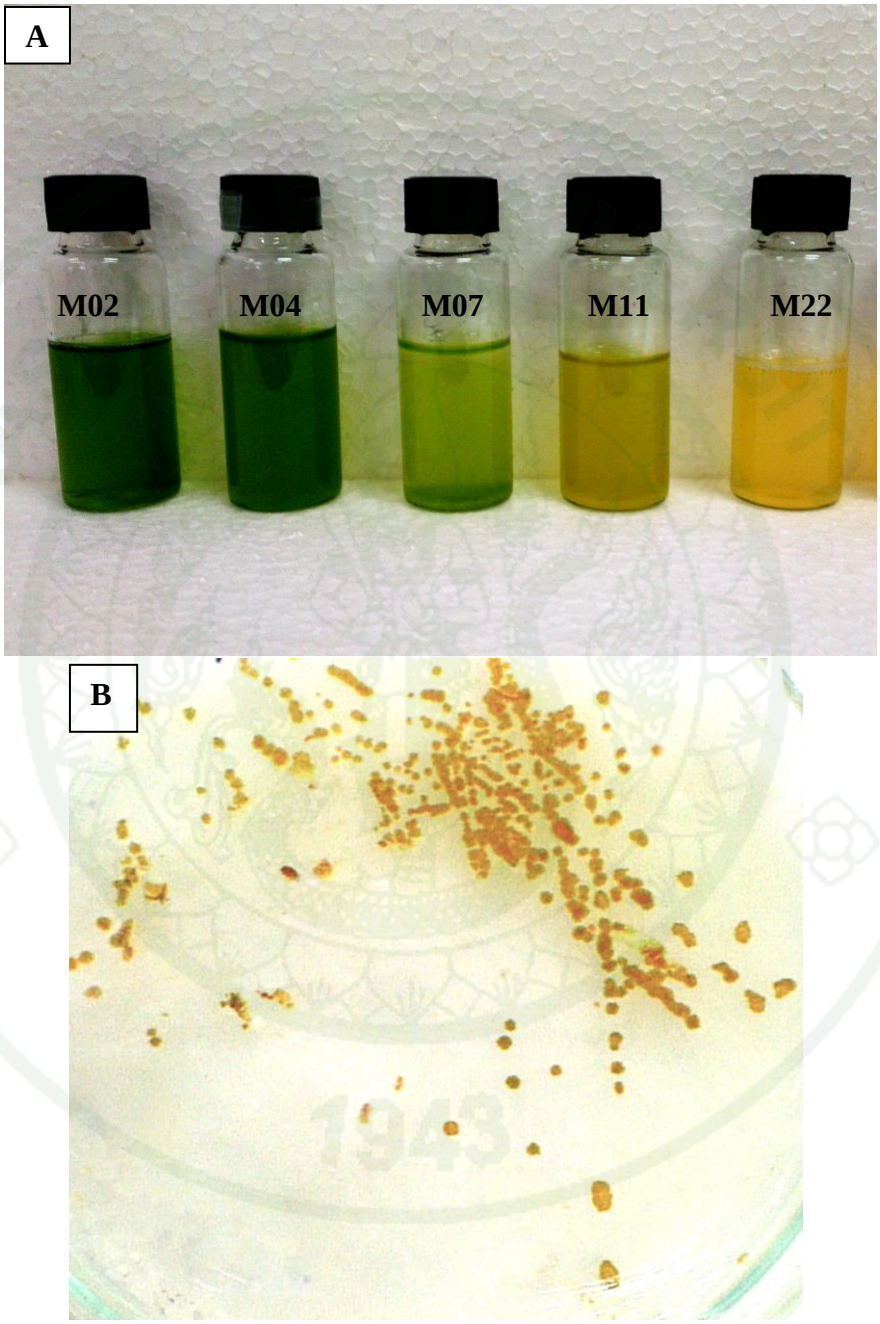


Figure 8 *Dunaliella* sp. strains M02, M04, M07, M11 and M22 were cultured in liquid 1.5 M NaCl modified Johnson's medium (A), the M22 strain turned orange after 2 months (B).

2. Growth and β-carotene accumulation of *Dunaliella* sp. M22 in flask culture

2.1 Growth

Among the four different NaCl concentrations (1.0, 1.5, 2.5 and 3.5 M) the *Dunaliella* sp. M22 reached the senescence phase within 7 days after inoculation in all NaCl concentrations and the highest cell density was obtained in 1.0 M NaCl and reached up to 2.7×10^6 cells ml^{-1} in modified Johnson's medium (Figure 9). The specific growth rate of *Dunaliella* sp. M22 was 0.66, 0.45, 0.31 and 0.26 d^{-1} in 1.0, 1.5, 2.5 and 3.5 M NaCl modified Johnson's medium, respectively (Appendix Table 2).

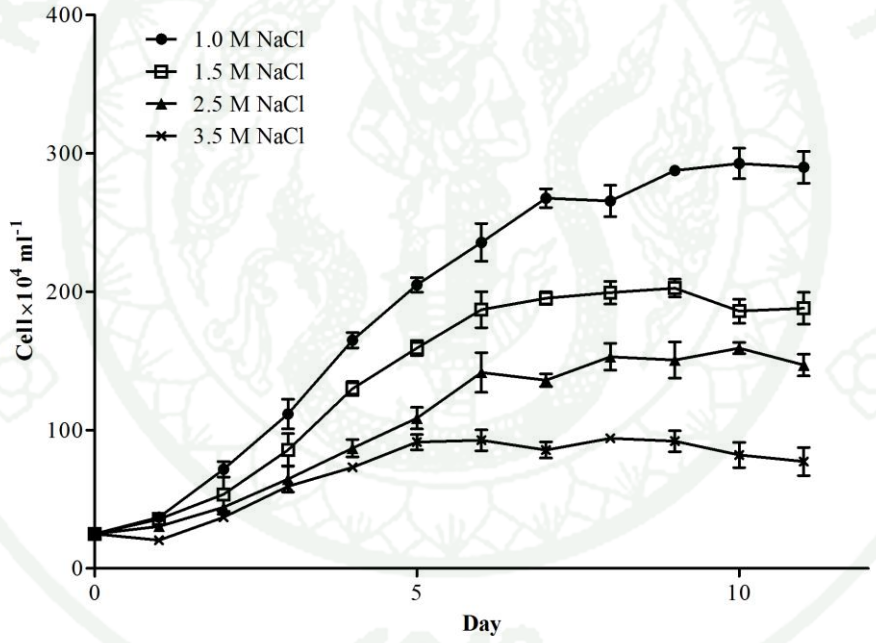


Figure 9 Growth of *Dunaliella* sp. M22 strain in modified Johnson's medium containing different NaCl concentrations in 250 ml Erlenmeyer flask under continuous illuminated cool daylight (80 μmol photon $m^{-2}s^{-1}$) at 25±2 °C. Plotted data are the averages ± SD of three replicates.

The growth of *Dunaliella* sp. M22 was also compared with remodified Johnson's medium containing different NaCl concentrations. In this medium, the strain M22 reached the exponential phase within 5 days, whereas in modified

Johnson's medium it took 7 days to reach exponential phase in all different concentrations of NaCl (Figure 10). The highest cell density was obtained in 1.0 M NaCl concentration (5.5 ±0.18 x10^6 cell ml^{-1}) whereas the lowest cell density was in 3.5 M cultures (1.95±0.13 xl0^6 cell ml^{-1}). The specific growth rate of this medium in 1.0 M, 1.5 M, 2.5 M and 3.5 M was 1.56, 1.41, 0.85 and 0.77 d^{-1}, respectively (Appendix Table 3). The specific growth rate in remodified Johnson's medium was 2 times higher than that in modified Johnson's medium.

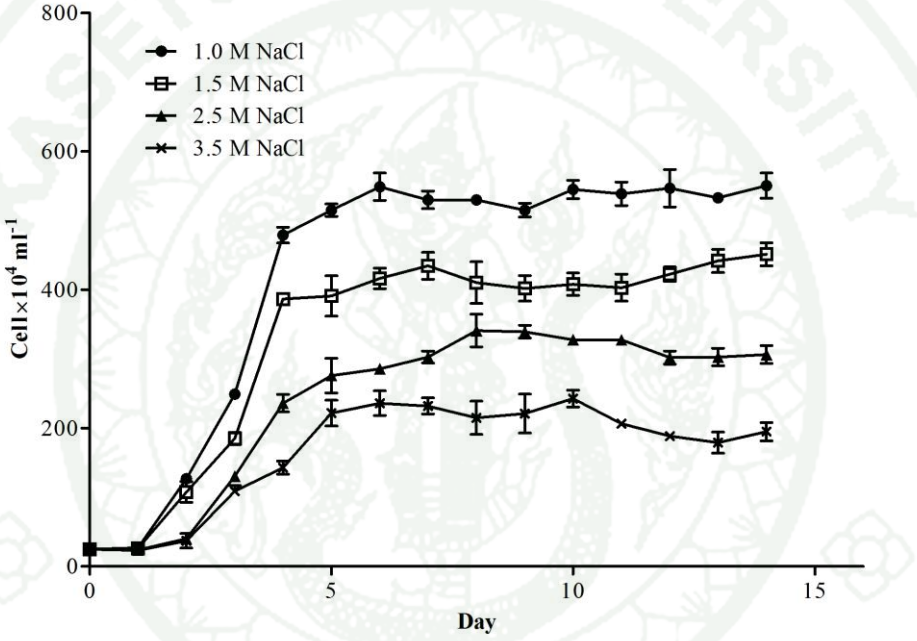


Figure 10 Growth of *Dunaliella* sp. M22 strain in remodified Johnson's medium containing different NaCl concentrations. The strain was grown in 250 ml Erlenmeyer flask under continuous cool daylight (80 μmol photon $m^{-2}s^{-1}$) at 25±2 °C. Plotted data are the averages ± SD of three replicates.

2.2 Determination of β-carotene in *Dunaliella* sp. M22.

The β-carotene accumulation on different NaCl concentrations in remodified Johnson's medium by *Dunaliella* sp. M22 was shown in Figure 11. Among all the different NaCl concentrations, until 12 days after inoculation the β-carotene content was higher in 1.0 M, 1.5 M and 2.5 M when compared to 3.5 M NaCl, whereas in day 13th and 14th the β-carotene content was increased in 3.5 M NaCl. At the end of the experiment, the highest β-carotene content was obtained in 3.5 M followed by 2.5, 1.5 and 1.0 M with 0.97, 0.85, 0.8 and 0.7 pg cell^{-1}, respectively.

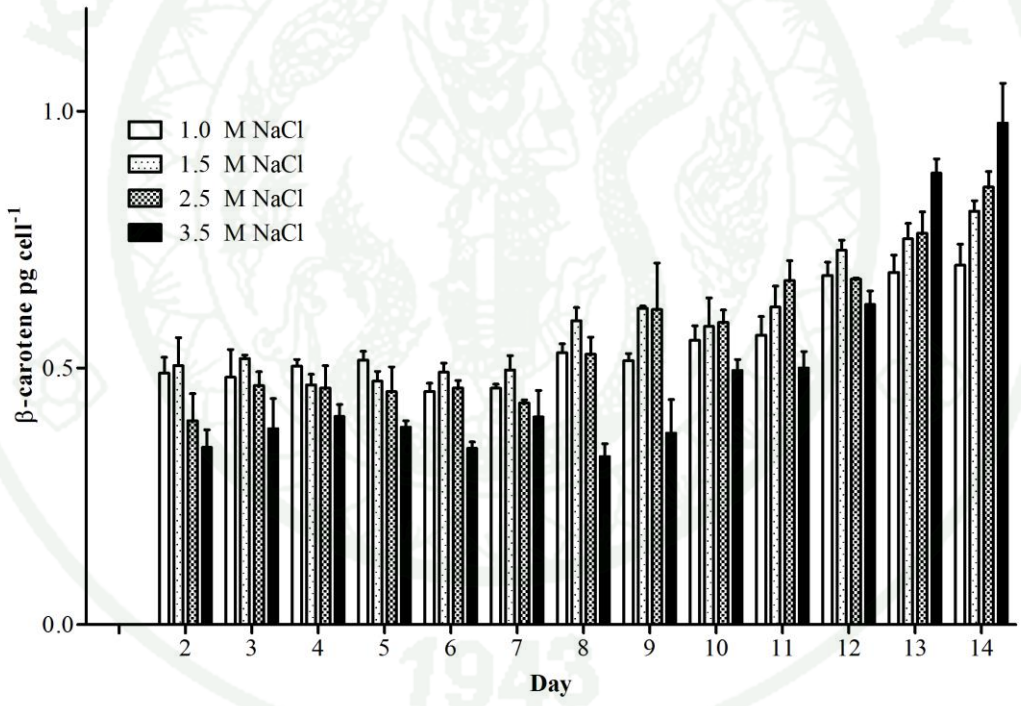


Figure 11 Accumulation of β-carotene by *Dunaliella* sp. M22 cultured in remodified Johnson's medium supplemented with 1.0, 1.5, 2.5 and 3.5 M NaCl concentrations. The strain was grown under continuous light intensity of 80 μmol photon $m^{-2}s^{-1}$ at 25±2 °C. Plotted data are the averages ± SD of three replicates.

3. Growth and β-carotene accumulation in photobioreactor

3.1 Growth rate

Dunaliella sp. M22 was grown in 1.0, 1.5, 2.5 and 3.5 M NaCl with an increasing of light intensity up to 200 μmol photon $m^{-2}s^{-1}$ and the cultures were sparged with air/CO_2 (ratio 95/5) at the flow rate of 30 ml min^{-1}. Growth of all cultures rapidly reached an exponential phase and late stage of senescence phase within 3 and 13 days, respectively (Figure 12). Maximum cell density occurred in 1.0 M NaCl ($2.03\pm40.37\times10^7$ cell ml^{-1}) with specific growth rate of 2.27 d^{-1} and the lowest cell density was $8.49\pm1.41\times10^6$ cell ml^{-1} in 3.5 M NaCl with specific growth rate of 0.89 d^{-1} (Appendix table 4).

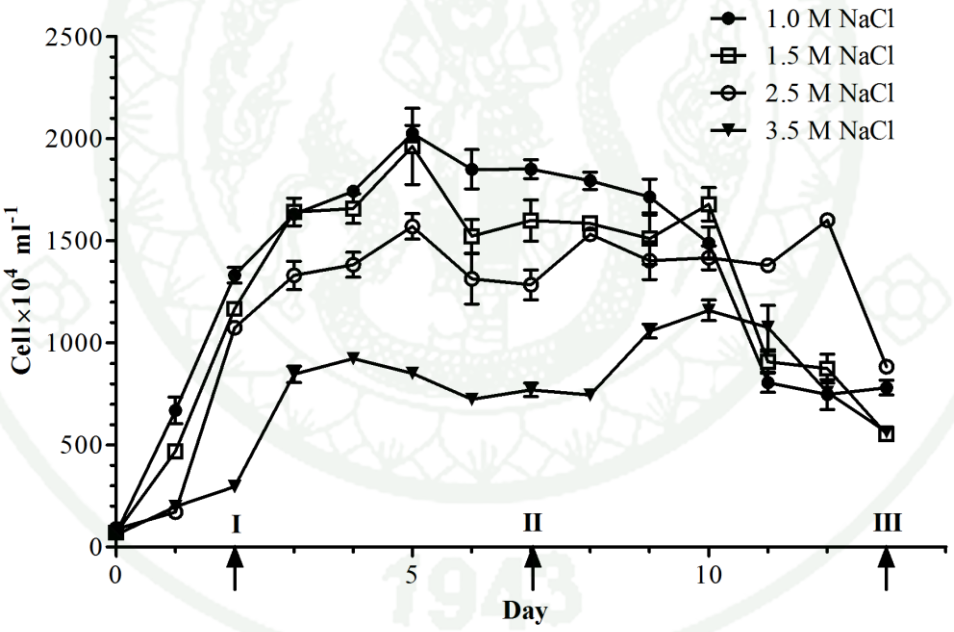


Figure 12 Growth of *Dunaliella* sp. M22 grown in remodified Johnson's medium containing different salinities (1.0, 1.5, 2.5 and 3.5 M NaCl). The cultures were grown under continuous light intensity of 80 μmol photon $m^{-2}s^{-1}$ at 25 ± 2 °C and the cultures were spurged with 5% CO_2. Arrows indicate day (I, II, III) when cultures were harvested for the estimation of β-carotene and mRNA transcript levels. Plotted data are the averages ± SD of three replicates.

3.2 β-carotene accumulation

The *Dunaliella* sp. M22 cells were cultured in different NaCl concentrations in a photobioreactor. The samples were collected at exponential growth phase (Period I), early stage (Period II) and late stage of senescence phase (Period III) for β-carotene pigment analysis (Figure 13). The β-carotene content gradually increased from the beginning (Period I) until the end of cultivation (Period III) (Figure 13a–f), ranging from 0.33 to 3.65 pg $cell^{-1}$ and 3.05 to 41.79 μg ml^{-1} in all treatments. At the Period I, 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.

During period II, higher significant amount of β-carotene up to 1.92 pg $cell^{-1}$ in culture containing 2.5 M NaCl when compared to other NaCl concentrations. At the end of the experiment (period III), cells grown in 2.5 M NaCl accumulated highest β-carotene pigment with 3.65 pg $cell^{-1}$, whereas the minimum β-carotene content was found in the 1.0 M NaCl culture with 1.14 pg $cell^{-1}$. It took 3 to 6 days to complete the color change of culture from green to orange (Figure 14).

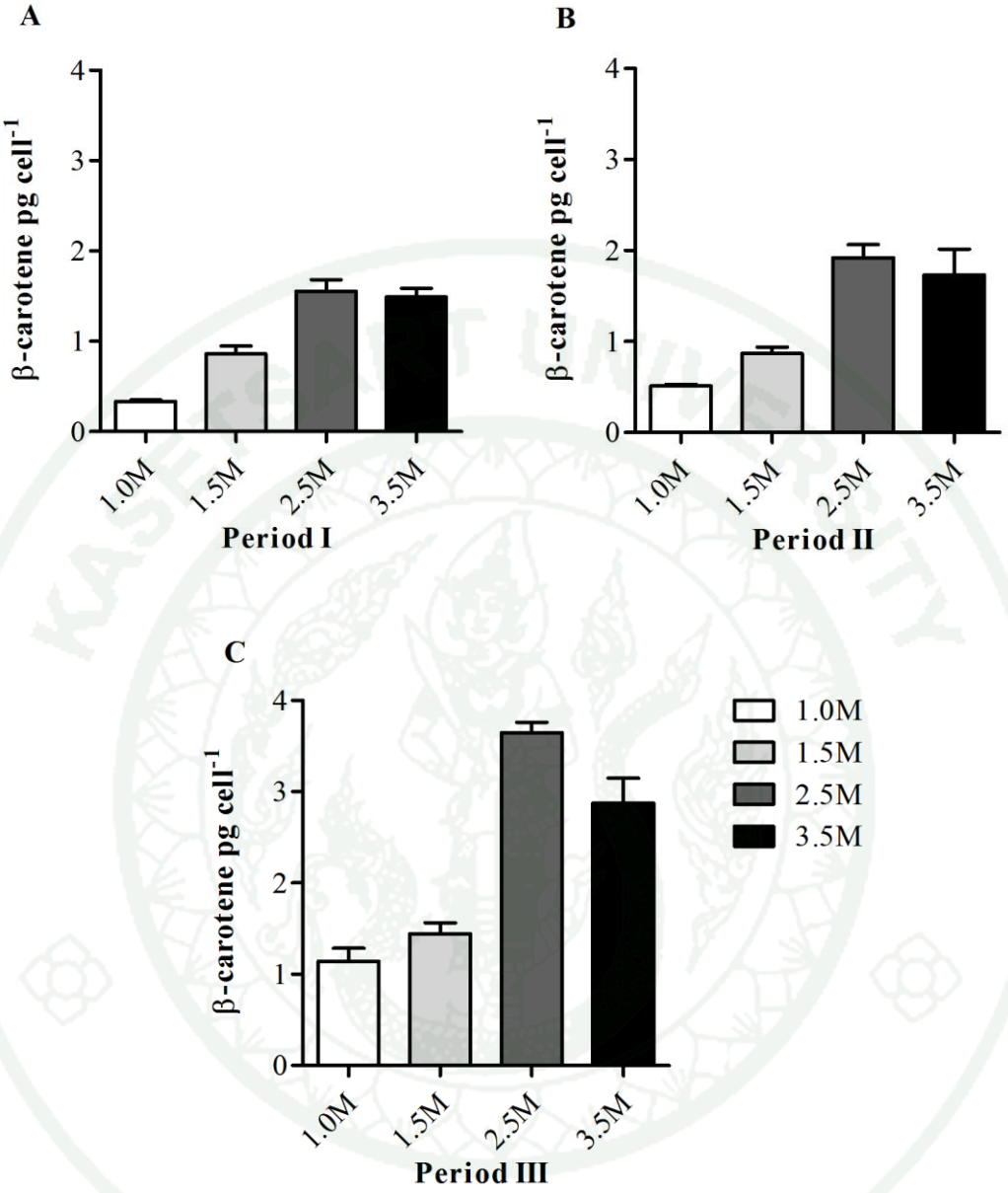


Figure 13 The effect of salinity on carotene accumulation in *Dunaliella* sp. M22. The culture grown at remodified Johnson's medium containing 1.0 M NaCl were transferred to fresh remodified Johnson's medium with 1.0, 1.5, 2.5 and 3.5 M NaCl. β-carotene accumulation per cell was determined at different stages of growth **A**) Period I **B**) Period II **C**) Period III and β-carotene accumulation per milliliter **D**) Period I **E**) Period II **F**) Period III.

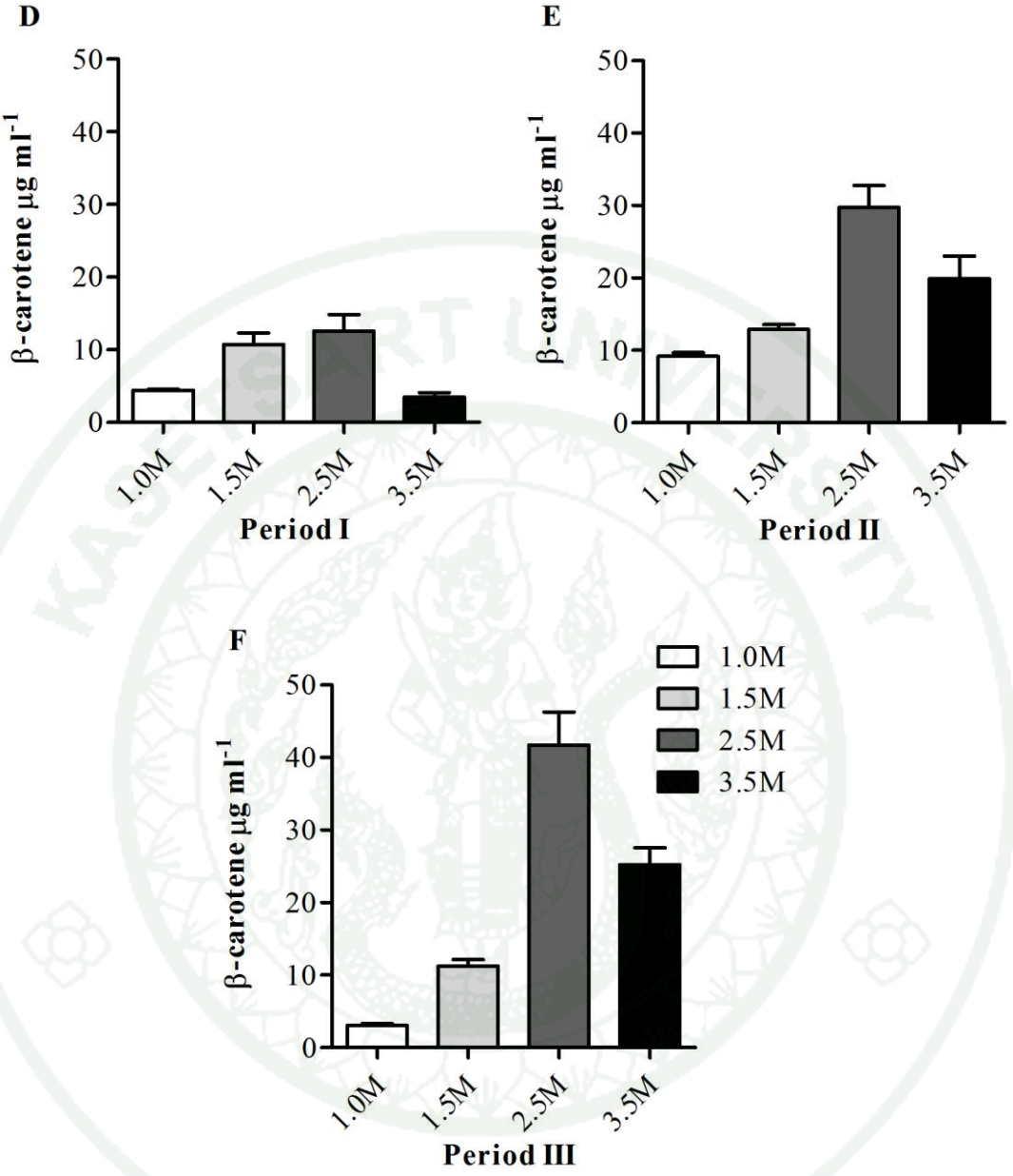


Figure 13 (Continued)

Figure 14 Salt stress accelerated the biosynthesis of orange pigment in *Dunaliella* sp. M22. The NaCl concentrations were 1.0, 1.5, 2.5 and 3.5 M from left to right tube. The initial cell density in each tube was 5.0×10^5cells ml^{-1}. All cultures were illuminated under 200 µmol photons $m^{-2}s^{-1}$ at 25±2 °C and were sparged continuously with filtered-sterile air and 5% CO_2.

4. Sequencing of full length of *lycopene β-cyclase* gene

4.1 Partial sequencing of *lycopene β-cyclase* gene

Partial fragment of *lycopene β-cyclase* gene was amplified with LcyβWF and LcyβNR degenerated primers. The gel electrophoresis result showed DNA band size of about 0.5 kb (Appendix Figure 3).

The DNA was purified and cloned into linearized RBC TA cloning vector (RBC Bioscience, Korea), overhanging 3´ deoxythymidine (T) residues. The colony PCR was performed to amplify the template using universal primers (M13F and M13R). The expected size of 0.5 kb was amplified from the template which was shown in Figure 15.

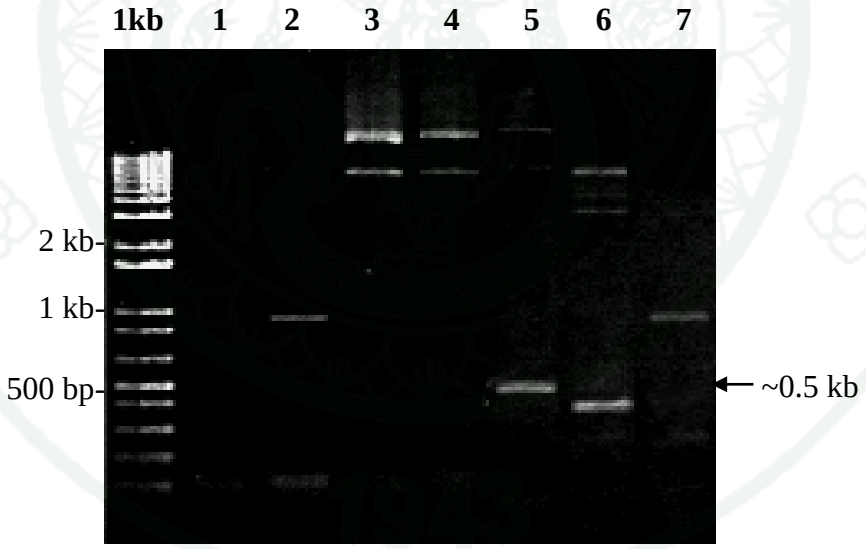


Figure 15 Screening of cloned PCR products using colony PCR (universal primers M13F and M13R), the expected size of 0.5 kb was amplified using the colony 5th as template.

Among the 7 colonies, the colony 5th showed the expected size about 0.5 kb when compared to 1 kb ladder (Figure 15, Lane 5). This indicated that the *Lcyβ* fragment was inserted into the vector. The figure 16 illustrated the sequencing result

of the partial *lycopene β-cyclase* gene with 458 bp in size. The figure 17 displayed a graphical view of the nucleotide sequence alignment of *Lcyβ* by BLASTN software from NCBI. The accession number, description, score and identity of the fragment of *Lcyβ* isolated from *Dunaliella* sp. M22 showed higher sequence similarity with *Dunaliella* sp. and other species from the NCBI in the Table 10.

The partial fragment of *Lcyβ* amplified with LcyβWF and LcyβNR primers showed the highest sequence similarity to *Dunaliella salina* (accession numbers EU327876.1) with 80% identity. The results also showed high sequence similarity to other organisms such as *Haematococcus pluvialis* (accession : AY182008.1) , *Muriella zofingiensis* (accession : FN563998.1) , *Chlamydomonas reinhardtii* (accession : AY860818.1) , *Ostreococcus tauri* (accession : XM_003082824.1) , *Coccomyxa subellipsoidea* (accession : XM_005648392.1) , *Volvox carteri* f. nagariensis (accession : XM_002957257.1) , *Lilium lancifolium* (accession : AB445121.1) , *Ostreococcus lucimarinus* CCE9901 (accession : XM_001422452.1), *Selaginella moellendorffii* (accession : XM_002971249.1), *Oryza sativa* (accession : AC108763.2) with the identity of 74, 71 73, 71, 72 , 71 , 67, 67 and 83%, respectively.

```
Name Lcyβ1
BASE COUNTS 458 bp
ORIGIN

        1     GGAGAAAAGT ACCTGAAGAG GGCGTACGGG CGCATGGACC GGCCCATGCT
        51    CAAAAAATTG CTGCTGCAAA AATGTGCATC CAACGGCGTG ACATTCTTGA
        101   CTAGTAAGGT GGACGGTGTG AGCCATGGGG GAGGCTGCTC CACAGTGTCA
        151   CTTACCGACG GGCGCACCAT TCAAGGCACC ATGGTCCTTG ATGCCACGGG
        201   CCATGCTCGC AAGCTGGTCA ACTTTGACCA GAAGTTTGAC CCGGGGTACC
        251   AAGGCGCGTA CGGCATTACA GCAGAGGTTG AATCCCATCC ATTTGAGCTG
        301   GACACAATGC TGTTCATGGA CTGGAGGGAT GAGCACACGC AATCGGATCC
        351   GGCGATGCGC GCATCAAATG AAGCATTGCC CACCTTCTTG TACGTCATGC
        401   CTTTCACAAA AAACAAGGTG TTCCTGGAAG AGACATCGTT GGTAGCGCGC
        451   CCAGCAGT
```

Figure 16 Partial sequence of *lycopene β-cyclase* gene (*Lcyβ*) amplified from *Dunaliella* sp. M22 using LcyβWF and LcyβNR degenerated primers.

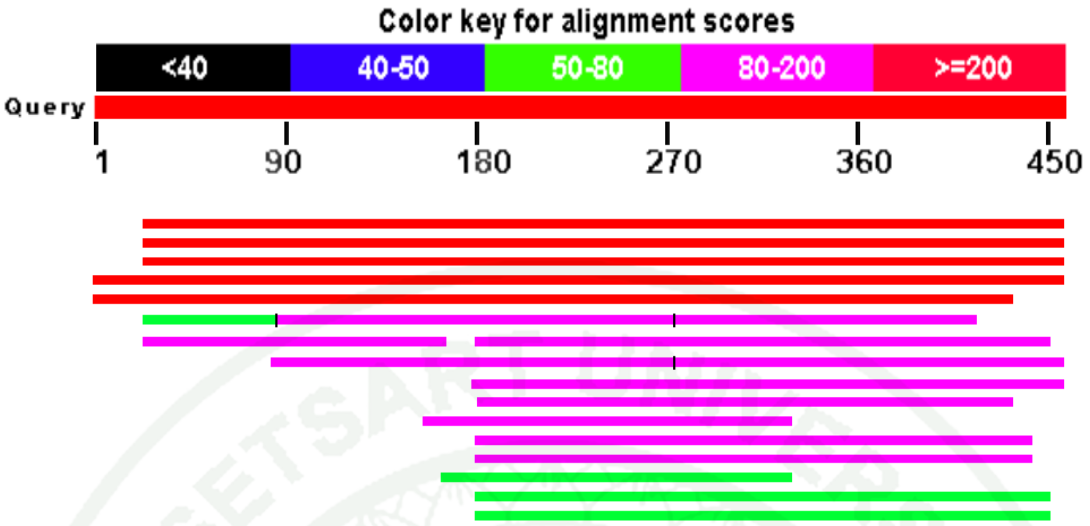


Figure 17 Graphical view of nucleotide sequence alignment of partial *Lcyβ* (458 bp) using BLASTN (2.2.29) program from NCBI.

Table 10 Sequence similarity result of partial *Lcyβ* fragment isolated from *Dunaliella* sp. M22 with other species from NCBI database.

Accession	Description	Strand	Score	E value	Identities
EU327876	*Dunaliella salina* chloroplast lycopene beta-cyclase (*LCYB*) mRNA, complete cds; nuclear gene for chloroplast product	Plus/ Plus	390	5e-105	347/434 (80%)
HQ728089	*Dunaliella* sp. ABRIINW-G4 chloroplast lycopene beta-cyclase enzyme mRNA, complete cds; nuclear gene for chloroplast product	Plus/ Plus	387	6e-104	346/434 (80%)

Table 10 (Continued)

Accession	Description	Strand	Score	E value	Identities
AY182008	*Haematococcus pluvialis* lycopene beta cyclase mRNA, partial cds	Plus/ Plus	259	2e-65	320/435 (74%)
FN563999	*Muriella zofingiensis* mRNA for chloroplast lycopene beta cyclase precursor strain SAG 211-14	Plus/ Plus	246	1e-61	337/462 (73%)
AY860818	*Chlamydomonas reinhardtii* putative chloroplast lycopene beta cyclase precursor (*LCYB*) mRNA, complete cds; nuclear gene for chloroplast product	Plus/ Plus	217	7e-53	315/441 (71%)
EU327877	*Dunaliella salina* chloroplast lycopene beta-cyclase (*LCYB*) gene, complete cds; nuclear gene for chloroplast product	Plus/ Plus	355	1e-37	151/190 (79%)
XM_003082-824	*Ostreococcus tauri* putative chloroplast *lycopene beta cyclase* precursor (ISS) (Ot14g00810) mRNA, complete cds	Plus/ Plus	134	6e-28	198/273 (73%)
FN563999	*Muriella zofingiensis lcyB* gene for chloroplast lycopene beta cyclase precursor, strain SAG 211-14	Plus/ Plus	227	3e-26	147/191 (77%)

Table 10 (Continued)

Accession	Description	Strand	Score	E value	Identities
XM_00564-8392	*Coccomyxa subellipsoidea* C-169 beta cyclase (COCSUDRAFT_36181) mRNA, complete cds	Plus/ Plus	122	4e-24	200/282 (71%)
JQ762457	*Dunaliella salina* chloroplast lycopene beta-cyclase enzyme (*LCYB*) mRNA, partial cds; nuclear gene for chloroplast product	Plus/ Plus	109	2e-20	110/143 (77%)
AB445121	*Lilium hybrid* division I *LCYB* mRNA for lycopene beta-cyclase, partial cds, cultivar: Connecticut King	Plus/ Plus	82.4	3e-12	126/176 (72%)

4.2 Sequencing of 5′ and 3′ end of *lycopene β-cyclase* gene

The 5′ and 3′ end of *lycopene β-cyclase* gene was identified by the protocol of Frohman (1994). To obtain 5′ and 3′ end fragments, the Q_O and Q_I primer which paired with the complementary Q_T primer region were used.

The 3′ end PCR product amplified by LcyβNF primer and Q_O primer showed a band of about 1.5 kb in size. The PCR product was smeared when visualized in agarose gel (Figure 18, Lane 2). To intensify the PCR product, nested PCR was carried out using Lcyβ33F and Q_I primer (Figure 18, Lane 3). The expected size of about 1.5 kb was obtained.

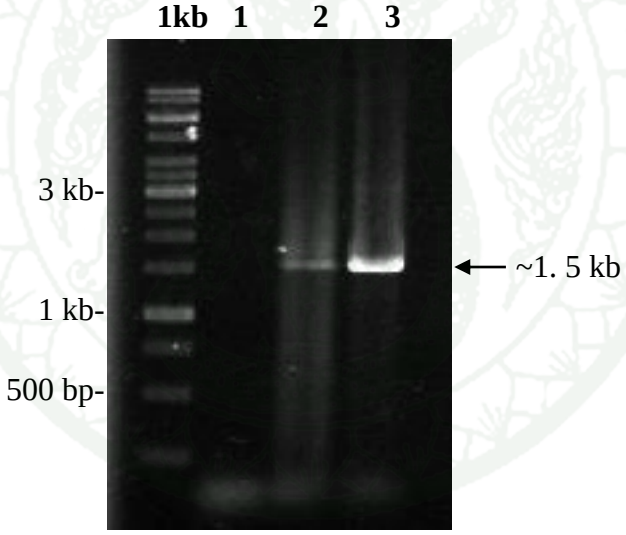


Figure 18 3′ RACE PCR of *lycopene β-cyclase* gene, Lane 1: negative control 2: 3′ RACE fragment was amplified using LcyβNF and Q_0 primers; Lane 3: 3′ nested PCR was carried out using Lcyβ33F and Q_1 primers.

Similarly, for 5′ end amplification, polyA was added to 5′ end cDNA and then the polyA added cDNA was amplified using LcyβNR and Q_T primers. The amplified band was smeared because of the different sizes of mRNA. Thus, to obtain 5′ end, the longest band (Figure 19, Lane 2) was cut, purified and used as a template for performing nested PCR. The nested PCR was carried out using Lcyβ124R and Q_1 primers which resulted in the amplification of about 900 bp fragment (Figure 19, Lane 3).

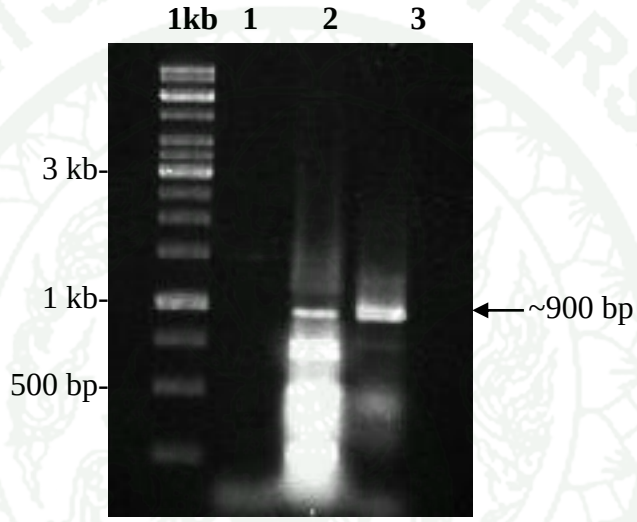


Figure 19 5′ RACE PCR of *lycopene β-cyclase* gene, Lane 1: negative control; Lane 2: 5′ RACE fragment was amplified using LcyβNR and Q_0 primers; Lane 3: 5′ nested PCR was performed by Lcyβ124R and Q_1 primers.

The 5′ RACE and 3′ RACE sequences were assembled to obtain the full-length sequence by DNA Baser Sequence Assembler software (Heracle BioSoft S.R.L., Romania) shown in Figure 20. Finally, LcyβfullFW and LcyβfullRW primers were designed for amplifying the full-length of Lcy*β* as shown in Appendix Figure 9.

Name Lcyβ
BASE COUNTS 2154 bp
ORIGIN GATGTCTCGGCAGTCAGACA

```
1       AAAAGCAAAG ACACCAGACA ACACACGCCA GAACAGCAAC AAAGAACACC
51      CCTAACCGGC TCCTCTAACA ACATGCGAGA TCAAACCTCG GCAGTATTAT
101     TACAGGAAGC ACTGACCGAG GCCCAGACAG TAGGGCATCC CAGTGGTAAA
151     CAACGACCCA CAGCAGCAAC CACCTTCAGA CCCCCAGACC CCGATGTCTC
201     GGCAGTCAGA CAAGGCAGTA ACAGAGCGTG TGTATACAGG TTGCACATCA
251     GTATGTGGCA TGTATATTGA AACATATACC GCCACAATCC CCAACTATGG
301     GTGCAGAGTA GAAACGCAGC AGACCAAGGG CCTTGAGGAG GGACTGGTGG
351     AGGGTTCACC GAACGAAAAA AATTTTTGTG GAGAAAAAAA GAGCCGGGAA
401     AAAATCCTGA AAAGGGCGTA CGGGCGCATG GACCGGCCCA TGCTCAAAAA
451     ATTGCTGCTG CAAAAATGTG CATCCAACGG CGTGACATTC TTGACTAGTA
501     AGGTGGACGG TGTGAGCCAT GGGGGAGGCT GCTCCACAGT GTCACTTACC
551     GACGGGCGCA CCATTCAAGG CACCATGGTC CTTGATGCCA CGGGCCATGC
601     TCGCAAGCTG GTCAACTTTG ACCAGAAGTT TGACCCGGGG TACCAAGGCG
651     CGTACGGCAT TACAGCAAAG GATGAATCCC ACCCATTTGA GCTGGACACA
701     ATGCTGTTCA TGGACTGGAG GGATGAGCAC ACGCAATCGG ATCCGGCGAT
751     GCGCGCATCA AATGAAGCAT TGCCCACCTT CTTGTACGTC ATGCCTTTCA
801     CAAAAAACAA GGTGTTCCTG GAAGAGACAT CGTTAGTAGC GCGCCCAGCA
851     GTTGGGTTTG AGGAATTAAA ACAAAGACTT GAAGCTCGCA TGAAGTGGTT
901     AGGCATCAAG GTGAAAAAGG TTGAGGATGA AGAATACTGC CTGATTCCCA
951     TGGGTGGTGT CCTACCACAG CACCCACAGC GTGTACTGGG CATCGGCGGA
1001    ACTGCCGGCA TGGTGCATCC TTCAACAGGC TTCATGATGA CCCGTATGCT
1051    GGGCTCTGCC CCTGTCGTTG CGGATGCCAT CATTGACCAA CTAGCCAAAC
1101    CAACAGACAA GGCCACGGAC TCAGGCACCT CACAGCAGCC ATTGACAGAG
1151    CAGGAGGCAG AAGAGATGGC AGCAGCTGTG TGGAGAGCCA CCTGGCCCGT
1201    GGAACGTATT CGGCAACGAG CCTTCTTTTG TTTTGGCATG GAGATGCTGC
1251    TCACCTTGAA CCTTGGCCAG ATGCGAGAGT TCTTTGCCGC CTTCTTCTCG
1301    CTCTCAGACT TTCACTGGCA AGGCTTCTTG TCAGCCCGCC TGTCCTTTAC
1351    GCAGCTCATC GGCTTTGGCC TGTCCCTCTT CACATCCGCC ACATCAGACA
1401    CTCGTCTGAA CCTGCTGCGG CTAGGCATCC CAGGCCTCAT ACAGATGCTG
1451    CTGGTGCTGT TCCCCACTGT AACTGGCTAC TACAAGGGTG ATCTCACTGT
1501    AAGGGACAAG AAGTTTGCGC ATGATGCTGC AGCTGCTGCT GCAAGCAAGC
1551    TAAAGCAGCC GCAAAGCCCA GCTCAGCCAT CGTAGCGAGG GGGGAGCTCG
1601    TCATCGTAGG TCATTGCTCC GTCAAGCTGT GTGCTGTTAA ACAGTAATTA
1651    AGTGTTGCAT AAATGTTGCA GAGGCATCGC GAGCAGGCTC GGGCTTACCC
1701    ATTGCCACGA GGAAGGGACA TGCCATCCTA GGTGTAGGTC TAGGGTTCTG
1751    GGTGGCTTCT GGTAATTAAT CGGGTGGGTT GTTCCAAGTG AAATATGCTG
1801    CAAACTGTCC ACATGCTTGC GACGAACCCC TTTCTTAATG GACACTTAGC
1851    TCAAACAGCA TCACCAGCTA TGTGATCAAT AATGCATGGG GTGTTGGAGA
1901    AGTTGCAAAA CGTTTTCTGG CCTTGGGGCC CATCTAAGGG GGAGGTTGCT
1951    AAACAAGCCC TCTTACAAGA AACCATTTGT CCACCTGAGA CACCCTTTTG
2001    TATGGGAGAT AAGGGTTCTT TTCCGAATTG GCCCCCTGGC CCAAGGGGTT
2051    TGATTTTGTT TTTTACCCAC ACAGGGGCGG CCTTCCACAG GAATATTTTT
2101    GCCCTCCCGG GGGGGGAACC AAAAATAGAG CTTTTTGCCA AAAAAAAAAA
2151    AAAA
```

Figure 20 Full-length nucleotide sequences with 2,154 bp in size of *lycopene β-cyclase* gene isolated from *Dunaliella* sp. M22. The sequence was submitted to the NCBI with the accession number KM016906.

```
atgtctcggcagtcagacaaggcagtaacagagcgtgtgtatacaggttgcacatcagta
 M  S  R  Q  S  D  K  A  V  T  E  R  V  Y  T  G  C  T  S  V
tgtggcatgtatattgaaacatataccgccacaatccccaactatgggtgcagagtagaa
 C  G  M  Y  I  E  T  Y  T  A  T  I  P  N  Y  G  C  R  V  E
acgcagcagaccaagggccttgaggagggactggtggagggttcaccgaacgaaaaaaat
 T  Q  Q  T  K  G  L  E  E  G  L  V  E  G  S  P  N  E  K  N
ttttgtggagaaaaaaagagccgggaaaaaatcctgaaaagggcgtacgggcgcatggac
 F  C  G  E  K  K  S  R  E  K  I  L  K  R  A  Y  G  R  M  D
cggcccatgctcaaaaaattgctgctgcaaaaatgtgcatccaacggcgtgacattcttg
 R  P  M  L  K  K  L  L  L  Q  K  C  A  S  N  G  V  T  F  L
actagtaaggtggacggtgtgagccatgggggaggctgctccacagtgtcacttaccgac
 T  S  K  V  D  G  V  S  H  G  G  G  C  S  T  V  S  L  T  D
gggcgcaccattcaaggcaccatggtccttgatgccacgggccatgctcgcaagctggtc
 G  R  T  I  Q  G  T  M  V  L  D  A  T  G  H  A  R  K  L  V
aactttgaccagaagtttgacccggggtaccaaggcgcgtacggcattacagcaaaggat
 N  F  D  Q  K  F  D  P  G  Y  Q  G  A  Y  G  I  T  A  K  D
gaatcccacccatttgagctggacacaatgctgttcatggactggagggatgagcacacg
 E  S  H  P  F  E  L  D  T  M  L  F  M  D  W  R  D  E  H  T
caatcggatccggcgatgcgcgcatcaaatgaagcattgcccaccttcttgtacgtcatg
 Q  S  D  P  A  M  R  A  S  N  E  A  L  P  T  F  L  Y  V  M
cctttcacaaaaaacaaggtgttcctggaagagacatcgttagtagcgcgcccagcagtt
 P  F  T  K  N  K  V  F  L  E  E  T  S  L  V  A  R  P  A  V
gggtttgaggaattaaaacaaagacttgaagctcgcatgaagtggttaggcatcaaggtg
 G  F  E  E  L  K  Q  R  L  E  A  R  M  K  W  L  G  I  K  V
aaaaaggttgaggatgaagaatactgcctgattcccatgggtggtgtcctaccacagcac
 K  K  V  E  D  E  E  Y  C  L  I  P  M  G  G  V  L  P  Q  H
ccacagcgtgtactgggcatcggcggaactgccggcatggtgcatccttcaacaggcttc
 P  Q  R  V  L  G  I  G  G  T  A  G  M  V  H  P  S  T  G  F
atgatgacccgtatgctgggctctgcccctgtcgttgcggatgccatcattgaccaacta
 M  M  T  R  M  L  G  S  A  P  V  V  A  D  A  I  I  D  Q  L
gccaaaccaacagacaaggccacggactcaggcacctcacagcagccattgacagagcag
 A  K  P  T  D  K  A  T  D  S  G  T  S  Q  Q  P  L  T  E  Q
gaggcagaagagatggcagcagctgtgtggagagccacctggcccgtggaacgtattcgg
 E  A  E  E  M  A  A  A  V  W  R  A  T  W  P  V  E  R  I  R
caacgagccttcttttgttttggcatggagatgctgctcaccttgaaccttggccagatg
 Q  R  A  F  F  C  F  G  M  E  M  L  L  T  L  N  L  G  Q  M
cgagagttctttgccgccttcttctcgctctcagactttcactggcaaggcttcttgtca
 R  E  F  F  A  A  F  F  S  L  S  D  F  H  W  Q  G  F  L  S
gcccgcctgtcctttacgcagctcatcggctttggcctgtccctcttcacatccgccaca
 A  R  L  S  F  T  Q  L  I  G  F  G  L  S  L  F  T  S  A  T
tcagacactcgtctgaacctgctgcggctaggcatcccaggcctcatacagatgctgctg
 S  D  T  R  L  N  L  L  R  L  G  I  P  G  L  I  Q  M  L  L
gtgctgttccccactgtaactggctactacaagggtgatctcactgtaagggacaagaag
 V  L  F  P  T  V  T  G  Y  Y  K  G  D  L  T  V  R  D  K  K
tttgcgcatgatgctgcagctgctgctgcaagcaagctaaagcagccgcaaagcccagct
 F  A  H  D  A  A  A  A  A  A  S  K  L  K  Q  P  Q  S  P  A
cagccatcgtag
 Q  P  S  *
```

Figure 21 Nucleotide and deduced amino acid sequence of full-length *lycopene β-cyclase* gene, start codon (ATG) and stop codon (TGA) are underlined (length 463 aa).

The full-length cDNA sequence of the *Lcyβ* gene was 2,154 bp in size, contained one open reading frame (ORF) of 1,392 bp from position of 194 to 1585. The ORF encoded a protein of 463 amino acids (Figure 21), with a predicted molecular weight of approximately 51.3 kDa by compute pI/Mw program. Analysis of the homology of the deduced amino acid sequences of *Lcyβ* from *Dunaliella* sp. M22 with other amino acid sequences retrieved from GenBank, revealed the degree of similarity ranging from 46-77% (Figure 22).

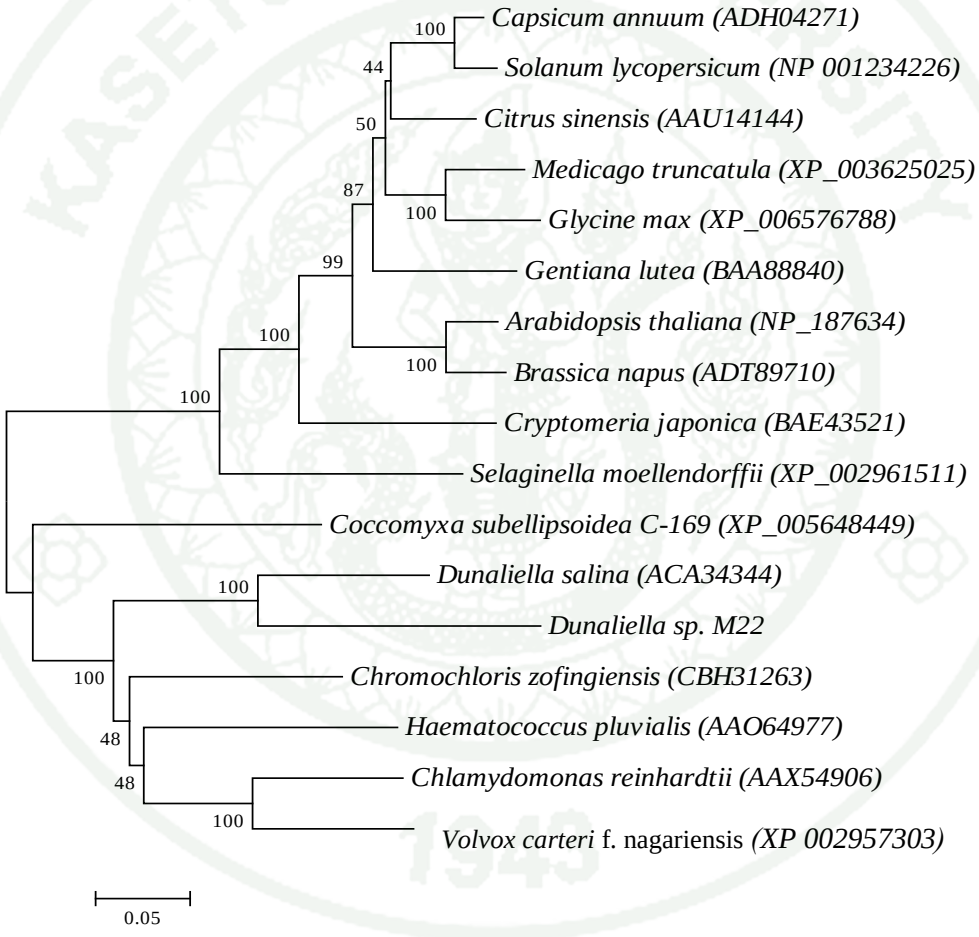


Figure 22 Phylogenetic analysis of the deduced amino acid sequences of *lycopene β-cyclase* from *Dunaliella* sp. M22 with other *lycopene β-cyclase* sequences from NCBI. The tree was constructed using MEGA version 5.2 software and conducted by the neighbor joining method. Bootstrap values were calculated from 1000 replicates. The GenBank accession numbers are indicated in brackets.

Phylogenetic analysis showed that *Dunaliella* sp. M22 is grouped to *D. salina* (Ramos *et al*., 2008), with amino acid identity of 77% (Figure 22). The deduced amino acid sequence of *Lcyβ* closely related with other green algae, including *Chromochloris zofingiensis* (GenBank CBH31263), *Haematococcus pluvialis* (GenBank AAO64977), *Chlamydomonas reinhardtii* (GenBank AAX54906) and *Volvox carteri* (GenBank XP_002957303) which showed identities of 66, 65, 63 and 62%, respectively. Lower identities were shown in higher plants; *Arabidopsis thaliana* (GenBank NP_001078131), *Selaginella moellendorffii* (GenBank XP_002961511) and *Capsicum annuum* (GenBank Q43415) with 51, 48 and 47%, respectively.

5. Expression of the *lycopene β-cyclase* gene

Total RNA was extracted from the *Dunaliella* sp. M22 culture grown in remodified Johnson's medium containing 1.0, 1.5, 2.5 and 3.5 M NaCl. The A_{260}/A_{280} ratio of the total RNA from each NaCl concentration ranged from 1.7 to 2.2. The total RNAs were analyzed by 1.2% (w/v) agarose gel electrophoresis for checking the intensity and quality of the RNA. No degradation of RNA in the agarose gel was observed (Figure 23). This indicated that RNA quality was good.

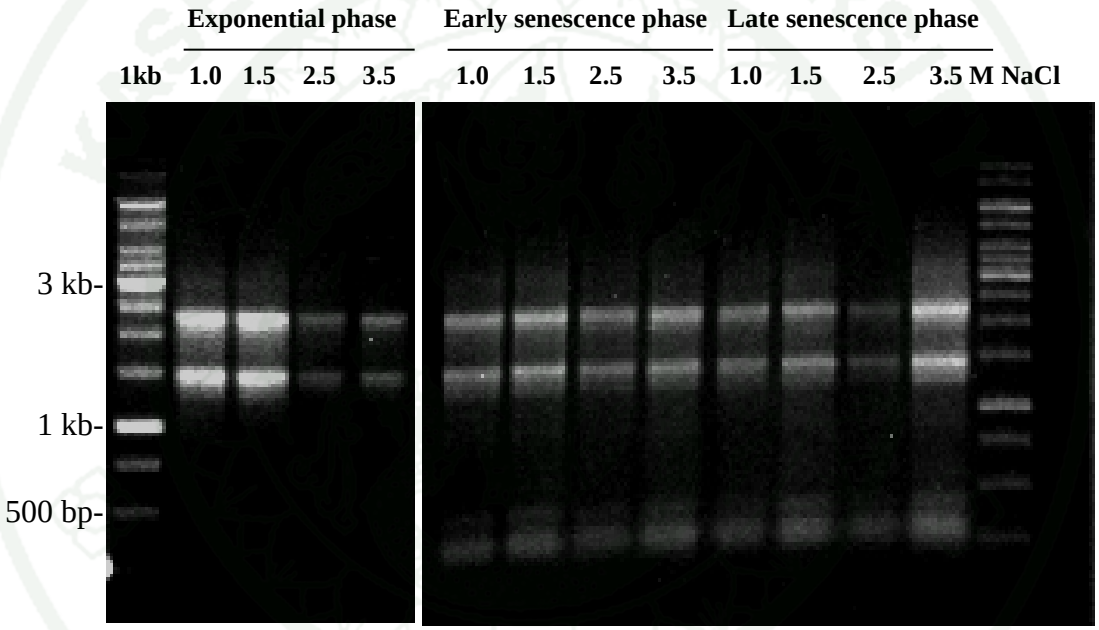


Figure 23 Total RNA was extracted from exponential phase, early senescence phase and late senescence phase. *Dunaliella* sp. M22 cultured in remodified Johnson's medium containing 1.0, 1.5, 2.5 and 3.5 M NaCl concentrations.

The 18S rRNA and *alpha-tubulin* genes were used as an internal control for checking the quality of the cDNA. For the amplification of the 18S rRNA and *Atub* genes, $MgCl_2$ concentrations were varied to 1, 2, 3, 4 and 5 mM. At 2 mM $MgCl_2$ and 1 mM $MgCl_2$ showed optimal amplification of *Atub* and 18S rRNA, respectively (Figure 24).

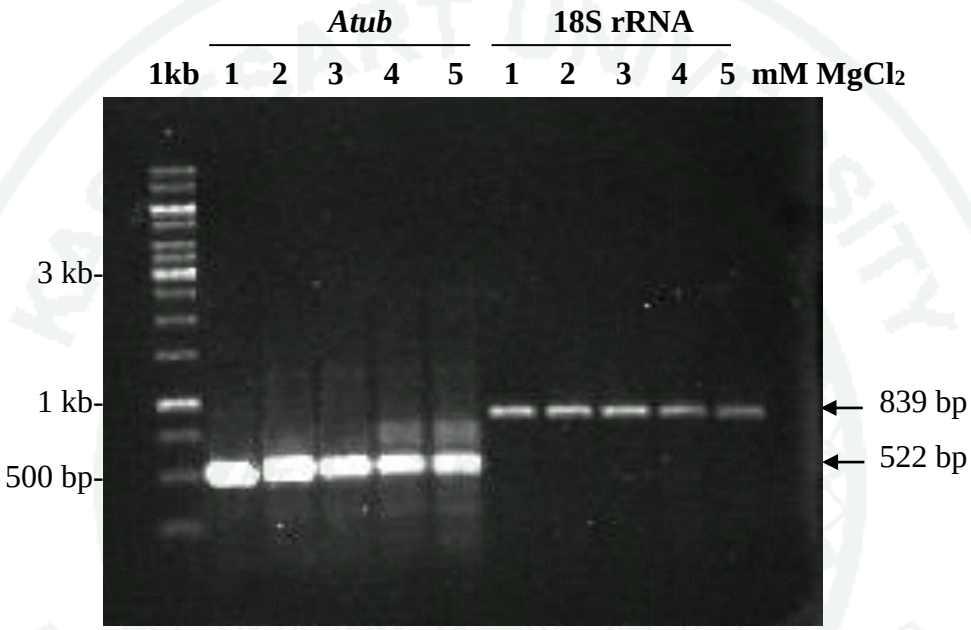


Figure 24 RT-PCR product of *Atub* and 18S rRNA gene amplified with different $MgCl_2$ concentrations of 1, 2, 3, 4 and 5 mM.

The *Lcyβ* full-length gene was sequenced to design the Lcyβ33Fw and Lcyβ33Rw primers. The expression of *Lcyβ* gene was determined from the culture grown in remodified Johnson's medium containing 1.0, 1.5, 2.5 and 3.5 M NaCl. As expected in all the grown culture conditions got an amplification of band at 264 bp. Magnesium concentrations and annealing temperature for the amplification of *Lcyβ* gene were tested. The result showed that the optimum condition for PCR reaction was 3.0 mM $MgCl_2$ and with the annealing temperature of 63 ℃ (Figure 25). Figure 26 showed that the suitable cycle number of *Lcyβ* and *Atub* amplification were 25 and 29 cycles, respectively.

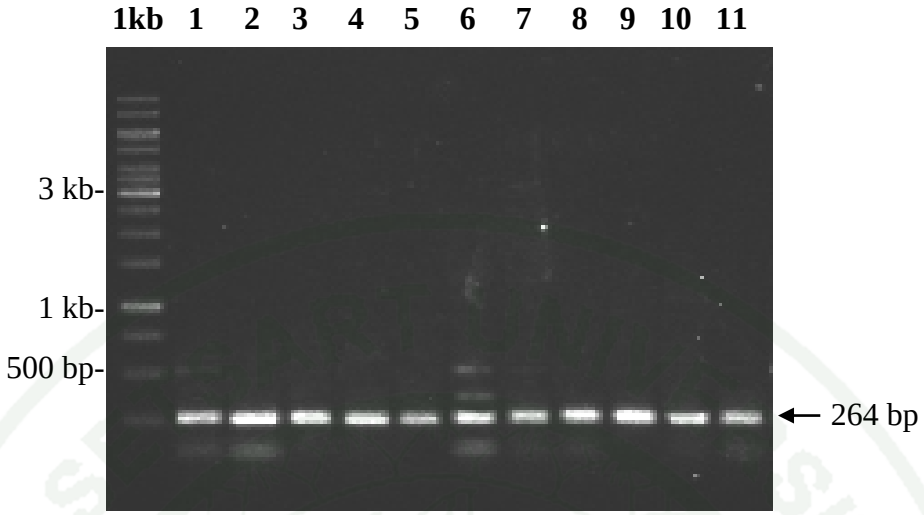


Figure 25 Lane 1-5: RT-PCR product of *Lcyβ* gene amplified with 1.0, 2.0, 3.0, 4.0 and 5.0 mM $MgCl_2$ concentrations at annealing temperature of 63 ℃; Lane 6-11: Different annealing temperatures were carried out to amplify the *Lcyβ* gene at 50, 54, 60, 63, 65 and 66.5 ℃, respectively, with 1.5 mM $MgCl_2$.

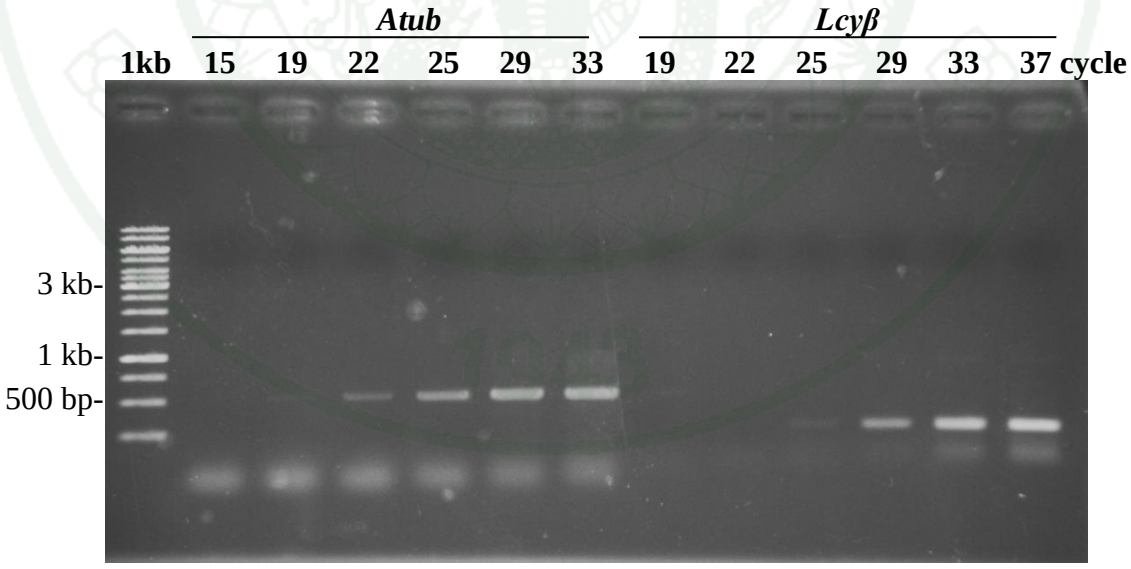


Figure 26 The different cycle numbers were carried out to amplify the *Atub* (15, 19, 22, 25, 29 and 33 cycles) and *Lcyβ* gene (19, 22, 25, 29, 33 and 37 cycles).

The cDNA from the first and second expression study were normalized using *Atub* gene as shown in figure 27 and 28, respectively.

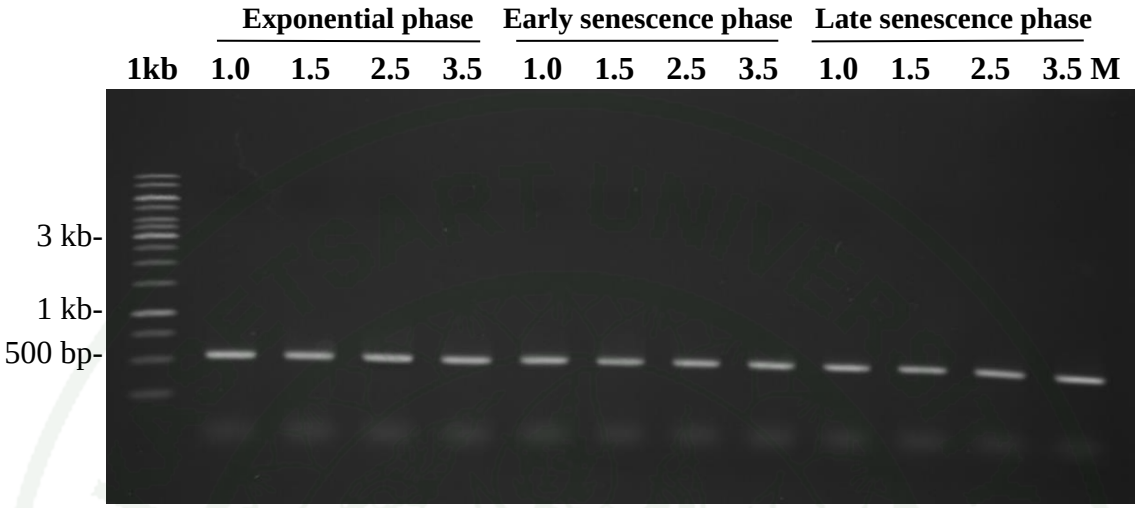


Figure 27 The cDNA normalization of *Atub* gene from cell grown in 1.0, 1.5, 2.5 and 3.5 M NaCl remodified Johnson medium in 3 periods of growth (exponential, early and late senescence phases).

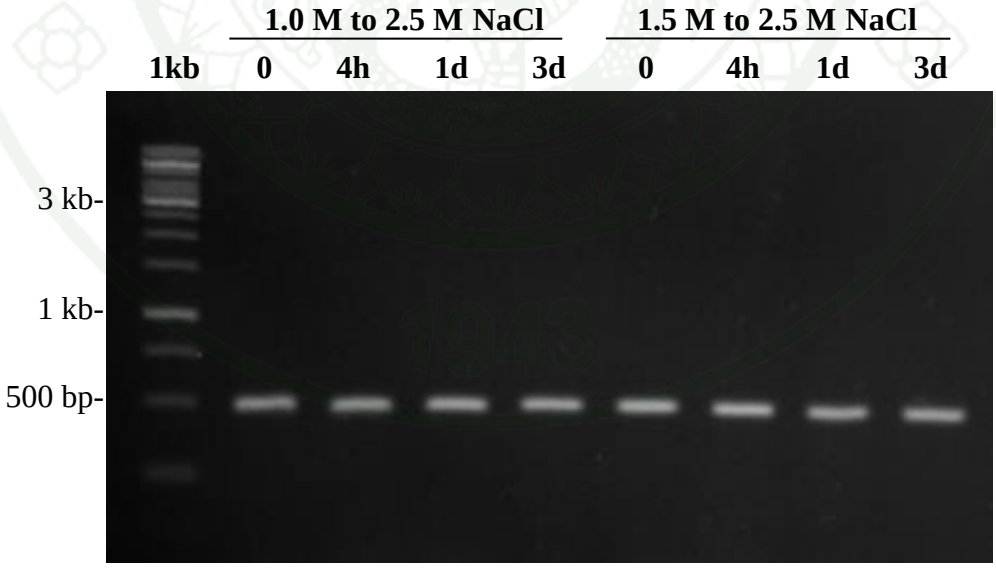


Figure 28 The cDNA normalization of *Atub* gene from cell grown in the culture shifted from 1.0 M to 2.5 M and 1.5 M to 2.5 M NaCl.

The expression study of *Lcyβ* gene from *Dunaliella* sp. M22 was carried out into 2 experiments. Firstly, the algae were grown in 1.0, 1.5, 2.5 and 3.5 M NaCl concentrations and collected for expression analysis in 3 periods of growth (exponential, early and late senescence phases) (Figure 29). Second experiment was carried out initially when the culture was grown in 1.0 M NaCl concentration until the culture reached up to exponential phase and then the culture was shifted to 2.5 M NaCl concentration for salinity shock (Figure 30).

During exponential phase (phase I), the high *Lcyβ* transcript level was found in the culture grown in 1.5, 2.5 and 3.5 M NaCl, while lower *Lcyβ* transcript level was occurred in 1.0 M NaCl. These expression patterns were correlated with β-carotene content (Figure 13a), indicating that *Lcyβ* is one of the salt-inducible genes. Interestingly, at early senescence phase (period II), the high expression of *Lcyβ* was found in 1.0 and 1.5 M NaCl when compared to 2.5 and 3.5 M NaCl. At late senescence phase (period III), the *Lcyβ* expression was high in 1.0 and 1.5 followed by 2.5 and 3.5 M NaCl, respectively (Figure 29).

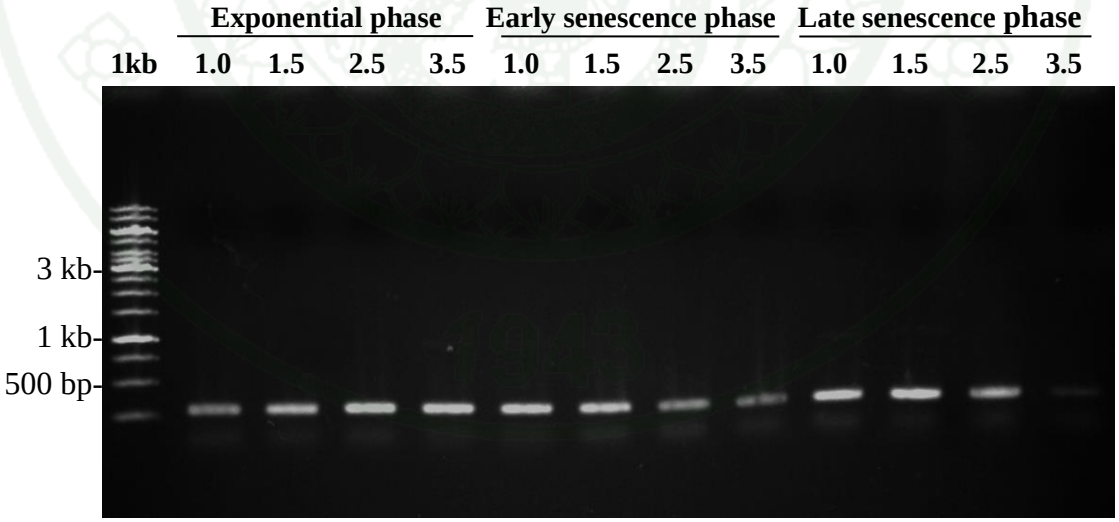


Figure 29 Expression patterns of *Lcyβ* at different growth periods (exponential phase, early senescence phase and late senescence phase) of *Dunaliella* sp. M22 was grown in 1.0, 1.5, 2.5 and 3.5 M NaCl were identified using the intensity of RT-PCR product.

The effect of hyper-saline shock from the culture shifted from 1.0 M to 2.5 M and 1.5 M to 2.5 M NaCl concentrations. *Lcyβ* transcript levels in senescence phase from both shifted culture were shown in Figure 30. After 4 h, *Lcyβ* transcript level was higher in both salinity shocked when compared to the non-salinity shocked cultures. Maximum transcript level was found about at 1 day after hyper-salinity shock from 1.0 to 2.5 M NaCl and 1.5 to 2.5 M NaCl. However, the *Lcyβ* transcript levels decreased after 3 days (1.0 to 2.5 M NaCl and 1.5 to 2.5 M NaCl).

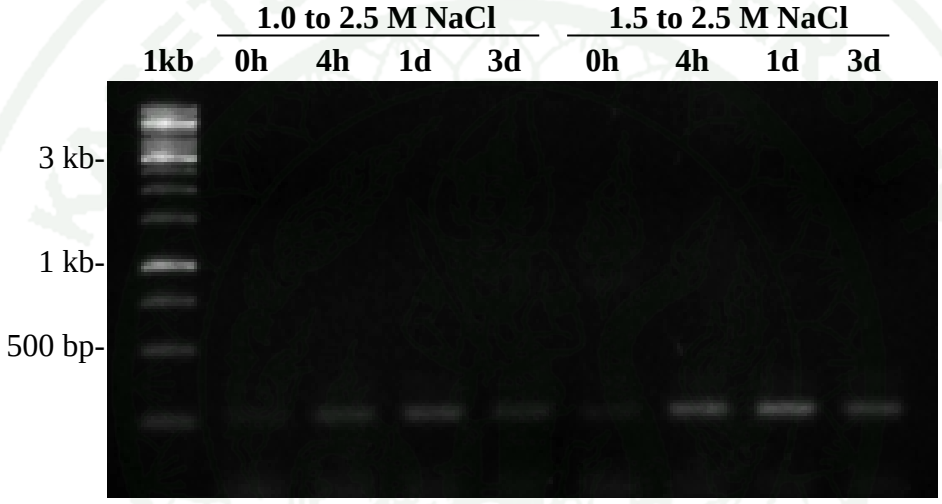


Figure 30 Expression of *Lcyβ* mRNA levels in hyper-saline shock treated *Dunaliella* sp. M22 cells. The culture was shifted from 1.0 to 2.5 M and 1.5 to 2.5 M NaCl at senescence phase. The RNA samples were collected at 0 h, 4 h, 1 day and 3 days after the salinity shock.

DISCUSSIONS

1. Screening the β-carotene producing *Dunaliella* sp. strain

The isolated strain M22 grew in the modified Johnson's medium containing higher salt concentration than 1.0 M NaCl and could produce orange pigment. This result indicated that the strain belongs to the genus *Dunaliella* (Borowitzka and Siva, 2007). For identification to the species, more detail information of molecular characteristics is required.

Oren (2005) revealed that *Dunaliella* was morphologically distinguished from its shape under light microscope. Its cell lacks rigid cell wall and has two flagella approximately equal to cell length (Ben-Amotz *et al.*, 1987). *Dunaliella* is very polymorphic and is distinguished from other halo-tolerant species by its ability to accumulate β-carotene more than 5% (up to 14%) of dry weight under high light intensity and high salinity conditions (Borowitzka, 1988; Borowitzka and Siva, 2007)

The selected colony turned orange in 8-10 weeks in remodified Johnson's medium containing 1.5 M NaCl (cool daylight 80 μmol $m^{-2}s^{-1}$) at 25±2 °C. The result is similar to the study of Lerche (1937) who found that *Dunaliella* sp. turned to yellow-green after several weeks and turned red after several months. The orange colony might result from nutrient limitation.

2. Growth and β-carotene accumulation of *Dunaliella* sp. in flask culture

2.1 Growth rate

The effect of different culture medium on growth rate of *Dunaliella* sp. M22 in modified Johnson's medium and remodified Johnson's medium was compared. It is clearly shown that growth of *Dunaliella* sp. M22 in remodified Johnson's medium approached an exponential phase faster than that in modified

Johnson's medium. The cell density in modified Johnson's medium was always lower than remodified Johnson's medium when compared with the same NaCl concentration. This result might come from the reason that the remodified Johnson's medium contained higher bicarbonate and boric acid than modified Johnson's medium (more than 25 and 34 folds, respectively). An increase of bicarbonate might increase photosynthesis efficiency, led to high growth rate of the algae. Borowitzka *et al.* (1990) found that *Dunaliella* sp. is able to uptake CO_2 and HCO_3^- during photosynthesis. *Dunaliella* sp. has a carbonic anhydrase enzyme, which converts HCO_3^- to CO_2, i.e. it can utilize HCO_3^-.

While, boric acid involved in pectin assembly and membrane function by complex formation with glycoproteins (Goldbach and Wimmer, 2007). Previously, it has been proposed the possible role of boric acid as a cellular signal through the interaction with transcription factors (Garcia-Gonzalez *et al.*, 1990).

2.2 Determination of β-carotene in *Dunaliella* sp. M22.

The relationship between different NaCl concentrations and β-carotene content in *Dunaliella* sp. M22 in remodified Johnson's medium was studied. During the exponential phase (in the first week- Figure 11), the amount of β-carotene content was low in 1.0 M, 1.5 M and 2.5 M, while the β-carotene content slightly reduced in 3.5 M compared to other NaCl concentrations. The β-carotene content was greatly elevated in all treatments after 7 days. The highest β-carotene content was found in 3.5 M, followed by 2.5, 1.5 and 1.0 M with 0.97, 0.85, 0.8 and 0.7 pg $cell^{-1}$, respectively. The result showed that the strain can produced more β-carotene under higher salinity. The result similar to Cifuentes *et al.* (2001) who found that the cell density was affected by salinity whereas total carotenoid production was increased. Similar, Fazeli *et al.* (2006) reported that the cell growth was repressed when NaCl concentration was elevated, although high salinity can increase total carotenoid production.

Marín *et al.* (1998) studied the effect of salinity on growth and pigment synthesis. At the death phase, amount of carotenoid in cell grown in 2.5 M and 3.5 M NaCl concentrations is significantly higher than 1.5 M. On the other hand, the highest carotenoid concentration was observed in the culture containing 1.5 M NaCl at low concentration of nitrate. Moreover, Phadwal and Singh (2003) revealed that the maximum content of β-carotene was accumulated under phosphate limitation followed by nitrate.

3. Growth and β-carotene accumulation in photobioreactor

3.1 Growth rate

Effect of different initial NaCl concentrations on β-carotene content was studied. The results showed that 1.0 M NaCl was suitable for increasing biomass, whereas 3.5 M NaCl has a negative effect on growth of *Dunaliella* sp. M22 (Figure 12). These results are in agree with the result of Borowitzka *et al.* (1990), Gomez *et al.* (2003) and Marín *et al.* (1998) that the higher salinity affect the cell density and growth rate. The cell grown culture containing 1.5 M NaCl was larger and more spherical than grown in 3.5 M NaCl (Marín *et al.*, 1998). However, the excess of salinity can cause cell shrinkage and damage of the cell (plasmolysis) (Dipak and Lele, 2005).

By comparing, cell density in photobioreactor with 5% CO_2 and 200 photons $m^{-2}s^{-1}$, was higher than cell density in flask cultivation. This result might come from the promoting effect of CO_2 and light intensity on an increase of photosynthesis. This result was consisted with Makino and Mae (1999), who revealed that abundant CO_2 condition could trigger an increasing rate of photosynthesis.

3.2 β-carotene accumulation

The β-carotene content in photobioreactor was approximately 3 times higher than in flask cultivation at the same NaCl concentration and harvesting time

(Figure 13) . The effect of light intensity on β-carotene accumulation was studied. Loeblich (1982) reported that carotene concentration is 4.6 pg $cell^{-1}$ at low irradiance (8 μmol photon $m^{-2}s^{-1}$), and 21 $pg.cell^{-1}$ at high irradiance (> 290 μmol photon m^{-2} s^{-1}). Ak *et al.* (2008) also reported in the same way that high light intensity retarded cell division, while increased β-carotene-to-chlorophyll ratio. As previously mentioned, CO_2 could directly elevate photosynthesis efficiency, resulting in the increase of cell density and nutrient depletion (Ramos *et al.*, 2008). The CO_2 played an indirect role on β-carotene accumulation.

The highest β-carotene content was obtained from culture containing 2.5 M NaCl in photobioreactor followed by 3.5, 1.5 and 1.0 M NaCl, respectively. The result indicated that higher salinity could increase an accumulation of β-carotene which acts as cell protective agent. Many reports described osmotic and ion effects of NaCl on stress induction. Sadka *et al.* (1989) revealed that *Dunaliella* is able to change their volume and shape in response to osmotic change, because *Dunaliella* is unique without a rigid polysaccharide cell wall, and the cell is only enclosed by a thin plasma membrane. Under high salinity, water will move out from cell and cell becomes shrunken. Subsequent synthesis of osmoprotectant, such as glycerol and β-carotene, permits the cells to regain their original volume and resume growth (Avron, 1992).

For ions effect, in range of 1–4 M NaCl, an intracellular *D. salina* contains only 20–100 mM Na^+ presenting that Na^+ is effectively excluded from the cells. Under osmotic upshocks, plasma membrane H^+-ATPase driven K^+ uptake, could cause Na^+ extrusion in *Dunaliella.* The Na^+ extrusion from the cells leads to internal alkalization, which is resulted by ATPase. For activation, the ATPase enzyme consumes ATP and converts ATP to ADP (Degani *et al.*, 1985; Pick *et al.*, 1986). The decline of ATP level in the cell stimulated an enzyme such as glucan phosphorylase, which catalyzes the synthesis of secondary metabolite, i.e. glycerol and carotenoid (Chen and Jiang, 2009).

However, this result showed that excess salinity (3.5 M NaCl) did not increase β-carotene accumulation. Similarly, Dipak and Lele (2005) reported that under extremely high salinity, cell turned completely white within 2 days after addition of 5 M NaCl salt to the culture. They found that the optimum salinity for carotenoid production was 3 M NaCl. In this study, we found that NaCl at 2.5 M concentration was suitable for β-carotene production and slightly affected growth inhibition.

4. Sequencing of full length of *lycopene β-cyclase* gene

In 5′ end amplification, broad smear and non-specific products were observed after the 5′ end amplification (Figure 19, lane 2) . This indicated that there were various sizes of mRNA. As described by Frohman (1994), after the first amplification, a broad smear of amplified cDNA with some discrete bands is normally observed using tailed templates. However, to obtain 5′ ends, PCR product should be visualized and the longest product was recovered to use as template in the nested PCR. For 3′ end amplification, the product was faint (Figure 18, lane 2). To intensify the product from the first amplification, nested PCR was carried out (Frohman, 1994).

Moreover, sequence similarity analysis with other *Lcyβ* from different organisms revealed a close relationship with other β-cyclases in green algae, for example, *Dunaliella salina* (GenBank ACA34344), *Chromochloris zofingiensis* (GenBank CBH31263) and *Haematococcus pluvialis* (GenBank AAO64977) which showed identities of 77, 66 and 65%, respectively (Figure 22). Although *Dunaliella* sp. M22 isolated from salt soil was grouped to *Dunaliella salina*, the similarity index was rather low. The lack of database sequences of β-cyclases in Genbank limited the similarity study result. Moreover, the difference of lycopene β-cyclases might come from different collected sources or from different *Dunaliella* species. The lycopene β-cyclase in bacteria (*Synechococcus* sp., *Deinococcus radiodurans* and *Rhodococcus erythropolis*) related to plant Lcyβ and LcyƐ (Sandmann, 2002; Tao *et al.*, 2004). The lycopene β-cyclase amino acid sequence from *Dunaliella* sp. M22 was similar to plant and bacterial lycopene β-cyclase (ref.)

5. Expression of the *lycopene β-cyclase* gene and carotenoid biosynthesis in high salinity medium

An optimal concentration of $MgCl_2$ for amplification of *Lcyβ*, *Atub* and 18S rRNA were 3, 2 and 1 mM, respectively. $MgCl_2$ is a metal activator of *Taq polymerase*, in a range of 1-5 mM, but the efficiency of amplification with specific primers is strictly sequence-dependent (Marone *et al.*, 2001).

The result revealed that in algae cell grown in high salinity culture (2.5 and 3.5 M NaCl) the *Lcyβ* expression levels were high at exponential and decreased at early and late senescence phase. In contrast, in low salinity culture (1.0 and 1.5 M NaCl), the *Lcyβ* expression levels in the algae cell were high at early and late senescence phases (Figure 29). Similar result was found in the second experiment where higher *Lcyβ* expression levels were found in the algae cell submitted to high salinity (Figure 30). The reason for supporting these results still obscure, but we supposed that at senescence phase, the high *Lcyβ* expression levels that found in low salinity culture might result from other factors, i.e. cell age and nutrient depletion. The rapid growth of the algae cells at low salinity during exponential phase caused a rapid exhaustion of nutrients, particularly nitrogen (Platt, 1981).

As described by Ben-Amotz and Avron (1983) that salinity has synergetic effect on carotenoid accumulation when nutrients are not depleted. Similar result was reported by Ramos *et al*. (2008) who found that the mRNA levels of *Lcyβ* gene increased when *D. salina* were submitted to abiotic stress conditions such as salinity and high light intensity.

Moreover, the cells in high salinity culture showed low *Lcyβ* expression levels at the senescence phase when compared to cells in low salinity culture, while β-carotene content in high salinity culture was very high at the senescence phase (Figure 13c). The result might be possible that the cells in high salinity culture initially accumulated β-carotene in exponential phase, whereas cells in low salinity culture began to accumulate β-carotene in senescence phase. Consequently, β-carotene

content of cells in high salinity culture was higher than in low salinity culture. Ramos *et al*. (2008) reported in the same way that *Lcyβ* mRNA levels were mostly regulated from nutrient limitation followed by an increasing of salinity. However, the high salinity always corresponded to β-carotene content.

CONCLUSIONS

1. The highest of β-carotene content in *Dunaliella* sp. M22 was found in remodified Johnson medium containing 2.5 M NaCl, followed by 3.5 M, 1.5 M and 1.0 M, respectively.

2. By RACE-PCR, The full-length sequences of *Lcyβ* cDNA contain 1392 bp open reading frames (ORF), 193 nucleotides of 5′ untranslated region (UTR), 569 nucleotides of 3′ UTR, and encode proteins of 463 amino acids. Phylogenetic analysis revealed that the Lcyβ was grouped to *Dunaliella salina* and amino acid identity was 77%.

3. *Lcyβ* mRNA transcript level in high salinity culture (2.5 M and 3.5 M NaCl) was higher than in low salinity culture (1.0 M and 1.5 M NaCl) in exponential phase.

LITERATURE CITED

Ak, i., S. Cirik and T. Goksan. 2008. Effects of light intensity, salinity and temperature on growth in Camaltl strain of *Dunaliella viridis* Teodoresco from Turkey. **J Biol Sci.** 8 (8): 1356-1359.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. **J Mol Biol.** 215 (3): 403-410.

Astorg, P. 1997. Food carotenoids and cancer prevention: An overview of current research. **Trends Food Sci Tech.** 8(12): 406-413.

Avron, M. 1992. Osmoregulation, pp. 135-164. *In* A. Ben-Amotz and M. Avron, eds. ***Dunaliella* : Physiology, biochemistry, and biotechnology.** CRC Press, Florida.

Bauernfeind, J.C. 1981. **Carotenoids as colorants and vitamin A precursors: technological and nutritional applications.** Academic Press, New York.

Ben-Amotz, A. and M. Avron. 1983. On the factors which determine massive β-carotene accumulation in the halotolerant alga *Dunaliella bardawil*. **Plant Physiol.** 72 (3): 593-597.

Ben-Amotz, A., J. Gressel and M. Avron. 1987. Massive accumulation of phytoene induced by norflurazon in *Dunaliella Bardawil* (Chlorophyceae) prevents recovery from photoinhibition 1. **J Phycol.** 23 (1): 176-181.

Ben-Amotz, A., A. Lers and M. Avron. 1988. Stereoisomers of β-carotene and phytoene in the alga *Dunaliella bardawil*. **Plant Physiol.** 86 (4): 1286-1291.

Bendich, A. 1996. Antioxidant Vitamins and Human Immune Responses. **Vitam Horm.** 52: 35-62.

Borowitzka, M., L. Borowitzka and D. Kessly. 1990. Effects of salinity increase on carotenoid accumulation in the green alga *Dunaliella salina*. **J Appl Phycol.** 2 (2): 111-119.

Borowitzka, M.A. 1988. Algal growth media and sources of cultures, pp. 456-465. *In*, eds. **Microalgal Biotechnology.** Cambridge University Press, Cambridge; UK.

Borowitzka, M.A. and C.J. Siva. 2007. The taxonomy of the genus *Dunaliella* (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. **J Appl Physiol.** 19 (5): 567-590.

Budavari, S. 1996. **The Merck index : an encyclopedia of chemicals, drugs, and biologicals.** 12th. Merck, Whitehouse Station, New Jersey.

Burton, G.W. and K.U. Ingold. 1984. β-carotene: an unusual type of lipid antioxidant. **Science.** 224 (4649): 569-573.

Chaudière, J. and R. Ferrari-Iliou. 1999. Intracellular antioxidants: from chemical to biochemical mechanisms. **Food Chem Toxicol.** 37 (9-10): 949-962.

Chen, H. and J.G. Jiang. 2009. Osmotic responses of *Dunaliella* to the changes of salinity. **J Cell Physiol.** 219 (2): 251-258.

Cifuentes, A.S., M.A. Gonzalez, I. Inostroza and A. Aguilera. 2001. Reappraisal of physiological attributes of nine strains of *Dunaliella* (Chlorophyceae): Growth and pigment content across a salinity gradient. **J Phycol.** 37 (2): 334-344.

Cuttriss, A.J., C.I. Cazzonelli, E.T. Wurtzel and B.J. Pogson. 2011. Carotenoids, pp. 1-36. *In* R. Fabrice and D. Roland, eds. **Advances in Botanical Research.** Academic Press, London, UK.

D'Ambrosio, C., G. Giorio, I. Marino, A. Merendino, A. Petrozza, L. Salfi, A.L. Stigliani and F. Cellini. 2004. Virtually complete conversion of lycopene into β-carotene in fruits of tomato plants transformed with the tomato *lycopene β-cyclase* (t*Lcy-b*) cDNA. **Plant Sci.** 166 (1): 207-214.

Darvin, M.E., I. Gersonde, H. Albrecht, S.A. Gonchukov, W. Sterry and J. Lademann. 2005. Determination of beta-carotene and lycopene concentrations in human skin using resonance Raman spectroscopy. **Laser Phys.** 15 (2): 295-299.

Degani, H., I. Sussman, G.A. Peschek and M. Avron. 1985. 13C- and 1H-NMR studies of osmoregulation in *Dunaliella*. **Biochim Biophys Acta.** 846 (3): 313-323.

Del Campo, J.A., M. García-González and M.G. Guerrero. 2007. Outdoor cultivation of microalgae for carotenoid production. **Appl Microbiol Biotechnol.** 74 (6): 1163-1174.

Dipak, S. and S. Lele. 2005. Carotenoid production from microalga, *Dunaliella salina*. **Indian J Biotechno.** 4 476-483.

Fazeli, M.R., H. Tofighi, N. Samadi and H. Jamalifar. 2006. Effects of salinity on beta-carotene production by Dunaliella tertiolecta DCCBC26 isolated from the Urmia salt lake, north of Iran. **Bioresour Technol.** 97 (18): 2453-2456.

Foote, C.S., Y.C. Chang and R.W. Denny. 1970. Quenching of singlet oxygen. **J Am Chem Soc.** 92 (17): 5218-5219.

Frohman, M.A. 1994. On beyond classic RACE (rapid amplification of cDNA ends). **PCR Methods Appl.** 4 (1): S40-58.

Garcia-Gonzalez, M., P. Mateo and I. Bonilla. 1990. Effect of boron deficiency on photosynthesis and reductant sources and their relationship with nitrogenase activity in anabaena PCC 7119. **Plant Physiol.** 93 (2): 560-565.

Goldbach, H.E. and M.A. Wimmer. 2007. Boron in plants and animals: Is there a role beyond cell-wall structure?. **J Plant Nutr Soil Sci.** 170 (1): 39-48.

Gomez, P.I., A. Barriga, A.S. Cifuentes and M.A. Gonzalez. 2003. Effect of salinity on the quantity and quality of carotenoids accumulated by *Dunaliella salina* (strain CONC-007) and *Dunaliella bardawil* (strain ATCC 30861) Chlorophyta. **Biol Res.** 36 (2): 185-192.

Goodwin, T.W. 1980. **The Biochemistry of the Carotenoids.** Chapman and Hall, London, UK.

Hao, H., L. Ma, H. Cong, Q. Li and X. Yu. 2012. Isolation and characterization of a muskmelon cDNA encoding lycopene beta-cyclase. **Gene.** 503 (1): 147-151.

Havaux, M. 1998. Carotenoids as membrane stabilizers in chloroplasts. **Trends Plant Sci.** 3 (4): 147-151.

Heldt, H.-W. and F. Heldt. 2005. 17 - A large diversity of isoprenoids has multiple functions in plant metabolism, pp. 413-433. *In*, eds. **Plant Biochemistry 3.** Academic Press, Burlington.

Hirschberg, J. 2001. Carotenoid biosynthesis in flowering plants. **Curr Opin Plant Biol.** 4 (3): 210-218.

Kopsell, D.A. and D.E. Kopsell. 2010. Carotenoids in Vegetables: Biosynthesis, Occurrence, Impacts on Human Health, and Potential for Manipulation, pp. 645-662. *In* W. Ronald Ross and R. P. Victor, eds. **Bioactive Foods in Promoting Health.** Academic Press, San Diego.

Krinsky, N.I. 1993. Actions of carotenoids in biological systems. **Annu Rev Nutr.** 13 (1): 561-587.

Krinsky, N.I. 1998. The antioxidant and biological properties of the carotenoids. **Ann NY Acad Sci.** 854 (1): 443-447.

Lamers, P.P., M. Janssen, R.C. De Vos, R.J. Bino and R.H. Wijffels. 2008. Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell-factory applications. **Trends Biotechnol.** 26 (11): 631-638.

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson and D.G. Higgins. 2007. Clustal W and Clustal X version 2.0. **Bioinformatics.** 23 (21): 2947-2948.

Lerche, W. 1937. Untersuchungen über Entwicklung und Fortpflanzung in der Gattung *Dunaliella*. **Arch Protistenk.** 88 236–268.

Loeblich, L.A. 1982. Photosynthesis and pigments influenced by light intensity and salinity in the halophile *Dunaliella Salina* (Chlorophyta). **J Mar Biol Assoc Uk.** 62 (03): 493-508.

Makino, A. and T. Mae. 1999. Photosynthesis and plant growth at elevated levels of CO_2. **Plant Cell Physiol.** 40 (10): 999-1006.

Marín, N., F. Morales, C. Lodeiros and E. Tamigneaux. 1998. Effect of nitrate concentration on growth and pigment synthesis of *Dunaliella salina* cultivated under low illumination and preadapted to different salinities. **J Appl Phycol.** 10 (4): 405-411.

Marone, M., S. Mozzetti, D. De Ritis, L. Pierelli and G. Scambia. 2001. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. **Biol Proced Online.** 3: 19-25.

Oren, A. 2005. A hundred years of *Dunaliella* research: 1905-2005. **Saline Systems.** 1: 2.

Phadwal, K. and P.K. Singh. 2003. Effect of nutrient depletion on β-carotene and glycerol accumulation in two strains of *Dunaliella* sp. **Bioresource Technol.** 90 (1): 55-58.

Pick, U. 2004. Adaptation of the halotolerant alga *Dunaliella* to high salinity, pp. 97-112. *In* A. Läuchli and U. Lüttge, eds. **Salinity: Environment - Plants - Molecules.** Springer Dordrecht, The Netherlands.

Pick, U., A. Ben-Amotz, L. Karni, C.J. Seebergts and M. Avron. 1986. Partial characterization of K and Ca uptake systems in the halotolerant alga *Dunaliella salina*. **Plant Physiol.** 81 (3): 875-881.

Platt, T. 1981. Physiological Bases of Phytoplankton Ecology. **Can Bull Fish Aquat Sci.** 210 (210): 346.

Rad, F.A., N. Aksoz and M. Hejazi. 2011. Effect of salinity on cell growth and β-carotene production in *Dunaliella* sp. isolates from Urmia Lake in northwest of Iran. **Afr J Biotechnol.** . 10 (12): 2282-2289.

Raja, R., S. Hemaiswarya, N.A. Kumar, S. Sridhar and R. Rengasamy. 2008. A perspective on the biotechnological potential of microalgae. **Crit Rev Microbiol.** 34 (2): 77-88.

Ramos, A., S. Coesel, A. Marques, M. Rodrigues, A. Baumgartner, J. Noronha, A. Rauter, B. Brenig and J. Varela. 2008. Isolation and characterization of a stress-inducible Dunaliella salina Lcy-beta gene encoding a functional lycopene beta-cyclase. **Appl Microbiol Biotechnol.** 79 (5): 819-828.

Rock, C.L. 1997. Carotenoids: Biology and treatment. **Pharmacol Ther.** 75 (3): 185-197.

Rodriguez-Saiz, M., C. Sanchez-Porro, J.L. De La Fuente, E. Mellado and J.L. Barredo. 2007. Engineering the halophilic bacterium *Halomonas elongata* to produce beta-carotene. **Appl Microbiol Biotechnol.** 77 (3): 637-643.

Romero, I., Y. Tikunov and A. Bovy. 2011. Virus-induced gene silencing in detached tomatoes and biochemical effects of *phytoene desaturase* gene silencing. **J Plant Physiol.** 168 (10): 1129-1135.

Rose, T.M., J.G. Henikoff and S. Henikoff. 2003. CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. **Nucleic Acids Res.** 31 (13): 3763-3766.

Sadka, A., A. Lers, A. Zamir and M. Avron. 1989. A critical examination of the role of de novo protein synthesis in the osmotic adaptation of the halotolerant alga *Dunaliella*. **FEBS Letters.** 244 (1): 93-98.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. **Mol Biol Evol.** 4 (4): 406-425.

Salvini, M., A. Bernini, M. Fambrini and C. Pugliesi. 2005. cDNA cloning and expression of the *phytoene synthase* gene in sunflower. **J Plant Physiol.** 162 (4): 479-484.

Sambrook, J. and D.W. Russell. 2001. **Molecular Cloning: A Laboratory Manual.** Cold Spring Harbor Laboratory Press, New York, USA.

Sandmann, G. 2002. Molecular evolution of carotenoid biosynthesis from bacteria to plants. **Physiologia Plantarum.** 116 (4): 431-440.

Schwartz, J.L., E. Flynn and G. Shklar. 1990. The effect of carotenoids on the antitumor immune response in vivo and in vitro with hamster and mouse immune effectors. **Annals of the New York Academy of Sciences.** 587 92-109.

Shariati, M. and M.R. Hadi. 2011. Microalgal biotechnology and bioenergy in *Dunaliella*, pp. *In* P. A. Carpi, eds. **Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications.** InTech, Rijeka, Croatia.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. **Mol Biol Evol.** 28 (10): 2731-2739.

Tao, L., S. Picataggio, P.E. Rouvière and Q. Cheng. 2004. Asymmetrically acting lycopene β-cyclases (CrtLm) from non-photosynthetic bacteria. **Mol Genet Genomics.** 271 (2): 180-188.

Tibaduiza, E.C., J.C. Fleet, R.M. Russell and N.I. Krinsky. 2002. Excentric cleavage products of β-carotene inhibit estrogen receptor positive and negative breast tumor cell growth in vitro and inhibit activator protein-1-mediated transcriptional activation. **J Nutr.** 132 (6): 1368-1375.

Tsuchihashi, H., M. Kigoshi, M. Iwatsuki and E. Niki. 1995. Action of β-carotene as an antioxidant against lipid peroxidation. **Arch Biochem Biophys.** 323 (1): 137-147.

Tuan, P.A., J.K. Kim, N.I. Park, S.Y. Lee and S.U. Park. 2011. Carotenoid content and expression of *phytoene synthase* and *phytoene desaturase* genes in bitter melon (*Momordica charantia*). **Food Chem.** 126 (4): 1686-1692.

Wang, F., J.G. Jiang and Q. Chen. 2007. Progress on molecular breeding and metabolic engineering of biosynthesis pathways of C(30), C(35), C(40), C(45), C(50) carotenoids. **Biotechnol Adv.** 25 (3): 211-222.

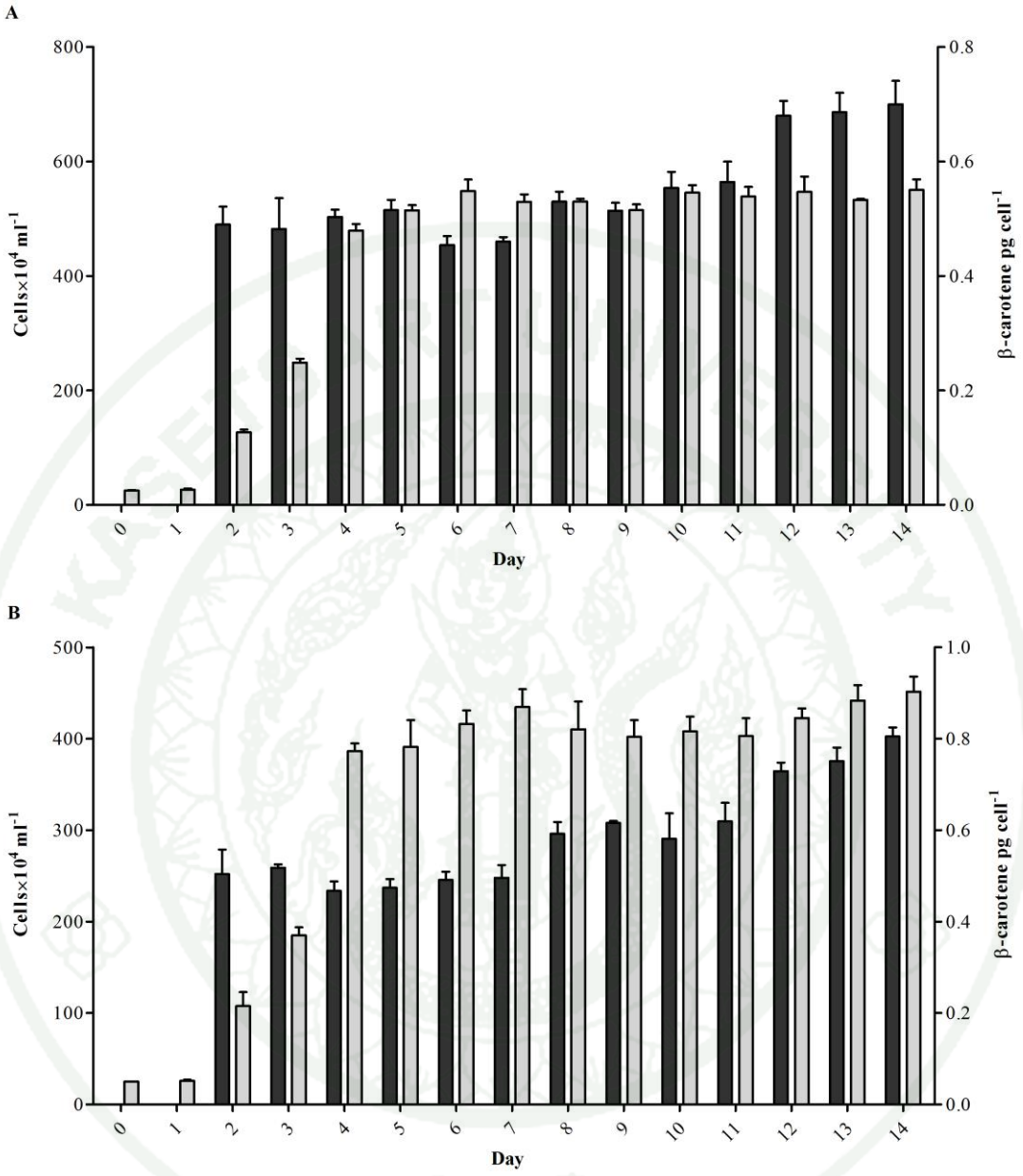
Ye, Z.-W., J.-G. Jiang and G.-H. Wu. 2008. Biosynthesis and regulation of carotenoids in *Dunaliella*: Progresses and prospects. **Biotechnol Adv.** 26 (4): 352-360.

Zhu, C., S. Yamamura, M. Nishihara, H. Koiwa and G. Sandmann. 2003. cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. **BBA-Gene Struct Expr.** 1625 (3): 305-308.

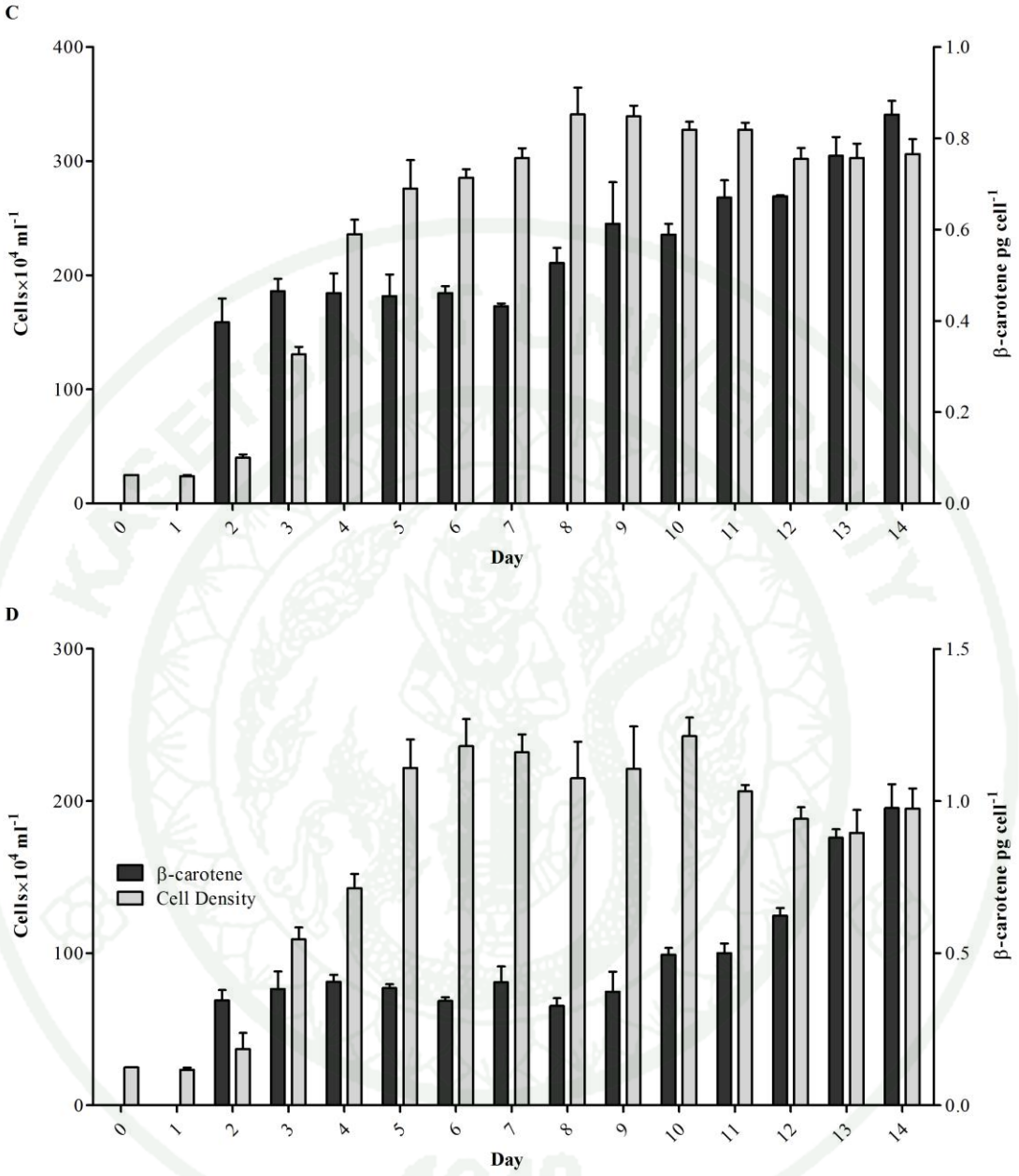
APPENDIX

Appendix Table 1 The recipe of modified Johnson's medium (Borowitzka, 1988) and remodified Johnson's medium.

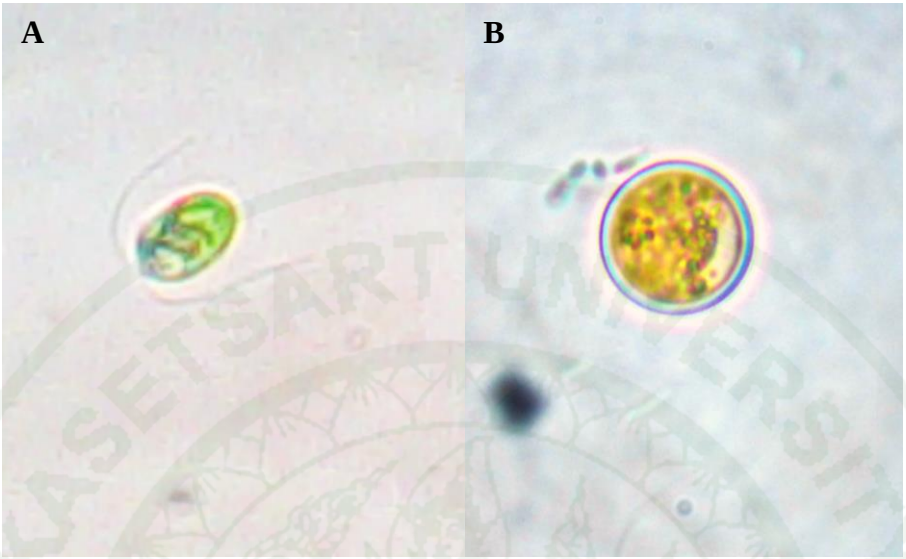
Chemical	Modified Johnson's medium	Remodified Johnson's medium
Macronutrients (mg/L)		
H_3BO_3	0.61	21.4
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	0.38	0.38
$CuSO_4\cdot 5H_2O$	0.06	0.06
$CoCl_2\cdot 6H_2O$	0.05	0.05
$ZnCl_2$	0.04	0.04
$MnCl_2\cdot 4H_2O$	0.04	0.04
Macronutrients (g/L)		
$MgCl_2\cdot 6H_2O$	1.5	1.0
$MgSO_4.7H_2O$	0.5	-
KCl	0.2	-
$CaCl_2.2H_2O$	0.2	0.05
KNO_3	1.0	1.0
KH_2PO_4	0.04	0.05
$FeCl_3.6H_2O$	0.0024	0.0005
Na_2EDTA	0.0018	0.0002
$NaHCO_3$	0.04	1.0



Appendix Figure 1 Illustration showed the relationship between cell density and β-carotene content under different salinity A) 1.0 M B) 1.5 M C) 2.5 M D) 3.5 M. The cell was grown in modified Johnson medium under continuous illumination (80 μmol $m^{-2}s^{-1}$). Plotted data are the averages ± SD of three replicates.



Appendix Figure 1 (Continued)



Appendix Figure 2 Illustration showed *Dunaliella* sp. M22 in culture containing A) 1.5 M and B) 2.5 M NaCl remodified Johnson's medium at late senescence phase under continuous illumination (200 μmol $m^{-2}s^{-1}$) and flow air with 5% CO_2 under light microscope (1000x).

Appendix Table 2 Effect of NaCl concentration (1.0, 1.5, 2.5 and 3.5 M) on *Dunaliella* sp. M22 growth in flask containing modified Johnson's medium.

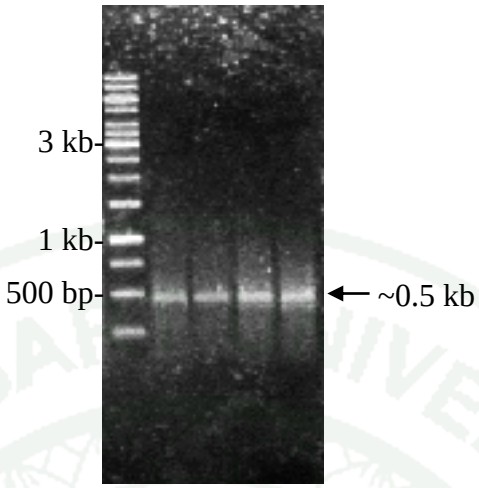
	NaCl concentration			
	1.0 M	1.5 M	2.5 M	3.5 M
Period (Days)	11	11	11	11
Specific growth rate (μ, per days)	0.66±0.01	0.45±0.01	0.31±0.01	0.26±0.01
Doubling time (T_d, days)	1.05±0.01	1.62±0.02	2.21±0.02	2.68±0.02
Doubling per day (k, per day)	0.95±0.01	0.65±0.03	0.45±0.07	0.37±0.09

Appendix Table 3 Effect of NaCl concentration (1.0, 1.5, 2.5 and 3.5 M) on *Dunaliella* sp. M22 growth in flask containing remodified Johnson's medium.

	NaCl concentration			
	1.0 M	1.5 M	2.5 M	3.5 M
Period (Days)	11	11	11	11
Specific growth rate (μ, per days)	1.56±0.05	1.41±0.01	0.85±0.03	0.77±0.02
Doubling time (T_d, days)	0.44±0.07	0.49±0.02	0.82±0.04	0.90±0.03
Doubling per day (k, per day)	2.25±0.01	2.04±0.03	1.22±0.02	1.11±0.01

Appendix Table 4 Effect of NaCl concentration (1.0, 1.5, 2.5 and 3.5 M) on *Dunaliella* sp. M22 growth in remodified Johnson's medium in photobioreactor

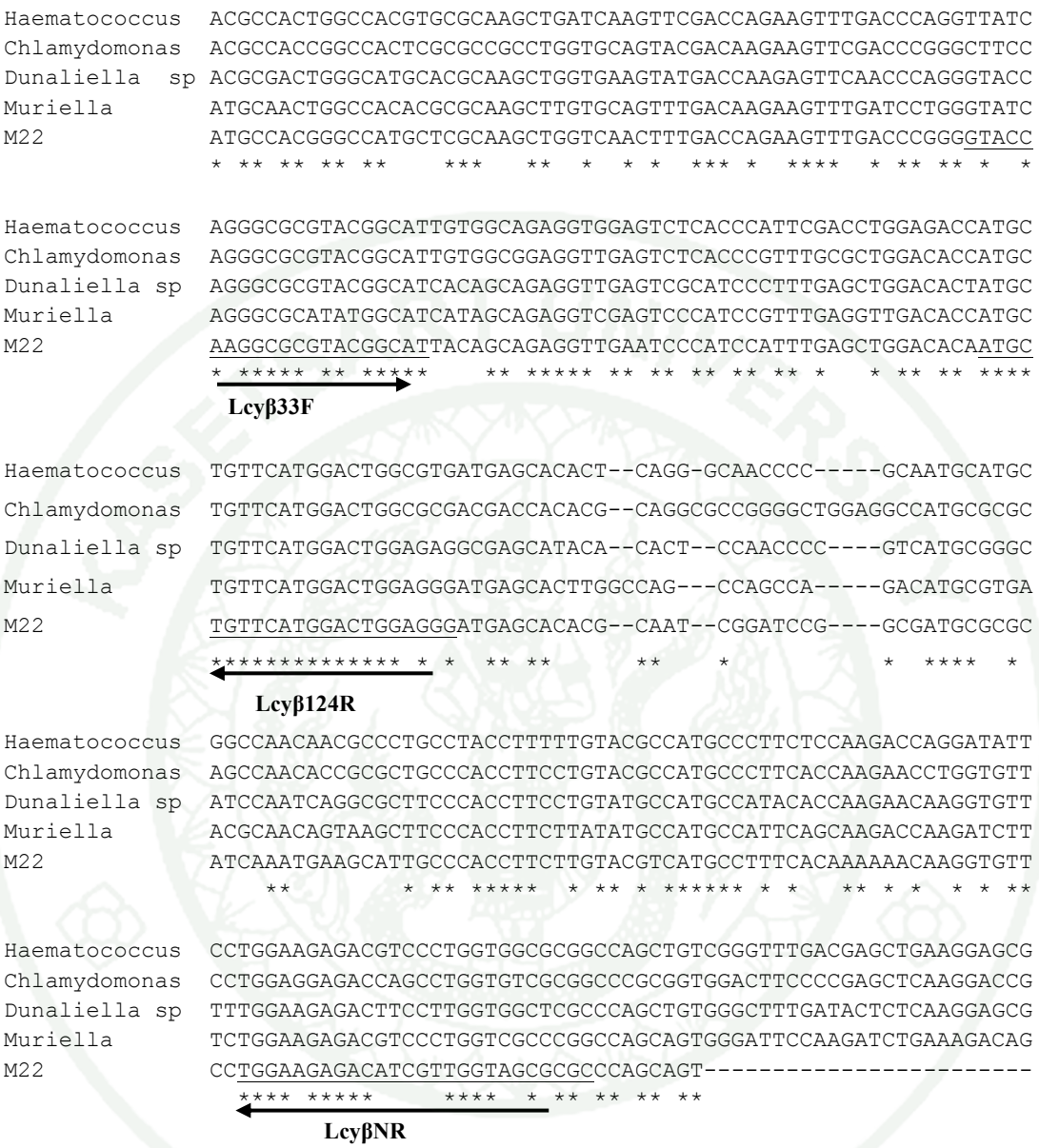
	NaCl concentration			
	1.0 M	1.5 M	2.5 M	3.5 M
Period (Days)	13	13	13	13
Specific growth rate (μ, per days)	2.27±0.16	1.40±0.00	1.25±0.02	0.89±0.03
Doubling time (T_d, days)	0.31±0.23	0.49±0.01	0.55±0.03	0.78±0.04
Doubling per day (k, per day)	3.27±0.01	2.02±0.00	1.80±0.01	1.28±0.02



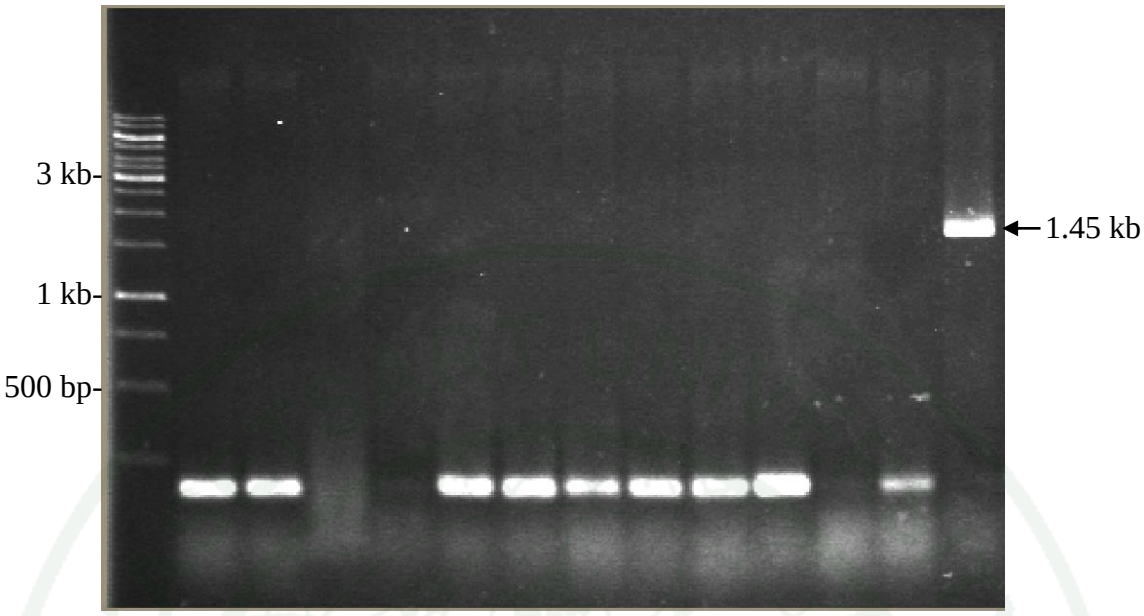
Appendix Figure 3 Amplification of partial *Lcyβ* gene with LcyβWF and LcyβNR primer.

```
Haematococcus  GGAGATTGTGTGGCCCAAGGCCTCGGTGCATCTCAGCAACAAGCCAGAGGGCGAGAAGTT
Chlamydomonas  GCACGTCATCTGGCCCAAGGCCAAGGTCTGGCTCAACAGCGAGGCCGACGGCGAGAAGTT
Dunaliella sp  AGAAGTGGTTTGGCCCAAAGCCAAGGTCTGGCTGGACAATGACAAGAGCGGAGAACGGTT
Muriella       GGAGGTCATTTGGCCAAAAGCAAAAGTGTGGCTGAACAATAGCAACGCAGGCGAAAAATT
Lcyβ1          --------------------------------------------------GGAGAAAAGTA
                                                                  ** **    *

Haematococcus  CCTAAGTCGCCCCTACGGTCGCGTGGACCGGCCACGCTTGAAAAGCATGCTGCTGAAGAA
Chlamydomonas  CCTGAACCGCCCCTTCGGCCGCGTGGACCGGCCCAAGCTGAAGCGCATCCTGCTGGAGCG
Dunaliella sp  TTTGAGACGGCCATATGGACGCATGGATCGGCCTATGCTCAAAAAGCTTCTGCTGCAGAA
Muriella       CCTGTCAAGACCATATGGTCGAGTGGACAGGCCCAAGCTTAAACGGAAGTTACTGGAGAG
Lcyβ1          CCTGAAGAGGGCGTACGGGCGCATGGACCGGCCCATGCTCAAAAAATTGCTGCTGCAAAA
                 *    *  * *  ** **  ****  ****    * **       * *** *

Haematococcus  GTGTGCGGCCCATGGAGTGACCTTTCTGGAAGGCAAAGTTGACGGG-GTCAACCATGGTG
Chlamydomonas  CTGCGTCGCCTCGGGCGTGACGTTCCTTGACGCCAAGGT-GTCGGGCGTGAGTCACGGCG
Dunaliella sp  GTGCGCTTCAAATGGTGTGACCTTCCTCACCAGCAAGGT-GTCTGGTGTGAGCCATGGGG
Muriella       ATGTGCAGCCAGCGGTGTGACATTCCTTACTGGCAAGGT-GGAGGGTGTAAGACATGGTG
Lcyβ1          ATGTGCATCCAACGGCGTGACATTCTTGACTAGTAAGGT-GGACGGTGTGAGCCATGGGG
               ** *   *    ** ***** **  *      ** ** *   ** ** *  ** ** *
                                                      LcyβNF

Haematococcus  AGGGACGCTCCGCCGTCAGCCTGGCTGATGGGCGCAGCATCCGCGGCTCTCTGGTGCTGG
Chlamydomonas  GCGGCTGCAGCGCCGTCAAGCTGGCGGACGGCCGCGAGATCCGCGGCAGCCTGGTCCTGG
Dunaliella sp  ATGGCACCTCCACTGTGACCCTCTCAGATGGGCGCACCATGCAAGGCACCATGGTGCTGG
M22            GAGGCTGCTCCACAGTGTCACTTACCGACGGGCGCACCATTCAAGGCACCATGGTCCTTG
Muriella       ATGGCTCATCAACAGTCAGCACAGCAGAGGGTGTCAGCCTACAAGGGTCATTGGTGTTGG
                 **       * **       * ** **   *    * *  **     ****  * *
```

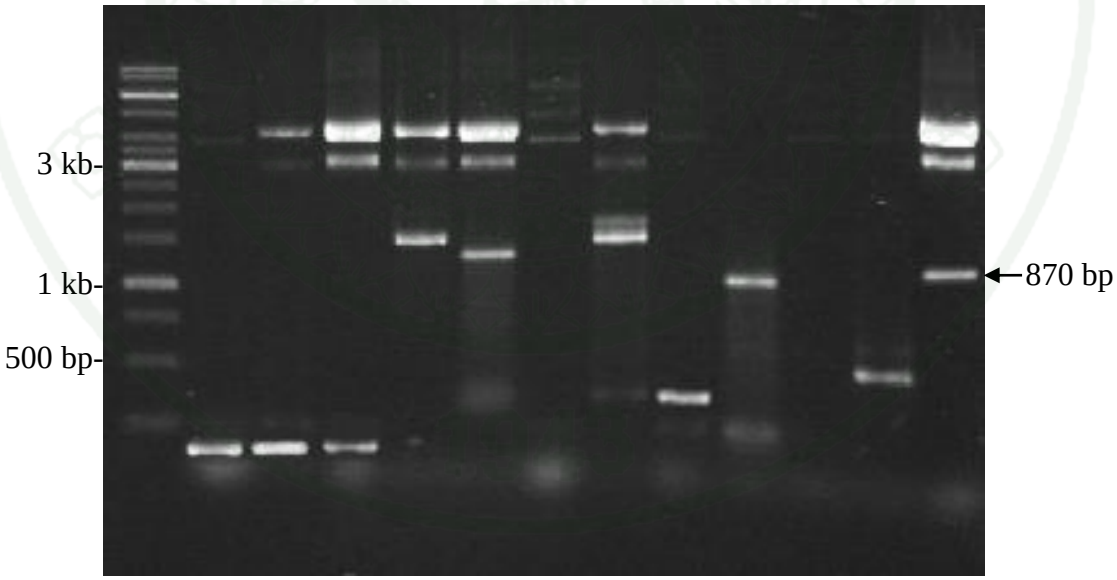
Appendix Figure 4 The multiple aligned sequences were used as template from design primer.



Appendix Figure 4 (Continued)



Appendix Figure 5 3′ end *Lcyβ* transformed clone was detected by colony PCR using M13F and M13R primer.



Appendix Figure 6 5′ end *Lcyβ* transformed clone was detected by colony PCR using M13F and M13R primer.

Name 5′Race Lcyβ
BASE COUNT 870 bp
ORIGIN

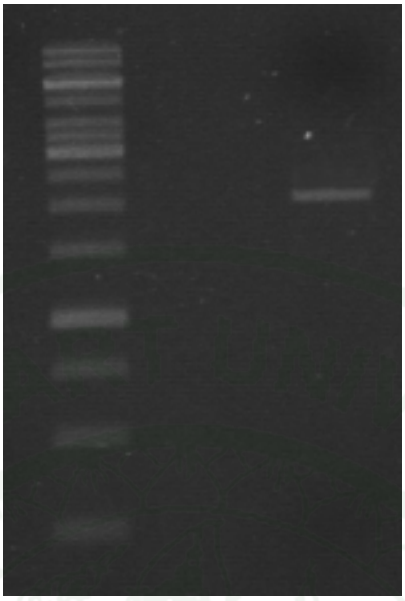
```
1     AAAAGCAAAG ACACCAGACA ACACACGCCA GAACAGCAAC AAAGAACACC
51    CCTAACCGGC TCCTCTAACA ACATGCGAGA TCAAACCTCG GCAGTATTAT
101   TACAGGAAGC ACTGACCGAG GCCCAGACAG TAGGGCATCC CAGTGGTAAA
151   CAACGACCCA CAGCAGCAAC CACCTTCAGA CCCCCAGACC CCGATGTCTC
201   GGCAGTCAGA CAAGGCAGTA ACAGAGCGTG TGTATACAGG TTGCACATCA
251   GTATGTGGCA TGTATATTGA AACATATACC GCCACAATCC CCAACTATGG
301   GTGCAGAGTA GAAACGCAGC AGACCAAGGG CCTTGAGGAG GGACTGGTGG
351   AGGGTTCACC GAACGAAAAA AATTTTTGTG GAGAAAAAAA GAGCCGGGAA
401   AAAATCCTGA AAAGGGCGTA CGGGCGCATG GACCGGCCCA TGCTCAAAAA
451   ATTGCTGCTG CAAAAATGTG CATCCAACGG CGTGACATTC TTGACTAGTA
501   AGGTGGACGG TGTGAGCCAT GGGGGAGGCT GCTCCACAGT GTCACTTACC
551   GACGGGCGCA CCATTCAAGG CACCATGGTC CTTGATGCCA CGGGCCATGC
601   TCGCAAGCTG GTCAACTTTG ACCAGAAGTT TGACCCGGGG TACCAAGGCG
651   CGTACGGCAT TACAGCAAAG GATGAATCCC ACCCATTTGA GCTGGACACA
701   ATGCTGTTCA TGGACTGGAG GGATGAGCAC ACGCAATCGG ATCCGGCGAT
751   GCGCGCATCA AATGAAGCAT TGCCCACCTT CTTGTACGTC ATGCCTTTCA
801   CAAAAAACAA GGTGTTCCTG GAAGAGACAT CGTTAGTAGC GCGCCCAGCA
851   GTTGGGTTTG AGGAATTAAA
```

Appendix Figure 7 5′ Race of *lycopene β-cyclase* gene sequence of *Dunaliella* sp. M22 strains.

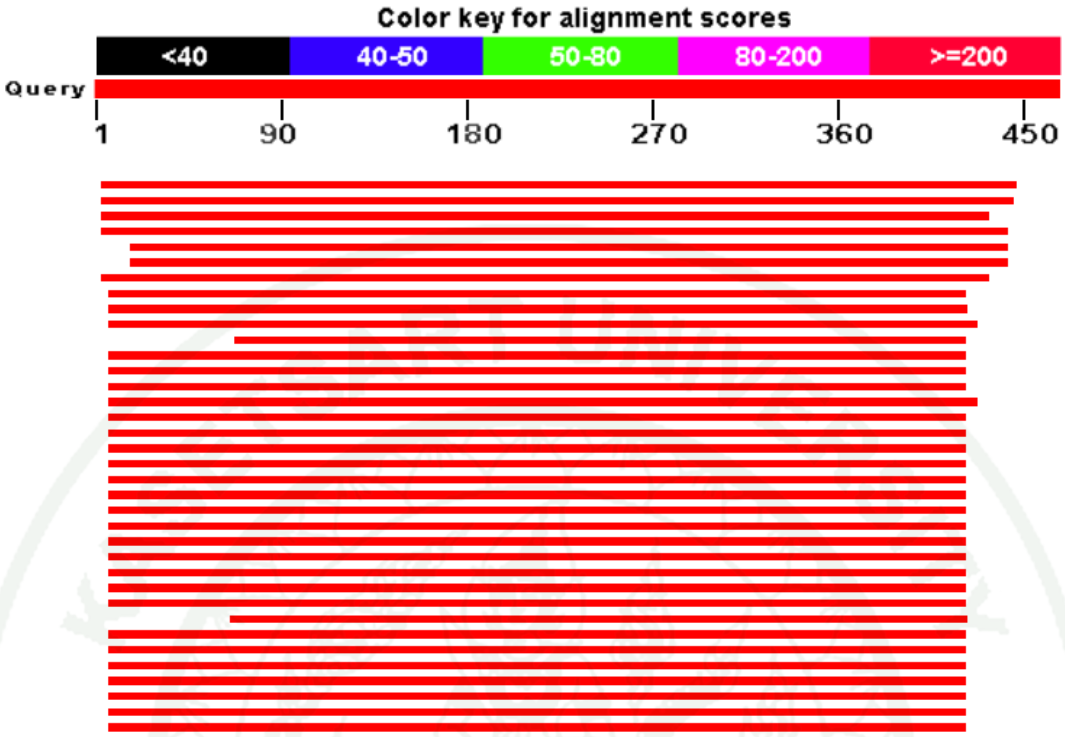
Name 3′Race Lcyβ
BASE COUNT 1454 bp
ORIGIN

```
1     ATGCTGTTCA TGGACTGGAG GGATGAGCAC ACGCAATCGG ATCCGGCGAT
51    GCGCGCATCA AATGAAGCAT TGCCCACCTT CTTGTACGTC ATGCCTTTCA
101   CAAAAAACAA GGTGTTCCTG GAAGAGACAT CGTTAGTAGC GCGCCCAGCA
151   GTTGGGTTTG AGGAATTAAA ACAAAGACTT GAAGCTCGCA TGAAGTGGTT
201   AGGCATCAAG GTGAAAAAGG TTGAGGATGA AGAATACTGC CTGATTCCCA
251   TGGGTGGTGT CCTACCACAG CACCCACAGC GTGTACTGGG CATCGGCGGA
301   ACTGCCGGCA TGGTGCATCC TTCAACAGGC TTCATGATGA CCCGTATGCT
351   GGGCTCTGCC CCTGTCGTTG CGGATGCCAT CATTGACCAA CTAGCCAAAC
401   CAACAGACAA GGCCACGGAC TCAGGCACCT CACAGCAGCC ATTGACAGAG
451   CAGGAGGCAG AAGAGATGGC AGCAGCTGTG TGGAGAGCCA CCTGGCCCGT
501   GGAACGTATT CGGCAACGAG CCTTCTTTTG TTTTGGCATG GAGATGCTGC
551   TCACCTTGAA CCTTGGCCAG ATGCGAGAGT TCTTTGCCGC CTTCTTCTCG
601   CTCTCAGACT TTCACTGGCA AGGCTTCTTG TCAGCCCGCC TGTCCTTTAC
651   GCAGCTCATC GGCTTTGGCC TGTCCCTCTT CACATCCGCC ACATCAGACA
701   CTCGTCTGAA CCTGCTGCGG CTAGGCATCC CAGGCCTCAT ACAGATGCTG
751   CTGGTGCTGT TCCCCACTGT AACTGGCTAC TACAAGGGTG ATCTCACTGT
801   AAGGGACAAG AAGTTTGCGC ATGATGCTGC AGCTGCTGCT GCAAGCAAGC
851   TAAAGCAGCC GCAAAGCCCA GCTCAGCCAT TGTCGCAAGG GGGGAGCTCG
901   TCATCGTAGG TCATTGCTCC GTCAAGCTGT GTGCTGTTAA ACAGTAATTA
951   AGTGTTGCAT AAATGTTGCA GAGGCATCGC GAGCAGGCTC GGGCTTACCC
1001  ATTGCCACGA GGAAGGGACA TGCCATCCTA GGTGTAGGTC TAGGGTTCTG
1051  GGTGGCTTCT GGTAATTAAT CGGGTGGGTT GTTCCAAGTG AAATATGCTG
1101  CAAACTGTCC ACATGCTTGC GACGAACCCC TTTCTTAATG GACACTTAGC
1151  TCAAACAGCA TCACCAGCTA TGTGATCAAT AATGCATGGG GTGTTGGAGA
1201  AGTTGCAAAA CGTTTTCTGG CCTTGGGGCC CATCTAAGGG GGAGGTTGCT
1251  AAACAAGCCC TCTTACAAGA AACCATTTGT CCACCTGAGA CACCCTTTTG
1301  TATGGGAGAT AAGGGTTCTT TTCCGAATTG GCCCCCTGGC CCAAGGGGTT
1351  TGATTTTGTT TTTTACCCAC ACAGGGGCGG CCTTCCACAG GAATATTTTT
1401  GCCCTCCCGG GGGGGGAACC AAAAATAGAG CTTTTTGCCA AAAAAAAAAA
1451  AAAA
```

Appendix Figure 8 3′ Race of *lycopene β-cyclase* gene sequence of *Dunaliella* sp. M22 strains.



Appendix Figure 9 Amplification of full-length *Lcyβ* gene using LcyβfullFW and LcyβfullRW.



Appendix Figure 10 Graphical view of amino sequences alignment of Lcyβ (463 amino acids) using BLASTP (2.2.29) program from NCBI.

```
CLUSTAL 2.1 multiple sequence alignment

Chlamydomonas   ---------------------------------MMLKAGNRPVALRSGRSATVSPISRVV
Volvox          ---------------------------------MLLQAGSCPSALRSG-LRPQRLARVTP
Coccomyxa       ------------------------------------------------------------
Chromochloris   -------------------------------MESKLLRNTGTLGATRQLVHASCTYHYRTA
Dunaliella      MLQTLSGRSTSLLSSPPQLPSQHVYSVKSTRICGAESLQSPQAALGTRTLNVTRSTTHAR
M22             ------------------------------------------------------------
Haematococcus   --------------------------MLSPLQRLCAVPGSSYSAGTALVLLPPAPRVCLS


Chlamydomonas   SRPQQLQRRICTAAAGQKDAFPSGPYPIPPGPVGHFYRETEKWPTSETVRLQPHDLNEVD
Volvox          LVPSLRRCTRAVIAAAQKAAFSSGPYPIPEGPASHYYRETESWPTEETLPLQQHNLEQQP
Coccomyxa       ------------------------------------------------MLPRYHQSIG
Chromochloris   VPGSQGGTFCVRHPRLPLKVQAAATLERPSTSGKSQFYVRDPAPWPTDVPIQQHDPKKTP
Dunaliella      EASRARTRAQALLQRTPETPYFSPPKPSVDGPVQYYYREPSPWPTQQDVQVAYHDLQRQP
M22             ------------------------------------------------------------
Haematococcus   RQHEQTTQWPRRRLVRASAATASEQRPARSSPSGYIMRDPMPWPTASDIALTQHDLQATP
```

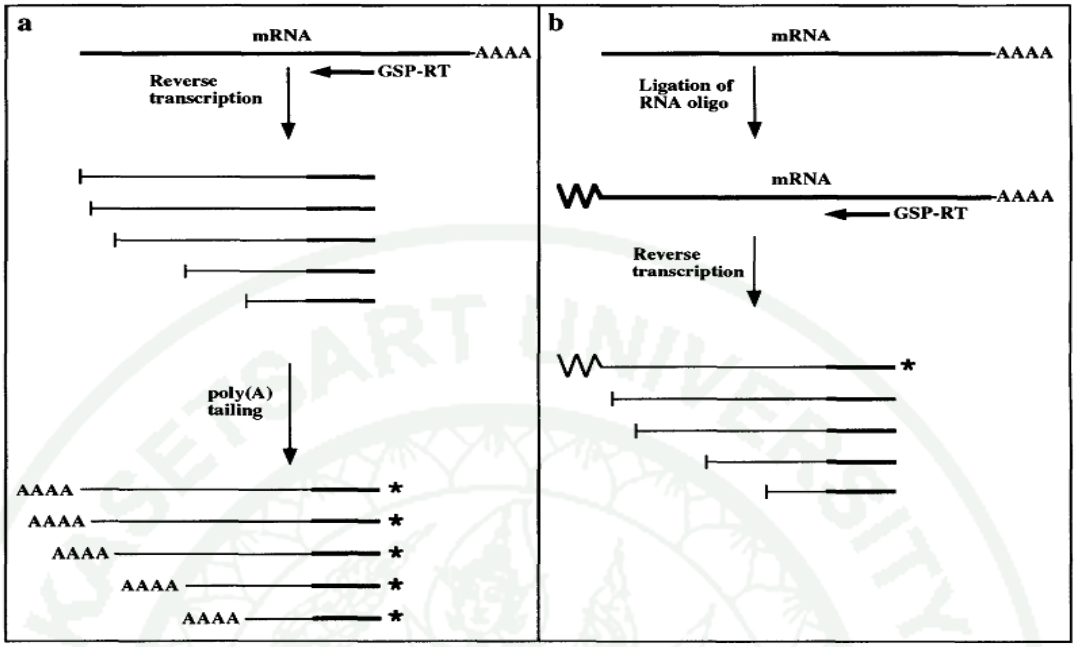
Appendix Figure 11 Results amino acid alignment of lycopene β-cyclase by CLUSTAL 2.1 multiple sequence alignment.

```
Chlamydomonas   YVDLVVAGAGPAGVAVASRVAAAGFSVCVVDPEPLAHWPNNYGVWLDEFQAMGLEDCLHV
Volvox          YVDLIVAGAGPAGVAAAGRVAAAGFSVCVIDPEPLAHWPNNYGVWLDEFQAMGLEDSLHV
Coccomyxa       KVELVVAGAGPSGLAVAERVSQAGYKVCVIDPSPLAAWPNNYGVWVDEFRAMGLDDCLDH
Chromochloris   FVDLVVAGAGPSGLAVAERVARAGFTVCIIDPNALGVWPNNYGVWVDEFQAMGLDDCLEV
Dunaliella      AADLLVVGSGPSGLAVAERVAAGGFSVCVIDLDPYAPMIPNYGCWVDEMQAMGLEECLEV
M22             -------MSRQSDKAVTERVYTGCTSVCGMYIETYTATIPNYGCRVETQQTKGLEEGLVE
Haematococcus   EVDLIVAGAGPSGIAVAERVAAAGFSVCVVDPEPLGIWPNNYGAWVDEFEAMGLGEYMEI
                        :  :. *.: **  .  .** :  ..      ***  ::  .: ** : :

Chlamydomonas   IWPKAKVWLNSEADGEKFLNRPFGRVDRPKLKRILLERCVASGVTFLDAKVSGVSHGGGC
Volvox          WPKAKVWLNSLPDGEKFLNRPFARVDRPKLKRMLLERCNAAGVTFLYGKVSGCCHGEGS
Coccomyxa       VWDRAEVFLDSSPSGLKHLARPYGRVDRAKLKRKLLQRCVAHGVVFQEARVEEVVHADGS
Chromochloris   IWPKAKVWLNNSNAGEKFLSRPYGRVDRPKLKRKLLERCAASGVTFLTGKVEGVRHGDGS
Dunaliella      VWPKAKVWLDNDKSGERFLRRPYGRMDRPMLKKLLLQKCASNGVTFLTSKVSGVSHGDGT
M22             GSPNEKNFCGEKKSREKILKRAYGRMDRPMLKKLLLQKCASNGVTFLTSKVDGVSHGGGC
Haematococcus   VWPKASVHLSNKPEGEKFLSRPYGRVDRPRLKSMLLKKCAAHGVTFLEGKVDGVNHGEGR
                   . .   ..     : * *.:.*:**. **  **::* : **.*  .:*.   *. *

Chlamydomonas   SAVKLADGREIRGSLVLDATGHSRRLVQYDKKFDPGFQGAYGIVAEVESHPFALDTMLFM
Volvox          SEVQLADGRKIRGSLVLDATGHSRRLVEYDKKFDPGFQGAYGIVAEVESHPFDIDTMLFM
Coccomyxa       SQITCSDGTRIPGCMVLDATGHARKLVEYDKPFNPGYQGAYGILAEVESHPFPTDAMLFM
Chromochloris   STVSTAEGVSLQGSLVLDATGHTRKLVQFDKKFDPGYQGAYGIIAEVESHPFEVDTMLFM
Dunaliella      STVTLSDGRTMQGTMVLDATGHARKLVKYDQEFNPGYQGAYGITAEVESHPFELDTMLFM
M22             STVSLTDGRTIQGTMVLDATGHARKLVNFDQKFDPGYQGAYGITAKDESHPFELDTMLFM
Haematococcus   SAVSLADGRSIRGSLVLDATGHVRKLIKFDQKFDPGYQGAYGIVAEVESHPFDLETMLFM
                * :  ::*  : * :******* *:*:::*: *:**:****** *: *****  ::****

Chlamydomonas   DWRDDHTQAPGLEAMRAANTALPTFLYAMPFTKNLVFLEETSLVSRPAVDFPELKDRLQA
Volvox          DWRDEHTHGPGLEAMRAANEKLPTFLYAMPFTKNKVFLEETSLVSRPAVGFPELKERLEA
Coccomyxa       DWRDEHTASN--LQMQESNRKLPTFLYAMPFSKTRIFLEETSLVARPAVPFLELKQRLEA
Chromochloris   DWRDEHLASQ--PDMRERNSKLPTFLYAMPFSKTKIFLEETSLVARPAVGFQDLKDRLEA
Dunaliella      DWRDEHTHSN--PVMRASNQALPTFLYAMPYTKNKVFLEETSLVARPAVGFDTLKERLMA
M22             DWRDEHTQSD--PAMRASNEALPTFLYVMPFTKNKVFLEETSLVARPAVGFEELKQRLEA
Haematococcus   DWRDEHTQGN--PAMHAANNALPTFLYAMPFSKTRIFLEETSLVARPAVGFDELKERLDA
                ****:*  .     *:  *  ******.**::*. :********:**** *  **:** *

Chlamydomonas   RLQHLGIKVTNVLEEEYCLIPMGGVLPKHPQRVLAIGGTAGMVHPSTGFMISRMMGAAPT
Volvox          RLKWLGIKVTRVEEEEYCLIPMGGVLPKHPQRVLALGGTAGMVHPSTGFMIYRMMSAAPT
Coccomyxa       RMAKYGIKIKAIEEEEYCLIPMGGVLPRLPQRTLGIGGTAGMVHPSTGYMVARMLGAAPV
Chromochloris   RMKWLGIKVKHIEEEEYCLIPMGGVLPKHPQRVLGIGGTAGMVHPSTGFMVSRMLGVAPT
Dunaliella      RMEWLGIKVTKVEDEEYCLIPMGGALPQHPQRVLGIGGTAGMVHPSTGFMMTRMLGSAPV
M22             RMKWLGIKVKKVEDEEYCLIPMGGVLPQHPQRVLGIGGTAGMVHPSTGFMMTRMLGSAPV
Haematococcus   RLKWLGIKVKAVEEEEYCLIPMGGVLPTHPQRVLGIGGTAGMVHPSTGFMMSRMLGAVPT
                *:   ***:. : :**********.**  ***.*.:************:*: **:. .*.

Chlamydomonas   VADTIVDQLSRPADKASESGAPLRPSSEAEAESMAAAVWAATWPLERVRQRAFFTFGMDV
Volvox          VADAIIDQLS----------CALRPADESEAVSMANAVWRATWPVERVRQRAFFTFGMDV
Coccomyxa       LADAIVEQLCAASDAAAQSHLPPGAVTESDADRLSAAVWHSIWPVERIRQRAFFSFGMDV
Chromochloris   IADAIIDQLSKPADRAADSAVALRPQSETEANNMAAAVWRTAWPVERLRQRAFFCFGMDV
Dunaliella      VADAIVDQLSRPTDKATTAGAARQLVTEQGAEEMAAAVWQAAWPVERIRQRIFMEFGMEV
M22             VADAIIDQLAKPTDKATDSGTSQQPLTEQEAEEMAAAVWRATWPVERIRQRAFFCFGMEM
Haematococcus   IADAIVDQLSAPADKATSLAARK-PGSEAEAEAMSAAVWRAAWPVERIRQRLFNTFGMEL
                :**:*::**.                *  *  :: *** : **:**:*** *  ***::
```

Appendix Figure 11 (Continued)

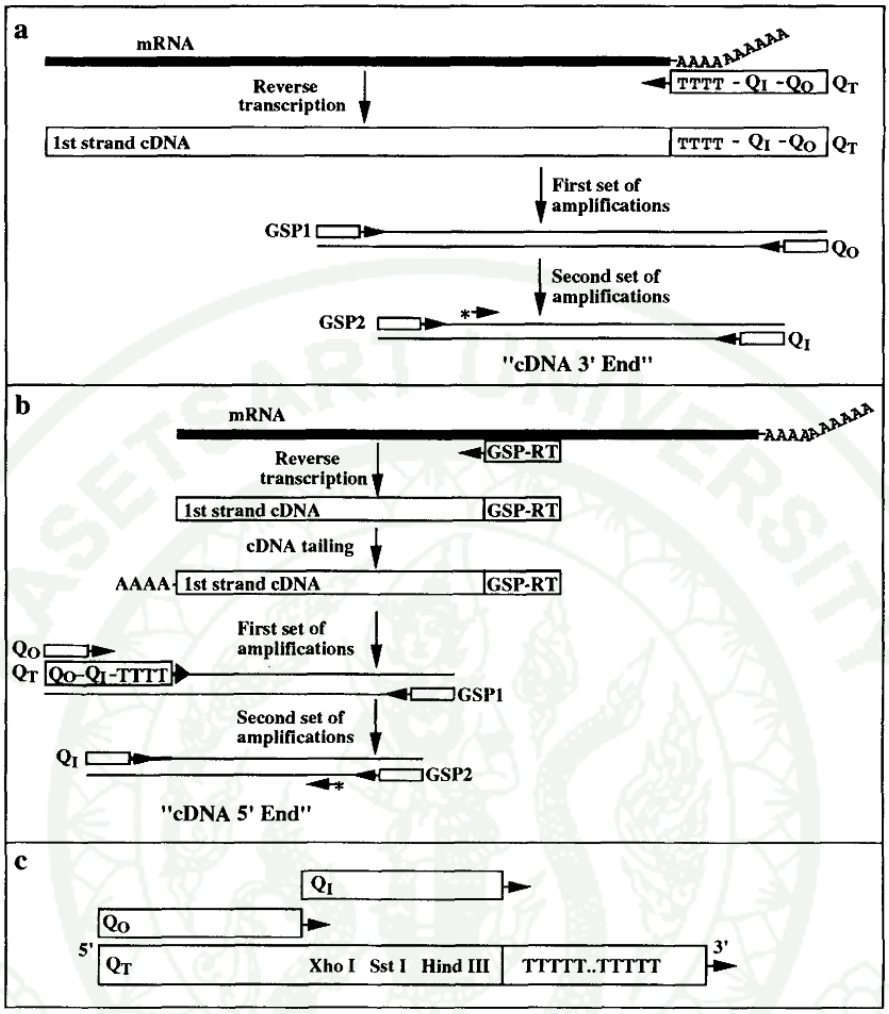
```
Chlamydomonas   LLKLNLPQIREFFRAFFSLSDFHWHGFLSTRLSLPQLIVFGLTLFWKSSNQARASLLQLG
Volvox          LLSLDLYEMREFFRAFFSLSSFHWHGFLSTRLSFPQLIVFGLSLFLKSSNAARSSLLLRG
Coccomyxa       LLSLDLTETRQFFAAFFALSDHHWQGFLSARLSFLELIGFGLSLFAKSSNEARLNLLVKG
Chromochloris   LLRLDLQQTREFFTAFFSLSDFHWHGFLSARLSFPQLIGFGLSLFTKSSNQARINLLAMG
Dunaliella      LLSLNLQQTRDFFAAFFSLSDFHWQGFLSSRLSFTQLIGFGLSLFVEASPETRLLLLRLG
M22             LLTLNLGQMREFFAAFFSLSDFHWQGFLSARLSFTQLIGFGLSLFTSATSDTRLNLLRLG
Haematococcus   LLSLDLQQTRDFFSAFFNLSDFHWHGFLSTRLSFSQLLGFGITLFFKSSNNIRIHLLKLG
                ** *:* : *:** *** **..**:****:***: :*: **::** .::   *  **  *

Chlamydomonas   IPGLVVMLSGLAPTLGGG-YYPDTMSLKERKDAVDAAARSAAAAARAAADVASDAAAFVS
Volvox          IPGLLAMLVELVPTLGGASYYPGMTSLKERKDAVDAAARQAAAATGAAVRQPLLATAGAV
Coccomyxa       VPGLVGMLAGLTGTIGYEKRLLGKKEQIR--------------------------------
Chromochloris   LPGLLSMLAGLAPTLGQYYKIPDGELGSLSKARAQVKS----------------------
Dunaliella      IPGVVQMLFVLLPTVTGYYKQDTTVKDKKFAHDRAAAASISAAKQQ---------------
M22             IPGLIQMLLVLFPTVTGYYKGDLTVRDKKFAHDAAAAAASKLKQPQSPAQPS--------
Haematococcus   VPGLVRILFMLAPTLRGYYKHAPTVRQQKAVADAAAAVAARTAAAAKPADLPPAMIK---
                :**:: :*  *  *:

Chlamydomonas   AN------------SSGADMAVVEVVEKAFSTSNTK--
Volvox          MTGPATAAGMPVGAAAAVPAAVVLEKEKEEEASVTVGN
Coccomyxa       ---------------------------------------
Chromochloris   ---------------------------------------
Dunaliella      ---------------------------------------
M22             ---------------------------------------
Haematococcus   ---------------------------------------
```

Appendix Figure 11 (Continued)



Appendix Figure 12 An illustration of the difference of using new RACE and classic RACE. a) In classic RACE, early termination in the reverse transcription step results in polyadenylation of less than full-length first-strand cDNAs, all of which can be amplified using PCR to generate less than full-length cDNA 5′ ends. (*) cDNA ends created that will be amplified in the subsequent PCR reaction. b) In new RACE, less than full-length cDNAs are also created but are not terminated by the anchor sequence; hence, they cannot be amplified in the subsequent PCR reaction.

Source : Frohman (1994)



Appendix Figure 13 Schematic representation of classic RACE. At each step, the diagram is simplified to illustrate only how the new product formed during the previous step is utilized. (GSP1) Gene-specific primer 1; (GSP2) gene-specific primer 2; (GSP-RT) gene-specific primer used for reverse transcription. a) Amplification of 3′ partial cDNA ends; b) amplification of 5′ partial cDNA ends; c) schematic representation of the primers used in classic RACE. The 52-nucleotide Q_T primer (5′ Q_O-Q_I-TTTT 3′) contains a 17-nucleotide oligo(dT) sequence at the 3′ end followed by a 35-nucleotide sequence encoding HindIII, SstI, and XhoI recognition sites.

Source: Frohman (1994)

CURRICULUM VITAE

NAME : Mr. Thanawat Boontip

BIRTH DATE : February 28, 1989

BIRTH PLACE : Ratchaburi, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE/DIPLOMA
2011	Silpakorn Univ.	B.Sc. (Biotechnology)

POSITION/TITLE : -

WORK PLACE : -

SCHOLARSHIP/AWARDS : -