Genetic Transformation of Green Alga *Haematococcus pluvialis* for the Regulation of Carotenoid Biosynthesis

The Thesis Submitted to the
Department of Studies in Biotechnology of
UNIVERSITY OF MYSORE
In fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in Biotechnology

By

Mr. S. Kathiresan, M.Sc. (Agri).,

Under the supervision of
Dr. R. Sarada Ph.D.,
Scientist

cftri

Plant Cell Biotechnology Department
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
(A constituent Laboratory of Council of Scientific and Industrial Research, New Delhi)
MYSORE - 570 020, INDIA

July 2009

Affectionately Dedicated.....

To My Parents, Relatives, Friends & Teachers

S. Kathiresan, M.Sc. (Agri).,
Senior Research Fellow (CSIR)
Plant Cell Biotechnology Department
Central Food Technological Research Institute
Mysore – 570 020, INDIA

DECLARATION

I **Kathiresan S,** certify that this thesis entitled **"Genetic Transformation of Green Alga *Haematococcus pluvialis* for the Regulation of Carotenoid Biosynthesis"** is the result of research work done by me under the supervision of **Dr. R. Sarada** at Plant Cell Biotechnology Department of Central Food Technological Research Institute, Mysore- 570 020, India during the period 2004 - 2009. I am submitting this thesis for possible award of **Doctor of Philosophy (Ph.D.)** degree in **BIOTECHNOLOGY** of the University of Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree / diploma of this or any other University.

S. Kathiresan
(Candidate)

Place: Mysore

Date:

Dr. R. SARADA,
Scientist
Department of Plant Cell Biotechnology
E-mail: sarada_ravi@yahoo.com
pcbt@cftri.res.in

CERTIFICATE

This is to certify that the thesis entitled **"Genetic Transformation of Green Alga *Haematococcus pluvialis* for the Regulation of Carotenoid Biosynthesis"** submitted by **Mr. S. Kathiresan,** to the **University of Mysore** for the award of the degree of **Doctor of Philosophy** in **Biotechnology**, is the result of work carried out by him in **Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore** under my guidance during the period March 2004 to July 2009.

Place: Mysore
Date:

R. Sarada
(Research Supervisor)

Acknowledgements

I take this opportunity to acknowledge my guide ***Dr. R. Sarada****, Scientist, Plant Cell Biotechnology Department, CFTRI, Mysore for her valuable guidance, constant supervision and co-operation. I am truly indebted to her for helpful attitude and the freedom to pursue my specific goals in the laboratory. For without her efforts, it is unlikely that the thesis would have been completed.*

I wish to express my heartfelt gratitude to ***Dr. V. Prakash****, Director, CFTRI, Mysore, for granting me the opportunity to utilize the excellent facilities available at CFTRI and submit the results of my work in the form of a thesis.*

I am ever grateful to ***Dr. G. A. Ravishankar****, Head, Department of Plant Cell Biotechnology, CFTRI, for his constant encouragement and support during the pursuit of my research work.*

I sincerely thank the, ***Dr. Arun Chandrashekar*** *for his constant encouragement and co-operation during my research work. I also wish to extend my gratitude to* ***Dr. N. Bhagyalakshmi*** *and* ***Dr. M.S. Narayan, Dr. P. Giridhar*** *a lot by way of scientific discussions, who were more than willing to lend their helping hand during the needy hours. I sincerely thank* ***Dr. B.P.R. Narasimha Rao*** *for his constant encouragement and valuable suggestions. My special thanks to the staff of PCBT****, Dr. M. Mahadevaswamy, Dr. T. Rajasekharan,*** *Srinivas Yella, Karuna, Shivanna, Palaksha, and others who have always been helpful.*

I thank my seniors ***Raju, Vinod, Murthy****,* ***Sathya*** *who as senior colleagues helped me when I joined CFTRI. Lovable thanks to all my dear, colleagues and dear friends,* ***Pari, Shakthi, Venki, Vidhya, Sandesh, Sreedhar, Vanitha, Anila, Shibin, Akitha, Lincy, Reeta Ranga, Danny, Guru, Ashwani, Kumudha, Padma, Kavitha, Rama, Mahendra, Lokesh, Harsha, Sridevi*** *and many others for creating friendly surroundings during my stay in this lab..*

I also thank the staff of FOSTIS, CIFS, I&P, HRD, Workshop, Stores & Purchase, Finance and Accounts, Administration, Canteen, Health Centre and Gymkhana for their kind support.

I reverentially express my gratitude towards to all my dear family - ***Appa, Amma, Periyappa Chiththppa, Sangeetha, Manikantan****, and my dear friend* ***Babu Rajendra Prasad*** *and all other relatives whose help and constant encouragement helped me in completing my studies.*

My deepest thanks to my dear lovable wife ***Sheela devi*** *for her unwavering support, patience and understanding throughout my study. Without her moral support, it would have been difficult to accomplish the task.*

I sincerely acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi for a research fellowship, which enabled me to undertake the research project.

Finally I would like to express my thanks to those who have directly or indirectly helped me in this venture.

S. Kathiresan

Abstract

Haematococcus pluvialis is a unicellular green alga which produces a ketocarotenoid, astaxanthin having pharmaceutical and nutraceutical applications owing to its high antioxidant activity. Biotechnological approaches like genetic transformation methods (*Agrobacterium* mediated), cloning strategies, etc are essential to improve/regulate this ketocarotenoid in *H. puvialis*. For the standardization of *Agrobacterium* mediated transformation procedures for algae, preliminary studies like, sensitivity of algae for different antibiotics, media selection for the co-cultivation for both algae and the bacteria were carried out. The sensitivity studies showed that the *H. pluvialis* was able to tolerate up to the concentrations of 2000 mg L^{-1} of antibiotics, cefotaxime and augmentin. Growth and multiplication of the algae was suppressed and ultimately killed when hygromycin concentration exceeded 2 mg L^{-1} in solid media. No growth was observed even after 4 weeks of inoculation when the hygromycin concentration exceeds 2 mg L^{-1}. Among the different cocultivation medium (BBM + half strength of LB medium, Z8 medium + half strength of LB medium, Z8 medium + 0.5% mannitol, Z8 medium only and Tris - acetate - phosphate (TAP)) medium were tested , only the TAP medium favoured the growth of both the alga and *Agrobacterium.* Colonies resistant to hygromycin at 10 mgL^{-1} expressed β-glucuronidase (GUS) and green fluorescence protein (GFP). PCR was used to successfully amplify fragments of the *hpt* (407 bp) and GUS (515 bp) genes from transformed cells while southern blots indicated the integration of hygromycin gene into the genome of *H. pluvialis.* Scanning electron microscopy indicated that the cell wall of *H. pluvialis* was altered on infection with *Agrobacterium*. The transformation achieved here by *Agrobacterium* does not need treatment with acetosyringone or the wounding of cells. The carotenoid profile of the transformed *H. pluvialis* showed no difference between the control cells.

The amplification of β-carotene ketolase (BKT) was observed for the different primers synthesized. No amplification was observed for the any of the BKH primers studied. The 1.8 kb amplicon of BKT gene was cloned to a cloning vector pRT100 in between the CaMV 35S promoter and the poly A region. The β-carotene ketolase gene from cloned plasmid pRT100 was further transferred to a binary vector pCAMBIA1304. Sequence analysis of cloned BKT for nucleotide and amino acid showed 99% similarity of the reported BKT gene accession number D45881. Six exons and five introns were observed for the BKT gene cloned from the *H. pluvialis.* Even though there were few nucleotide

polymorphism, there is no shift in the reading frame. The cloned plasmid pRT100 and pCAMBIA1304-BKT were further confirmed by restriction digestion. Standardized *Agrobacterium* mediated genetic transformation protocol was followed to transform the cloned binary vector having BKT (pCAMBIA1304-BKT) gene to the *H. pluvialis.* Confirmation of the transformation of cloned BKT to the *H. pluvialis* was studied by analyzing the GUS, GFP expression. PCR analysis for the CaMV 35S primers showed 2.5 kb of the BKT gene with the promoter and poly A region. For the BKT forward primer and the CaMV 35S reverse primer also the exact size of amplicon was observed. Southern blotting also showed the difference in the banding pattern of the enzyme digested plasmid and the transformed *H. pluvialis*. No bands were observed for the control cells. Transcript level analysis of the BKT showed 3 to 4 fold higher expression of BKT in transformants than the control cells. The transformed cells were subjected to different stress condition for the induction of secondary carotenoids. Total carotenoids and astaxanthin content in transformed cells showed 2-3 folds higher when compared to the control. Astaxanthin was found to be higher (7.96 mg/g) in sodium acetate (4.4 mM) treated culture followed by sodium acetate 4.4mM and NaCl 0.25% (4.99 mg/g). The intermediates like canthaxanthin and echinenone were the major carotenoids which are present more in the transformants but not in the control *H. pluvialis.* It shows that echinenone and canthaxanthin content were approximately 8 to 10 fold higher in sodium acetate 4.4mM and NaCl 0.25% treated culture. The NaCl + sodium acetate treated culture in transformed cells showed higher level of expression for all the enzymes studied. It was observed that the expression levels of PSY, PDS, LCY, BKT and BKH were 8.8, 5.8, 11.7, 8.1 and 6.4 fold higher respectively when compared to the control green cells. But when compared to the transformed green cells it is 6.1, 1.7, 22.7, 4.0 and 2.9 fold higher respectively.

This first successful *Agrobacterium* mediated transformation in green micro alga *Haematococcus pluvialis* would pave the way for manipulation of many important pathways relevant to food, pharmaceutical and nutraceutical industries. Further the cloned BKT gene from *H. pluvialis* will be used for higher production of carotenoids through transformation in the heterologous host like *Duanliella* sp, *Daucus* sp, *Lycopersicum* sp to regulate the carotenoid biosynthesis. Therefore this study helps in production of higher level of carotenoids through the *Agrobacterium* mediated transformation system in carotenoid producing organisms using the cloned BKT gene.

CONTENTS

Section	Title	Page No
	List contents	vi – viii
	List of Tables	ix - x
	List of Figures	xi - xiii
	List of Abbreviations	xiv - xv
I	Introduction	1 - 4
II	Review of Literature	5 - 45
III Chapter 1	Genetic transformation of *Haematococcus pluvialis* using selectable marker genes	46 - 76
IV Chapter 2	Cloning of genes responsible for enzymes (β-carotene ketolase and β- carotene hydroxylase) and transformation to *Haematococcus pluvialis*	77 - 110
V Chapter 3	Analysis of carotenoid profile in transformants	111 - 135
VI	Summary and Conclusions	136 - 141
VII	Bibliography	142 - 173
VIII	Appendix	174 - 181

List of contents

Legend		Title	Page No.
I	**Introduction**		1
II	**Review of Literature**		
	A1.0	Introduction	5
	A 1.1	Preview for the utilization of algae	6
	A 1.2	Carotenoids	8
	A 1.2.1	General biosynthesis of carotenoids	9
	A 1.3	Astaxanthin	15
	A 1.3.1	Physical and chemical properties of astaxanthin	16
	A 1.3.2	Molecular structure and forms of Astaxanthin	16
	A 1.4	Applications on astaxanthin	17
	A 1.4.1	Astaxanthin as an antioxidant	18
	A 1.4.2	Astaxanthin and eye health	18
	A 1.4.3	Astaxanthin as a human dietary supplement	19
	A 1.4.4	Anticancer Activity	19
	A 1.4.5	Prevention of Cardiovascular Diseases	20
	A1.4.6	Astaxanthin and neurodegenerative diseases	21
	A1.4.7	Astaxanthin effect against *Helicobacter pylori* infections	21
	A1.4.8	Astaxanthin as a modulator of the immunological system	21
	A1.4.9	Astaxanthin in aquaculture	22
	A1.4.10	Additional Benefits	23
	A1.4.11	Industrial applications of astaxanthin	24
	A1.5	Types of Astaxanthin	25
	A1.5.1	Synthetic astaxanthin	25
	A1.5.2	Astaxanthin in nature	25
	A1.5.3	Current market status of astaxanthin	27
	A1.6	*Haematococcus pluvialis*	29
	A1.6.1	Classification	29
	A1.6.2	History and Distribution of *Haematococcus pluvialis*	29
	A1.6.3	General biology, ultrastructure and life cycle	30
	A1.6.4	Biosynthesis of secondary carotenoids especially astaxanthin in *Haematococcus pluvialis*	32
	A1.7	Carotenogenesis	34
	A1.7.1	Regulation of Carotenoid biosynthesis	34
	A1.8	Genetic Engineering	38
	A1.8 .1	Metabolic Engineering of Astaxanthin Biosynthesis	39
	A1.9	Genetic transformation in algae	40
	A1.9.1	Methods to introduce DNA into algal cells	43
	A1.10	Safety of *Haematococcus pluvialis* astaxanthin	44

III Chapter 1

	Genetic transformation of *Haematococcus pluvialis* using selectable marker genes	46
1.0	Introduction	47
1.1	Materials and Methods	48
1.2	Methodolgy	49
1.2.1	Maintenance of stock culture	49
1.2.2	Growth condition for *H. pluvialis*	51
1.2.3	Sensitivity test for antibiotics	51
1.2.4	Cocultivation media for *Agrobacterium* and *H. pluvialis*	52
1.3	*Agrobacterium* mediated genetic transformation	52
1.4	Confirmation of Transformation	55
1.4.1	Growth of hygromycin resistant cells	55
1.4.2	GUS Assay	56
1.4.3	Detection of GFP	56
1.4.4	Scanning Electron Microscopy	57
1.4.5	Stability analysis	57
1.5	Molecular confirmation	57
1.5.1	Genomic DNA isolation and detection by PCR	57
1.5.2	Southern blotting analysis	58
1.6	Growth measurement and pigment extraction from transformanted *H. pluvialis*	59
1.7	Results	61
1.7.1	Growth in cocultivation medium	61
1.7.2	Sensitivity for antibiotics and selection of resistant colonies of *H. pluvialis*	62
1.8	Detection of reporter genes	67
1.9	Scanning Electron Microscopy	69
1.10	Molecular Confirmation	69
1.11	Analysis of astaxanthin in transformants and control *H. pluvialis*	72
1.12	Discussion	73

IV Chapter 2

	Cloning of genes responsible for enzymes (β-carotene ketolase and β- carotene hydroxylase) and transformation to *Haematococcus pluvialis*	77
2.0	Introduction	78
2.1	Materials and Methods	79
2.1.2	DNA and RNA extraction	79
2.1.3	Isolation and cloning of astaxanthin biosynthetic genes	79
2.1.4	Designing and synthesis of primers for BKT and BKH genes	80

2.1.5	PCR amplification of the BKT and BKH gene	82
2.1.6	Cloning	82
2.1.7	Transformation	89
2.2	Results	91
2.2.1	Isolation of BKT and BKH genes from *H. pluvialis*	91
2.2.1.2	Amplification of BKT gene from genomic DNA of *H. pluvialis*	92
2.2.1.3	Amplification of BKH gene from total RNA	92
2.2.1.4	Cloning of BKT to the cloning vector pRT100	94
2.2.2	Confirmation of cloning	95
2.2.2.3	Sequence result of the genomic BKT in cloned pRT100	96
2.2.3	Sub-cloning of the cloned BKT from pRT100 to pCAMBIA1304	106
2.3	Discussion	108

V Chapter 3

	Analysis of carotenoid profile in transformants	**111**
3.0	Introduction	112
3.1	Materials and Methods	113
3.2	Methodolgy	113
3.2.1	Plasmid constructs and bacterial strains	113
3.2.3	*Agrobacterium* mediated genetic transformation	113
3.3	Confirmation of Transformation	113
3.3.1	Molecular confirmation	113
3.4	Growth measurement and pigment extraction from transformed *H. pluvialis*	114
3.5	Expression analysis of carotenoid biosynthetic genes	116
3.5.1	RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)	116
3.6	Experimental design and data analysis	117
3.7	Results	118
3.7.1	Growth in cocultivation medium	118
3.7.2	Confirmation of the transformants	118
3.7.3	Carotenoid analysis under different stress conditions	123
3.7.4	Effect of inhibitors on control cells and Transformed cells	129
3.7.5	Expression analysis of carotenoid biosynthetic genes	131
3.8	Discussion	133
VI	**Summary and conclusions**	136
VII	**Bibliography**	142
VIII	**Appendix**	174

LIST OF TABLES

Table No.	Title	Page No
A1.1	Important Algal species for production of high value metabolites of biological significance	8
A1.2	Major carotenoids of biotechnological importance from microlgal sources	12
A1.3	Compared effectiveness of astaxanthin versus Vitamin E and other carotenoids	14
A1.4	Natural sources of astaxanthin	26
A1.5	Some major producers of natural astaxanthin from *H. pluvialis* and their merchandise brand name	28
A1.6	Percentage of astaxanthin under different nutrient condition	36
A1.7	Environmental factors affecting astaxanthin over accumulation in the alga *H. pluvialis*.	36
A1.8	Comparison of the regulation of all known enzymes of the carotenoid biosynthesis pathway of the alga *H. pluvialis*	37
A1.9	Transformable algal species. Nuclear transformation unless otherwise noted	42
1.1.	Composition of media for *H. pluvialis* growth	50
1.2.	Composition of trace elements for *H. pluvialis* growth	50
1.3	Composition of TAP Media	51
1.4	Composition of hunters trace elements	51
1.5.	Growth of both *Haematococcus pluvialis* and *Agrobacterium tumefaciens* in different cocultivation medium	61
1.6.	Growth of control and cocultivated *H. pluvialis* in selection medium using different concentration of hygromycin	62
1.7.	Growth of cocultivated *H. pluvialis* in selection medium (solid) using different concentration of hygromycin	64
1.8	Transformation frequency of *H. pluvialis*	65
1.9.	Growth of cocultivated *H. pluvialis* and control cells in selection medium having different concentration of hygromycin	66
1.10.	Growth of transformed and control *H. pluvialis* in liquid selection medium at different inoculum density	67

2.1	Primers used for the of amplification of BKT	80
2.2	Primers used for the of amplification of BKH	80
3.1	Specific primers, annealing temperatures, and total numbers of amplification cycles used for RT-PCR	117
3.2	Amplicon size of the BKT from transformed *H. pluvialis* and recombinant pCAMBIA1304 using different combinations	121
3.3	Amount of total chlorophyll and total carotenoids in of both control and transformed *H. pluvialis*	124
3.4	Effect of different inhibitors in the transformed and control *H. pluvialis* for chlorophyll and carotenoid production	129

LIST OF FIGURES

Fig. No.	Title	Page no
A1.1	General biosynthesis pathways of carotenoids.	10
A1.2	Enzymes and genes in the carotenoids biosynthesis of plants and algae	11
A1.3.	Chemical structure of astaxanthin molecules and other carotenoids	13
A1.4	Isomers of astaxanthin	17
A1.5	Life cycle of *H. pluvialis*	31
A1.6	General biosynthetic pathway of astaxanthin	33
A1.7	Regulation of astaxanthin overaccumulation in *H. pluvialis*	35
1.1	*H. Pluvialis* stock culture	51
1.2	Linear map of the T-DNA region of the binary vector pSK 53 and circular map of pCAMBIA1301	53
1.3	Schematic illustration showing co-cultivation of Agrobacterium tumefaciens and *H. pluvialis*	55
1.4	Different stages of the growth of transformed *H. pluvialis* in selection medium after co-cultivation	63
1.5.	Different stages of the growth of control *H. pluvialis* in medium without hygromycin	63
1.6	Growth of *H. pluvialis* at different concentration of hygromycin containing liquid medium	66
1.7	Fluorescent microscopic observation of non-transformed and transformed cells of *H. pluvialis*	68
1.8	Microscopic observations of the non-transformed *H. pluvialis* for GUS analysis	68
1.9	Microscopic observations of the transformed *H. pluvialis* for GUS analysis	68
1.10	Scanning electron microscopic photograph of the control and co-cultivated *H. pluvialis*	69
1.11	PCR analysis for hpt gene from *H. pluvialis*	70
1.12	PCR analysis for GUS gene from *H.pluvialis*	71
1.13	Southern blot analysis *H. pluvialis* DNA	71
1.14.	HPLC profile of the carotenoid extracts from control (A) and transformed *H. pluvialis*	72
1.15	Astaxanthin percentage of the control and transformed *H. pluvialis*	73
2.1	Flowchart showing the cloning procedure of BKT from genomic DNA of H. pluvialis to a pRT100	83
2.2	pRT100 vector map with promoter and poly A	85

2.3	Flowchart showing the cloning of BKT gene from pRT100 to binary vector pCAMBIA1304	87
2.4	Binary vector pCAMBIA1304	88
2.5	Total RNA isolated from *H. pluvialis*	91
2.6	Amplification of BKT gene from total RNA of *H. pluvialis* through RT-PCR	91
2.7	Amplification BKT gene from genomic DNA of *H. pluvialis*	92
2.8	Amplification of BKH gene from total RNA of *H. pluvialis* through RT-PCR	93
2.9	Amplification of BKH gene from genomic DNA of *H. pluvialis*	93
2.10	Amplification of BKT gene from genomic DNA of *H. pluvialis* using BKT-A primer	94
2.11	Plasmid pRT100 extracted from *E. coli*	94
2.12	Plasmid pRT100 double digested with *XhoI* and *XbaI*	95
2.13	Gel photograph showing the cloned and wild plasmid pRT100	95
2.14	Gel photograph of the *HindIII* digested wild and cloned pRT100	96
2.15	Gene sequence of the cloned BKT from pRT100	97
2.16	Dialign output of the cloned BKT (genomic) with the gene sequence ID D45881	102
2.17	Dialign output of the cloned BKT (cDNA) with the cDNA gene sequence ID D45881	104
2.18	Dialign output of the aminoacid sequence of the cloned BKT	105
2.19	PCR amplification of the BKT gene from recombinant pRT100	105
2.20	Plasmid extracted from the wild and recombinant binary vector pCAMBIA1304	106
2.21	Restricted digested wild pCAMBIA1304 and cloned pCAMBIA1034-BKT with *HindIII*	107
3.1	Growth of control and BKT transformants in the TAP medium	118
3.2	GFP observation in control and Transformed *H. pluvialis*	119
3.3	GUS assay for the control and Transformed *H. pluvialis*	119
3.4	Scanning electron microscopic photograph of the control and co-cultivated *H. pluvialis* with pCAMBIA 1304-BKT	120
3.5	PCR Amplification of the BKT gene cloned using different primer combinations	121
3.6	Southern blot analysis of the BKT transformed *H. pluvialis*	122

	and pCAMBIA1304-BKT	
3.7	RT-PCR analysis for the *H. pluvialis*	123
3.8	Amplification intensity of the BKT from the transformed cells and control cells of *H. pluvialis*	123
3.9	Effect of different stress condition on control and Transformed cells	125
3.10	HPLC profile of the carotenoid extracts from control and BKT transformed *H. pluvialis*	126
3.11	Effect of different stress in control and transformed cells	127
3.12	Effect of inhibitors on control cells and Transformed cells	130
3.13	Effect of inhibitors on control cells and Transformed cells	130
3.14	Expression of carotenoid biosynthetic genes in *H. pluvialis* under stress conditions	131
3.15	Expression of carotenoid biosynthetic genes in *H. pluvialis* under stress conditions	132

LIST OF ABBREVIATIONS

%	Percent
α	alfa
β	beta
µ	micro
µg	Microgram
µM	Micromolar
^{0}C	Degree centigrade
3’	Hydroxyl- terminus of DNA molecule
5’	Phosphate-terminus of DNA molecule
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cDNA	Complementary Deoxyribonucleic Acid
Chl	chlorophyll
cm	Centimeter
D. bardawil	*Dunaliella bardawil*
D. salina	*Dunaliella salina*
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DNA	: Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double strand Deoxyribonucleic Acid
DW	Dry weight
EDTA	Ethylene diamine tetra acetic acid
FW	Fresh weight
g	Gram
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
hrs	Hours
IPTG	Isopropyl- β̃-D-thiogalactopyranoside
kb	Kilobase
kg	Kilogram
Klux	Kilolux
L	Litre
LB	Luria- Bertani (medium)
M	Molar
mg	Milli gram
min	Minute(s)
ml	Millilitre
mM	Millimolar
mRNA	messenger RNA
NBT	Nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information

OD	Optical Density
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Revolution per minutes
rRNA	:ribosomal RNA
RT	Reverse transcription
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SC	secondary carotenoid
SD	Standard deviation
SD	Standard deviation
SE	Standard error
SEM	Scanning Electron Microscopy
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TE	Tris-EDTA buffer
TLC	Thin Layer Chromatography
Tris	Tris (hydroxymethyl) amino methane
UV	Ultra Violet
w/v	Weight per volume
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	Micro gram
µl	Micro litre
µM	Micro molar

Introduction

Introduction

In recent years microalgae (green algae) are realized for their potential for production of high value compounds such as carotenoids and compounds of nutraceutical and pharmaceutical importance. They produce wide spectrum of carotenoids viz., lutein, zeaxanthin, astaxanthin, canthaxanthin and β-carotene. Among the commercially important carotenoids, astaxanthin has the potential clinical application in human health, because of its higher antioxidant capacity compared to β-carotene and vitamin-E (α-tocopherol). Apart from this, it is mainly used as feed supplement in marine fish aquaculture and as a pigment source for egg yolks. Its beneficial effects are reduction of gastric inflammation and bacterial load in *Helicobacter pylorii* infected mice and human, prevention of age related macular degeneration and several other degenerative diseases (Pulz and Gross 2004; Higuera-Ciapara et al. 2006; Guerin et al. 2003).

Astaxanthin, a keto carotenoid produced in limited number of organisms like *Agrobacterium auranticum*, *Phaffia rhodozyma*, *Haematococcus pluvialis* a green alga, *Adonis aestivalis* and *Viola tricolor* (plants) etc. *Haematococcus pluvialis* is a potent organism which accumulates highest astaxanthin content (2-3% of dry weight) and it is one of the most promising sources for production of natural astaxanthin (Johnson and An 1991). There is growing interest in the commercial exploitation of green alga such as *Haematococcus* for production of astaxanthin.

During the past ten years the biosynthetic pathway of astaxanthin and genes involved have been well studied in *Haematococcus pluvialis*, but the molecular regulatory mechanism of astaxanthin biosynthesis has not been investigated intensively. Also, efforts have been focused to elevate the astaxanthin from available species through manipulation of cultural conditions, mutation, stimulate astaxanthin production by the addition of specific compounds or precursors of carotenogenesis. Strain improvement aiming to optimize astaxanthin production on an industrial scale has not received much attention. Little work has been done so far in identification of enzymes and cloning of genes involved in the carotenoid biosynthesis leading to their increased production. In contrast to the large number of genetically modified bacteria, yeast and even higher plants, only few species of microalgae have been successfully transformed with efficiency. Therefore there is a need to study the regulatory

mechanism at molecular level using genetic manipulation methods leading to enhanced astaxanthin production. Efficient genetic transformation system in *H. pluvialis is* therefore necessary to enhance its potential and utility.

Among the transformation protocols in green alga *Chlamydomonas reinhardtii,* the use of particle bombardment, glass beads and *Agrobacterium* mediated transformation, the *Agrobacterium* mediated genetic transformation has its own advantage. Different common transformation methodology, Electroporation and particle bombardment are also reported in *Chlorella* sp and *Dunaliella* sp. The transformation frequency and the stability of the gene in subsequent generations have been the limiting factors. In *H. pluvialis* also, the transformation was achieved by using particle bombardment. The recent developments in algal transformations suggest the possibility of using *Agrobacterium tumefaciens* for delivering desired traits in microalgae. Since the stability of the transgene and frequency of transformation is higher in *Agrobacterium tumefaciens* mediated genetic transformation, the study mainly focused on the *Agrobacterium tumefaciens* mediated transformation in *H. pluvialis* for expression of marker, reporter genes. Subsequent studies were made to transform with genes responsible for the enzymes in astaxanthin biosynthesis to regulate the carotenoid level especially astaxanthin.

For genetic transformation studies for the regulation of carotenoid levels in *H. pluvialis*, basic techniques of *Agrobacterium* mediated genetic transformation is necessary. At present, no appropriate genetic tools are available for *H. pluvialis*. Therefore the present study was undertaken to standardize the *Agrobacterium* mediated transformation protocols in *H. pluvialis* and further cloning of β-carotene ketolase and β-carotene hydroxylase gene for the expression of astaxanthin. Since the alga belongs to the order chlorophyta, this transformation system/protocol will be very much useful for the other algal systems which produce the high value compounds. Further the cloned genes from *H. pluvialis* will be useful for the regulation of the carotenoid biosynthesis in the species which produces high level of β-carotene (such as *Dunaliella* sp). With this background knowledge, the objectives of the present research work were laid as follows:

Objectives

1. Genetic transformation of *H. pluvialis* using selectable marker genes
2. Cloning of genes responsible for enzymes (β-carotene ketolase and β-carotene hydroxylase) involved in carotenoid biosynthesis and their expression in *H. pluvialis.*

The results of the research work done systematically on the above objectives are compiled in the thesis, as Introduction, review of literature, results in Chapter I, Chapter II, Chapter III, summary and conclusions and finally the references. The chapters are as follows

Chapter I **:** Genetic transformation of *H. pluvialis* using selectable marker genes.

Chapter II **:** Cloning of genes responsible for enzymes (β-carotene ketolase and β- carotene hydroxylase) and transformation to *H. pluvialis.*

Chapter III **:** Analysis of carotenoid profile in transformants.

Review of Literature

A 1.0 Introduction

Algae are remarkably diverse and fascinating group of organisms that are of fundamental ecological importance as primary producers and as basic components of food chain. They are accountable for the net primary production of ~52,000,000,000 tons of organic carbon per year, which is ~50% of the total organic carbon produced on earth each year (Field et al. 1998). They are also of commercial importance in food industry, aquaculture and as a natural source of high value products such as carotenoids, long chain polyunsaturated fatty acids, and phycocolloids (Apt and Behrens 1999; Tseng 2001). The diversified traits and living conditions of algae make them extremely attractive for commercial utilization particularly if the desired candidate alga is accessible to genetic manipulation. Because algal transgenics and bio-technology primarily utilizes small, lab-suited species, genetic engineering of algae, both prokaryotic (Koksharova and Wolk 2002; Vioque 2007) and eukaryotic (Pulz 2001; McHugh 2003; Olaizola 2003; Franklin and Mayfield 2004; León-Banares et al. 2004; Pulz and Gross 2004; Ball 2005; Grossman 2005; Montsant et al. 2005; Qin et al. 2005; Walker et al. 2005a, 2005b; Chan et al.2006; Spolaore et al. 2006) is one of the most efficient biotechnological approach to make this diverse group to exploit their potential for high value compounds to commercialize for the benefits of mankind.

A 1.1 Preview for the utilization of algae

The first traceable use of microalgae by humans dates back to 2000 years. The Chinese, who used *Nostoc* to survive during famine (Spolaore et al. 2006). The use of macroalgae as food has been traced back to the fourth century in Japan and the sixth century in China (McHugh 2003). The first report on collection of a macroalga, "nori", i.e. algae of the genus *Porphyra*, dates back to the year 530 AD. The first known documentation of cultivation of this alga occured in 1640 (Pulz and Gross 2004). At about the same time, in the year 1658, people in Japan started to process collected Chondrus, Gelidium, and Gracilaria species to produce an agar-like product (Pulz and Gross 2004). In the eighteenth century, iodine and soda were extracted from brown algae, like Laminaria, Macrocystis and Fucus. In the 1860s, Alfred Nobel invented dynamite by using diatomaceous earth (diatomite), which consists of the fossil silica cell walls of diatoms, to stabilize and absorb nitroglycerine into a portable stick (Dolley and Moyle 2003). So dynamite, in all respects, one of the most effective algal products.

In the 1940s, microalgae became more and more important as live feeds in aquaculture (shellfish or fish farming). After 1948, applied algology developed rapidly, starting in Germany and extending into the USA, Israel, Japan, and Italy, with the aim of using algal biomass for producing protein and fat as a nutrition source (Burlew 1953). In addition, in the 1970s, the first large-scale *Spirulina* production plant was established in Mexico (Borowitzka 1999). In the 1980s, there were already 46 large-scale algae production plants in Asia mainly producing *Chlorella*. Large scale production of Cyanobacteria (*Spirulina*) began in India, and large commercial production facilities in the USA and Israel started to process the halophilic green alga *Dunaliella salina* as a source of β-carotene (Spolaore et al. 2006). In the 1980s, the use of microalgae as a source of common and fine chemicals was the beginning of a new trend (De la Noue and De Pauw 1988). In the 1990s in the USA, few plants were started with large-scale production of *H. pluvialis* as a source of the carotenoid astaxanthin, which is used in pharmaceuticals, nutraceuticals, agriculture, and animal nutrition (Olaizola 2000; Spolaore et al. 2006).

Nowadays about 10^7 tons of algae are harvested each year by algal biotechnological industries through non-transgenic, commercial algal biotechnology for several applications to the mankind viz., human nutrition, animal feed, aquaculture, production of chemicals and pharmaceuticals, pigments, polysaccharides, fatty acids, biomass, diatomite, fertilizers, cosmetics, fuel etc (Pulz and Gross 2004; Grossman 2005). But recent progress in algal transgenics promises a much broader field of application: molecular farming, the production of proteins or metabolites that are valuable to pharmaceutical, seems to be feasible with transgenic algal systems. Indeed, the ability of transgenic algae to produce recombinant antibodies, vaccines, insecticidal proteins, or bio-hydrogen has already been demonstrated (Hallmann 2007). Some of the commercially important algal species and their importance as a valuable product have been summarized in Table 1.1 The major biologically active constituents present in algae belong to the following groups.

Table A1.1 Important Algal species for production of high value metabolites of biological significance

Algal sp	Compounds of Interest	Reference
Haematococcus pluvialis	Astaxanthin	Del campo et al. 2007; Higuera-Ciapara et al. 2006
Dunaliella sp	β-carotene, Glycerol,	Del campo et al. 2007;
	Protein	Ben-Amotz and Avron 1990
Spirulina	High protein, Essential amino acids, vitamin B complex and E, Gamma linolenic acid, β-carotene, Phycocyanin, Chlorophyll	Becker 2007; Becker and Venkataraman, 1982
Botryococcus	Hydrocarbon	Banerjee et al 2002; Dayanand et al. 2005
Porphyridium	Phycoerythrin	Dufosse et al. 2005; Kathiresan et al. 2007
Chlorella	Lutein, Protein, minerals	Becker and Venkataraman, 1982
Scenedesmus	Protein, Essential amino acids	Becker and Venkataraman, 1982
Kappaphycus	Protein, iron, α-tocopherol, ascorbic acid, β - carotene	Moore et al. 1988 Fayaz et al. 2005
Enteromorpha	Protein, ascorbic acid, Iron	Moore et al. 1988
Porphyra	Protein, Essential amino acids, vitamins	Moore et al. 1988

1.2 Carotenoids

Carotenoids are 40-carbon isoprenoid organic pigments that are naturally occurring in plants and other photosynthetic organisms like algae, some types of fungi and bacteria (Ikan 1991; Mastuno and Hirao 1989). There are over 600 known carotenoids, which are split into two classes, xanthophylls and carotenes (Cunningham and Gantt 1998). Carotenes are made up of carbon and hydrogen, without the oxygen group are collectively called as carotenes; e.g., lycopene, α-carotene, and β-carotene. Carotenoids with molecules containing oxygen, such as lutein, zeaxanthin, cryptoxanthin, β-cryptoxanthin and **astaxanthin**, are known as xanthophylls (Cunningham 2002; Hirschberg 2001). Carotenoids are synthesized de novo by higher plants, algae, mosses, liverworts, photosynthetic and non-photosynthetic bacteria and fungi (Del campo et al. 2007; Armstrong 1994).

A1.2.1 General biosynthesis of carotenoids

Biosynthesis of carotenoids are shown in Figure 1.1 Plants use both the methylerythritol phosphate pathway (MEP) and the mevalonic acid (MVA) pathway for isoprenoid biosynthesis, although they are localized in different compartments. The MEP pathway synthesizes isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) in plastids, whereas the MVA pathway produces cytosolic IPP. Mitochondrial isoprenoids are synthesized from MVA-derived IPP that is imported from the cytosol. Some exchange of IPP or a common downstream intermediate does also appear to take place between the plastids and the cytoplasm (Lichtenthaler et al. 1997; Lichtenthaler 1999; Rohmer 1999; Hirschberg et al. 1997). Plant carotenoids have been classified as primary or secondary carotenoids. Primary carotenoids are the compounds required by plants in photosynthesis and function within the photosynthetic machinery (neoxanthin, violaxanthin, lutein, zeaxanthin and β-carotene). Secondary carotenoids are those carotenoids that are not exclusively required for photosynthesis and are not localized in the thylakoid membranes of the chloroplast. Many genes and enzymes are involved in the biosynthesis of primary and secondary carotenoids in plants and algae (Fig. 1.2). They almost exclusively accumulate under stress conditions (e.g. nutrient starvation, salinity, high temperature etc.) and this process is species specific. The physiological function of secondary carotenoid remains to be clarified (Boussiba 2000). However, it is generally believed that they function as passive photoprotectants (i.e., as a filter) reducing the amount of light which can reach the light-harvesting pigment complex of PSII (Hagen et al. 1994).

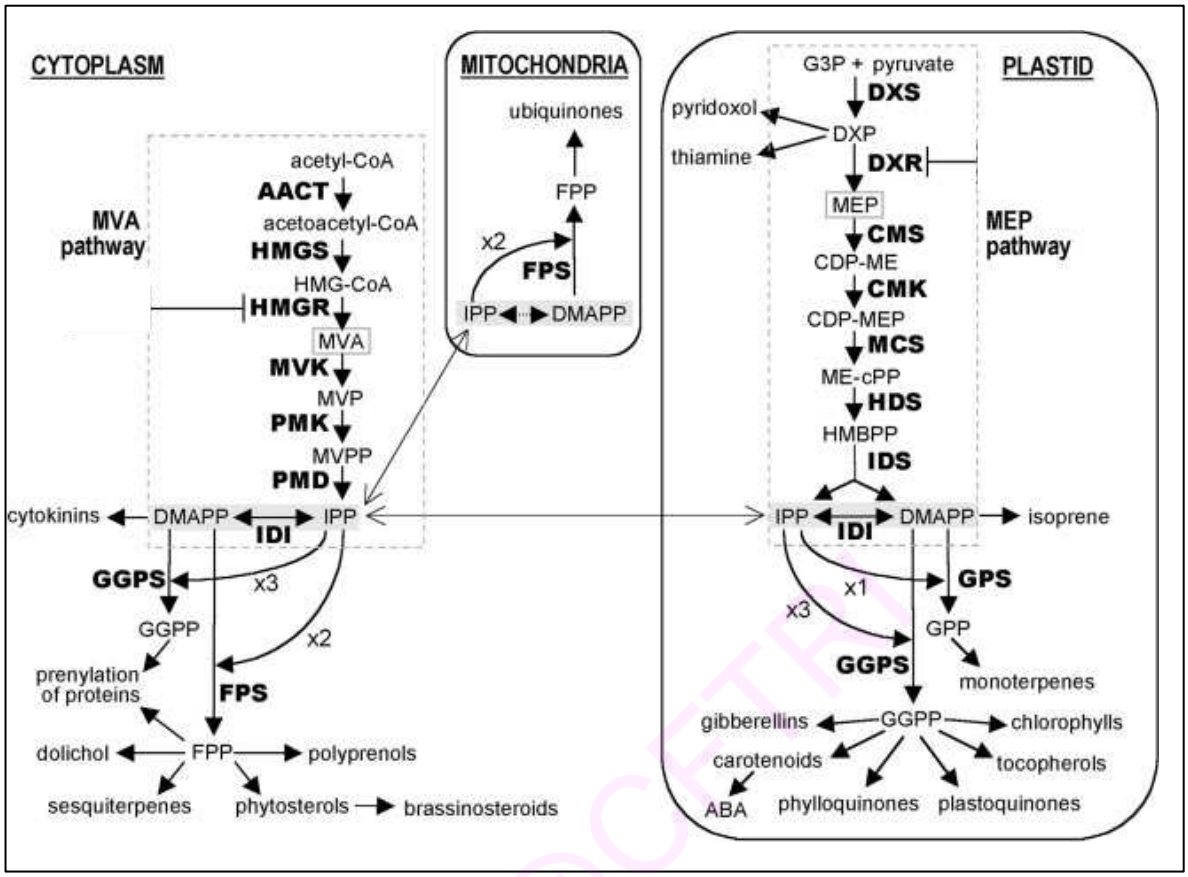


Figure A1.1 General biosynthesis pathways of carotenoids. HMG-CoA, Hydroxymethylglutaryl CoA; MVP, 5-phosphomevalonate; MVPP, 5-diphosphomevalonate; HBMPP,hydroxymethylbutenyl 4-diphosphate; FPP, farnesyl diphosphate; ABA, abscisic acid. The first intermediate specific to each pathway is boxed. Enzymes are indicated in bold: **AACT**, acetoacetyl CoA thiolase; **HMGS**, HMG-CoA synthase; **HMGR**, HMG-CoA reductase; **MVK**, MVA kinase; **PMK**, MVP kinase; **PMD**, MVPP decarboxylase; **IDI**, IPP isomerase; **GPS**, GPP synthase; **FPS**, FPP synthase; **GGPS**, GGPP synthase; **DXS**; **DXR**, DXP reductoisomerase; **CMS**; **CMK**; **MCS**; **HDS**; **IDS**, IPP/DMAPP synthase. (Adopted from Rodríguez-Concepción and Boronat 2002)

The total carotenoid production in nature has been estimated to be approximately 100 million tonnes per annum by all the living organisms (Krinsky and Johnson 2005). All the carotenoids in photosynthetic tissues are located in the grana of the chloroplast and consist of the same major group of pigments. Major ones are β -carotene, lutein, violaxanthin and neoxanthin and smaller amount of δ -carotene, δ-cryptoxanthin, zeaxanthin, astaxanthin and antheraxanthin (Ladygin 2000).

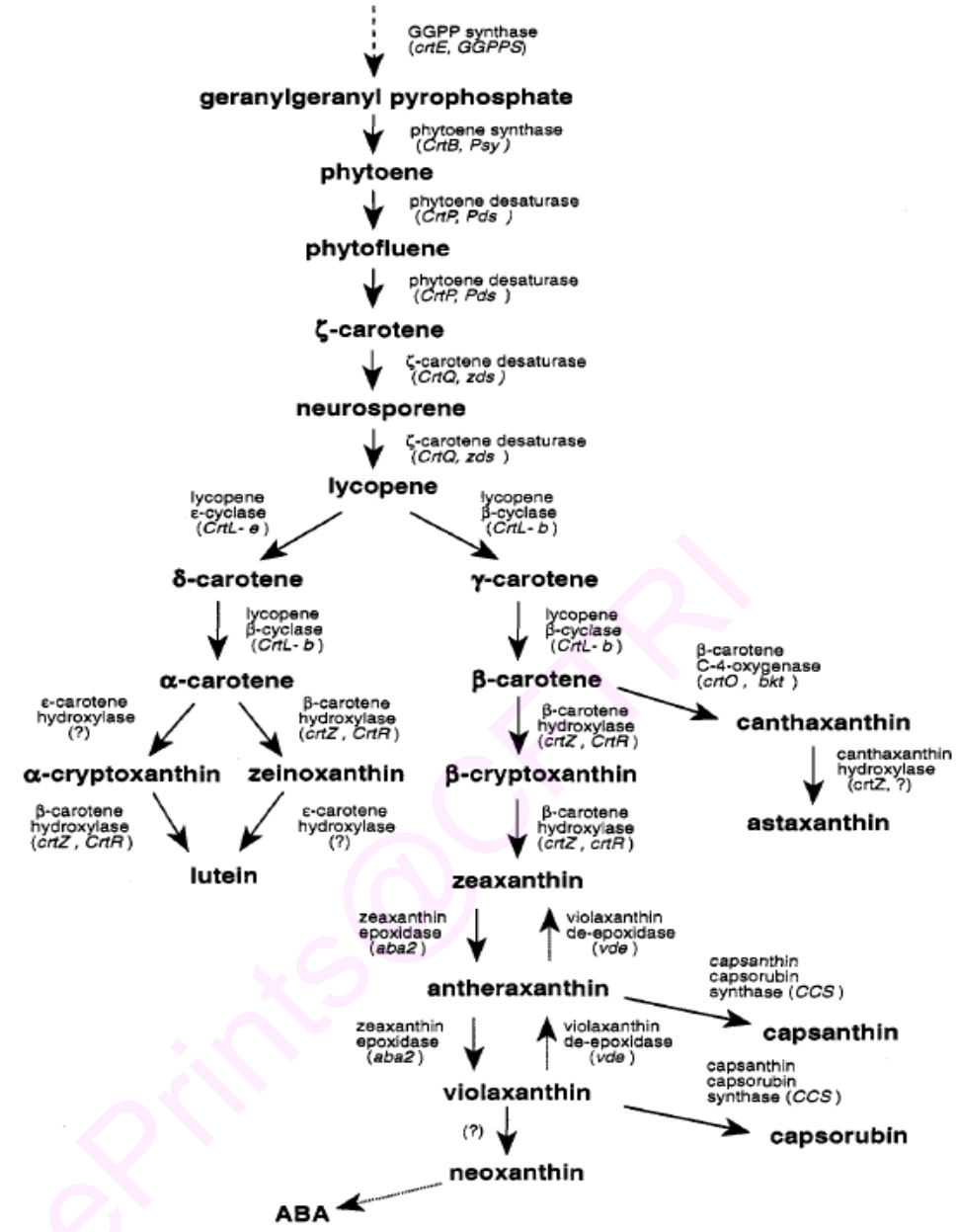


Figure A1.2 Enzymes and genes in the carotenoids biosynthesis of plants and algae. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCY-B, lycopene β-cyclase; LCY-E, lycopene ε-cyclase; CRTR-B, β-carotene hydroxylase; CRTR-E, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, Violaxanthin de-epoxidase; NXS, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase.

The major carotenoids of biotechnological importance from microbial sources are listed in Table 1.2 and their chemical structures are shown in Figure 1.3 They are a group of molecules which are responsible for diverse functions, ranging from their original evolutionary role as photosynthetic or light quenching pigments to

antioxidants, precursors of vitamin A, or pigments involved in the visual attraction of animals such as flower pollinators (Johnson and Schroeder 1995).

Table A1.2 Major carotenoids of biotechnological importance from microlgal sources

Carotenoids	Sources	Yield	References
Astaxanthin	*Haematococcus pluvialis* *Chlorella zofingiensis*	30 mg/g <1 mg/g	Lorenz and Cysewski 2000 Ip and Chen 2005
Lutein	*Chlorella zofingiensis* *Chlorella protothecoides* *Muriellopsis sp.*	21 μg/ml 225 μg/ml l35 μg/ml	Del Campo et al. 2004 Shi et al. 1999 Del Campo et al. 2001
Zeaxanthin	*Dunaliella salina* *Microcystis aeruginosa* *Nannochloropsis*	6 mg/g Not reported Not reported	Jin et al. 2003 Chen et al. 2005 Lee et al. 2006
β-**Carotene**	*Dunaliella salina* *Dunaliella bardawil*	100mg/g >100mg/g	Garcia-Gonzalez et al. 2005 Lers et al. 1990
Canthaxanthin	*Chlorella emersonii*	0.6 μg/ml	Arad et al. 1993

Modified from Bhosale and Bernstein (2005).

Carotenoids are essential components of the photosynthetic apparatus. In the chloroplast they function in the protection against photo-oxidative damage and participate in the light harvesting process (Demmig-Adams et al. 1996). They play a key role in oxygenic photosynthesis, as accessory pigments for harvesting light or as structural molecules to stabilize protein folding in the photosynthetic apparatus (Siefermann-Harms 1987). Some carotenoids have protective functions, either as direct quenchers of reactive oxygen species (Edge et al. 1997) or playing a role in the thermal dissipation of excess energy in the photosynthetic apparatus (Havaux and Niyogi 1999). carotenoids, due to their high antioxidant properties which has been reported to surpass those of β-carotene or even α-tocopherol (Miki 1991). Due to its high antioxidant activity it has been attributed with extraordinary potential for protecting the organisms against a wide range of diseases such as cardiovascular problems, different types of cancer and immunological system. This has stirred great interest in astaxanthin and prompted numerous research studies concerning its

potential benefits to humans and animals. Carotenoids form an important group of colorant too. In certain non-photosynthetic organs of higher plants, carotenoids accumulate in large amounts in chromoplasts and lead to the bright colours of many flowers and fruits (Hirschberg 2001).

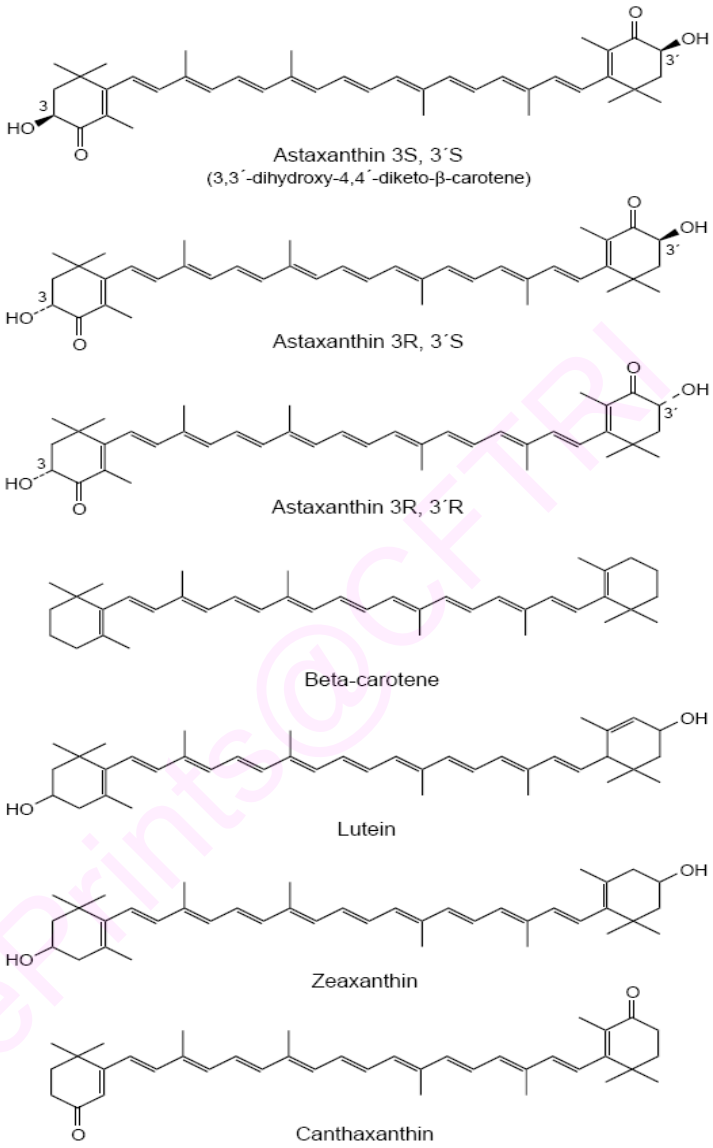


Figure A1.3. Chemical structure of astaxanthin molecules and other carotenoids (Urich 1994).

Table A1.3 Comparative effectiveness of astaxanthin versus Vitamin E and other carotenoids

Astaxanthin effectiveness	Compared to	Test	References
87 X better	Vitamin E	Quenching singlet oxygen from the $NDPO_2$ dissociation in a chemical solution	DiMascio et al. 1990
100-50 X better	Vitamin E	Inhibit Fe^{2+}-induced lipid peroxidation of mitochondria of liver cells from	Kurashige et al. 1990
Better	Vitamin E	Vitamin E deficient rats	
80 times better	Vitamin E	Protecting the cells from herbicide-induced oxidative stress, in tissue culture model (chicken embryo fibroblasts)	Shimidzu et al. 1996
		In vitro, methylene blue and light mediated, singlet oxygen quenching and lipid peroxidation of linolenic acid	Miki 1991
550 times better	Vitamin E	Quenching singlet oxygen in chemical solution ($CDCl_3$)	
67% better	Vitamin E	Peroxyl radical scavenging in liposomal suspension	Shimidzu et al. 1996 Naguib 2000
2 X better	β-carotene	*In vitro*, inhibition of lipid peroxides in liposomes	Goto et al 2001
Better than	β-carotene	Protecting the cells from herbicide-induced oxidative stress, in tissue culture model	Lawlor and Brien 1995
50 to 200 times	β-carotene	Preventing UV-A light mediated oxidative stress in rat kidney fibroblasts	O'Connor and Brien 1998
38 times better	β-carotene	Quenching singlet oxygen in chemical solution ($CDCl_3$/CD_3OD)	Shimidzu et al. 1996
4.8 times better	β-carotene	*In vitro*, heme protein and heat mediated, free radical scavenging and lipid peroxidation of linolenic acid	Miki 1991
50 % better	β-carotene	Prevent fatty acid peroxidation in chemical solution	Terao 1995
14% better	Cantaxanthin	Quenching singlet oxygen in chemical solution	Di Mascio et al. 1990
4 X better	Cantaxanthin	Delay lipid peroxidation in membrane model	Lim et al . 1992
Equal	Cantaxanthin	Prevent fatty acid peroxidation in chemical solution	Terao 1989
3 X better	Lutein	Quenching singlet oxygen from the $NDPO_2$ dissociation in a chemical solution	Di Mascio et al. 1990
3.5 times better	Lutein	*In vitro*, heme protein and heat mediated, free radical scavenging and lipid peroxidation of linolenic acid	Miki 1991
67% better	Lutein	Peroxyl radical scavenging in liposomal suspension	Naguib 2000
10 to1000 times	Lutein	Preventing UV-A light mediated oxidative stress in rat kidney fibroblasts	O'Connor and Brien. 1998
3 X better	Lutein	Quenching singlet oxygen in chemical solution ($CDCl_3$)	Shimidzu et al. 1996
2.5 to 2 X better	Lycopene	Peroxyl radical scavenging in organic solution	Naguib 2000
2.4 X better	Zeaxanthin	Quenching singlet oxygen in chemical solution	Di Mascio et al. 1990
2 X better	Zeaxanthin	Delay lipid peroxidation in membrane model	Lim et al. 1992
Better	Zeaxanthin	*In vitro*, methylene blue and light mediated, singlet oxygen quenching and lipid peroxidation of linolenic acid	Miki 1991
2 times better	Zeaxanthin	*In vitro*, heme protein and heat mediated, free radical scavenging and lipid peroxidation of linolenic acid	Miki 1991
15 times better	Zeaxanthin	Quenching singlet oxygen in chemical solution ($CDCl_3$/CD_3OD) Quenching	
18% better	Zeaxanthin	singlet oxygen in chemical solution ($CDCl_3$)	Shimidzu et al. 1996
50 % better	Zeaxanthin	Prevent fatty acid peroxidation in chemical solution	Shimidzu et al. 1996 Terao 1989

Among the carotenoids, **astaxanthin** a pigment that belongs to the xanthophylls, the oxygenated derivatives of carotenoid gaining more importance than any other carotenoids, due to their high antioxidant properties which has been reported to surpass those of β-carotene or even α-tocopherol (Miki 1991). Due to its outstanding antioxidant activity it has been attributed with extraordinary potential for protecting the organism against a wide range of ailmants such as cardiovascular problems, different types of cancer and some diseases of the immunological system. This has stirred great interest in astaxanthin and prompted numerous research studies concerning its potential benefits to human and animals.

A comparision of astaxanthin ability to quench singlet oxygen and scavenge free radicals has been demonstrated by a number of *in vitro* and *invivo* studies (Table 1.3). Most studies showed that the astaxanthin is a stronger antioxidant than vitamin E and other carotenoids like β-carotene or lutein. In fact, astaxanthin has been shown to have up to 500 fold stronger free radical antioxidant activity than vitamin E and 38 times more than β-carotene (Shimidzu et al. 1996; Kurashige et al. 1990). The antioxidant properties of astaxanthin are believed to play a key role in a number of other properties such as protection against UV-light photooxidation, inflammation, cancer, ulcer, *Helicobacter pylorii* infection, aging, and age-related macular diseases, or the promotion of the immune response, heart health, eye health, liver function, joint health, and prostate health (Guerin et al 2003).

A1.3 Astaxanthin

Astaxanthin (3.3'-dihydroxy-β,β'-carotene-4,4'-dione) (Fig 1.4) is a oxygenated ketocarotenoid (Kunn and Sorenson 1983; Andrewes et al. 1974) closely related to other well-known carotenoids, such as β-carotene, zeaxanthin and lutein, thus they share many of the metabolic and physiological functions attributed to carotenoids. Astaxanthin was first chemically identified by Khun and Sorensen (Davis et al. 1960) based on Davis and Weedon later confirmed the structure by partial synthesis of its derivative astacene from canthaxanthin. The total synthesis of optically pure astaxanthin from racemic intermediates has also been accomplished (Bernhard 1991; Becher et al. 1981) mainly by Hoffmann-LaRoche, Basel, Switzerland.

A1.3.1 Physical and chemical properties of astaxanthin

The molecular formula of astaxanthin is $C_{40}H_{52}O_4$ and has a molecular weight of 596.86 (Fopppe 1971; Straub 1976). The crystalline astaxanthin has the appearance of a fine, dark reddish-brown powder. Its melting point is approximately 224^{o}C. It is insoluble in aqueous solutions and most organic solvents but can be dissolved at room temperature in dichloromethane (~30g/l), chloroform (~10g/l), acetone (~0.2g/l), dimethylsulfoxide (DMSO) (~0.5g/l), and other non polar solvents. Its absorption spectrum represents a conjugated polyene structure, λ_{max} = 489nm in chloroform, 478 nm in ethanol and 480nm in acetone (Davis 1976).

A1.3.2 Molecular structure and forms of Astaxanthin

The structure of astaxanthin is derived from β-carotene. The majority are hydrocarbons of 40 carbon atoms which contain two terminal ring systems joined by a chain of conjugated double bonds (Urich, 1994). The presence of the hydroxyl and keto groups (Fig. 1.4) on each ionone ring, explains some unique features, such as the ability to be esterified, a higher anti-oxidant activity and a more polar configuration than other carotenoids (Urich, 1994). The astaxanthin molecule has two asymmetric carbons located at the 3 and 3" positions of the benzenoid rings on either end of the molecule. Different enantiomers of the molecule result from the exact way that the hydroxyl groups (-OH) are attached to the carbon atoms at these centers of asymmetry (Fig. 1.4). If the hydroxyl group is attached so that it projects above the plane of the molecule it is said to be in the R configuration and when the hydroxyl group is attached to project below the plane of the molecule it is said to be in the S configuration. Thus the three possible enantiomers are designated R,R', S,S' and R,S' (meso). Free astaxanthin and its mono- and diesters from *H. pluvialis* have optically pure (3S,3'S)-chirality (Grung et al., 1992 and Renstrom et al. 1981). The carotenoid fraction of green vegetative cells consists of mostly lutein (75-80%) and β-carotene (10-20%), whereas in red cysts, the predominate carotenoid is astaxanthin (Renstrom et al. 1981). Various astaxanthin stereoisomers are found in nature that differs in the configuration of the two hydroxyl groups on the molecule (Fig 1.4).

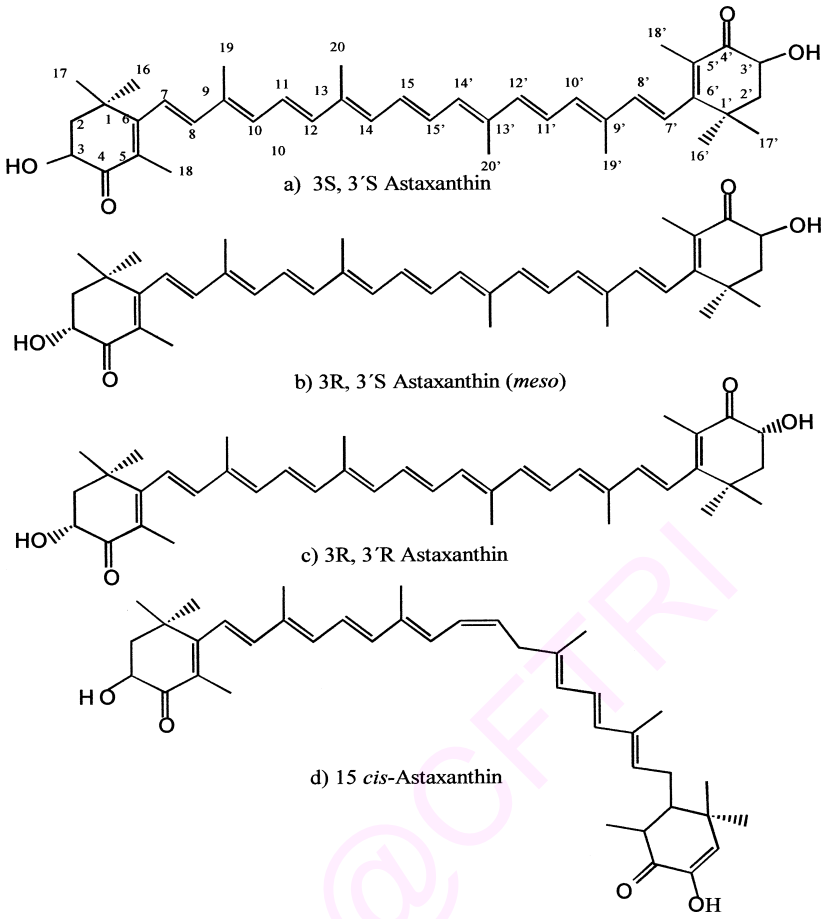


Figure A1.4 Isomers of astaxanthin (a–c) configurational isomers and (d) geometric *cis* isomer (Turujman 1997; Osterlie et al. 1999).

The 3S,3'S stereoisomer is the main form found in wild salmon (Turujman 1997) and in green microalga *H. pluvialis* especially as monoester (Lorenz and Cysewski 2000). Free astaxanthin is particularly sensitive to oxidation. In nature, it is found either conjugated to proteins, such as in salmon muscle or lobster exoskeleton (Miki et al. 1982), or esterified with one or two fatty acids, which stabilize the molecule.

A1.4 Applications on astaxanthin

The beneficial role of astaxanthin and its applications has been reviewed by Guerin et al. (2003) and Higuera-Ciapara et al. (2006). Research on the health benefits has mostly been performed *in vitro* or at the pre-clinical level with humans. Astaxanthin has drawn more and more attention due to its multiple functions and its great antioxidant potential. The effectiveness of astaxanthin with other carotenoids like, vitamin E, *β*-carotene, lutein, lycopene, canthaxantin and zeaxanthin has been

compared in Table 1.3. The applications of astaxanthin in different areas of human health and other benefits are described below.

A1.4.1 Astaxanthin as an antioxidant

Normal aerobic metabolism in organisms generates oxidative molecules, that is, free radicals (molecules with unpaired electrons) such as hydroxyls and peroxides, as well as reactive oxygen species (singlets) which are needed to sustain life processes. However, excess quantities of such compounds are dangerous due to their very high reactivity because they may react with various cellular components such as proteins, lipids, carbohydrates, and DNA (Di Mascio et al. 1991). This situation may cause oxidative damage through a chain reaction with devastating effects causing protein and lipid oxidation and DNA damage *in vivo*. Such damage has been associated with different diseases such as macular degeneration due to the aging process, retinopathy, carcinogenesis, arteriosclerosis, and Alzheimer disease, among other ailments (Maher 2000). An antioxidant is a molecule which has the ability to remove free radicals from a system either by reacting with them to produce other innocuous compounds or disrupting the oxidation reactions (Britton 1995).

Astaxanthin has an antioxidant activity as high as 10 times more than other carotenoids such as zeaxanthin, lutein, canthaxantin, and *β*-carotene; and 100 times more that *α*-tocopherol. Thus, astaxanthin has been dubbed a "super vitamin E" (Miki 1991). It has been demonstrated that astaxanthin is significantly more effective than β-carotene in neutralizing free radicals and gives better protection against the peroxidation of unsaturated fatty acid methyl esters than canthaxanthin, β-carotene or zeaxanthin (Lee 1986; Jorgensen and Skibsted 1993). It was found that astaxanthin has a higher antioxidant activity than lutein, lycopene, *α* and *β*-carotene, and *α*-tocopherol (Table A1.3). Astaxanthin exists in equilibrium, with the enol form of the ketone, thus the resulting dihydroxy conjugated polyene system possesses a hydrogen atom capable of breaking the free radical reaction in a similar way to that of α-tocopherol. As a result of its particular molecular structure, astaxanthin has a potent neutralizing or 'quenching' effect against singlet oxygen, as well as a powerful scavenging ability for free radicals, and it serves as an extremely potent antioxidant against these reactive species.

A1.4.2 Astaxanthin and eye health

Two of the leading causes of visual impairment and blindness are age-related macular degeneration (ARMD) and age related cataracts (Gerster 1991; Jacques

1999). Lutein and zeaxanthin, are two carotenoid pigments closely related to astaxanthin, are concentrated in the macula and give it its yellow color (Bone et al. 1985). These pigments are known to absorb blue light and have the potential to quench singlet oxygen (Landrum et al. 1999). Astaxanthin has not been isolated in the human eye, yet it is found in the eye or eye parts of a number of animals (Egeland 1993). Furthermore, an animal study has demonstrated that astaxanthin is capable of crossing the retinal blood-brain barrier, and like lutein will deposit in the retina of mammals if included in the diet (Tso and Lam 1996). The composition of the astaxanthin is very close to that of lutein and zeaxanthin, yet it has demonstrated, in *in vitro* studies, a stronger antioxidant activity and UV-light protection effect than other carotenoids (O'Connor and O'Brien 1998). It could therefore be inferred that deposition of astaxanthin in the eye may provide superior protection against UV light and oxidation of retinal tissues. Interestingly, in the study by Tso and Lam (1996) the retinal photoreceptors of rats fed astaxanthin were less damaged by a UV-light injury and recovered faster than animals fed no astaxanthin, confirming the potential of astaxanthin for eye health. No human clinical study on effect of astaxanthin in specific eye diseases like macular degeneration or cataract has been published yet.

A1.4.3 Astaxanthin as a human dietary supplement

Manufacturers of natural astaxanthin have long tried to penetrate the aquaculture market niche with very little or no success at all. In recent years, their attention has shifted towards another growing industry: the nutraceuticals market (McCoy 1999). Currently there is a wide variety of astaxanthin products sold in health food stores in the form of nutritional supplements. Most of these products are manufactured from algae or yeast extracts. Due to their high antioxidant properties these supplements have been attributed with potential properties against many diseases. Thus, research on the actual benefits of astaxanthin as a dietary supplement is very recent and basically has thus far has been limited to *in vitro* assays or pre-clinical trials.

A1.4.4 Anticancer Activity

Activity of carotenoids against cancer has been the focus of much attention due to the association between low levels of these compounds in the body and cancer prevalence. Several research groups have studied the effect of astaxanthin

supplementation on various cancer types showing that oral administration of astaxanthin inhibits carcinogenesis in mice urinary bladder (Tanaka et al. 1994), in the oral cavity (Tanaka et al. 1995a) and rat colon (Tanaka et al. 1995b). This effect has been partially attributed to suppression of cell proliferation. Furthermore, Jyonouchi et al. (2000) found that when mice were inoculated with fibrosarcoma cells, the dietary administration of astaxanthin suppresses tumor growth and stimulates the immune response against the antigen which expresses the tumor. Astaxanthin activity against breast cancer has also been studied in female mice. Chew et al. (1999) fed mice with a diet containing 0, 0.1% and 0.4% astaxanthin, β-carotene or canthaxanthin during three weeks before inoculating the mammary fat pad with tumor cells. Tumor growth inhibition by astaxanthin was shown to be dependent on the dose and more effective than the other two carotenoids tested. It has also been suggested that astaxanthin attenuates the liver metastasis induced by stress in mice thus promoting the immune response though the inhibition of lipid peroxidation (Kurihara et al. 2002). Kang et al. (2001) also reported that astaxanthin protects the rat liver from damage induced by CCl_4 through the inhibition of lipid peroxidation and the stimulation of the cell antioxidant system.

A1.4.5 Prevention of Cardiovascular Diseases

The risk of developing arteriosclerosis in humans correlates positively with the cholesterol content bound to Low Density Lipoprotein (LDL) or "bad cholesterol" (Golstein and Brown, 1977). Many studies have documented that high levels of LDL are related to prevalence of cardiovascular diseases such as angina pectoris, myocardial infarction, and brain thrombosis (Maher 2000). Inhibition of oxidation of LDL has been postulated as a likely mechanism through which antioxidants could prevent the development of arteriosclerosis. Several studies have looked at carotenoids, mainly astaxanthin as inhibitors of LDL oxidation (Iwamoto et al. 2000). The studies performed *in vivo* and *ex vivo* and their results suggest that astaxanthin inhibits the oxidation of LDL which presumably contributes to arteriosclerosis prevention. Miki et al. (1998) proposed the manufacture of a drink containing astaxanthin whose antioxidant action on LDL would be useful for the prevention of arteriosclerosis, ischemic heart disease or ischemic encephalopathy. While it is feasible that oxidation of LDL may be decreased by antioxidant consumption, more

research is needed to establish the true effect on coronary heart disease (Jialal and Fuller 1995).

A1.4.6 Astaxanthin and neurodegenerative diseases

The nervous system is rich in both unsaturated fats (which are prone to oxidation) and iron (which has strong prooxidative properties). These, together with the intense metabolic aerobic activity and rich irrigation with blood vessels found in tissues of the nervous system, make tissues particularly susceptible to oxidative damage (Facchinetti 1998). There is substantial evidence that oxidative stress is a causative or at least ancillary factor in the pathogenesis of major neurodegenerative diseases (Alzheimer's, Huntington's, Parkinson's and amyotrophic lateral sclerosis) and that diets high in antioxidants offer the potential to lower the associated risks (Grant 1997; Borlongan 1996; De Rijk 1997; Ferrante 1997). The above-mentioned study with rats fed natural astaxanthin (Tso and Lam 1996) demonstrated that astaxanthin can cross the blood brain barrier in mammals and can extend its antioxidant benefits beyond that barrier. Astaxanthin, is therefore an excellent candidate for testing in Alzheimer's disease and other neurological diseases.

A1.4.7 Astaxanthin effect against *Helicobacter pylori* infections

H. pylori is considered an important factor inducing acute gastritis, peptic ulcers, and stomach cancer in humans. The antibacterial action of astaxanthin has been shown in mice infected with this bacterium. When mice are fed with an astaxanthin rich diet, the gastric mucous inflammation is reduced as well as the load and colonization by the bacterium (Bennedsen et al. 1999; Wang et al. 2000). The mechanism of astaxanthin action to produce this effect is not known but it is suspected that its antioxidant properties play an important role in the protection of the hydrophobic lining of the mucous membrane making colonization by *H. pylori* much more difficult (Wadstron and Alejung 2001). The use of astaxanthin could represent a new and attractive strategy for the treatment of *H. pylori* infections. The dry biomass of *H. pluvialis* also showed antiulcer property (Sandesh 2007).

A1.4.8 Astaxanthin as a modulator of the immunological system

The studies led by Jyonouchi et al. (1996) has performed the large majority of investigations regarding the potential activity of astaxanthin as a booster and modulator of the immunological system. Astaxanthin increases the production of T-

helper cell antibody and increases the number of antibody secretory cells from primed spleen cells (Jyonouchi et al. 1996). They also studied the effect of astaxanthin in the production of immunoglobulins *in vitro* by human blood cells and found that it increases the production of IgA, IgG, and IgM in response to T-dependent stimuli (Jyonouchi et al. 1995). Other studies performed *in vivo* using mice have shown the immunomodulating action of astaxanthin and other carotenoids for humoral responses to T-dependent antigens, and suggested that the supplementation with carotenoids may be useful to restore immune responses (Jyonouchi et al. 1994). In agreement with the above results, various foods and drinks with added astaxanthin have been prepared to increase the immune response mediated by T lymphocytes, to alleviate or prevent the decrease of immunological functions caused by stress (Asami et al. 2001). Due to its immunomodulating action, astaxanthin has also been utilized as a medication for the treatment of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and Crohn's disease (Lignell and Bottiger 2001).

A1.4.9 Astaxanthin in aquaculture

Salmonid and crustacean coloring is perceived as a key quality attribute by consumers. The reddish-orange color characteristic of such organisms originate in the carotenoids obtained from their feeds which are deposited in their skin, muscle, exoskeleton, and gonads either in their original chemical form or in a modified state depending on the species (Meyers and Chen 1982). The predominant carotenoid in most crustacean and salmonids is astaxanthin (Yamada et al. 1990; Shahidi and Synowiecki 1991; Gentles and Haard 1991). For instance, from the total carotenoids in crustacean exoskeleton, astaxanthin comprises 84–99%, while in the internal organs it represents 70–96% (Tanaka et al. 1976). In the aquatic environment, the microalgae biosynthesize astaxanthin which are consumed by zooplankton, insects, or crustacean and later it is ingested by fish, thereby getting the natural coloration (Lorenz 1998). Farmed fish and crustacea do not have access to natural sources of astaxanthin, hence the total astaxanthin intake must be derived from their feed. The use of astaxanthin as pigmenting agents in aquaculture species has been well documented through many scientific publications for more than two decades (Meyers and Chen 1982; Torrisen 1989; Yamada et al. 1990; No and Storebakken 1991; Putnam 1991; Storebakken and No 1992; Smith et al. 1992; Choubert and Heinrich, 1993; Coral et al. 1998; Lorenz 1998; Gouveia et al. 2002; Bowen et al. 2002).

Currently, the synthetic form of pigments represents the most important source for fish and crustacean farming operations. Astaxanthin is available under the commercial brand name Carophyll Pink^{TM} owned by Hoffman-LaRoche. In spite of the fact that canthaxanthin provides a fairly good pigmentation, astaxanthin is widely preferred over it due to the higher color intensity attained with similar concentrations (Storebakken and No 1992). Additionally, astaxanthin is deposited in muscles more efficiently probably due to a better absorption in the digestive tract (Torrisen 1989). It has also been reported that when a combination of astaxanthin and canthaxanthin are used, a better pigmentation is obtained than when using either pigment separately (Torrisen 1989; Bell et al. 1998). However, in a more recent study of Buttle et al. (2001) found that the absortion of these two pigments is species dependent. In spite of the fact that astaxanthin is widely used with the sole purpose of attaining a given pigmentation, it has many other important functions in fish related mainly to reproduction: acceleration of sexual maturity, increasing fertilization and egg survival, and a better embryo development (Putnam 1991). It has also been demonstrated that astaxanthin improves liver function, it increases the defense potential against oxidative stress (Nakano et al. 1995) and has a significant influence on biodefense mechanisms (Amar et al. 2001). Similarly, several other physiological and nutritional studies have been performed in crustaceans, mainly on shrimp, which have suggested that astaxanthin increases tolerance to stress, improves the immune response, acts as an intracellular protectant, and has a substantial effect on larvae growth and survival (Gabaudan 1996; Darachai et al. 1999). Chien et al. (2003) proposed that astaxanthin is a "semi-essential" nutrient for tiger shrimp (*Penaeus monodon*) because the presence of this compound can be critical to the animal when it is physiologically stressed due to environmental changes. According to the above information, the use of astaxanthin in the aquaculture industry is important not only from the standpoint of pigmentation to increase consumer acceptance but also as a necessary nutrient for adequate growth and reproduction of commercially valuable species.

A1.4.10 Additional Benefits

Ultraviolet radiation is a significant risk factor for skin cancer due to the activation of a chain reaction which generates peroxides and other free radicals from lipids. These molecules damage the cell structures like DNA thus increasing the risk

for cancer development. As we discussed previously, astaxanthin is a potent antioxidant which stimulates and modulates the immune system. These effects are capable of preventing or delaying sunburns. The ability of astaxanthin extracted from algae to protect against DNA damage by UV radiation has been shown in studies with cultured rat kidney fibroblasts (O'Connor and O'Brien 1998) and human skin cells (Lyons and O'Brien 2002). Various astaxanthin supplements consisting of injectable solutions, capsules, or topical creams have been manufactured for sunburn prevention from UV exposure (Lorenz 2002). Additional beneficial effects attributed to astaxanthin include anti-inflammatory activity (Uchiumi 1990; Nakajima 1995), anticataract prevention activity (Guyen et al. 1998), as a treatment against rheumatoid arthritis and also carpal tunnel syndrome (Lignell and Bottiger 2001; Cyanotech 2002). The large majority of the studies to support the multiple potential benefits of astaxanthin have been performed with animal models. A few clinical trials have been performed with voluntary patients by the manufacturing companies. For instance, Cyanotech (2002) has performed extensive work on the preventative effects of astaxanthin on the development of rheumatoid arthritis and carpal tunnel syndrome.

A1.4.11 Industrial applications of astaxanthin

Industrial applications of carotenoids include their use as colorants for human food, as feed additives to enhance the pigmentation of fish, eggs, cosmetics, and pharmaceutical products. The major market for astaxanthin is its use as a pigmentation source in aquaculture and poultry industries (Lorenz and Cysewski 2000). It specifically provides a characteristic pink color to salmonoids, trout and shrimp (Jin et al. 2006). Astaxanthin sells for around U.S. $2500/kg with an annual worldwide market estimated at over U.S. $200 million. The astaxanthin used by the fish farmers represents 10 to 20% of the feed cost. Although more than 95% of this market consumes synthetically derived astaxanthin, consumer demand for natural products makes the synthetic pigments less desirable and provides an opportunity for the production of natural astaxanthin by the alga *H. pluvialis. H. pluvialis* is a potential source producing 1.5 to 3.0% astaxanthin. It has already gained acceptance in aquaculture and other markets as a concentrated form of natural astaxanthin. The Canadian Food Inspection Agency and the U.S. FDA have approved the use of this alga as a color additive in salmonid feeds. Astaxanthin is used in aquaculture as it can function as antioxidant, hormone precursor, immune enhancement, provitamin A

activity, reproduction, growth, maturation, and photoprotection (Lorenz and Cysewski 2000; Margalith 1999). In general, astaxanthin is used as a nutraceutical ingredient. The algal meal has been approved as a natural red food color in Japan and some European countries as well as a dietary supplement ingredient in the United States (Lorenz and Cysewski 2000). Because of the ability of *H. pluvialis* to accumulate higher amounts of astaxanthin and multidimensional applications of its astaxanthin in various industries, it is recognized as a potential candidate for commercial production.

A1.5 Types of Astaxanthin

A1.5.1 Synthetic astaxanthin

Synthetic astaxanthin consists of a racemic mixture of the two enantiomers and the meso form (Turujman et al. 1997). Three types of optical isomers can be found in crustacean (Cort´es 1993). Depending on their origin, astaxanthin can be found in association with other compounds. It may be esterified in one or both hydroxyl groups with different fatty acids such as palmitic, oleic, stearic, or linoleic: it may also be found free, that is, with the hydroxyl groups without esterification; or else, forming a chemical complex with proteins (carotenoproteins) or lipoproteins (carotenolipoproteins). Synthetic astaxanthin is not esterified, while found in algae is always esterified (Johnson and An 1991; Yuan et al. 1997). Crustacean astaxanthin on the other hand, is a mixture of the three forms as previously described in the section 1.3.2.

Astaxanthin cannot be synthesized by animals and must be acquired from the diet. Although mammals and most fish are unable to convert other dietary carotenoids into astaxanthin, crustaceans (such as shrimp and some fish species including koi carp) have a limited capacity to convert closely related dietary carotenoids into astaxanthin, although they benefit from being fed astaxanthin directly. Mammals lack the ability to synthesize astaxanthin or to convert dietary astaxanthin into vitamin A: unlike β-carotene, astaxanthin has no pro-vitamin A activity in these animals (Jyonouchi et al. 1995).

A1.5.2 Astaxanthin in nature

Astaxanthin can be found in many of our favorite seafood such as salmon, trout, red seabream, shrimp, lobster and fish eggs (Torissen et al. 1989). It is also found in birds like flamingoes, quails, and other species (Egeland 1993; Inbor 1998). The astaxanthin producing organisms are listed in the Table 1.4.

Table A1.4 Natural sources of astaxanthin

	Organism	Astaxanthin content (% w/w dry wt)	Reference
Green Algae	***Haematococcus pluvialis***	**2.3-7.7**	Kang et al. 2005
	Neochloris wimmeri	0.6	Orosa et al. 2000
	Chlorococcum	< 0.2	Zhang et al. 1997
	Nannochloropsis gaditana	<0.3	Lubian et al. 2000
	Scenedesmus vacuolatus	0.01	Orosa et al. 2000
	Chlorella zofingiensis	0.02-0.15	Ip et al. 2005
	Chlamydomonas nivalis	0.04	Bidigare et al. 1993
	Monoraphidium sp. GK12	0.25	Fujii et al. 2008
	Scenedesmus komarekii	Not reported	Hanagata and Dubinsky 1999
Fungi	*Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*)	0.4	Jacobson et al. 2000
	Yeast-Candida utilis	0.04	Miura et al. 1998
Bacteria	*Mycobacterium lacticola*	0.003	Simpson et al. 1981
	Agrobacterium aurantiacum	0.01	Yokoyama et al. 1995
	Paracoccus carotinifaciens	Not reported	Tsubokura et al. 1999
	Brevibacterium sp	0.003	Neils and Leenheer 1991
Animals	Shrimp-*Pandalus clarkii*	0.015	Meyers and Bligh 1981
	Shrimp-*Pandalus borealis*	0.014	Shahidi and Synowiecki 1991
	Backs snow crab – *Chinoecetes opilio*	0.011	Shahidi and Synowiecki 1991

A1.5.3 Current market status of astaxanthin

Annual growth of the global market for astaxanthin for human use is thought to be at least 15 per cent, with current estimates valuing the market at $15-20m (€12.4-16.6m) per year (www.nutraingredients-usa.com). Upon approval of *Haematococcus* alga by the US Food and Drug Administration (21 CFR 190.6) and clearance for marketing as a new dietary ingredient, various products of *Haematococcus* have entered the world market. US based Cyanotech Corporation, Mera Pharmaceuticals Inc., Israel based Alga technologies Ltd, Sweden based BioReal AB are the major producers of *Haematococcus* algae meal and astaxanthin products. Mera Pharmaceuticls Inc. employs a fully enclosed 25,000L computer-controlled outdoor photobioreactor for large-scale biomass production of *Haematococcus*. (Olaizola, 2000). BioReal (Sweden) AB has the BioDome technology for cultivation of *Haematococcus* (www.fujihealthscience.com). In India, Chennai based Parry Nutraceuticals is engaged in development of process for production of astaxanthin from *Haematococcus* (www.parrynutraceuticals.com). The range of astaxanthin products produced and marketed by various companies has been listed in Table 1.5. Significant price difference exists between the synthetic and natural astaxanthin, synthetic variety costs $2000 per kg where as the natural astaxanthin is still expensive at between $10,000 - 15,000 per kg (www.nutraingredients-usa.com).

Astaxanthin, like other carotenoids, cannot be synthesized by animals and must be provided in the diet. While mammals and fish such as salmon or trout are unable to convert other dietary carotenoids into astaxanthin, crustaceans like shrimp and some fish species like koi carp have a limited capacity to convert closely related dietary carotenoids into astaxanthin, although they benefit strongly from being fed astaxanthin directly (Meyers 1993). Mammals lack the ability to synthesize astaxanthin, or to convert dietary astaxanthin into vitamin A: unlike β-carotene, astaxanthin has no pro-vitamin A activity in these animals (Jyonouchi et al. 1995). Among the astaxanthin producers, *H. pluvialis* a Chlorophyte alga, is believed to be accumulate highest levels of astaxanthin in nature. Commercially grown *H. pluvialis* can accumulate as much as 30 g of astaxanthin per kg of dry biomass and a number of companies are producing nutraceuticals from this alga. Numerous research reports exist concerning the study of microalgae, particularly *H. pluvialis* with the aim of optimizing the astaxanthin production processes.

Table A1.5 Some major producers of natural astaxanthin from *H. pluvialis* and their merchandise brand name

Company	Trademark	Use	Web site
Mera Pharmaceuticals (former Aquasearch), U.S.A.	Astafactor	Antioxidant	http://www.merapharm.com, http://www.astafactor.com,
Cyanotech Corp., U.S.A.	BioAstin, NatuRose	Antioxidant, neutraceutical, aquaculture	http://www.cyanotech.com, http://www.bioastin.com
Nutrex Hawaii Inc., U.S.A.	BioAstin	Neutraceutical, aquaculture, pharmaceutical	http://www.nutrex-hawaii.com
BioReal, Inc. (former MicroGia and subsidiary of Fuji Chemical Industry Co., Ltd.), U.S.A.	AstaXin	Dietary supplement containing *Haematococcus* - crushed and dried algae meal	http://www.bioreal.com
Valensa International, U.S.A.	Zanthin	Antioxidant	http://www.usnutra.com/
AstaReal (subsidiary of Bioreal, Inc.), Sweden	AstaCarox	Super critical fluid-oil extract derived from crushed algae	http://www.astareal.com
Algatechnologies Ltd., Israel	AstaPure	Animal feed, neutraceutical	www.algatech.com
Natural Astaxanthin Co. Ltd. (PA-Shun Group), China	NaturAsta	Animal feed, neutraceutical	http://www.pa-shun.com/xiaqingsu/astaxanthin.htm
Stazen Inc., USA	Stazen	Dietary supplement containing *Haematococcus* - crushed and dried algae meal	www.stazen.com
Parry Neutraceuticals (Murugappa Group), India	OleoResin	Neutraceutical	http://www.parrynutraceuticals.com

The main focus of these efforts has been the assessment of various factors and conditions which affect *H. pluvialis* growth and the production of astaxanthin (Kakizono et al. 1992; Kobayashi et al. 1992, 1993; Harker et al. 1995, 1996; Fabregas et al. 1998, 2000; Gong and Chen 1997; Boussiba et al. 1999; Hata et al. 2001; and Orosa et al. 2001).

A1.6 *Haematococcus pluvialis*

The green microalga *Haematococcus pluvialis* Flotow (Chlorophyceae) is a unicellular biflagellate living in fresh water. It is now known that the alga occurs in nature worldwide, where environmental conditions for its growth are favorable. *H. pluvialis*, also referred to as *H. lacustris* or *Sphaerella lacustris*, is a ubiquitous green alga of the order Volvocales, family Haematococcaceae. *H. pluvialis* is an ubiquitous green algae classified as

A1.6.1 Classification

Phylum: Chlorophyta

Class: Chlorophyceae

Order: Volvocales

Family: Haematococcaceae

Genus: *Haematococcus*

Species: *pluvialis*

A1.6.2 History and Distribution of *H. pluvialis*

The observations of *H. pluvialis* began in 1797 by Girod-Chantrans and were continued by other Europeans. The first description of *H. pluvialis* was conducted by Flotow in 1844, and in 1851 Braun added to the details and corrected a few errors of earlier observations (Lorenz 1999). Peebles (1909a, 1909b) published a life history of the alga with detailed drawings of changes occurring in the "haematochrom" throughout the life cycle. In 1934, Elliot added details of the cellular morphology to the life history of the alga. During the life cycle four types of cells were distinguished: microzooids, large flagellated macrozooids, non-motile palmella forms; and haematocysts, which are large red cells with a heavy resistant cell wall. Pocock (1937, 1961) described the distribution and life history of *H. pluvialis* strains isolated in Africa. Almgren (1966) described the ecology and distribution of *H. pluvialis* in Sweden, where the alga is found in ephemeral rain pools made of rock, generally of small dimensions and based upon firm material, impermeable to water. Droop (1961) also noted that *H. pluvialis* typically inhabited rock pools, often, though not necessarily, within a few feet of the sea. The widespread occurrence of *H. pluvialis* in

temporary rather than permanent bodies of water is due, at least in part, to the fact that such pools are usually free of other competing algae, and not to any inherent characteristic of the pools. *H. pluvialis* is considerably better suited for survival under conditions of expeditious and extreme fluctuations in light, temperature and salt concentration than most algae, due to its rapid ability to encyst (Proctor 1957). Recent reports show its existence in small artificial pool in poland (Burchardt et al. 2006) and natural and man-made ponds in Himachal Pradesh, India (Suseela and Toppo 2006)

A1.6.3 General biology, ultrastructure and life cycle

The general life cycle of *H. pluvialis* under favourable growth conditions is illustrated in Figure 1.5. In its growth stages, it has both motile (Fig 1.5A) and non-motile (Fig 1.5D) forms. In the former, a pear-shaped cell ranges from 8 to 25 µm in diameter, which exist as a single biflagellate swimmer capable of photosynthetic autotrophic growth. The cellular structure of this stage is similar to most of its family members: a cup-shaped chloroplast with numerous and scattered pyrenoids, contractile vacuoles which are often numerous and apparently quite irregularly distributed near the surface of the protoplast, a nucleus and 2 flagella of equal length emerging from the anterior papilla. The structure's uniqueness is marked by its cell wall which is strongly thickened, gelatinous, and is usually connected to its protoplast by simple or branched strands. Once growing conditions become unfavorable, the cells initiates carotenogenesis where the cells increase their volume drastically and enter a resting stage in which the cell is surrounded by a heavy resistant cellulose wall, comprised in part by sporopolinine-like substances (Goodwin and Jamikorn 1954; Boussiba 1992) and undergoes morphological transformation from green vegetative cells to deep-red, astaxanthin-rich, immotile aplanospores. The enlarged cysts containing many new germinating cells (Fig 1.5 E) may be observed (Kobayashi et al. 1997).

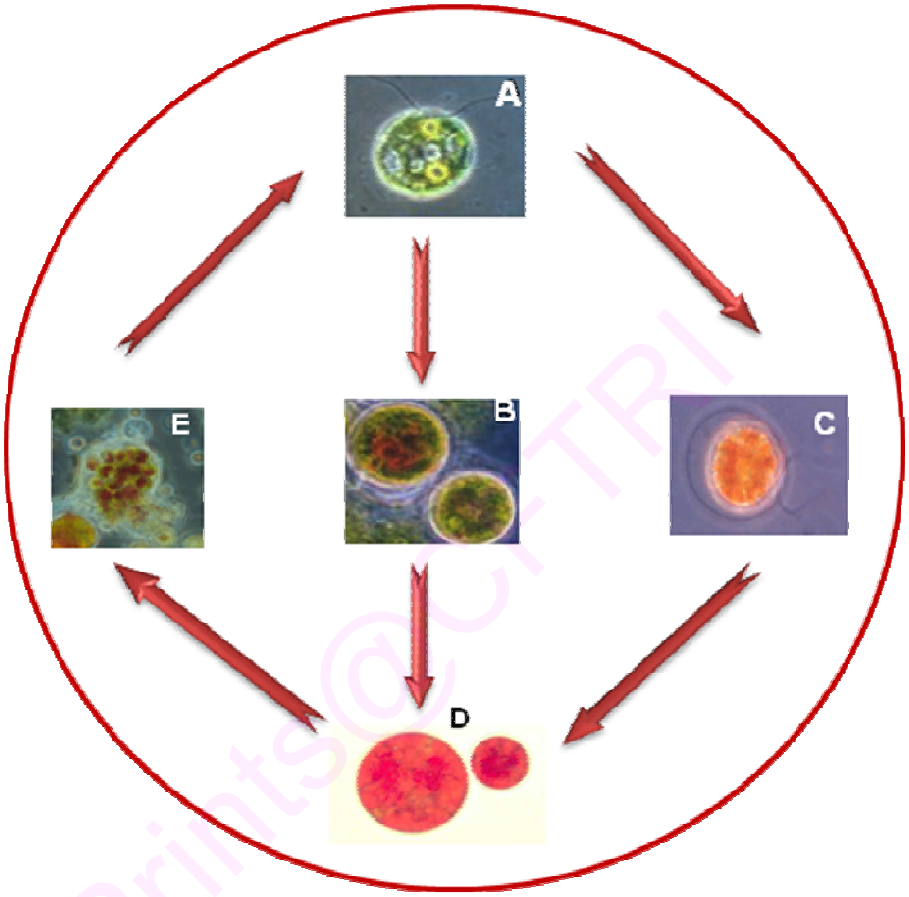


Figure A1.5 Life cycle of *H. pluvialis* showing green vegetative motile cells (A), vegetative palmella cells (B), astaxanthin accumulating flagellated cells (C), astaxanthin accumulating cyst cells (D) and germinating cells (E).

The volume of the algae increases to giant red aplanospores with a diameter of over 40-50 µm (Figure 1.5 D). This is ten times the diameter of the vegetative cell. This overall process is termed 'encystment'. The protoplast is then a markedly red color, determined to be a secondary carotenoid, astaxanthin (Harker et al. 1996). Under nonstressed conditions after maturation, the cysts germinate, releasing mostly flagellated cells and leaving behind the typical cell wall. Reproduction is usually by cell division throughout the vegetative stage. The duration of the growth cycle of *H.*

pluvialis varies according to nutritional and environmental conditions, but it is usually between less than 10 days to several weeks.

A1.6.4 Biosynthesis of secondary carotenoids especially astaxanthin in *H. pluvialis*

During unfavourable growth conditions, *H. pluvialis* initiates carotenogenesis and undergoes morphological transformation from green vegetative cells to deep-red, astaxanthin-rich, immotile aplanospores (Harker et al. 1996). Astaxanthin, is biosynthesized through the isoprenoid pathway which is also responsible for the vast array of lipid soluble molecules such as sterols, steroids, prostaglandins, hormones, vitamins D, K and E.

The key steps in the astaxanthin biosynthesis pathway in *H. pluvialis* is illustrated schematically in Figure 1.6. The key building block of carotenoids is isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) (Fig. 1.6) (Grünewald et al. 2001; Lichtenthaler 1999). The enzyme isopentenyl pyrophosphate isomerase (IPI) carries out this reversible isomerization reaction. Two *H. pluvialis* cDNAs for IPP isomerase genes have been identified (Sun et al. 1996; Sun et al.1998). Isopentenyl pyrophosphate is produced in the cytosol through the acetate/mevalonate pathway and in the chloroplast through the 1-deoxy-D-xylulose- 5-phosphate (DOXP) pathway. Genes for the enzymes participating in the biosynthetic pathway of plastidal DOXP are localized in the nucleus and the gene products are imported to the chloroplast (Lichtenthaler 1999). In contrast to higher plants, there is no evidence for activity of the cytosolic acetate/mevalonate biosynthetic pathway of isoprenoids in unicellular green algae belonging to the class chlorophyceae (Lichtenthaler 1999; Schwender et al. 2001).

Thus, it is proposed that *H. pluvialis* produce their isoprenoids only through the DOXP pathway that operates in both the cytosol and plastids (Disch et al. 1998; Schwender et al. 2001).Another important enzyme of the carotenoid biosynthesis pathway is phytoene synthase (PSY) (Fig 1.6, right). It catalyzes the first committed step in carotenoid biosynthesis by condensing two 20-carbon geranylgeranyl pyrophosphate (GGPP) molecules to form the 40-carbon molecule phytoene, the precursor molecule for all other carotenoids.

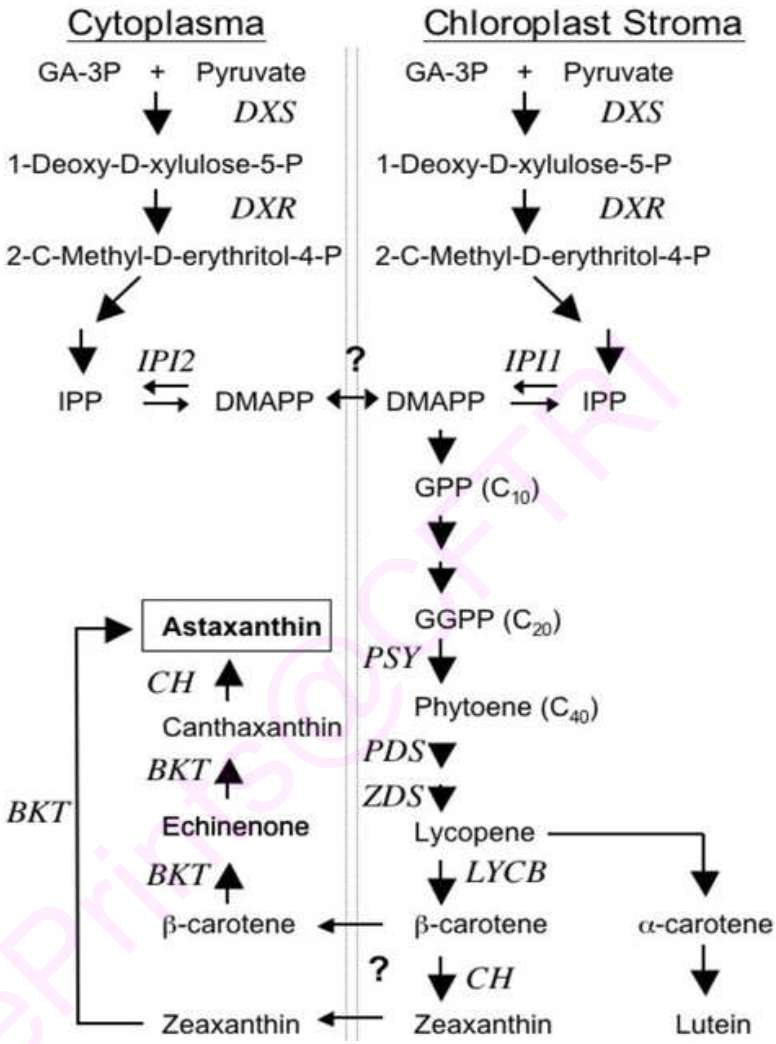


Figure A1.6 General biosynthetic pathway of astaxanthin. Enzymes are designated as DXS (Deoxy Xylulose-5-P Synthase), DXR (Deoxy Xylulose-5-P Reductase), IPI (Isopentenyl Pyrophosphate Isomerase), LCYB (Lycopene β-Cyclase), PSY (Pytoene synthase), PDS (Phytoene Desaturase), ZDS (ζ-Carotene Desaturase), CH (β-Carotene Hydroxylase), BKT (β-Carotene ketolase).

Two structurally and functionally similar enzymes, phytoene desaturase PDS and ζ-carotene desaturase (ZDS), convert phytoene to lycopene via ζ-carotene. Carotenoids in the photosynthetic apparatus and secondary carotenoids are bicyclic compounds. Therefore, cyclization of lycopene to α-carotene and β-carotene is an important branch point in carotenoid biosynthesis (Fig 1.6). Cyclization of lycopene

to β-carotene involves lycopene β-cyclases (LYCB) carrying out two cyclization reactions, thus introducing two β-rings to form β-carotene or its derivatives which are accumulated upon environmental stress. As shown in Figure 1.6, β-carotene is the precursor of astaxanthin that accumulates in lipid globules in the cytosol. The enzymes carrying out the necessary oxygenation reactions in the alga *H. pluvialis* are β-carotene hydroxylase (CH) and β-carotene ketolase (BKT) (Boussiba 2000; Grünewald et al. 2001; Hirschberg 2001). In summary, several genes for the secondary carotenoid biosynthesis pathway in the alga *H. pluvialis* are known.

A1.7 Carotenogenesis

A1.7.1 Regulation of Carotenoid biosynthesis

Although many nuclear-encoded genes for enzymes of the carotenoid biosynthetic pathway have been identified in several species including algae (Cunningham 2000; Steinbrenner and Linden 2001; Yan et al. 2005; Zhu et al. 2005), not much is known about regulation of carotenogenesis in vivo. Nevertheless, expression studies of several genes involved in carotenoid biosynthesis indicate upregulation of genes participating in carotenoid biosynthesis in the alga *H. pluvialis* at either the mRNA level, the protein level, or both (Grünewald et al. 2000; Sun et al. 1996; Steinbrenner and Linden 2001; Vidhyavathi et al. 2008). The regulation of carotenogenesis is summarized in Fig. 1.7 and Table 1.5.

In general, for *H. pluvialis*, all investigated genes that code for enzymes involved in carotenoid biosynthesis were shown to be upregulated at the mRNA level in response to environmental stress conditions. Concerning the biosynthesis of precursors of the carotenoid biosynthesis pathway, Sun et al. (1996) showed that both isopentenyl pyrophosphate isomerase (IPI) genes are upregulated at the mRNA level (Table 1.5). The PSY is the enzyme that catalyzes the entry-step into the carotenoid biosynthesis pathway. As expected, the mRNA of the phytoene synthase is upregulated in response to various stress conditions (Steinbrenner and Linden 2001; Pulz and Gross 2004).

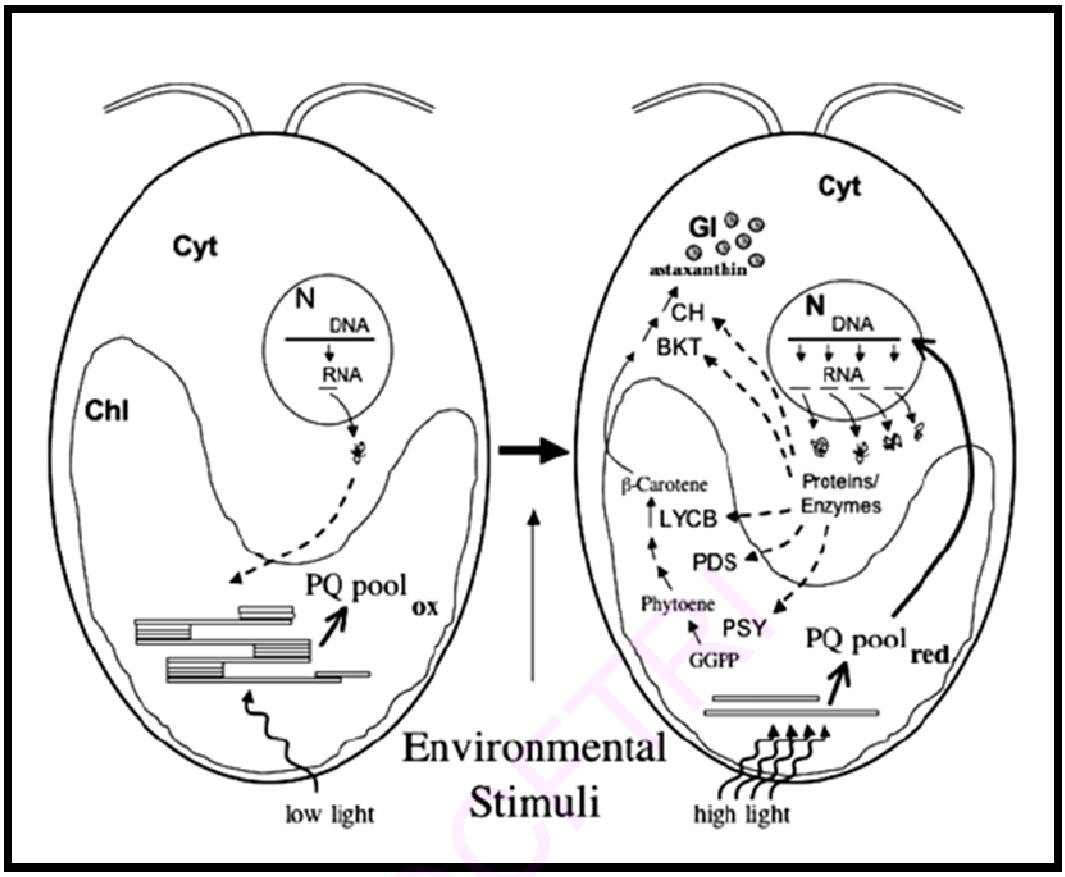


Figure A1.7 Regulation of astaxanthin overaccumulation in *H. pluvialis.* The left panel shows a cell that does not overaccumulate astaxanthin when under nonstressed growth conditions.

Upon environmental stress, cells overaccumulate astaxanthin through a mechanism that involves the induced expression of all genes of the carotenoid biosynthesis pathway studied so far. One hypothesis is that the redox state of the plastoquinone pool in the thylakoid membrane is involved in regulation of gene expression. The addition of sodium acetate, Fe^{2+}, and growth under high light leads to a strong induction of steady-state mRNA levels for the PSY gene (Steinbrenner and Linden 2001). The regulation of astaxanthin from *H. pluvialis* in different nutritional and environmental stimuli were given in table 1.6 and 1.7

Table A1.6 Percentage of astaxanthin under different nutrient condition

Modifications			% of Astaxanthin	Reference
Light	**Temperature**	**Nutrients**		
80µmol $m^{-2}s^{-1}$	-	-	1.9%	Boussiba and Vonshak, 1991
-	30^0C 20^0C	-	1.9% 0.8%	Tjahjono et al., 1994
-	-	0.2%NaCl	3.0%	Cordero et al., 1996
-	-	KM2 Medium with Trace elements and Vitamin B	2.3%	Usha et al., 1999
-	-	Malonate 2g/l Acetate 2g/l	2.6% 2.0 %	Orosa, 2001
-	-	1/4 & 1/10 of Nitrogen and Phosphate of normal Concentration	2.0 %	Brinda et al., 2004

Table A1.7 Environmental factors affecting astaxanthin over accumulation in the alga *H. pluvialis*.

Environmental factor	Stimulus	Reference
Light	Increased irradiance	Hagen et al. 2001; Orosa et al. 2001
Salt (NaCl)	High concentration	Sarada e al. 2002a; Sarada et al. 2002b,2002c
Nutrients		
Phosphate	Deficiency	Boussiba et al. 1999
Nitrogen	Deficiency	Boussiba et al. 1999 Hagen et al. 2005 Orosa et al. 2000 Zhu et al. 2005
CaNO3	Presence	Sarada et al. 2002a
Acetate	Presence	Kobayashi et al 1993 Orosa et al. 2001 Orosa et al. 2000 Sarada e al. 2002a
Malonate	Presence	Orosa et al. 2001 Orosa et al. 2000

One way to increase the production of carotenoids in biological systems is to use recombinant DNA techniques. The potential commercial interest for the production of carotenoids and the cloning of genes encoding biosynthetic enzymes has led to all kinds of examples of metabolic pathway engineering. These examples

include the overexpression of a gene encoding for the specific enzyme, the expression of carotenogenic genes in noncarotenogenic heterologous hosts, the increase of the carbon flux into the carotenoid biosynthetic pathway, and the combination of genes and modification of catalytic activities in order to improve and/or modify carotenoid biosynthetic pathways.

Table A1.8 Comparison of the regulation of all known enzymes of the carotenoid biosynthesis pathway of the alga *H. pluvialis*

Enzyme	Level of expression after exposure to environmental stress	
	mRNA	Protein
Isopentenyl pyrophosphate isomerase 1	+	o
Isopentenyl pyrophosphate isomerase 2	+	+
Phytoene synthase	+	nk
Phytoene desaturase	+	+
Lycopene β-cyclase	+	o
β-Carotene hydroxylase	+	nk
β-Carotene oxygenase	+	+

+, upregulation; o, no change of mRNA or protein level; nk, not known (Jin et al 2006)

Grünewald et al. (2000) report that PDS is found exclusively in the chloroplast. This suggests that the carotenoid precursors of astaxanthin are made exclusively in the chloroplast. Because astaxanthin accumulates in lipid vesicles in the cytoplasm, the export of carotenoids from chloroplast to cytoplasm must occur. Because lycopene β-cyclase (LYCB) controls the metabolic flux towards zeaxanthin, a precursor of astaxanthin, the expression of LYCB was examined during the induction of astaxanthin biosynthesis (Linden 1999; Steinbrenner and Linden 2001; Sun et al. 1996). However, only one gene has been found for LYCB in *H. pluvialis*. Although the level of mRNA increased upon exposure to environmental stress conditions, the protein level of the LYCB remained constant (Sun et al. 1996; Sun et al. 1998). This raises the question of whether more of the LYCB enzyme present in nonstressed cells becomes active in response to the exposure of cells to stress conditions. Once the precursor β-carotene is made, two slightly different pathways for generation of astaxanthin are postulated. In both pathways, the enzymes β-carotene ketolase (BKT)

and β-carotene hydroxylase (BKH) are required. CRTO/BKT is upregulated at the mRNA level as well as at the protein level (Grünewald et al. 2001; Grünewald et al. 2000; Sun et al. 1996).

Grünewald et al. (2001) showed that the β-carotene ketolase is located in the chloroplast and in the cytosolic vesicles. Moreover, BKT activity is demonstrated *in vitro* in the lipid vesicles, indicating that BKT acts not only in the chloroplast, but also in the cytosol. For BKH, only upregulation at the mRNA level has been demonstrated so far (Linden 1999; Steinbrenner and Linden 2001; Sun et al. 1996). It has been reported previously that reactive oxygen species (ROS)-generating compounds such as Fe^{2+}, methyl viologen, and methylene blue result in increased astaxanthin accumulation. This leads to the hypothesis that the stress response in *H. pluvialis* may be mediated by ROS [Boussiba 2000; Fan et al. 1998; Kobayashi et al. 1993). However, results from Steinbrenner and Linden (2001) suggested that ROS generators are not involved in the transcriptional regulation of PYS and carotenoid hydroxylase. In corroboration with this finding, previous reports showed that the effect of Fe^{2+} on astaxanthin accumulation is independent of de novo protein biosynthesis and it is suggested that there is a function of ROS at the posttranslational level (Kobayashi et al. 1993). The hydroxylase activity appears to be cytochrome P450- dependent. When hydroxylase activity is inhibited by ellipticine, which is a specific inhibitor of cytochrome P450 enzyme activity, only canthaxanthin accumulates (Schoefs et al. 2001). Overall, the summary of results concerning enzyme functioning in the carotenoid biosynthetic pathway, shown in Table 1.5, demonstrates that all genes investigated so far are upregulated at the level of mRNA in response to stress. However, there exist at least two types of regulation at the translational level. These two types can be distinguished as (1) No change in the protein level of an enzyme (e.g., lycopene β-cyclase) and (2) upregulation at the translational level (example, phytoene desaturase).

A1.8 Genetic Engineering

The progress in the algal genetic engineering was extremely slow until recently little work has been done by adopting a genetic engineering approach to improve the algae. The methods successfully used for transformation in other systems failed when applied to algae. Techniques to introduce DNA into algal cells with suitable promoters, new selectable marker genes, and expression vectors have to be

standardized. Currently, all these requirements have been fulfilled for the diatom *Phaeodactylum*, the green alga, *Chlamydomonas* and the blue green algae, *Synechococcus* and *Synechocystis* (Boussiba 2000). The success of genetic engineering lies in the improvement of nutritional value, product yield with optimal production parameters.

A1.8 .1 Metabolic Engineering of Astaxanthin Biosynthesis

Metabolic engineering is generally carried out to improve the production of existing compounds, to mediate the degradation of compounds, or to produce new compounds by redirecting one or more enzymatic reaction. Approaches for achieving genetic/metabolic engineering include over expression of a single gene, multiple gene combinations or a transcription factor to establish single gene or multigenes control in the biosynthesis pathway for carotenoids, or use of RNAi/antisense knockout of a pathway in order to increase the content or change the composition of carotenoids. There is growing interest worldwide in manipulating carotenoid biosynthesis in carotenoid producing organisms. Cloning of most of the astaxanthin biosynthesis genes in *H. pluvialis* has now opened the door to genetically manipulate this pathway not only in algae, but also in other organisms. Production of natural astaxanthin by genetically engineered microorganisms has been reported (Misawa and Shimada 1998). The highest yield achieved in *E. coli* is 1.4 mg/g dry weight (0.14%) (Wang et al. 1999). Mutants of the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) accumulate (3R, 3R) astaxanthin up to 0.5% of the dry weight (Chen et al. 2003). Since the supply of the common precursors, IPP, DMAPP, GPP, FPP, or GGPP are limited in the bacterium *E. coli*, a host strain that increases the supply of GGPP gave the highest astaxanthin yield of 1.25 mg/g dry weight (Wang et al. 1999). This is 50-fold higher than that of previous reports (Breitenbach et al. 1996). Tobacco plants that expressed BKT in a regulated manner accumulated a high concentration of ketocarotenoids, including astaxanthin, in the chromoplasts of the nectar tissue in their flowers. This changed the flower color from yellow to red (Mann et al. 2003). The concentration of the red ketocarotenoids in the nectary reached 0.2 mg/g fresh weight, which corresponds to ~2 mg/g dry weight (0.2%). The petals of many plants contain considerably higher concentrations of carotenoids. However, in contrast to other organisms, cells of the alga *H. pluvialis* can naturally accumulate (3S, 3'S) astaxanthin up to 4-5% of their dry weight (Boussiba et al. 1999; Yuan and Chen

1999). The reason that plants and algae accumulate carotenoids at concentrations that are 10- to 50-fold higher than other microorganisms is that they possess special mechanisms for storing large amounts of carotenoids inside lipid vesicles located in the cytoplasm or in lipid globules within plastids. In the nectarines of transgenic plants, canthaxanthin and adonirubin are more abundant than zeaxanthin and adonixanthin, suggesting that the BKT preferentially uses β-carotene as a substrate and is more active than the hydroxylase. The gene BKT of *Chlorella zofingiensis* have been cloned and characterized and found to be transiently up-regulated upon glucose treatment (Huang et al. 2006a). Trace amounts of ketocarotenoids found in the leaves of transgenic plants can be explained by the relatively low expression of BKT, driven by the tomato PDS promoter, as indicated from the GUS assay. Unavailability of proper substrates for the β-carotene ketolase in chloroplasts and a lack of the ketocarotenoid-accumulating mechanism, i.e. binding proteins and lipid globules, could also affect astaxanthin formation in plant cells.

The potential commercial interest for the production of carotenoids, cloning of genes encoding biosynthetic enzymes and genetic transformation of gene of interest has led to all kinds of examples of metabolic pathway engineering. These examples include the overexpression of a gene encoding a rate-limiting enzyme (Hoshino et al. 1994; Kajiwara et al 1997), the expression of carotenogenic genes in noncarotenogenic heterologous hosts (Farmer et al. 2000; Misawa and Shimada 1998; Wang et al.1999), the increase of the carbon flux into the carotenoid biosynthetic pathway (Albrecht et al. 1999; Farmer et al. 2000; Kajiwara et al 1997; Wang et al.1999), and the combination of genes and modification of catalytic activities in order to improve and/or modify carotenoid biosynthetic pathways (Mann et al. 2003; Sandmann et al 1999; Schmidt-Dannert 2000 ; Schmidt-Dannert et al. 2000 ; Wang et al.1999). Thus cloning and genetic transformation of the particular gene of interest is the most modern approach for the higher production of novel proteins which are useful for pharmaceuticals and nutraceuticals.

A1.9 Genetic transformation in algae

Genetic transformation in algae is a complex and fast-growing technology. In general, today's non-transgenic, commercial algal biotechnology produces food additives, cosmetics, animal feed additives, pigments, polysaccharides, fatty acids, and biomass. But recent progress in algal transgenics promises a much broader field of application:

molecular farming, the production of proteins or metabolites that are valuable to medicine or industry, seems to be feasible with transgenic algal systems. Indeed, the ability of transgenic algae to produce recombinant antibodies, vaccines, insecticidal proteins, or bio-hydrogen has already been demonstrated. Genetic modifications that enhance physiological properties of algal strains and optimization of algal production systems should further improve the potential of this auspicious technology in the future. In the last few years, successful genetic transformation of ~25 algal species has been demonstrated (Table 1.8); most of these were achieved by nuclear transformation. Ten species of green algae have been transformed, stable transformation has been shown for seven of them, one of which was the unicellular model organism *Chlamydomonas reinhardtii* (Debuchy et al. 1989; Kindle et al. 1989), and transient transformation was demonstrated in the other three. All of these green algae are unicellular species except for *Volvox carteri*, for which stable transformation has been shown (Schiedlmeier et al. 1994), and *Ulva lactuca*, which was transiently transformed (Huang et al. 1996). Species of red algae have also transformed so far (Table A1.9), two of which are unicellular, and one of which is the ultrasmall unicell *Cyanidioschyzon merolae* (Minoda et al. 2004). All four of the multicellular species are macroalgae. Two multicellular species come from the genus *Porphyra*; stable transformation was demonstrated in one of these species, *Porphyra yezoensis* (Cheney et al. 2001).

Table A1.9 Transformable algal species. Nuclear transformation unless otherwise noted.

Groups	Species	Transformation	Reference
Green algae	*Chlamydomonas reinhardtii*	Stable	Debuchy et al. 1989; Kindle et al. 1989
	Haematococcus pluvialis	Stable	Steinbrenner and Sandmann 2006
	Dunaliella salina	Stable	Geng et al. 2003, 2004; Tan et al. 2005
	Dunaliella viridis	Stable	Sun et al. 2006
	Volvox carteri	Stable	Schiedlmeier et al. 1994
	Chlorella sorokiniana	Stable	Dawson et al. 1997
	Chlorella' ellipsoidea	Transient	Jarvis and Brown 1991
	Chlorella' kessleri	Stable	El-Sheekh 1999
	Chlorella vulgaris	Transient	Chow and Tung 1999
	Ulva lactuca	Transient	Huang et al. 1996
Red algae	*Porphyra yezoensis*	Stable	Cheney et al. 2001
	Porphyra miniata	Transient	Kübler et al. 1993
	Kappaphycus alvarezii	Transient	Kurtzman and Cheney 1991
	Gracilaria changii	Transient	Gan et al. 2003
	Porphyridium sp.	stable (chloroplast)	Lapidot et al. 2002
Brown algae	*Laminaria japonica*	Stable	Qin et al. 1999
	Undaria pinnatifida	Stable	Qin et al. 2003
Diatoms	*Phaeodactylum tricornutum*	Stable	Apt et al. 1996; Falciatore et al. 1999, 2000; Zaslavskaia et al. 2000, 2001
	Navicula saprophila	Stable	Dunahay et al. 1995
	Cylindrotheca fusiformis	Stable	Fischer et al. 1999
	Cyclotella cryptica	Stable	Dunahay et al. 1995
	Thalassiosira weissflogii	Transient	Falciatore et al. 1999
Euglenids	*Euglena gracilis*	stable (chloroplast)	Doetsch et al. 2001
Dinoflagellates	*Amphidinium* sp.	Stable	Ten Lohuis and Miller 1998
	Symbiodinium microadriaticum	Stable	Ten Lohuis and Miller 1998
Cyanobacteria	*Spirulina platensis*	Stable	Kawata et al. 2004
	Anabaena sp.	Stable	Thiel and Poo 1989
	Synechocystis sp.	Stable	Dzelzkalns and Bogorad 1986

A1.9.1 Methods to introduce DNA into algal cells

The basis of almost all algal transformation methods is to cause, by various means, temporal permeabilization of the cell membrane, enabling DNA molecules to enter the cell.

Entrance of the DNA into the nucleus and integration into the genome occurs without any external help. DNA integration mainly occurs by illegitimate recombination events, resulting in ectopic integration of the introduced DNA and, thus, culminates in stable genetic transformation. In actuality, it is not difficult to permeabilize a cell membrane in order to introduce DNA; however, the affected reproductive cell must survive this life-threatening damage and DNA invasion and resume cell division. There are a couple of working transformation methods for algal systems that enable the recovery of viable transformants. The most popular method is micro-particle bombardment, also referred to as

micro-projectile bombardment, particle gun transformation, gene gun transformation, or simply biolistics. This method makes use of DNA-coated heavy-metal (mostly gold) micro-projectiles and allows transformation of almost any type of cell, regardless of the thickness or rigidity of the cell wall, and it also allows transformation of organelles. This course of action, was successfully applied in green microalgae like *Chlamydomonas reinha*

rdtii, Haematococcus pluvaialis and other algae groups (Table 1.8) like red algae, brown algae, diatoms, euglenids, dinoflagellates and cyanobacteria. Another less complex and less expensive transformation procedure involves preparation of a suspension of (micro) algae that is then agitated in the presence of micro- or macro-particles, polyethylene glycol and DNA. Several investigators have used silicon carbide (SiC) whiskers (~0.3- 0.6 μm thick and ~5-15 μm long) as micro-particles. These hard and rigid micro-particles allowed transformation of cells with intact cell walls including *Chlamydomonas reinhardtii* (Dunahay 1993), *Symbiodinium microadriaticum* (ten Lohuis and Miller 1998), and *Amphidinium* sp. (Ten Lohuis and Miller 1998); however, cell wall reduced algae seem to be more appropriate when applying this method. Cell-wall free protoplasts of the green alga *'Chlorella' ellipsoidea* can be transformed without any micro- or macro-particles (Jarvis and Brown 1991); agitation of the protoplast in the presence of polyethylene glycol and DNA is sufficient. Naked cells, protoplasts, cell wall reduced mutants and other cells

with thin walls can also be transformed by electroporation. This large electronic pulse temporarily disturbs the phospholipid bilayer of the cell membrane, allowing molecules like DNA to pass. Cells of *Chlamydomonas reinhardtii* (Brown et al. 1991), *Cyanidioschyzon merolae* (Minoda et al. 2004), *Dunaliella salina* (Geng et al. 2003), and *Chlorella vulgaris* (Chow and Tung 1999) have been transformed in this way. Two algal species have been genetically modified in a different way, namely by *Agrobacterium tumefaciens*-mediated transformation via tumor inducing (Ti) plasmids, which integrate semi-randomly into the genome of infected plant cells. The *Agrobacterium* infection causes tumors ("crown galls") in dicots and some monocots, and, astonishingly, some algae become infected, but they do not develop tumors. *Agrobacterium*-commended mediated transformation was demonstrated to work in the multicellular red alga *Porphyra yezoensis* (Cheney et al. 2001) and, most surprisingly, in the unicellular green alga *Chlamydomonas reinhardtii* (Kumar et al. 2004).

A1.10 Safety of *H. pluvialis* astaxanthin

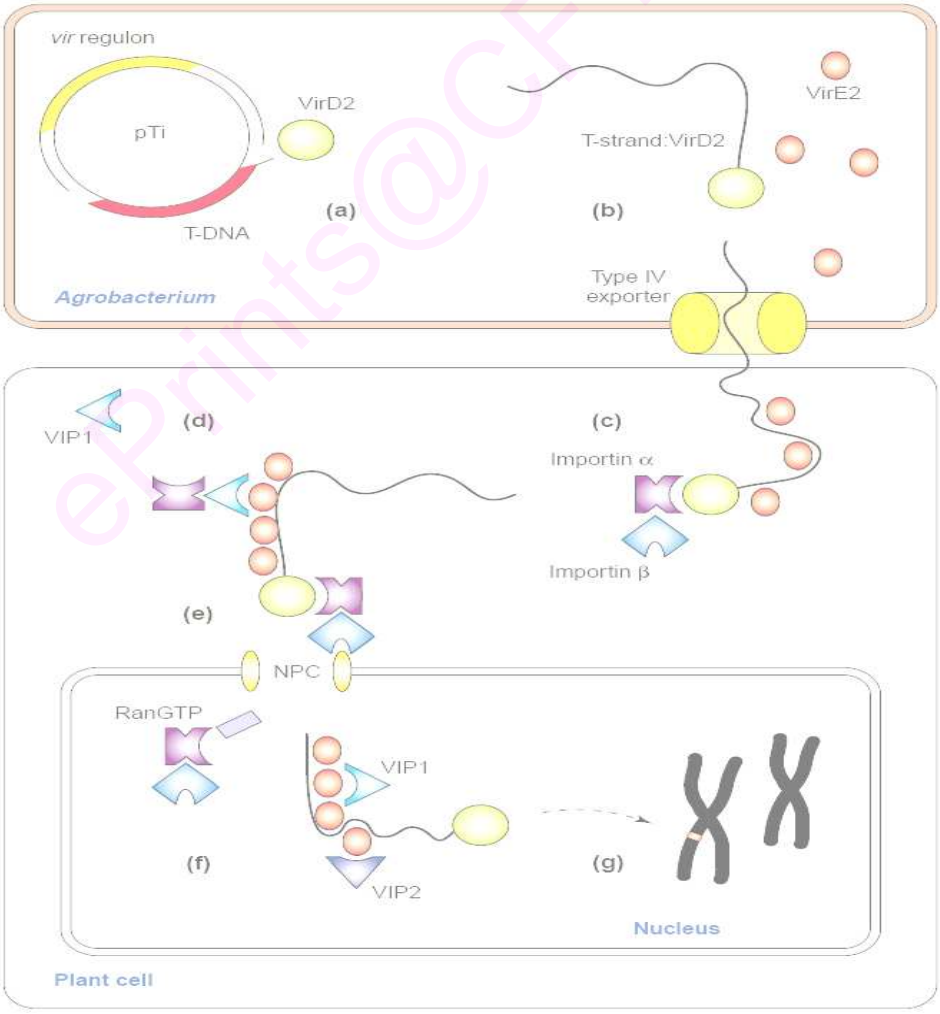
Recent studies support that *H. pluvialis* astaxanthin does not possess any health risks at the tested dosages (Mera Pharmaceuticals 1999). *H. pluvialis* astaxanthin supplements have been available to the public for the last ten years. A recent survey of consumers of a commercial *H. pluvialis* astaxanthin supplement indicates several benefits from astaxanthin supplementation. An improvement as a result of *H. pluvialis* astaxanthin supplementation was observed in 85% of the health conditions reported (Martin et al 2003). Of 26 comparisons with popular brands of prescription drugs, *H. pluvialis* astaxanthin supplementation was reported to be as effective as or more effective than the anti-inflammatory drugs in 92% of the comparisons. Of 62 comparisons with over-the-counter (OTC) drugs including aspirin or ibuprofen, astaxanthin supplementation was reported as effective or more effective in 76% of the comparisons. The large percentage of responses indicating a positive effect of *H. pluvialis* astaxanthin supplementation on health conditions that have or might have a strong inflammation component as well as the positive comparisons of the efficacy of the supplementation with that of anti-inflammatory drugs are indicative of strong anti-inflammatory properties for astaxanthin (Kurashige et al.1990; Bennedsen et al. 1999). The exact mode of action and circumstances under which astaxanthin can help fight inflammation remains to be clarified, whether it is by breaking the chain formation of free radicals aggravating inflammation or through

modulation of enzyme-mediated inflammation mechanisms. These results, however, support the unique potential of astaxanthin to be used as the nutritional component in treatment or prevention strategies against several health problems caused by oxidative stress, UV-light photooxidation or inflammation.

In view of the importance of astaxanthin for their multiple applications, the present studies have focused on *H. pluvialis* for enhanced production through genetic transformation and cloning of BKT and BKH genes.

Chapter I

Genetic transformation of *Haematococcus pluvialis* using selectable marker genes



1.0 Introduction

Progress in genetic engineering has been spectacular since the recovery of the first transformed plants in the early 1980s. Molecular techniques have now been applied to an array of species, resulting in the generation of numerous transgenic species. These species were initially transformed with marker genes, but subsequently with commercially important genes including those enabling pharmaceuticals, nutraceuticals, agronomic improvement, easier processing and other alternative uses. Presently the genetic transformation of unicellular green alga has been reported for some of the green alga like *Chlamydomonas reinhardii* (Kumar et al. 2004), *Chlorella* sp (Maruyama et al. 1994; Dawson et al. 1997), *Spirulina* sp (Toyomizu et al. 2001), *Volvox carteri* (Schiedlmier et al. 1994), *Synechocystis* (Dzelzkalns and Bogorad 1986), *Haematococcus pluvialis* (Teng et al. 2002 and Steinbrenner and Sandman 2006) using different transformation methods like electroporation, particle bombardment, PEG, glass beads, silicon carbide, cell wall-deficient mutants or protoplasts (Kindle 1990; Lumbreras et al. 1998; Dawson et al. 1997). But *Agrobacterium* mediated genetic transfer which is a highly efficient, stable method of genetic transformation was followed only for *Chlamydomonas reinhardii* and no other algae have been attempted using this method due to its compatibility between the hosts for its adherence, induction of vir genes, integration of T-DNA. *Agrobacterium tumefaciens* is a gram-negative pathogenic soil bacteria, cause crown gall disease in a wide range of plants by transferring the T-DNA from its tumour inducing plasmid (Ti plasmid) to the genome of plants. The trans-kingdom gene transfer is initiated by the activity of Ti plasmid encoded virulence (*vir*) genes in response to low molecular weight phenolic compounds like acetosyringone (Gelvin 2000) that are released from the wound region of plants. The ease of the procedure, the transfer of relatively large segment of DNA (up to 150 kb) with little rearrangements, preferential insertion of T-DNA into potentially transcribed regions and the integration of mostly single copy of the transgene(s) into plant chromosomes (Hamilton et al. 1996; Hiei et al. 1994; Kumria et al. 2001) have made this a fast method for gene transfer to a large number of plant species including dicots, monocots and recently the capacity of this inter-kingdom gene transfer has been extended to a variety of fungi (Bundock et al. 1995; De Groot et al. 1998) and also for the green alga *Chlamydomonas reinhardii* (Kumar et al. 2004). So far, *Agrobacterium* mediated genetic transformation is not reported in

the green alga *Haematococcus pluvialis* which produces a high value ketocarotenoid, astaxanthin that has been shown to have higher antioxidant activity than β-carotene and α-tocopherol (Kobayashi and Sakamoto 1999), enhance the immune responses (Jyonouchi et al. 1995) and also act against cancer (Tanaka et al. 1995b). The highlighted mass production of astaxanthin by *Haematococcus* remains problematic since inhibition of cell division occurs while astaxanthin is produced (Boussiba and Vonshak 1991). During the past ten years, the pathway of astaxanthin synthesis has been well studied (Fraser et al. 1998), but the molecular regulatory mechanism of astaxanthin synthesis has not been investigated intensively. Therefore there is an urgent need to develop a genetic transformation system for *Haematococcus* to understand further the molecular regulatory mechanism and to enhance astaxanthin production using genetic manipulation. Although Teng et al. 2002 and Steinbrenner and Sandman 2006 have reported transformation of *H. pluvialis* through electroporation and particle bombardment, the stability and the transformation frequency has not been discussed. Here we report for the first time, genetic transformation of *H. pluvialis* a commercially important micro alga using *Agrobacterium tumefaciens* as the transforming agent.

1.1 Materials and Methods

1.1.1 Green alga *Haematococcus pluvialis*

The *Haematococcus pluvialis* (SAG-19a) culture was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institüt, Universität Göttingen, Göttingen, Germany.

1.1.2 Glass wares

All the glassware, such as conical flasks, test tubes, culture tubes, measuring cylinders, pipettes etc., used for the experiments, were from Vensil Ltd., Mumbai or Borosil Glass works Limited, Mumbai, India.

1.1.3 Plastic wares

The microcentrifuge tubes, microtips and screwcap centrifuge tubes were from Tarsons Products Pvt. Ltd. Kolkata. The polyethylene (LDPE) bags and tubes were procured from local market.

1.1.4 Chemicals

All the media chemicals used for the experiments were analytical grade, obtained from companies - HiMedia Laboratories Pvt. Ltd.,-Mumbai; Sisco Research

Laboratories Pvt. Ltd.,-Mumbai; Mumbai; Ranbaxy Fine Chemicals Ltd.,-New Delhi; Loba Chemie Pvt. Ltd.,-Mumbai. Qualigens Fine Chemicals-Mumbai. Authentic standards and fine chemicals such as astaxanthin, β-carotene, were obtained from Sigma-Aldrich Chemicals - USA. Antibiotics used were obtained from HiMedia Laboratories Pvt. Ltd.,-Mumbai and Ducheffa-The Netherlands. Molecular biology chemical and enzymes were obtained from Fermentas International Inc., Burlington - Canada, Bangalore Genei - Bangalore, Sigma-Aldrich Chemicals - USA, Different kits used for molecular analysis were obtained from Qiagen PCR purification kit and Qiagen Gel elution kit from Qiagen, GmpH - Germany, Brightstar psoralen-Biotin nonisotopic Labelling kit from Ambion Inc, Texas - USA, Commercial nitrogen gas cylinders were procured from Kiran Corporation - Mysore.

1.2 Methodolgy

1.2.1 Maintenance of stock culture

Stock cultures of *H. pluvialis* were maintained in autotrophic liquid Bold's basal medium (BBM) (Kanz and Bold 1969) and solid Z8 medium (Sivonen et al. 1989). The composition of the BBM and Z8 medium are provided in Table 1.1. and Table 1.1. For cocultivation procedure for *H. pluvialis* and *Agrobacterium*, Tris acetate phosphate (TAP) medium (Harris 1989) was used (Table 1.3 and Table 1.4). The constituents of each medium were dissolved in distilled water and the pH was adjusted accordingly to the particular media using 0.1N HCl or 0.1N NaOH. To prepare solid medium for plates and slants, agar at the rate of 15g/L was added to the medium. The media was distributed into 150/250ml conical flasks, closed with cotton plugs and sterilized by autoclaving at 121°C for 20 min and allowed to cool at room temperature before inoculation. Inoculation was carried out under aseptic conditions in laminar air flow hood. The *H. pluvialis* slants and the liquid cultures were subcultured at every 4 week and 2 week intervals respectively.

Table 1.1. Composition of media for *H. pluvialis* growth

Constituents (gL^{-1})	BBM	Modified BBM	Z8 Medium	Nutrient limiting medium
$NaNO_3$	0.25	-	0.466	-
Ammonium carbonate	-	0.159	-	0.0159
K_2HPO_4	0.075	0.075	0.031	0.0075
KH_2PO_4	0.175	0.175	-	0.0175
$MgSO_4.7H_2O$	0.073	0.073	0.024	0.073
$CaCl_2.2H_2O$	0.024	0.024	-	0.024
$Ca(NO_3)_2\ 4H_2O$	-	-	0.059	-
NaCl	0.025	0.025	-	0.025
Na_2CO_3	-	-	0.021	-
$FeSO_4.7H_2O$	0.005	0.005	-	0.005
$FeCl_3$	-	-	0.0016	-
EDTA	0.045	0.045	-	0.045
Disodium EDTA	-	-	0.0033	-
Trace elements	1 ml	1 ml	1 ml	1 ml
pH	7.0	7.0	7.0	7.0

Table 1.2. Composition of trace elements for *H. pluvialis* growth

Constituents	g/100ml
H_3BO_3	0.3100
$MnSO_4.4H_2O$	0.2230
$ZnSO_4.7H_2O$	0.0287
$(NH_4)_6MoO_{24}.4H_2O$	0.0088
$(CoNO_3)_2.4H2O$	0.0146
$Na_2WO_4.2H_2O$	0.0033
KBr	0.0119
KI	0.0083
$Cd(NO_3)_2.4H_2O$	0.0154
$NiSO_4(NH_4)_2SO_4.6H_2O$	0.0198
$VoSO_4.2H_2O$	0.0020
$AlCl_3.6H_2O$	0.00237

Table 1.3 Composition of TAP Media

Constituents	g/100ml
Ammonium chloride	0.400
$MgSO_4.7H_2O$	0.100
$CaCl_2.2H_2O$	0 .500
K_2HPO_4	0.108
KH_2PO_4	0.540
Tris	2.400
Glacial acetic acid	1 ml/l
Hunter's trace elements	1 ml/l
pH	6.7

Table 1.4 Composition of hunters trace elements

Composition of hunters trace elements	mg L^{-1}
EDTA Disodium	50.00
$ZnSO_4.7H_2O$	22.00
H_3BO_3	11.00
$MnCl_2.4H_2O$	5.06
$FeSO_4.7H_2O$	4.99
$Co\ Cl_2$	1.61
$CuSO_4$	1.57
$(NH_4)_6MoO_{24}.4H_2O$	1.10
pH	6.7

1.2.2 Growth condition for *H. pluvialis*

The inoculated *H. pluvialis* in liquid cultures, plates and slants (Fig 1.1) were incubated in culture room under controlled temperature at 25±1°C and light intensity 18.75 ± 2.5 µmol m^{-2} s^{-1} under 16/8 h light/dark cycle. Light was provided by cool white fluorescent set of lamps (40W; Phillips India Ltd, Kolkata, India) and the light intensity was measured using lux meter (TES 1332, Taiwan). The liquid cultures were shaken manually once a day. The *H. pluvialis* liquid cultures were subcultured at every 15-20 days intervals and the slants cultures were subcultured at every 45 days intervals.

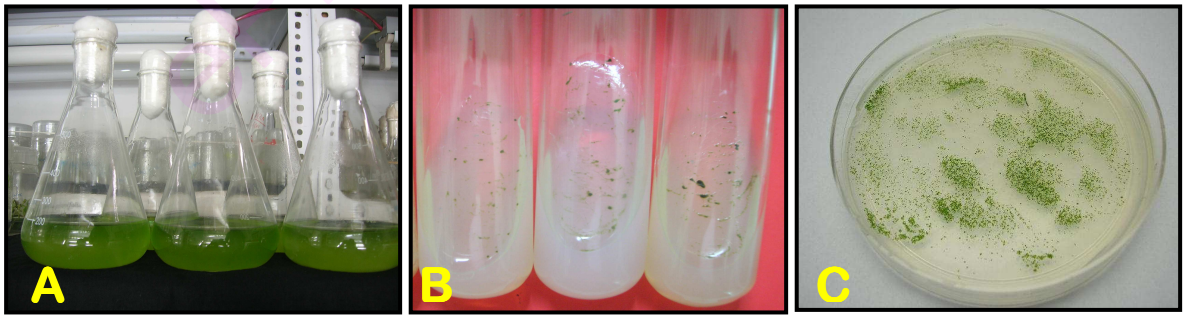


Figure 1.1 *H. Pluvialis* stock culture maintained in liquid (A) slants (B) and plates (C).

1.2.3 Sensitivity test for antibiotics

Initially the alga was tested for antibiotics like cefotaxime, augmentin at the concentration of 250, 500, 1000 and 2000 mg L^{-1}. The alga was tested for its sensitivity to hygromycin at 1 to 10, 25, 50 and 100 mg L^{-1} of concentration. The

sterile Z8 agar medium was melted and cooled to a temperature of 50^0 C to 55^0 C and the hygromycin was added to get a required concentration and plated in the plates. The plates were allowed to cool and the alga was inoculated at 10^6 cells per ml. The growth was monitored up to 8 weeks.

1.2.4 Cocultivation media for *Agrobacterium* and *H. pluvialis*

Different cocultivation media were tested for the growth of both alga and bacterium. The solid cocultivation medium viz., BBM + Half strength of LB (Luria-Bertaini) medium, Z8 medium + half strength of LB medium, Z8 medium + mannitol (0.5%), Z8 medium only and TAP (Tris acetate phosphate) medium alone were tested. The plates were prepared separately by plating the molten medium in the plates. The algae and bacterium were separately plated in petri plates containing these cocultivation medium and growth was monitored at weekly intervals.

1.3 *Agrobacterium* mediated genetic transformation

1.3.1 Plasmid constructs and bacterial strains

The binary vector pSK53 (Fig 1.1 A) was introduced into *Agrobacterium tumefaciens* strain EHA 101 (nopaline type). The plasmid pSK53, harbours *hpt* (hygromycin phosphotransferase) as selection marker gene and GFP (green fluorescence protein) and *UidA* (β-glucuronidase) as reporter genes interrupted by an intron both driven by the CaMV 35S promoter and has been constructed from pCAMBIA 1301. Kanamycin 100 mgL^{-1} was used which is the bacterial selection marker while DH5α was the *E. coli* strain used. The binary vector pCAMBIA1301 (Fig 1.1B) (Genbank Accession: AF234290 - AF234316 (http://www.cambia.org/daisy/cambia/585.html#dsy585 References) was also used for the study which is having the same selectable marker and reporter genes but without the green fluorescent protein.

1.3.2 *Agrobacterium* mediated transformation procedure

Agrobacterium mediated transformation was carried out by using both the vectors separately. The protocol for the cocultivation of *Agrobacterium tumefaciens* and *H. pluvialis* is given in Fig 1.3 Approximately 10^6 cells of *H. pluvialis* were plated on the solid TAP medium in petri plates and incubated under 16:8 light dark cycle with the light intensity of 18.75 ± 2.5 μmol $m^{-2}s^{-1}$ at 25 ± 1^0C temperature until a lawn of cells was observed (~5-7 days).

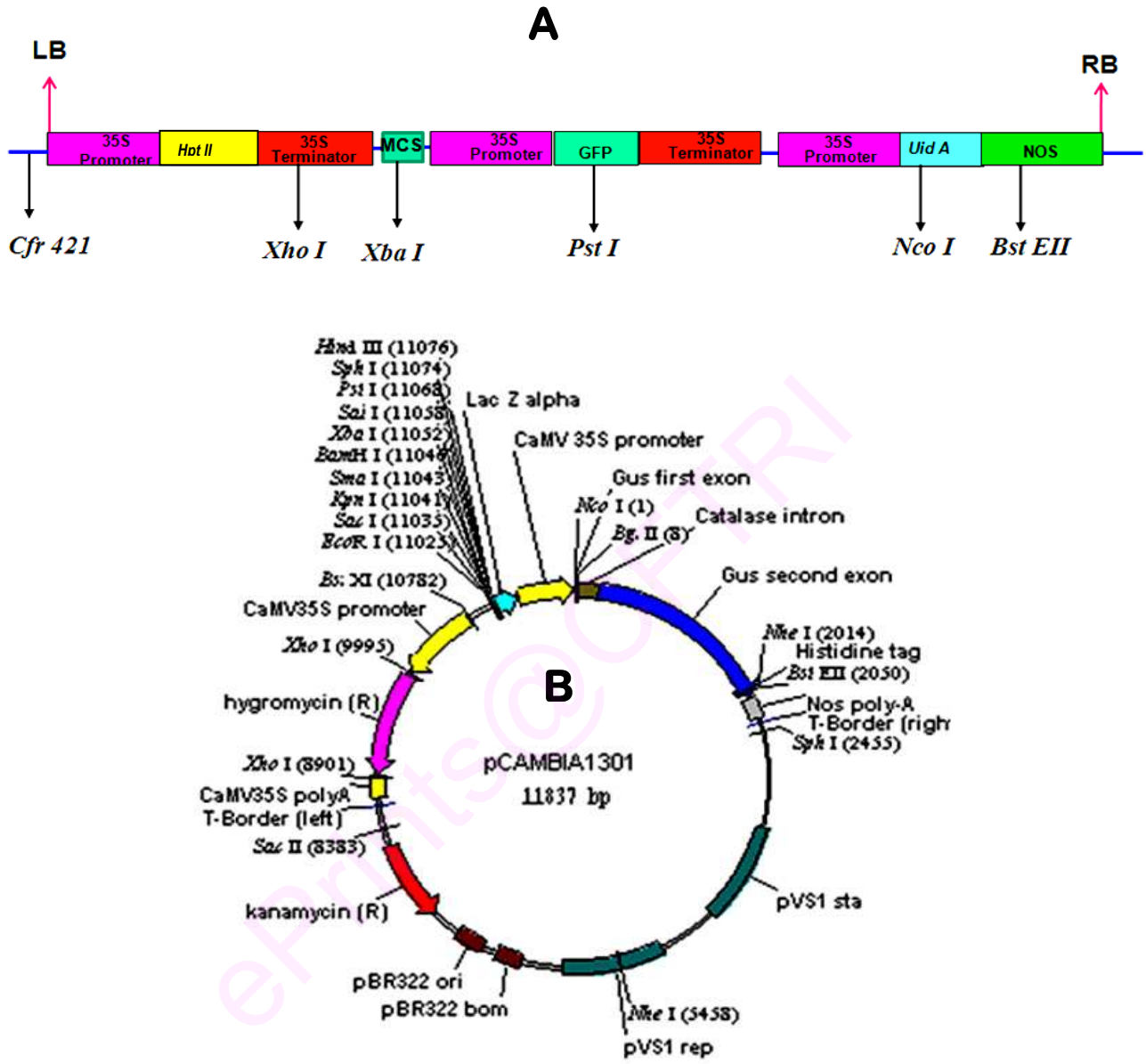


Figure 1.2. Linear map of the T-DNA region of the binary vector pSK 53 (A) and circular map of pCAMBIA1301 (B) both having the selectable marker gene *hpt* (hygromycin phospho transferase) and *UidA* (β-glucoronidase) as reporter genes both driven by the CaMV 35S promoter. pSK 53 also having the selection marker gene and GFP (green fluorescence protein). The restriction sites in the T-DNA region are approximately marked. LB- Left border of the T-DNA region, RB- Right border of the T-DNA region, *Hpt II* – hygromycin phosphotransferase gene, MCS- multiple cloning sites and NOS – nopaline synthase terminator gene.

Agrobacterium (A600 - 0.5) bearing pSK53/pCAMBIA1301 plasmid, grown overnight in liquid LB medium was harvested by centrifugation at 5000rpm for 10

min. The pellet was further resuspended in the TAP medium. A 200µL aliquot of the bacterial culture suspended in TAP medium was plated on a lawn of *H. pluvialis* already grown on plates containing TAP medium. The plates were incubated under continuous light intensity of 18.75 ± 2.5 µmol $m^{-2}s^{-1}$ at 22±1^{0}C temperature. After 48hrs of co-cultivation the cells were harvested, washed with liquid TAP medium (for 30 min) containing 500 mgL^{-1} of cefotaxime + 200 mgL^{-1} augmentin to kill *Agrobacterium*. *H. pluvialis* was recovered by centrifugation at 1000 rpm for 5 min, washed with sterile distilled water several times and resuspended in liquid TAP medium The cells were plated on solid Z8 medium containing 10 mg $L^{-1}$of hygromycin and a mixture of cefotaxime (500 mgL^{-1}) and augmentin (200 mg L^{-1}). After the colonies were observed on the plate the growth was monitored up to 6-8 weeks. Colonies were selected from plates and maintained in a selection medium. Hygromycin resistant cells grown in selection medium were further cultured in liquid medium without hygromycin and used for molecular analysis. The effect of phenolic, acetosyringone (AS) (3', 5'-Dimethoxy-4'-hydroxy-acetophenone, Sigma- Aldrich, USA) during co-cultivation was also studied. The TAP medium containing acetosyringone at 100µM and 250µM concentration was prepared (Appendix) for the cocultivation.

1.3.3 Growth analysis of resistant cells in liquid selection medium

The hygromycin resistant cells have been maintained on solid media with 10 mg L^{-1} of hygromycin. The hygromycin resistant *H. pluvialis* cells from solid selection media were inoculated to the fresh BBM medium (liquid) with the initial cell count of 1 x 10^6 cells per ml without hygromycin and the culture was maintained with regular subculturing in the flask at 15 day intervals. The stability was observed by plating these cells (grown in liquid BBM non selection medium) in the hygromycin containing plates. The hygromycin resistant cells and control cells were also cultured in liquid medium with hygromycin at 1 to 10 mg L^{-1} and the growth of the culture in terms of cell count was monitored on 1st, 3rd, 5th and 7th day after inoculation

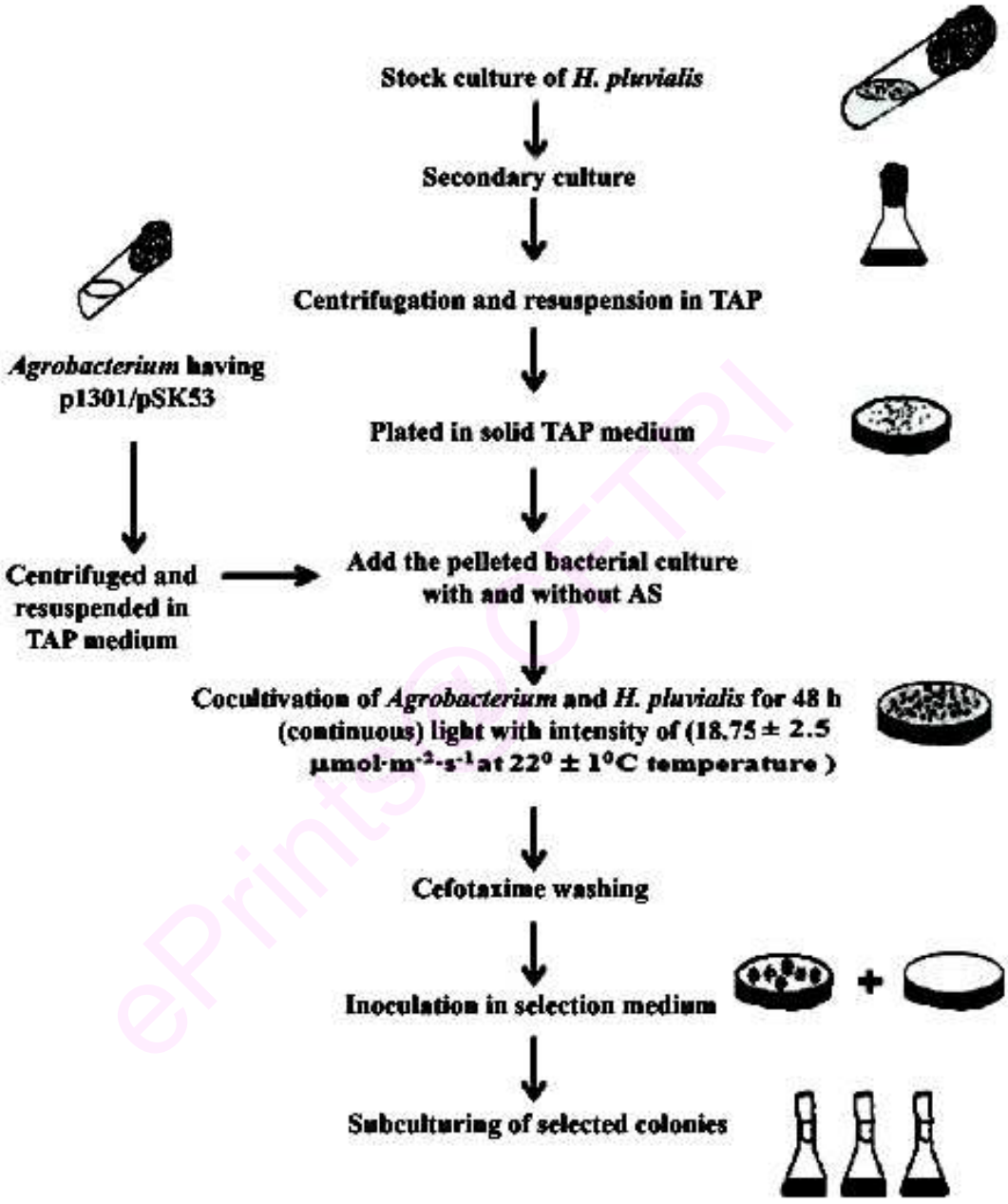


Figure 1.3 Schematic illustration showing co-cultivation of *Agrobacterium tumefaciens* and *H. pluvialis*

1.4 Confirmation of Transformation

1.4.1 Growth of hygromycin resistant cells

The transformation was initially confirmed by observing the growth of hygromycin resistant and susceptible cells in the selection medium having the hygromycin10 mgL^{-1} in the solid medium. The numbers of colonies observed on the hygromycin containing plates were counted. Transformation frequency was calculated based on the number of colonies that are resistant to hygromycin, in proportion to the number of colony forming units taken for transformation. Cells not subjected to co-cultivation are referred to as control cells/non-transformed cells.

$$\textbf{Transformation frequency} = \frac{\text{Number of cells inoculated x } 10^6}{\text{Number hygromycin resistant colony forming units}} \text{ X } 100$$

1.4.2 GUS Assay

Cells resistant to hygromycin were harvested after centrifugation at 5000 rpm for 5 min. The harvested cells were incubated with pectinase (0.2%) and cellulase (0.1%) in 0.01M phosphate buffer (Appendix) for 2hrs at 37^0C and subsequently washed with phosphate buffer and sterile water several times. The enzyme treated cells were destained with 70% ethanol several times and fixed with FAA [10: 5: 85 of formaldehyde: glacial acetic acid: ethanol]. GUS expression was visualized by incubating the cells with staining buffer [50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 3mg X-Gluc (5-bromo-chloro-indolylglucuronide cyclo-hexammonium salt Sigma-Aldrich chemicals, USA) 5ml Triton X-100, pH 7.0 overnight (Jefferson et al. 1987). The stained cells were observed under light microscope (OLYMPUS OPTICAL Co. Ltd. Tokyo, Japan. BX40 F4).

1.4.3 Detection of GFP

H. pluvialis cells resistant to hygromycin were collected after centrifugation at 5000 rpm for 5 min. Chlorophyll was removed from the cells by heating with tetrahydrofuran and methanol (1:1) at 60^0C. The presence or absence of fluorescence was observed under fluorescent microscope (OLYMPUS OPTICAL Co. Ltd. Tokyo, Japan. CKX41) under the excitation filter (BP460-490C) and barrier filter (BA520IF).

1.4.4 Scanning Electron Microscopy

Samples were prepared using the procedure followed by Whittaker and Drucker, 1970. *H. pluvialis* cells cocultivated with *Agrobacterium* and control cells pelleted at 4000 rpm for 5 min, washed with distilled water and fixed in 3% glutaraldehyde in 0.01M phosphate buffer (Appendix) for 2 hours at 2^0C. The fixative was removed with four changes of distilled water and the cells were incubated overnight in sterile water. Cells were then washed with isopentane, frozen in liquid nitrogen, freeze dried, coated with gold using a sputter coater (Polaram Ltd., Watford, England. E5100) and examined on a scanning electron microscope (Leo Electron Microscopy Ltd., (now Carl Zeiss) Cambridge, UK. Jeol JSM-35C).

1.4.5 Stability analysis

Transformed cells have been maintained on solid media with 10 mgL^{-1} of hygromycin. The transformed *H. pluvialis* cells were inoculated to the fresh BBM medium (liquid) with the initial cell count of 1 x 10^4 cells per ml without hygromycin and the culture was maintained with regular subculturing at 15 day intervals. The DNA extracted (Gene Elute-Plant Genomic extraction kit, Sigma, USA) from the transformant culture in non selective liquid medium were analyzed for its stability by PCR using specific primers (section 1.5.1) for a portion of the *hpt* gene at 3 - 4 months intervals. The transformants were also cultured in liquid medium with hygromycin at 1to 10 mgL^{-1} and growth of the culture in terms of cell count was monitored. The minimum load of transformants required for sustenance in liquid medium containing 5 and 10 mgL^{-1} hygromycin was also tested by varying the initial inoculum from 1 x 10^4 to 8 x 10^4 colony forming units per ml.

1.5 Molecular confirmation

1.5.1 Genomic DNA isolation and detection by PCR

Genomic DNA from hygromycin resistant and non-transformed cells of *H. pluvialis* were extracted using a DNA extraction kit (Gene Elute-Plant Genomic extraction kit, Sigma-Aldrich chemicals, USA). The part of *hpt* DNA was amplified using the PCR thermal cycler (Eppendorf Mastercycler personal, Germany) with the designed primers (synthesized by MWG biotech pvt Ltd. Bangalore). The primer pairs were as follows, forward: 5'GATGTTGGCGACCTCGTATT3' and reverse: 5'GTGTCACGTTGCAAGACCTG3'. The primer pairs used to amplify the part of the GUS gene is as follows,

forward: 5'AATTGATCAGCGTTGGTGG3' and reverse 5'GCAAGACTGTAACCA CGCGT3'. PCR was performed with 200ng of genomic DNA and 100 pmol of each of the primers (Sigma-Aldrich Chemicals, USA) using Taq polymerase (MBI Fermentas, Lithuania). The cycling parameters were: 4 min initial denaturation at 94^0C and 35 cycles involving 1 min denaturation at 94^0C, 1 min annealing at 55^0C and 1 min at 72^0C extension. The PCR products were separated on agarose gels (1.5%) using TAE buffer (Appendix) and stained with ethidium bromide (Appendix). Images of gels were recorded with a gel documentation system (Hero Lab, GmbH, Wiesloch, Germany). The expected size of amplicon when using the *hpt* and GUS primers is 407 bp and 515 bp respectively.

1.5.2 Southern blotting analysis

1.5.2.1 Probe preparation and Labeling

A portion of the hygromycin gene was amplified using plasmid (pCAMBIA1301/pSK53) DNA as template and then purified using a Qiagen PCR purification kit (Qiagen GmpH, Germany). The fragment was labeled with psoralen - biotin labeling kit (from Ambion Inc, Texas, USA), according to the method prescribed by the manufacturer. The *hpt* labeled probe was used for the detection of T-DNA in the transformants. The procedure followed for the southern blotting like restriction digestion of DNA, transformation of digested DNA to nylon membrane, purination, repurination, washing, prehybridization, post hybridization, blocking, detection etc were carried out by following the procedures in Sambrook and Russel 2001 ; Ckezhk et al. 2004 (appendix). The reagents, stock solutions, buffers etc for the southern blotting were described in appendix.

1.5.2.2 Digestion, hybridization and detection

Aliquots of 25µL of genomic DNA (25µg) from transformed and nontransformed cells were digested with *Cfr* 421 (*Sac II*) and *XbaI* enzymes separately as described by Sambrook et al. 1989. Digested fragments of DNA were separated on 0.8% agarose gel (Appendix), transferred to positively charged nylon membrane (BrightstarTM Plus, Ambion Inc, Texas, USA) and hybridized at 58^0C with biotin labeled *hpt* gene fragments. The probe hybridized fragments in the membrane was blocked with Strep-alkaline phosphatase by incubation and then

washed two to three times. The washed membrane was incubated in the detection buffer with NBT substrate for colour development.

1.6 Growth measurement and pigment extraction from transformed *H. pluvialis*

1.6.1 Cell number

Algal cell number was determined by counting algal cells using Neubauer haemacytometer (Thoma neu, Germany) and expressed as number of cells/ml.

1.6.2 Dry weight

Known volume of culture was centrifuged at 3000 × *g* for 10 min and the algal biomass was washed with distilled water and dried in a hot air oven (Sanyo, Electrical Biomedical Co. Ltd., Japan) at 60°C till constant weight was obtained. Biomass weight was expressed as gL^{-1}.

1.6.3 Pigment extraction

For pigment analysis, an aliquot of culture was harvested by centrifugation at 3000 x g for 10 min and freeze-dried. A known quantity of biomass was extracted with 90% acetone in a mortar and pestle using neutralized sand. The extraction was repeated till the pellet became colorless. The extracts were centrifuged at 8800 x g for 5 min and the supernatants were pooled. Aliquot of extract is flushed with N_2 gas and stored at -20^0C preferably at -80^0C for spectrophotometric analysis, and another aliquot of extract was evaporated to dryness using N_2 gas and stored at -20^0C or -80^0C for HPLC analysis. All operations were carried out under dim light.

1.6.4 Spectrophotometric estimation of pigments

The acetone extracts absorbance was recorded at 470, 480, 645 and 661.5 nm using spectrophotometer (Shimadzu 160A). Chlorophyll and total carotenoid contents were calculated using following Lichtenthaler (1987) equations.

Chlorophyll a C_a (µg/ml) = $11.24\ OD_{661.5} - 2.04\ OD_{645}$

Chlorophyll b C_b (µg/ml) = $20.13\ OD_{645} - 4.19\ OD_{661.5}$

Total Chlorophyll C_{a+b} (µg/ml) = $7.05\ OD_{661.5} + 18.09\ OD_{645}$

Total carotenoid (µg/ml) = $[1000 \times OD_{470} - (1.9 \times C_a + 63.14 \times C_b)]/214$

Pigment (mg/g) = (Pigment content (µg/ml) × volume of extract × dilution factor) / biomass taken for extraction (mg)

Astaxanthin content was determined at 480 nm using an absorption coefficient, A1% of 2500 by the method of Davies (1976).

Astaxanthin content (mg) = (OD_{480} x volume of extract x dilution factor x 10) / 2500

1.6.5 High Performance Liquid Chromatograph (HPLC) of pigments

The *H. pluvialis* extracts were subjected to HPLC analysis in Shimadzu LC-10AT liquid chromatograph instrument using reverse phase C18 column (Supelco, 25 cm × 4.6 mm). Gradient solvent system consisting of acetone (10%) and 90% (v/v) methanol at a flow rate of 1.25 ml/minute was used. The separated carotenoids were identified by comparing retention times and spectra against known standards. Echinenone and astaxanthin esters (mono and di) were identified using a photodiode array detector (SPD-M10AVP, Shimadzu) by comparing their spectra and retention time with published data (Yuan and Chen 1999; Grünewald and Hagen 2001; Miao et al. 2006). The peaks were integrated by Class VP version 6.14 SP1 software (Shimadzu, Singapore) at 476 nm to quantify ketocarotenoids and 445nm to quantify other carotenoids. The peaks were also integrated at 645nm to detect chlorophylls. Standard β-carotene, lutein and astaxanthin were purchased from Sigma-Aldrich (St.Louis, MO, USA) and canthaxanthin was obtained from ChromaDex, Inc. (SantaAna, CA, USA). Neoxanthin and Violaxanthin were gift from Dr. Akhihiko Nagao of Food Research Institute, Tsukuba, Japan.

1.7 Results

1.7.1 Growth in cocultivation medium

Initial studies focused on selection of the medium that would allow the simultaneous growth of both *Agrobacterium* and *H. pluvialis.* The *H. pluvialis* and *Agrobacterium* were inoculated separately in the different cocultivation medium (Table 1.5). Among the cocultivation media tested, BBM + Half strength of LB medium and Z8 medium + half strength of LB medium favoured the profuse growth for *Agrobacterium* (full mat like appearance of bacteria was observed in the plates within 24 hrs of inoculation) but limited growth for the alga (visible growth of the alga was not observed in the plates even after one week of inoculation). Whereas Z8 medium + mannitol (0.5%) and Z8 medium alone favoured good growth of alga (single, many minute colonies were observed after one week of inoculation) but no growth of the *Agrobacterium* (growth was not observed in the plates even after 48 hrs of inoculation). Only the TAP medium favoured growth of both the alga and *Agrobacterium*.

Table 1.5. Growth of both *Haematococcus pluvialis* and *Agrobacterium tumefaciens* in different cocultivation medium

Sl.No	Media used for cocultivation	Growth of *Haematococcus pluvialis*	Growth of *Agrobacterium tumefaciens*
1	BBM + Half strength of LB medium	Limited growth	Profuse growth
2	Z8 medium + Half strength of LB medium	Limited growth	Profuse growth
3	Z8 medium + mannitol (0.5%)	Good growth	No growth
4	Z8 medium only	Good growth	No growth
5	TAP (Tris acetate phosphate) medium	Good growth	Good growth

Limited growth – Visible growth of the alga was not observed in the plates even after one week of inoculation

Profuse growth – Full mat like appearance of bacteria was observed in the plates within 24 hrs of inoculation

Good growth – Single, enormous minute colonies of alga was observed after one week of inoculation and growth bacteria was observed like a thin slimy layer on the medium after 2 days of inoculation.

No growth – Growth of the bacteria was not observed in the plates even after 48 hrs of inoculation

1.7.2 Sensitivity for antibiotics and selection of resistant colonies of *H. pluvialis*

The effect of cefotaxime and augmentin on the growth of both algae and bacterium in solid medium was studied. *H. pluvialis* was able to tolerate up to the concentrations of 2000 $mgL^{-1}$of both cefotaxime and augmentin. Growth and multiplication of the algae was suppressed and cells ultimately killed when the hygromycin concentration exceeded 2 mg L^{-1} (Table 1.6). The cocultivated alga (with *Agrobacterium* having the plasmid pCAMBIA1301) was subjected to grow in selection medium at 1 to 10, 25, 50 and 100 mg L^{-1} of hygromycin. The colonies appeared after 3 – 4 weeks of incubation. Growth was observed upto the concentration of 10 mg L^{-1} of hygromycin. No growth was observed for the concentrations of 25, 50 and 100 mg L^{-1} of hygromycin (Table 1.7). These cells eventually were grown in concentrations of hygromycin attaining 10 mgL^{-1}. There was no growth, even after 4 weeks of inoculation when the hygromycin concentration exceeds 2 mg L^{-1} for the control cells. The colonies of control cells in nonselective medium were more in number when compared to the colonies of hygromycin resistant cells in selection medium (having hygromycin 10 mg L^{-1}).

Table 1.6. Growth of control and cocultivated *H. pluvialis* in selection medium using different concentration of hygromycin

Concentration of Hygromycin (mg L^{-1})	Growth of *H. pluvialis* at weekly intervals			
	I week	II week	III week	IV week
Control	+	+	+	+
1	+	+	+	+
2	+	+	+	+
3	+	+	+	-
4	+	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
25	-	-	-	-
50	-	-	-	-
100	-	-	-	-

+ → Growth of cells were observed **- → No growth of cells were observed**

1.7.3 Cocultivation of *Haematococcus* and *Agrobacterium*

The cocultivation protocol followed here (Fig 1.3) gave the single isolated colonies on plates containing 10 mg L^{-1} of hygromycin after 3-4 weeks of incubation. A different stage of the growth of hygromycin resistant *H. pluvialis* in selection medium was observed (Fig 1.4). Initially the colonies were very tiny and more in number in the selection medium. Thin mat like appearance of cocultivated *H. pluvialis* was observed after 1 week of incubation (Fig 1.4A), minute single cell colonies after 2 weeks (Fig 1.4B) and clear single cell colony of hygromycin resistant *H. pluvialis* after 4 weeks of incubation (Fig 1.4C). The number of colonies appeared after 4 weeks were not reduced

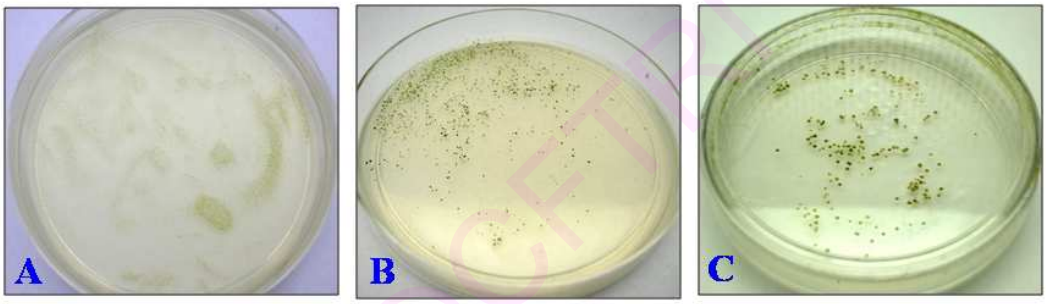


Figure 1.4 Different stages of the growth of transformed *H. pluvialis* in selection medium after co-cultivation (A) Thin mat like appearance of *H. pluvialis* after 1 week of co-cultivation (B) Minute single cell colony of *H. pluvialis* after 2 weeks of co-cultivation (C) Clear single cell colony of transformed *H. pluvialis* grown in selection media after 4 weeks of co-cultivation

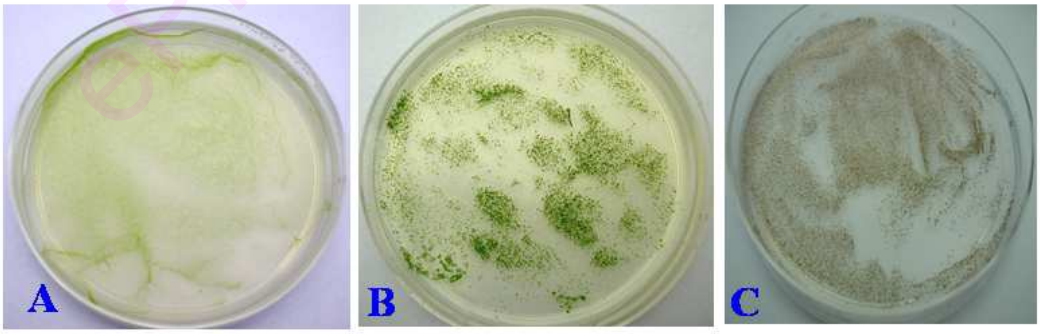


Figure 1.5. Different stages of the growth of non transformed *H. pluvialis* in medium without hygromycin (A) Thin mat like appearance of *H. pluvialis* after 1 week of co-cultivation (B) Minute single cell colony of *H. pluvialis* after 2 weeks of co-cultivation (C) Clear single cell colony of transformed *H. pluvialis* grown in selection media after 4 weeks of co-cultivation

The growth pattern of control cells in non selection medium was differed with cocultivated cells (Fig 1.5). The control cells in nonselective medium grows like thin mat after 1 week (Fig 1.5A), minute single cell colonies after 2 weeks (Fig 1.5B) and profuse growth of the single cell colonies after 4 weeks (Fig 1.5C).

Table 1.7. Growth of cocultivated *H. pluvialis* in selection medium (solid) using different concentration of hygromycin

Concentration of Hygromycin (mgl^{-1})	Growth of *H. pluvialis* at weekly intervals			
	I week	II week	III week	IV week
0	+	+	+	+
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9 & 10	+	+	+	+
25,50,100	-	-	-	-

1.7.4 Transformation frequency

The frequency of transformation by pCAMBIA1301/pSK53 was calculated as the number of cells that survived on plates (containing 10 mgL^{-1} of hygromycin) from a count of 10^6 colony forming units that were initially plated (Table 1.8). The transformation frequency of cells grown in the presence of 100µM and 250µM of acetosyringone was 153 ± 4.5 and 128 ± 5.2 colony forming units respectively per 10^6 cells while that observed in cells co-cultivated with *Agrobacterium* in the absence of acetosyringone was 109 ± 4.0 cfu per 10^6. There was no significant difference between the cells cocultivated with and without acetosyringone.

Table 1.8 Transformation frequency of *H. pluvialis*

Treatments	No. of Cells plated	No. of Hygromycin resistant colonies observed	Transformation frequency (per 10^6 cells ± S.D)
Co-cultivation on TAP	2.8 x 10^6	307	109 ± 4.0
Co-cultivation on TAP+ 100 µM AS	3.1 x 10^6	476	153 ± 4.5
Co-cultivation on TAP+ 250 µM AS	3.4 x 10^6	437	128 ± 5.2

1.7.5 Growth of resistant cells in liquid selection medium

The hygromycin resistant cells in solid TAP medium showed different growth pattern in liquid TAP medium having different concentration of hygromycin viz, 1, 2, 4, 5 and 10 mg L^{-1} of hygromycin. The growth of hygromycin resistant cells and control cells were monitored in liquid selection medium (Fig. 1.6 A & B). The growth of resistant cells were observed in hygromycin concentrations upto 2 mg L^{-1} and the cell count was found to be 10 x 10^4 cells/ml (6 fold increase) at 7 days after inoculation. The cells were found to be viable up to the concentrations of 5 and 10 mg L^{-1} of hygromycin (2 x 10^4 cells/ml at 7 days after inoculation), while they were unable to multiply. The ability to multiply was resurrected when these cells were transferred to nonselective medium. This may be due to the differences in membrane permeability among the cells grown in solid medium and those grown in liquid. Control cells were unable to survive even in 1 mg L^{-1} of hygromycin (Table 1.9 and Fig. 1.6B) since the number of control cells in 1 mg L^{-1} of hygromycin got decreased to 0.5 x 10^4 from the initial number of cells inoculated 1.5 x 10^4. Hence the resistant cells are being maintained in non selection medium. The stability of the resistant (having *hpt* gene) cells in non selection medium over a period of two and half years has been confirmed by inoculating them in solid selection medium having 10 mg L^{-1} of hygromycin at intervals of 3 - 4 months and the growth was observed.

Table 1.9. Growth of cocultivated *H. pluvialis* and control cells in selection medium having different concentration of hygromycin.

Conc. of Hygromycin mg L^{-1}	Number of cells x 10^4							
	1st Day		3rd Day		5th Day		7th Day	
	T. cells	C. cells	T. cells	C. cells	T. cells	C. cells	T. cells	C. cells
0	1.5	1.5	38.0	29	46	42	51	48
1	1.5	1.5	9.0	7.0	12	2.5	13	0.5
2	1.5	1.5	8.5	3.0	11	1.5	10	0
4	1.5	1.5	4.5	1.0	4	0.5	4	0
5	1.5	1.5	2.5	1.0	3	0	2	0
10	1.5	1.5	2.0	0	2	0	2	0

T. cells – Transformed cells (Cocultivated cells) **C. cells – Control cells**

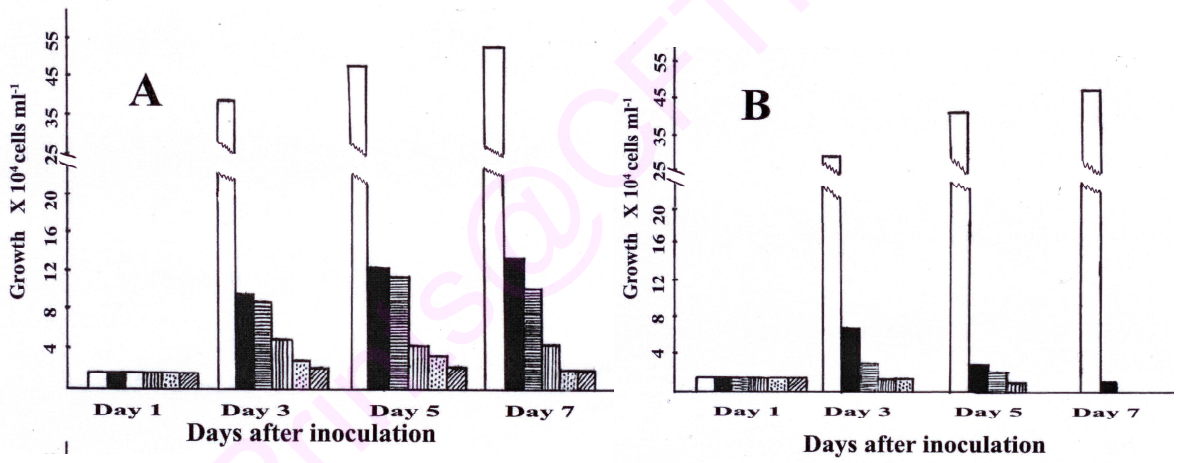


Figure 1.6 Growth of *H. pluvialis* at different concentration of hygromycin containing liquid medium. A – Cocultivated cells. B – Control cells. ☐ - No hygromycin, ■ - 1 mg L^{-1} of hygromycin, ⊟ - 2 mg L^{-1} of hygromycin , ◫ - 4 mg L^{-1} of hygromycin , ⊠ 5 mg L^{-1} of hygromycin and ▨ 10 mg L^{-1} of hygromycin.

1.7.6 Stability of the transformants

Since the hygromycin resistant cells were showed a slow growth rate, the initial inoculum level of resistant cells in different concentrations of selection medium was studied. Inoculum loads greater than 4 x 10^4 transformants were needed for their survival in liquid medium containing 5 and 10 mgL^{-1} of hygromycin (Table 1.10). Control cells were unable to survive even in 1 mgL^{-1} of hygromycin at any inoculum density studied. The stability of the integrated *hpt* gene in transformants grown in

non selection medium over a period of two and half years has been confirmed through PCR undertaken at intervals of 3 - 4 months (data not shown). Fluorescence of GFP protein was observed consistently in cells transfomed with pSK53 over the same time period (data not shown).

Table 1.10. Growth of transformed and control *H. pluvialis* in liquid selection medium at different inoculum density

Days after inoculation	Conc. of hygromycin mgL^{-1}	Initial inoculum density level of *H. pluvialis* inoculated x 10^4 cells ml^{-1}									
		T.C	C.C	T.C	C.C	T.C	C.C	T.C	C.C	T.C	C.C
1st Day	5	1	1	2	2	4	4	8	8	10	10
	10	1	1	2	2	4	4	8	8	10	10
3rd Day	5	0	0	1	0	5	0	11	0	13	0
	10	0	0	0	0	4	0	10	0	12	0
5th Day	5	0	0	0	0	6	0	14	0	15	0
	10	0	0	0	0	8	0	13	0	14	0

T.C – Transformed cells C.C – Control cells

1.8 Detection of reporter genes

1.8.1 GFP expression

The bright green fluorescence was characteristic of hygromycin resistant cells when observed under a fluorescence microscope. The fluorescence was distributed throughout the cell (Fig 1.7A and 1.7B). Control cells emitted only the characteristic red fluorescence of chloroplasts (Fig 1.7 C).

1.8.2 GUS Assay

GUS assay was performed for both control and transformed *H. pluvialis*. There was no positive GUS activity was detected in control cells (Fig 1.8 A, B and C). Cells that were resistant to hygromycin were analyzed for GUS activity. The positive GUS activity was observed as blue colour in the hygromycin resistant cells (Fig 1.9 A, B and C).

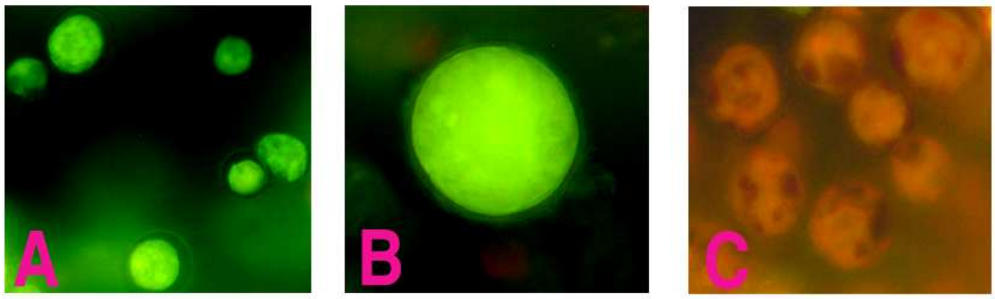


Figure 1.7 Fluorescent microscopic observation of non-transformed and transformed cells of *H. pluvialis.* A bright greenish fluorescence of the GFP was observed in the transformed *H. pluvialis* (A and B). No bright greenish fluorescence, was observed in non transformed *H. pluvialis*. Only a red colour due to the autofluorescence of chlorophyll was observed non transformed cells (C).

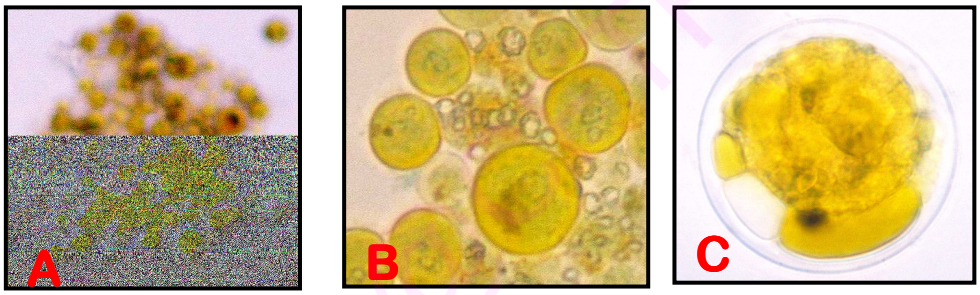


Figure 1.8 Microscopic observations of the non-transformed *H. pluvialis* for GUS analysis. D, E and F, are the non-transformed *H. pluvialis* observed under 10, 40 and 100X showing no blue colour inside the cells.

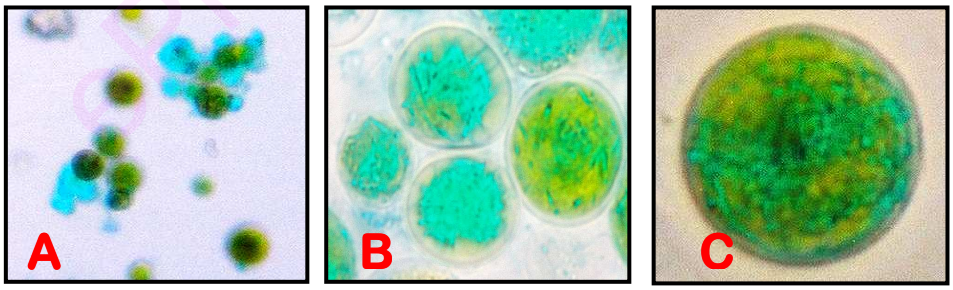


Figure 1.9 Microscopic observations of the transformed *H. pluvialis* for GUS analysis. A, B and C are the transformed *H. pluvialis* observed under 10, 40 and 100X showing clear blue colour inside the cells which shows the presence of GUS gene

The GUS positive transformants when observed under microscope showed scattered blue coloured spots in some cells and complete blue colour (Fig 1.9 B) in others. Activity of GUS differed among different cells. In some cells expression was strong throughout the cell while in other cells expression was weak or variegated (Fig

1.9B). The whole cells turned blue when the cell wall was digested completely with cellulose and pectinase. But in case of the partially digested cell wall the blue spots were observed in scattered manner. This may be due to the intact/thick cell wall of the cells, which may not be digested fully by the enzyme cellulase and pectinase. This aberrant GUS expression was observed in all the transformed colonies that were maintained in autotrophic medium.

1.9 Scanning Electron Microscopy

Cells co-cultivated with *Agrobacterium* when observed under the SEM there were numerous bacteria which are freely lying on the cell surface (Fig. 1.10A, B and C). The cell surface showed typical pore-like openings, which are present only in cocultivated cells but not in, the control cells. Hair like structures/protrusions (Fig. 1.10B, arrow) appeared on the cell surface of cocultivated cells. Control cells grown in the absence of *Agrobacterium* (Fig. 1.10D and E) presented a smooth cell surface.

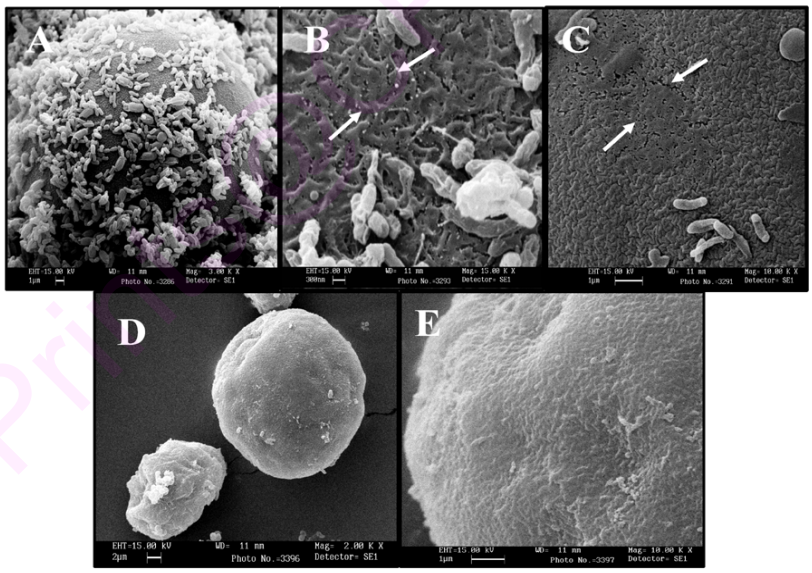


Figure 1.10 Scanning electron microscopic photograph of the control and co-cultivated *H. pluvialis.* A - Single cocultivated cell showing a number of *Agrobacterium tumefaciens* adhering on the surface of the cells. B and C- Closer view of the cell surface of co-cultivated *H. pluvialis* (Arrows indicating the pore like structures formed due to the co-cultivation of *Agrobacterium tumefaciens*). D and E are the single cell of *H. pluvialis* without co-cultivation showing a smooth cell surface.

1.10 Molecular Confirmation

1.10.1 PCR (Polymerase Chain Reaction) analysis

The *hpt* and GUS primer pairs were able to amplify a 407bp (Fig. 1.11) and 515 bp (Fig. 1.12) fragments respectively from the DNA of different resistant

colonies grown on hygromycin, indicating the presence of the hygromycin and GUS gene in DNA isolated from them. Amplicons were obtained from the DNA of transformants obtained after co-cultivating *H. pluvialis* cells with the *Agrobacterium* having pCAMBIA1301 and pSK53 respectively (Fig 1.11, lanes 2 to 6 and 7 to 11). The expected size of the 407 bp was obtained from DNA isolated from cells transformed with either vector is indicative of integration of the *hpt* gene in these cells. The amplicons from both the transformants and the positive control (pCAMBIA 1301; Lane 12) were of the same size (407bp). PCR was negative for the DNA isolated from hygromycin sensitive cells (Fig. 1.11, lane 1). Lane 13 is the 3 kb DNA marker.

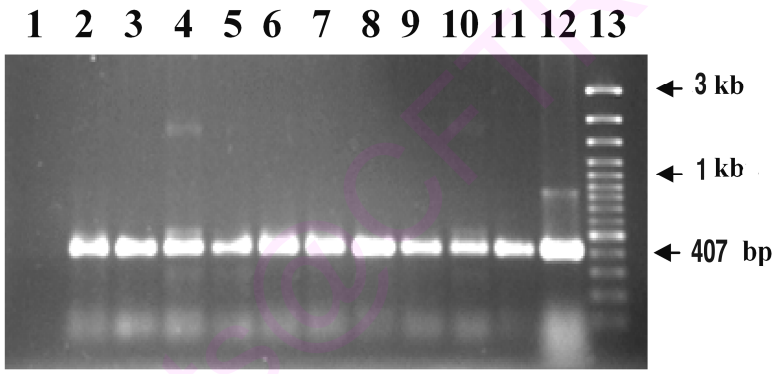


Figure 1.11 PCR analysis for *hpt* gene from *H. pluvialis*. Lane 1 – DNA isolated from nontransformed *H. pluvialis*, lanes 2 to 11 are the DNA isolated from transformed *H. pluvialis*, and lane 12 is the positive control (pCAMBIA 1301). Lane 13 shows the 3kb marker.

Amplicons from a portion of the GUS gene (515 bp) was obtained from transformants obtained by cocultivating cells of *H. pluvialis* with the *Agrobacterium* bearing pCAMBIA1301 and pSK53 respectively (Fig 1.12: Lanes 1 to 3 and 5and 6). Size of the amplicon (515bp) from both transformants and the positive control, pCAMBIA1301 were similar. No amplification was observed for the DNA isolated from the nontransformed *H. pluvialis* (Fig. 1.12, lane 4).

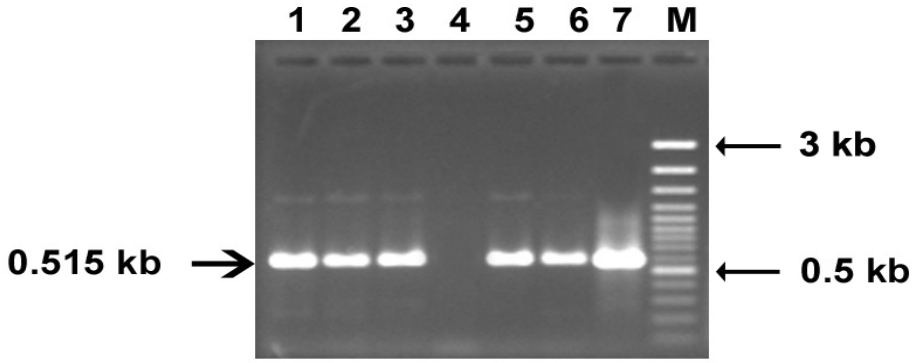


Figure 1.12 PCR analysis for GUS gene from *H.pluvialis*. DNA isolated from transformed *H. pluvialis* (lanes 1 to 3 and 5 and 6); DNA isolated from non transformed *H. pluvialis* (lane 4); and lane 7 is the positive control (pCAMBIA 1301). M is the 3kb marker.

1.10.2 Southern analysis of Transgenics

A fragment of the *hpt* gene amplified by PCR was labeled using the Ambion's psoralen biotin. The transformed *H. pluvialis* DNA and the plasmids were digested with the restriction enzymes *XbaI* and *Cfr421*. It is expected that, in the two binary vectors used, the restriction enzyme *XbaI* would cut once through the multiple cloning site and the enzyme *Cfr421* would cut outside the T-DNA area.

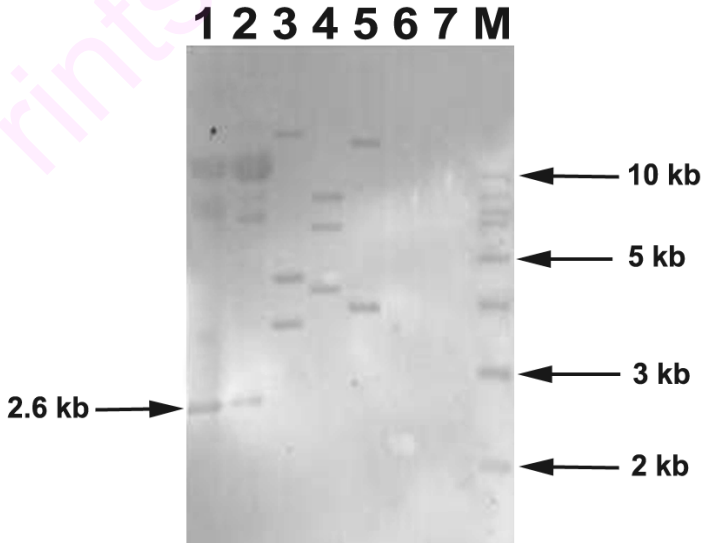


Figure 1.13 Southern blot analysis *H. pluvialis* DNA. Lane 1 and 2 are the positive control DNA of plasmid pCAMBIA1301 and pSK53. Lane 3 to 5 are the transformed *H. pluvialis* and lane 6 and 7 are the non transformed *H. pluvialis*. M is the 10 kb marker.

An intense single band (1.6 kb) on the lower end of the gel hybridizing with the probe (hygromycin gene fragment) was seen with either plasmids pCAMBIA1301 or pSK53

digested with *XbaI* and *Cfr421*. Two other reactive bands larger in size were also observed (Fig. 1.13 lane 1 and 2) and these would be expected to correspond uncut and linearised (partially digested) plasmid DNA. Two to three bands of different sizes were obtained (Fig. 1.13 lane 3, 4 and 5) when genomic DNA of hygromycin resistant cells were digested and probed as above.

The sizes of these bands were different from those obtained when the plasmids pCAMBIA1301 and pSK53 were digested and probed. No bands were observed when DNA from control cells digested with the same enzymes was probed (Fig. 1.13 lane 6&7).

1.11 Analysis of astaxanthin in transformants and control *H. pluvialis*

To compare the astaxanthin profile in the transformants and non transformed *H. pluvialis*, the cells were subjected to continuous high light intensity without any stress condition under normal medium. The transformants showed the same carotenoid profile (Fig 1.14A) like the non transformants(Fig 1.14B) and there is no significant change in astaxanthin content in transformants (Fig 1.15) when compared to the control cells.

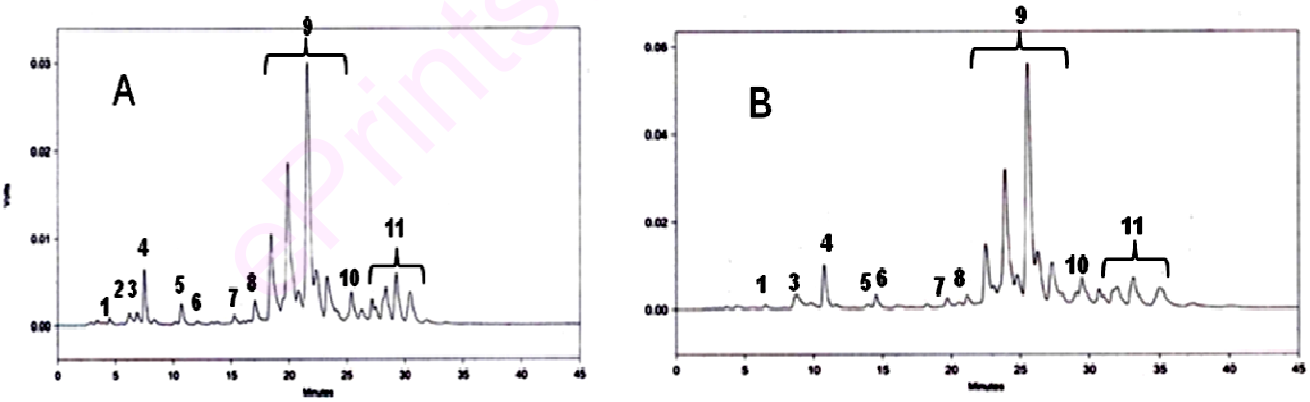


Figure 1.14. HPLC profile of the carotenoid extracts from control (A) and transformed *H. pluvialis* (B) Peaks were identified as (1) Neoxanthin, (2) Violaxanthin, (3) Free astaxanthin, (4) Lutein, (5) Canthaxanthin, (6) Chlorophyll b, (7) Chlorophyll b', (8) Echinenone, (9) Astaxanthin monoesters, (10) β-carotene and (11) Astaxanthin diesters.

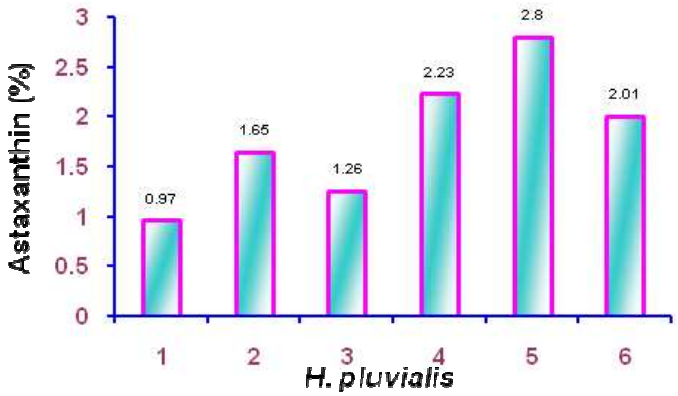


Figure 1.15 Astaxanthin percentage of the control and transformed *H. pluvialis.* 1 to 4 are the transformants. 5 and 6 are the non transformed *H. pluvialis*

1.12 Discussion

Genetic transformation method is one of the important technique to improve the economically important species. Among the genetic transformation methods, *Agrobacterium* mediated is one of the most popular method for its efficiency, stability, and reliability (Schell et al 1984). The inheritance pattern of *Agrobacterium* mediated gene transfer is simple Mendelian principle, and no silencing of the gene in the T_1 populations. Before starting the *Agrobactrium* mediated genetic transformation studies in microalgae, it is important to identify the sensitivity of algae for different concentrations of antibiotics for the selection of resistant colonies and also the different cocultivation media for the growth of both alga and bacterium. The TAP medium used here for cocultivation favoured the good growth of both *Haematococcus* and *Agrobacterium*. This TAP medium differs from the other media by the presence of acetic acid as carbon source and ammonium chloride as nitrogen source allowing the growth of both algae and bacteria. The same medium was also used for the cocultivation of *Agrobacterium* with the green microalga *Chlamydomonas reinhardti* in the genetic transformation studies (Kumar et al. 2004).

Since the alga *H. pluvialis* showed high sensitivity to hygromycin, the hygromycin phosphotransferase (*hpt*) gene can be used as selectable marker gene to select the transformants in the hygromycin containing media. The same *hpt* gene is also used for green algal transformation of *Chlamydomonas reinhardtii* by *Agrobacterium* mediation (Kumar et al. 2004), glass bead (Berthold et al 2002) and by electroporation (Brown et al 1991: Ladygin VG 2004). Reports have also shown

that hygromycin is an effective antibiotic in a number of plant cell culture selections (Ortiz et al. 1996; Lin et al. 1996) which prompted us to use the hygromycin resistance marker gene carrying plasmid (pCAMBIA1301) to explore the feasibility of transformation studies in *H. pluvialis*.

The growth of resistant cells obtained from the plates (having hygromycin 10 mg L^{-1}), when grown in liquid medium containing hygromycin, growth was limited and the cells were not able to multiply. To identify the exact concentration of hygromycin in liquid medium for the growth of hygromycin resistant cells, they were cultured in different concentrations of hygromycin like 1, 2, 4, 5 and 10 mg L^{-1} (Table 1.9, Fig 1.6). The result showed differences in growth of cocultivated cells in hygromycin containing liquid and solid media. This may be due to differences in membrane permeability among the cells grown in the two media. Higher inoculum cell densities allowed greater tolerance to hygomycin in liquid medium (Table 1.10). This may be due to quicker degradation of hygomycin to tolerable levels by higher inoculum load than by lower inoculum levels. Transformants of *H. pluvialis* grew slowly on selection medium while their growth was normal and comparable when transferred to non selection medium. Therefore the transformants were grown in liquid nonselection medium. The same kind of different response of green alga in hygromycin containing solid and liquid media was reported by Berthold et al 2004 for *Chlamydomonas reinhardtii* and subsequently the resistant cells were grown in liquid nonselection medium. Kumar et al. 2004 for *Chlamydomonas* and Steinbrenner and Sandman 2006 for *Haematococcus* also reported maintenance of the hygromycin resistant cells in non selection medium and the stability of the gene was found to be stable for 18 months and 80 generations respectively.

Integration of the hygromycin gene into the genome of the algae was confirmed using southern blotting procedures and through PCR. Cells resistant to hygromycin were shown to express the GUS and GFP genes. There was aberrant expression of GUS gene in the transformed cells. Some cells became completely stained while some cells were partially stained. Transformed cells were treated with the enzymes pectinase (0.2%) and cellulase (0.1%) prior to staining for GUS activity (Fig. 1.9). GUS activity was observed only in the enzyme treated cells and not in the untreated cells. This may be due to thick cell wall which hindered the penetration of the GUS substrate. Variegated staining was seen probably in cells with partially

digested cell walls. Gene silencing or improper integration of the transgene may be postulated as responsible for the weak and spatially restricted expression of the GUS gene (Meyer, 1995). However the GFP protein is clearly visible requiring no entry of substrate (Fig. 1.8).

It may be predicted that restriction enzymes *Cfr 421* and *Xba I* would cut the plasmid pCAMBIA 1301 into fragments of 2.66 kb and 9.16 kb in size if completely digested. Three bands were observed in the Southern blot of the double digested vectors. The two upper bands in the plasmid (Fig.1.13, lane 1 and 2) probably represent uncut and linearised (partially digested) plasmid. The lowest band 2.6kb in size represents the insert from the T-DNA region of the plasmid which hybridized with the *hpt* probe. Bands of different size (not same as the bands observed from plasmid) were observed when Southern blots were carried out with DNA isolated from hygromycin resistant cells. The different banding pattern obtained with DNA from hygromycin resistant cells as compared to that from plasmid DNA clearly indicates that integration of the hygromycin gene into the genomic DNA of *H. pluvialis* has occurred. The presence of multiple bands in southern blots may possibly arise from multiple copy integration or from the presence of a mixture of cells with different integrations or through incomplete digestion by the restriction enzymes used.

The transformation in *H. pluvialis* was achieved without injury to cell or through wounding. Escudero and Hohn 1997 reported, that non dividing or intact mesophyll cells could take up and integrate T-DNA. Zambre et al. 2003 reported that light plays an important role in *Agrobacterium* mediated transformation process and that continuous light enhanced transformation frequency. In the present study continuous light was used. Dillen *et al* 1997 claimed that a temperature of 22^{o}C was optimal for the *Agrobacterium* mediated transformation of plants. In the present study also the cultures were maintained at this temperature during co-cultivation. The transformation of the algae resulted in altered cell wall morphology as observed with the Scanning Electron Microscope. This may be a resultant of the formation of "bacterium-to-host cell channels" following activation of the *vir* genes (Tzfira and Citovsky, 2002).

It is interesting to note that acetosyringone has been reported to be effective in increasing transformation efficiency by activating the vir genes of the *Agrobacterium* (Stachel et al. 1985). *Chlamydomonas* secretes phenolic compounds into the medium

(Harris 1989). It may be presumed that these compounds may activate the vir genes. Kumar et al. 2004 reported significantly higher transformation frequency in *Chlamydomnas reinhardtii* while using acetosyringone ($311–355\times10^6$) than without ($7–8\times10^6$). In our work, there was only little difference in transformations efficiencies with (153 ± 4.5 per10^6 cells) and without the use of acetosyringone (109 ± 4.0 per10^6 cells). Hence the transformation achieved here by *Agrobacterium* does not need treatment with acetosyringone or the wounding of cells.

Different methods have been attempted for the genetic transformation of green algae. The use of particle bombardment (Kindle et al. 1989), glass beads (Hall et al. 1993) and *Agrobacterium* mediated (Kumar et al. 2004) for the transformation of *Chlamydomonas reinhardtii* while Chow and Tung 1999 and Chen et al. 2001 electroporated DNA into *Chlorella sp.* Geng et al. 2003 used the electroporation technique for the transformation of *Dunaliella*. Transformation frequency and the stability of the gene in subsequent generations have been limiting factors except where *Agrobacterium* mediated transformation has been used (Kumar et al. 2004). So far the genetic transformation method studied in green alga *H. pluvialis* was particle bombardment (Teng et al. 2002; Steinbrenner and Sandmann 2006). Teng et al. 2002 noted transient expression of the *Lac Z* gene in *H .pluvialis* subjected to particle bombardment. Steinbrenner and Sandman (2006) have reported the use of particle bombardment for transformation of *H. pluvialis*. These results and those of Kumar et al. 2004 substantiates that efficient transformation can be attained through *Agrobacterium* mediation in green algae in general.

The method described here is highly efficient in developing genetically transformed *H. pluvialis* through *Agrobacterium* mediated transformation system in this commercially important microalga. This robust transformation method for this alga would also pave the way for manipulation of many important pathways relevant to food, pharmaceutical and nutraceutical industries. Further this result will be very much useful for the *Agrobacterium* mediated transformation studies in this other green microalgae like *Dunaliella* sp, *Botryococcus* sp, *Chlorella* sp etc which are having the high commercial and economic value.

Chapter II

Cloning of genes responsible for enzymes (β-carotene ketolase & β-carotene hydroxylase) involved in carotenoid biosynthesis

2.0 Introduction

Development of strain improvement for the sustainable increase in value added products from the available biological sources both in terms of quality and quantity is essential for the hunger world. The developmental studies include many biotechnologically related adventures like identification and isolation of the functional compounds (protein/genes) from the biological sources, characterization of the compounds, *in vitro* expression of the isolated protein/genes, cloning and transferring the identified gene to the suitable host, increasing the production/productivity through modern techniques like inducing stress, mutation studies, modification of the culturing condition etc. *Haematococcus pluvialis* is the green micro alga produces a high value ketocarotenoid, astaxanthin which have been shown to have higher antioxidant activity than β-carotene and α-tocopherol (Kobayashi and Sakamoto 1999), enhance the immune responses (Jyonouchi et al. 1995) and also act against cancer (Tanaka et al. 1995b). The genes responsible for the synthesis of astaxanthin from the β-carotene in *Haematococcus pluvialis* has been well studied. Also the cloning of the genes like β-carotene ketolase (BKT), β-carotene hydroxylase (BKH), phytoene synthase, phytoene desaturase, lycopene cyclase etc have been well studied and also expressed *in vitro*. The critical enzymes involved in the astaxanthin biosynthesis are BKT and BKH where their *invitro* studies were attempted by several workers (Lotan and Hirschberg 1995; Kajiwara et al. 1995; Meng et al. 2005; Huang et al. 2006; Tao et al. 2006; Vidhyavathi et al. 2008).

Several environmental stress conditions have been demonstrated to induce astaxanthin accumulation. Among them high irradiance, nutrient deficiency, high salinity, and high temperature (Kobayashi et al. 1992, Tjahjono et al. 1994, Harker et al. 1996, Boussiba 2000, Sarada et al. 2002b).

Metabolic engineering is generally carried out to improve the production of existing compounds, to mediate the degradation of compounds, or to produce new compounds by redirecting one or more enzymatic reaction. Approaches for achieving genetic/metabolic engineering include over expression of a single gene. Multiple gene combinations or a transcription factor to establish single gene or multigenes control in the biosynthesis pathway for carotenoids, or use of RNAi/antisense knockout of a

pathway in order to increase the content or change the composition of carotenoids. There is growing interest worldwide in manipulating carotenoid biosynthesis in carotenoid producing organisms. Cloning of most of the astaxanthin biosynthesis genes in *H. pluvialis* has now opened the door to genetically manipulating this pathway not only in algae, but also in other organisms. So, the cloning of the gene BKT which convert β-carotene to astaxanthin, in astaxanthin biosynthesis in *H. pluvialis* has been presented here.

2.1 Materials and methods

2.1.1Culture and growth condition

The culture and growth conditions for *H. pluvialis* were followed as in the section 1.1 and 1.2 of chapter II

2.1.2 DNA and RNA extraction

DNA and RNA techniques were followed according to the standard methods (Sambrook et al. 2001). DNA was extracted using a DNA extraction kit (Gene Elute-Plant Genomic extraction kit, Sigma-Aldrich, USA). RNA was isolated from aliquots of about 10^7 cells harvested at different growth conditions using RNAqueous RNA isolation kit (Ambion Inc, Texas - USA,) according to the manufacturer's instructions. The concentration of total DNA and RNA was determined spectrophotometrically at 260 nm.

2.1.3 Isolation and cloning of astaxanthin biosynthetic genes

The genes involved in astaxanthin biosynthesis pathway have been elucidated and published by different workers (Grünewald et al. 2001; Sun et al. 1998: Hirschberg 1997; Linden 1999). Based on those proposed biosynthetic sequences, the gene sequence for beta carotene ketolase (BKT) (GenBank accession number: D45881, Kajiwara et al 1995) and beta carotene hydroxylase (BKH) (GenBank accession number: AF162276 – Linden 2005; and AY187011 – Teng et al. 2003) genes were obtained from NCBI database. The sequences of BKT and BKH were aligned with Clustal X using the FASTA format (Pearson and Lipman 1988). The divergent 3' ends of the cDNA were selected as targets of PCR amplification. Primers tagged with restriction sites were designed with the computer software Primer3 (Rozen and

Skaletsky 2000). All the enzymes involved in cloning were procured from MBI fermentas, Germany unless otherwise stated.

2.1.4 Designing and synthesis of primers for BKT and BKH genes

The primer sequences had been tagged with the restriction enzymes *XhoI* and *XbaI* in the left and right primers for the cloning of BKT and BKH gene (Table 2.1 and Table 2.2) in between the CaMV 35S promoter and poly A region of the cloning vector pRT 100 (Topfer et al 1987) and further to a binary vector pCAMBIA1304 (Nguyen and Jefferson, 2001; Centre for the Application of Molecular Biology to the International Agriculture, Canberra, Australia) developed by

Table 2.1 Primers used for the of amplification of BKT

Primer name	Discription	Gene bank Accession number	Size of Amplicon from cDNA	Primers used
BKT-A	**BKT(*XhoI*)-246 F BKT(*XbaI*)**	**D45881**	**908bp**	**F-TGACTCGAGTGGGCGACACAGTATCACAT R-ACTCTAGAACCAGGTCATGCCAAG**
BKT-B	**BKT(*XhoI*)-168 F BKT(*XbaI*)-1130 R**	**D45881**	**987bp**	**F- GCAAGCCTCGAGATGCACGTCGCATCG GCACTA R- CGAGACTCTAGATCATGCCAAGGCAGG CACCAGGCC**
BKT-C	**BKT internal BKT internal**	**D45881**	**657bp**	**F-TGGGCGACACAGTATCACAT R-GTAGAAGAGGCGGAATGCTG**

Table 2.2 Primers used for the of amplification of BKH

Name of the primers	Gene bank Accession number	Amplicon size from cDNA (bp)	Primers used
BKH-A1	**AF162276**	**995**	**F - GCAAGCCTCGAGCTACATTTCACAAGCCCGTGAGC R – CGAGACTCTAGACTACCGCTTGGACCAGTCCAGTTCC**
BKH-A2	**AY187011**	**3226**	**F - GCAAGCCTCGAGATTACCACGATGCTGTCGAAGCT R - CGAGACTCTAGACTACCGCTTGGACCAGTCCAGTTCC**
BKH-B1	**AY187011**	**3222**	**F - ATTACCACGATGCTGTCG R - CAACAGCCCTAGGTGAATAG**
BKH-B2	**AF162276**	**1511**	**F - CCACCTCCTCATCTCCAT R - AGACAGTGCATCTCACCTG**
BKH-B3	**AF162276**	**1529**	**F - ACTGGATCCCCACCTCCTCATCTCCAT R - CTGTCTAGAAGACAGTGCATCTCACCTG**
BKH-B4	**AY187011 & AF162276**	**571**	**F - AGTCAATCAGCGTCAAGG R - CAGAAGCCAAAGGTACACAG**

Hoekema et al. (1983) – and are constructed in a diverse way to suit varied applications in transformation (Jefferson et al. 1987; Jefferson et al. 1998). As reported by Lotan and Hirschberg 1995, regarding the cloning of BKT for the expression studies of canthaxanthin in *E. coli*, BKT gene was isolated using genomic DNA and cDNA that had been extracted from the *H. pluvialis* culture for gene isolation. The cDNA of BKT was amplified for the confirmation of the primer sequence to check its exact sequence length. For cloning purpose the genomic DNA had been used as the template to amplify the gene of interest including introns using the designed primers for isolation of both BKT and BKH genes using the primer sequences (Table 2.1 and Table 2.2). The amplicon that are produced using the above primer sequence are initially cloned into the T-tailed vector – the strategy as given by Hu (1993) – as mentioned in the following section.

2.1.4.1 BKT primers

All the primers were synthesized from the Sigma-Aldrich chemicals USA and MWG Biotech pvt Ltd, Bangalore. Among the different BKT primers sequences mentioned, primers A, B, and C has to amplify the fragment size of 908, 987 and 657 base pairs respectively from the cDNA of *H. pluvialis*. BKT-A and BKT-B are the primers with restriction sites *XhoI* (CTCGAG) in left and *XbaI* (TCTAGA) in right primer. The primer BKT-C is without restriction sites which will be used for the confirmation of the BKT gene fragments amplified from the primers BKT A and B. These above mentioned primers are used to amplify the BKT and BKH gene from genomic DNA extracted from the *H. pluvialis* for cloning in T-tail vector, then to a cloning vector pRT100 and finally to the binary vector pCAMBIA1304.

2.1.4.2 BKH primers

For the amplification of BKH gene from genomic DNA *H. pluvialis*, the primers were synthesized with and without restriction sites. Initially the primer synthesized were BKH A1 and A2 (from Gene Accession number AF162276 (Linden 2005) and AY187011 (Teng et al. 2003)**)** which were adapted with restriction sites *XhoI* (CTCGAG) in left and *XbaI* (TCTAGA) in right primer (Table 2.2). Further some more primers were also synthesized using the gene accession number AF162276 and AY187011. The amplicon size of the respective primer pairs were mentioned in the table 2.2.

2.1.5 PCR amplification of the BKT and BKH gene

The amplification reactions were in volumes of 25.0 µL each containing 14.0 µL of sterilized double distilled water, 2.5 µL of 10X *taq* assay buffer, 2.0 µL of dNTP (2.5 mM each in the dNTP mix; obtain 0.2 mM final concentration), 2.0 µL each of left and right primer (0.08 µM each in final concentration), and 0.5 µL (~2.5 U) of *Taq* polymerase (MBI Fermentas International Inc., Burlington - Canada) with 2.0 µL of the template DNA. Amplification were performed using 0.2 mL PCR tubes (Axygen Inc.,USA) in an Eppendorf mastercycler personal (Eppendorf, Germany) programmed for the initial denaturation of four minutes at 94^0C and step of 35 cycles consisting the denaturation of one minute at 94^0C, annealing of one minute at 60^0C and extension of one minute at 72^0C followed by a final extension of 10 minutes at 72^0C. Then it is programmed to maintain at 4^0C till we use it for electrophoresis. Amplified products were resolved based on their molecular weight by running the products on a 1.0% agarose gel matrix using a submarine electrophoresis (Consort E861, Germany) with 1X TAE buffer at 5V cm^{-1}. 100 bp-10.0 kbp ladder (MBI fermentas, Germany) was used to identify the size of the amplicon. After the run, the gel was stained using ethidium bromide (Sharp et al. 1973) solution for five to ten minutes and was destained using dH_2O for two to three minutes. Ethidium bromide (Sigma-Aldrich, USA) solution was prepared once in four days at 5 mg L^{-1} concentration. The stained gel image was viewed on an UV transilluminator (Fotodyne 3440). The intercalating agent ethidium bromide fluoresces orange upon UV illumination (310 – 320 nm). The gel image has been documented using a Herolab documentation unit (Herolab 442K, E.A.S.Y., Germany).

2.1.6 Cloning

2.1.6.1 T-tail cloning of BKT gene.

Amplified fragments of BKT was eluted from the gel matrix using Qiagen minelute gel elution kit (Qiagen, GmbH, Germany) and were cloned in a pRSET T-tailed vector (Himedia Laboratories Pvt. Ltd.,-Mumbai) using the principle as mentioned by Hu (1993) by keeping for overnight ligation at 4^0C. Hence, ligated fragments were transformed into the *E. coli* DH5α strain using the chemically prepared competent cells (Cohen et al. 1972) as described in the appendix. The transformed

colonies were identified using the blue-white selection (Ullmann et al. 1967) by adding X-gal (40 µL of 2% w/v per plate for spreading) and IPTG (7 µL of 20% w/v per plate for spreading) onto the LB solid ampicillin plates (100 mg L^{-1}) and incubated at 37^0C for two hours before plating. Preparation of X-gal and IPTG stocks were described in appendix. After transformation (appendix), the culture were centrifuged at 4000 rpm for five minutes and the supernatant were removed and the last 100 µL of cultures were plated and incubated in 37^0C for overnight for development of colonies. After overnight incubation, the colonies that are white are selected singly and grown in LB broth with ampicillin (100 mg L^{-1}) for plasmid isolation and its further confirmation of the recombinant clones. Isolated plasmids were run on a 0.8% agarose gel as mentioned above and the positive clones were further inoculated in 10 mL of LB broth with ampicillin for further isolation using miniprep plasmid isolation kit (Qiagen, GmbH, Germany) and the release of insert.

2.1.6.2 Cloning of BKT gene fragment to pRT100 vector.

The flow chart of cloning of BKT gene from genomic DNA of *H. pluvialis* to cloning vector pRT100 is illustrated in the Figure 2.1. The cloning procedure having the steps like amplification of the BKT gene, digestion, ligation and transformation.

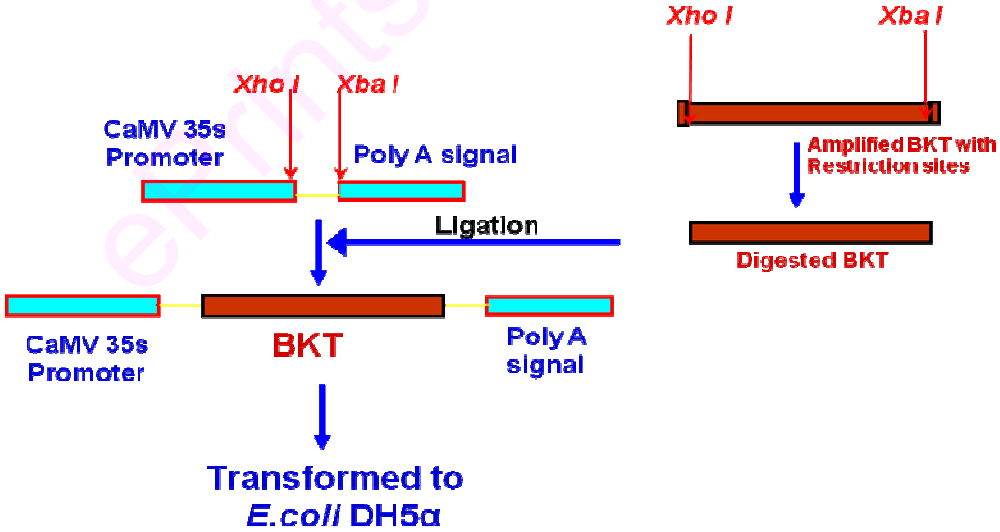


Figure 2.1 Flowchart showing the cloning procedure of BKT from genomic DNA of *H. pluvialis* to a pRT100

2.1.6.3 Amplification of BKT gene from genomic DNA of *H. pluvialis*

The cloned T-tailed vector was used only for the purpose of maintenance and also for further use. To clone the BKT gene into the pRT100 the amplified fragment

from the primer BKT-A was directly cloned between the CaMV35S promoter and a poly A tail of the pRT100 (Figure 2.2). A 3340 bp plasmid vector pRT100 was used as a cloning vector for the study since it has the CaMV 35S promoter, poly A tail, ampicillin resistance gene and restriction sites *XhoI* and *XbaI*. The amplification of the BKT gene was performed by the BKT-A primer as mentioned in the section 2.2.1. Since the primers were tagged with *XhoI* and *XbaI* restriction enzymes, amplified BKT was cloned directly to the *XhoI* and *XbaI* sites of the vector.

2.1.6.4 Restriction digestion

The *E.coli* strain DH5α which having the plasmid pRT100 was extracted and purified. The extracted plasmid was confirmed under the 0.8% gel electrophoresis. After extraction and purification of the plasmid, the plasmid was double digested with enzymes *XhoI* and *XbaI* (MBI Fermentas International Inc., Burlington - Canada). Simultaeously the amplified BKT fragment were also double digested with the same enzymes *XhoI* and *XbaI*. The digestion procedure was followed as per the instruction manual of MBI Fermentas (MBI Fermentas International Inc., Burlington - Canada). The reaction was kept for 12hrs at 37^0C. After the completion of double digestion the reaction was inactivated by boiling at 80^0C for 20 min. The inactivated reaction mixture was purified and checked by 0.8% gel electrophoresis. The double digested product of palsmid and BKT were further used for the ligation reaction

2.1.6.5 Ligation reaction

The Ligation reactions were carried out with the enzyme ligase (MBI Fermentas and Bangalore Genei) as per the instruction manual. The digested fragments (amplified BKT gene fragment and 3340 bp linearised vector backbone of pRT100) were eluted from the 0.8% agarose gel electrophoresis using Qiagen gelelute kit (Qiagen, GmbH, Germany). Eluted products were confirmed by running 2 mL of the eluted products on a 0.8% agarose gel electrophoresis as mentioned in section 2.2.1. Since, the intensity of pRT100 linearized vector was around three to five times less intense than the gene fragment eluted ones; ligation set up was done at 2:1 (v/v) ratio of gene and insert. After confirmation of elution, ligation mixture of 20 µL containing 10X ligation buffer 2.0 µL; linearized pRT100 vector - 5.0 µL; amplified BKT gene fragment 10.0 µL; T4 DNA Ligase 2.0 µL (100 Weiss units μL^{-1}; MBI Fermentas International Inc.,

Burlington - Canada) sterile dH_2O 1.0 µL. The ligation mixture was kept at room temperature (~25^0C) for overnight.

2.1.6.6 Transformation and selection of cloned pRT100

The ligated mixture was transformed into *E coli* DH5α strain competent cells as mentioned in the appendix. Since the vector doesn't contain *lacZ* fragment, blue-white selection is not possible. Randomly ten single colonies were selected and grown in 5 mL of LB medium containing ampicillin (100 mg L^{-1}) and plasmids were isolated manually as mentioned appendix and used for digestion to confirm the presence of insert. Restriction digestion of the plasmids isolated from the ten colonies were digested using *HindIII*, for the confirmation of insert. Diagram of pRT100 with their gene cassette with promoter and poly A is given in the Figure 2.2.

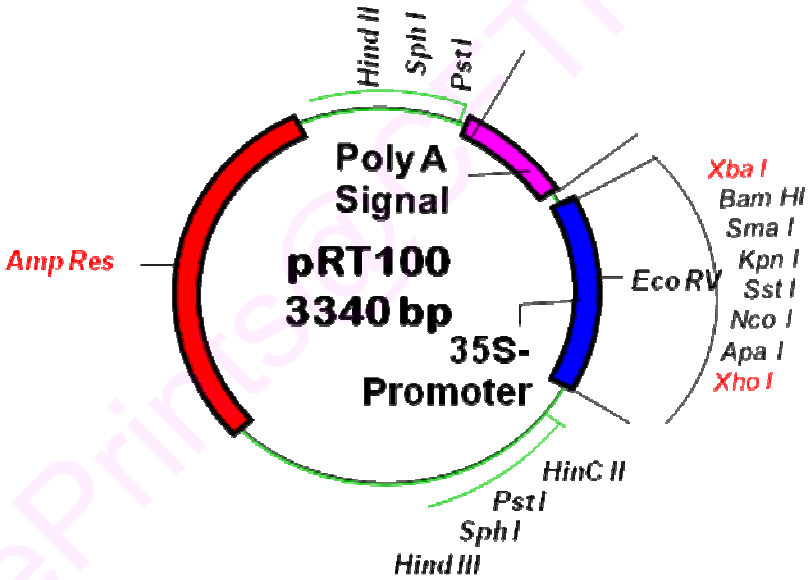


Figure 2.2 pRT100 vector map with promoter and poly A

2.1.6.7 Confirmation of cloning in pRT100

2.1.6.7.1 Enzymatic digestion

The clones were further inoculated in 10 mL of LB medium containing ampicillin (100 mg L^{-1}). Overnight grown cultures were used for plasmid isolation using Qiagen miniprep plasmid isolation kit (Qiagen, GmbH, Germany). Isolated plasmids were digested separately with different enzymes like *HindIII, SphI, PstI HinCII* and double digestion with *XhoI* and *XbaI* to know the approximate size of the release and also to know the presence of any of these restriction enzyme sites in the

amplified fragment. Since the primer was designed based on the cDNA sequence and the restriction analysis of the cDNA of BKT will not be same of the amplified BKT from genomic DNA of *H. pluvialis*. The amplified BKT may have the introns which will be present in the genomic DNA. Conditions for digestion were provided in appendix. Digested fragments were electrophoresed on a 0.8% agarose gel electrophoresis and documented as mentioned earlier in section 2.2.1. They were further used for the isolation of gene cassette from the recombinant clones of pRT100 vector and its cloning in the binary vector p1304.

2.1.6.7.2 PCR analysis for the cloned pRT100

The extracted plasmid from pRT100 was analyzed for the amplicon length/size by using the CaMV 35S forward primer and poly A reverse primer. The primers pairs were as follows

CaMV 35S – F 5' ATGGTGGAGCACGACACTCT 3'
Poly A - R 5' GCTCAACACATGAGCGAAAC 3'

For this above primer the wild pRT100 plasmid will give 583 bp of amplicon size and the recombinant pRT100 has to give more than the size of wild pRT100 (CaMV35 and poly A region (583bp) + actual cDNA length for the primer (908bp) + intron regions amplified from the genomic DNA of *H. pluvialis*). The PCR condition for this primer having initial denaturation of four minutes at 94^0C and step of 35 cycles consisting the denaturation of one minute at 94^0C, annealing of one minute at 55^0C and extension of one minute at 72^0C followed by a final extension of 10 minutes at 72^0C. Then it was programmed to maintain at 4^0C till use for electrophoresis. The same plasmid was also used for the PCR amplification of the BKT gene with different primer combination. The different combination followed were

CaMV 35S - F 5' ATGGTGGAGCACGACACTCT 3'
BKT-A - R 5' ACTCTAGAACCAGGTCATGCCAAG 3'

BKT-A - F 5' TGACTCGAGTGGGCGACACAGTATCACAT 3'
Poly A - R 5' GCTCAACACATGAGCGAAAC 3'

CaMV 35S - F 5' ATGGTGGAGCACGACACTCT 3'
BKT-C - R 5' GTAGAAGAGGCGGAATGCTG 3'

The PCR condition followed for these above primers were as like the conditions of primer CaMV35S and poly A. The amplified fragments were analyzed under the gel electrophoresis for its exact length/size.

2.1.6.7.3 Recombinant plasmid isolation and its sequencing.

Recombinant plasmids were isolated using miniprep plasmid isolation kit (Qiagen, GmbH, Germany) and were confirmed by agarose gel electrophoresis using a 0.8% agarose gel as mentioned earlier in the section 1.2.1 of this chapter. Hence isolated plasmids were digested for the insert release using double digestion with *XhoI* and *XbaI* restriction enzymes and were confirmed by running a 0.8% agarose gel electrophoresis. The cloned plasmids were sequenced at (Smith et al. 1986; Ansorge et al. 1987) at MWG sequencing services, Bangalore, Inda.

2.1.6.7.4 Sequence BLAST.

Obtained sequences from MWG sequencing services, the DNA sequence were subjected to BLAST (Altschul et al. 1990) using the BLAST program from the NCBI database for the confirmation of the gene and the vector backbone. Also, a restriction map for the gene sequence has been done by using NEB cutter2.0 (Vincze et al. 2003).

2.1.6.8 Cloning of BKT gene cassette from pRT100 to pCAMBIA1304

The flow chart of cloning of BKT gene from pRT100 to binary vector pCAMBIA1304 is illustrated in the Figure 2.3.

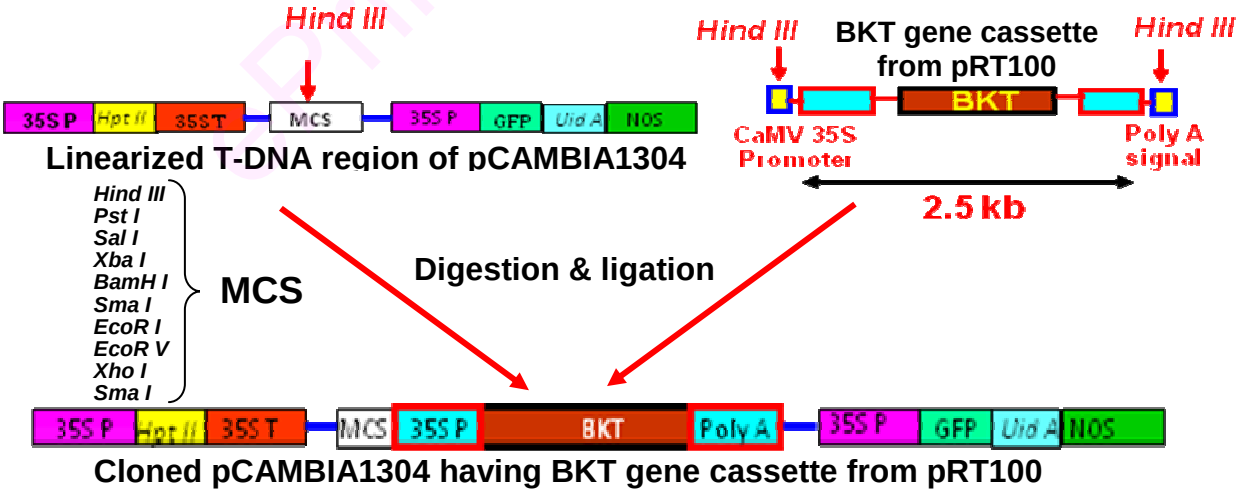


Figure 2.3 Flowchart showing the cloning of BKT gene from pRT100 to binary vector pCAMBIA1304. Linear map of the T-DNA region the plasmid p1304 having the selectable marker gene *hpt* (hygromycin phospho transferase) and GFP (green fluorescence protein) and *UidA* (β-glucoronidase) as reporter genes both driven by the CaMV 35S promoter

The cloning procedure having the steps like amplification of the BKT gene, digestion, ligation and transformation. The sequence result of the cloned BKT gene from genomic DNA showed a single restriction site of *HindIII* (A-AGCTT) inside the gene. Hence partial digestion procedure was followed to release the full gene of the BKT from the cloned pRT100.

2.1.6.8.1 Partial digestion of pRT100 with *HindIII*

The cloned vector pRT100 was extracted from the *E. coli* using miniprep plasmid isolation kit (Qiagen, GmbH, Germany) and were confirmed by agarose gel electrophoresis using a 0.8% agarose gel as mentioned earlier in the section 2.2.1 of this chapter. The genomic BKT from pRT100 was restriction digested with *HindIII* (MBI Fermentas International Inc., Burlington - Canada) partially at 27^0C for 30 min. The partial digested fragments of cloned pRT100 was run on a 0.8% agarose gel to elute the appropriate sized fragments. The elution was carried out using the Qiagen gel elution kit (Qiagen, GmbH, Germany).

2.1.6.8.2 Digestion of pCAMBIA1304 with *HindIII*

Simultaneously the binary vector pCAMBIA1304 (Fig 2.4) also digested with the restriction enzyme *HindIII* (MBI Fermentas International Inc., Burlington – Canada).

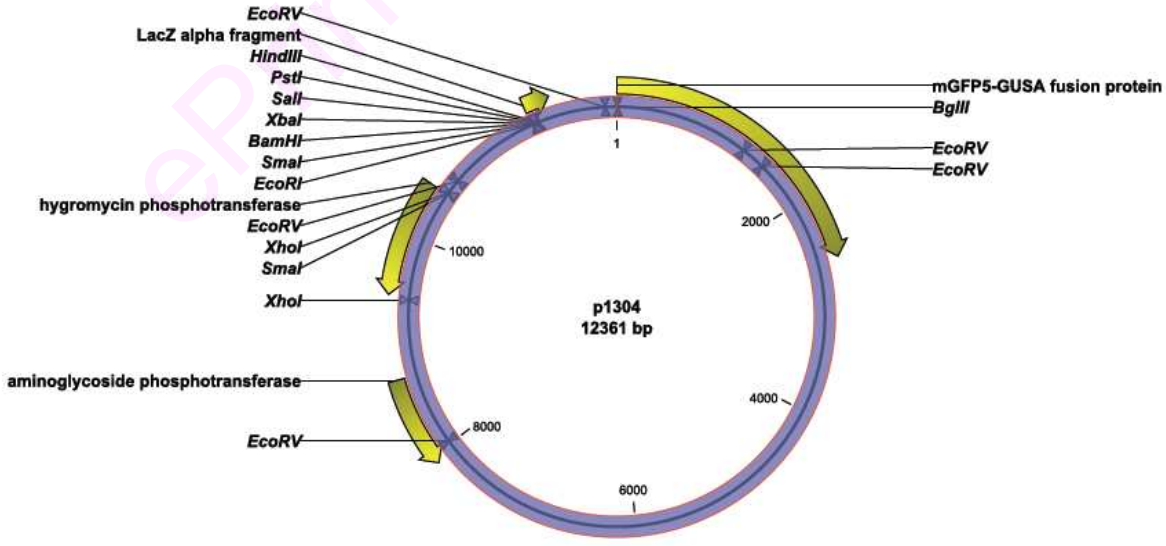


Figure 2.4 Binary vector pCAMBIA1304 having the selectable marker gene *hpt* (hygromycin phospho transferase) and GFP (green fluorescence protein) and *UidA* (β-glucoronidase) as reporter genes both driven by the CaMV 35S promoter

The vector harbours the selectable marker gene *hpt* (hygromycin phospho transferase) and *UidA* (β-glucoronidase) and GFP (green fluorescence protein) as reporter genes both driven by the CaMV 35S promoter. The restriction sites in the T-DNA region are marked in the multiple cloning site (MCS) of the vector. *HindIII* is one of the restriction site in the MCS region of the vector. The digestion of the binary vector is to linearize the circular plasmid. The digestion reaction mixture having the total of 20 µL volume consisting of 4.0 µL of binary vector, 2.0 µL of 10X reaction buffer, 2.0 µL of restriction enzyme *HindIII* and the final volume make up to 20 µL with sterile distilled water. The reaction was kept for 8 – 12 hrs at 37^0C and reaction was deactivated after the complete digestion by heating the reaction mixture at 80^0C for 20 min.

2.1.6.8.3 Ligation of the digested pCAMBIA1304 with BKT

Ligation mixture of 20 µL containing 10X ligation buffer - 2.0 µL; linearized pCAMBIA 1304 vector - 7.2 µL; released gene cassette from pRT100 vector - 7.2 µL; T4 DNA Ligase- 1.0 µL (200 Weiss units μL^{-1}; MBI Fermentas International Inc., Burlington - Canada) sterile dH_2O – 4.6 µL. The ligation mixture was kept at room temperature (~25^0C) for overnight.

2.1.7 Transformation

2.1.7.1 Transformation of recombinant pCAMBIA1304 to the wild *E.coli* strain

The enzyme digested and ligated product of recombinant pCAMBIA 1304-BKT was mixed in the competent cells of *E.coli* for the transformation. The sample was kept in ice for 30 min and then at 42^0C for 90 sec and then in ice for 60-90 sec. After this the mixture was incubated in shaker for 45 min at 37^0C by adding 700µL of LB media. After the incubation period, the mixture was centrifuged at 4000rpm for 5 min and the pellets were resuspended in 200µL of same LB medium. The suspension was plated to the selection media containing antibiotic kanamycin (100 mg L^{-1}) along with X-gal and IPTG for blue-white selection (appendix) and incubated overnight at 37^0C. Based on the blue and white colony screening, the white colonies were isolated and extracted the plasmid for further analysis.

2.1.7.2 Transformation of recombinant pCAMBIA 1304 vector to *Agrobacterium*

Recombinant pCAMBIA 1304-BKT vector containing the gene cassette of BKT has been isolated using miniprep plasmid isolation kit (Qiagen, GmbH, Germany) from *E coli* DH5α strain and was transformed into *Agrobacerium tumefaciens* EHA101 by freezing and thawing method (Sambrook and Russel. 2001) and they were plated onto the LB solid medium containing kanamycin (100 mg L^{-1}) and incubated at 28^0C for 18-24 hrs. Colonies obtained were selected and confirmed for the presence of plasmid restriction digestion and also by PCR. Those colonies that are confirmed for the presence of recombinant vector were also used for *Agrobacterium* mediated transformation of BKT to *H. pluvialis*.

2.1.7.3 Confirmation of transformation

2.1.7.3.1 Confirmation by plasmid extraction

The transformation of the recombinant plasmid pCAMBIA 1304-BKT to *E. coli* and *Agrobacterium* were confirmed by extraction of the plasmid from the different single white colonies in the plates, by inoculating in 5 mL of LB medium separately. Overnight grown cultures were used for plasmid isolation manually to analyse the presence of insert by digesting with *HindIII*. Recombinant pCAMBIA 1304 plasmid containing the BKT gene cassette is herein called as pCAMBIA 1304-BKT. The control binary vector pCAMBIA 1304 vector is 12361 bp.

2.1.7.3.2 Confirmation by PCR

The recombinant plasmid pCAMBIA 1304-BKT extracted from *E. coli* and *Agrobacterium* was further confirmed by restriction digestion with different restriction enzymes as described in section 1.2.3.1. The detailed digestion procedure has been given in appendix. And also the extracted plasmids were used for the amplification of different segments of the BKT gene using different combination of primers as described in section 1.2.3.2.

2.2 Results

2.2.1 Isolation of BKT and BKH genes from *H. pluvialis*

2.2.1.1 Amplification of BKT gene from total RNA

The genomic DNA of *H. pluvialis* was used for the amplification of the BKT gene. Initially amplification of the designed primers were analyzed and confirmed with the total RNA extracted from *H. pluvialis* to confirm the correct size of the amplification. The yield of the total RNA can be observed from the Figure 2.5. From this total RNA, RT-PCR was performed directly using the Qiagen's one step RT-PCR kit.

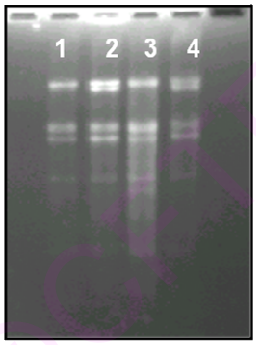


Figure 2.5 Total RNA isolated from *H. pluvialis* 1 & 2 – from continuous light, 3 & 4 – from 12 light and 12 hrs dark

The different primers used to amplify the BKT gene fully or partially were mentioned in the table 2.1 of this chapter. The BKT-A forward primer starts from the 264th position of the nucleotide sequence (D45881) which is having the *XhoI* restriction site in its 3 prime end, while the reverse primer having the *XbaI* restriction site in the 3 prime end.

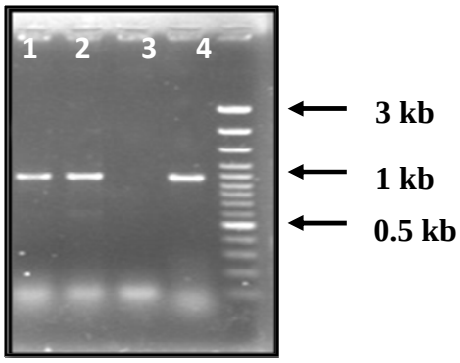


Figure 2.6. Amplification of BKT gene from total RNA of *H. pluvialis* through RT-PCR. Lane 1 and 2 are amplification using BKT-A primer, lane 4 is the amplification of BKT using BKT-B primer. M is the 3 kb marker

This primer gives the amplification size of 908 bp (Fig 2.7, lane-1 and 2). The BKT-B forward primer starts from the 168th position of the nucleotide sequence (D45881) also having the same *XhoI* restriction site at the 3 prime end and the reverse primer having the restriction site *XbaI* which gave amplification of 987bp (Fig 2.6, lane 4).

2.2.1.2 Amplification of BKT gene from genomic DNA of *H. pluvialis*

The exact size of the amplification of designed primers of BKT gene was confirmed by RT-PCR. After confirming, the same primer was used to amplify the BKT gene from the genomic DNA of *H. pluvialis*. All the three BKT primers gave the amplification (Fig 2.7). An approximately 1.8 kb amplicon size was observed for the BKT-A and BKT-B primer (Fig 2.7, lane).

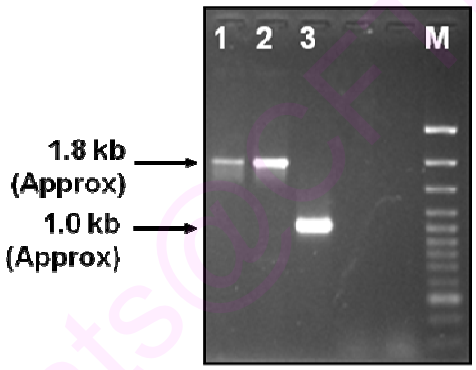


Figure 2.7 Amplification of BKT gene from genomic DNA of *H. pluvialis.* 1, 2 and 3 are the amplification of BKT gene using BKT-B, BKT-A and BKT-C primers respectively. M is the 3 kb marker

For BKT-C primer an approximately 1.0 kb amplicon was observed (Fig 2.7, lane – 3). For cloning of BKT gene, the BKT-A primer was used to amplify the BKT gene to clone to the cloning vector pRT100.

2.2.1.3 Amplification of BKH gene from total RNA

The different primers used for the amplification of the BKH primers were listed in the table 2.2. Initially the PCR was performed using the primers BKH-A1 and BKH-A2. If cDNA is used as the template, these primers should give the amplicon size of 995 bp and 3226 bp. But the none of these two prime gave the exact size of the amplification (Fig 2.8). The same primer was also used to amplify the BKH gene from genomic DNA of *H. pluvialis.* No amplification was observed for the genomic DNA.

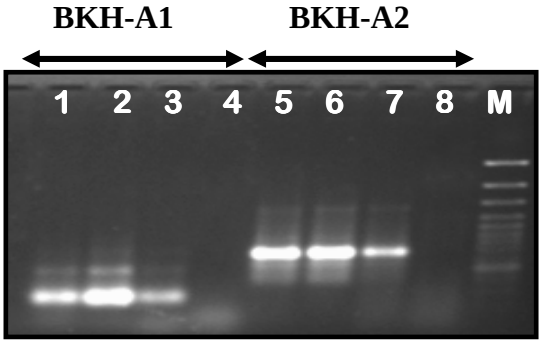


Figure 2.8 Amplification of BKH gene from total RNA of *H. pluvialis* through RT-PCR. Lane 1 to 4 – No amplification of BKH gene using BKH-A1 primer, lane 5 to 8 – No amplification of BKH gene using BKH-A2 primer. M is the 3 kb marker.

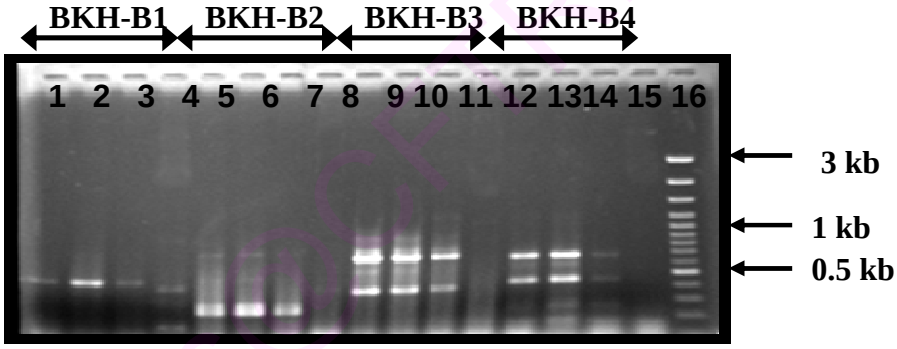


Figure 2.9 Amplification of BKH gene from genomic DNA of *H. pluvialis* using the primer BKH-B1 (lane 1 to 4), BKH-B2 (lane 5 to 8) and BKH-B3 (lane 9 to 12). BKH-B1 (lane 13 to 16). The exact size of amplification of BKH gene was not observed from all of the primers. M is the 3 kb marker.

Further some more primers were synthesized to amplify the BKH from genomic DNA of *H. pluvialis.* The primer BKH-B1, BKH-B2 and BKH-B3 were used to amplify the BKH gene. But none of the primers were not able to amplify the BKH gene of the exact size. The amplification observed are not the exact size of the BKH gene (Fig 2.9).

Since there were no amplification for BKH primers for the PCR conditions used (section 2.1.7), the alteration of PCR condition was performed at different annealing and extension time and temperature. But for all of the PCR conditions performed, no exact size of amplification was observed. Hence the cloning procedure was further continued only with BKT.

2.2.1.4 Cloning of BKT to the cloning vector pRT100

Although the amplification of BKT was performed by both the primers BKT-A and BKT-B, for cloning purpose the primer BKT-A was used. Figure 2.11 shows the amplification

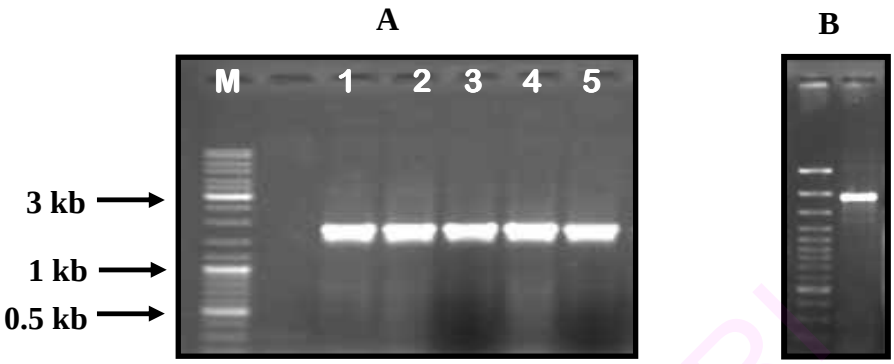


Figure 2.10 A - Amplification of BKT gene from genomic DNA of *H. pluvialis* using BKT-A primer. Lanes 1 to 5 are the approximately 1.8 kb amplicon, M is the 10 kb marker. B – Purified product of the BKT gene from the amplified products.

of the BKT gene from the genomic DNA of *H. pluvialis*. A good yield of approximately 1.8 kb fragment (Fig 2.10) was observed in the gel. This amplified fragment linked with the *XhoI* and *XbaI* restriction sites in its 3' and 5' region. Hence, the amplified fragment was double digested with *XhoI* and *XbaI* restriction enzymes. Simultaneously the plasmid pRT100 extracted from the *E. coli* was double digested with the same enzymes and purified (Fig 2.12).

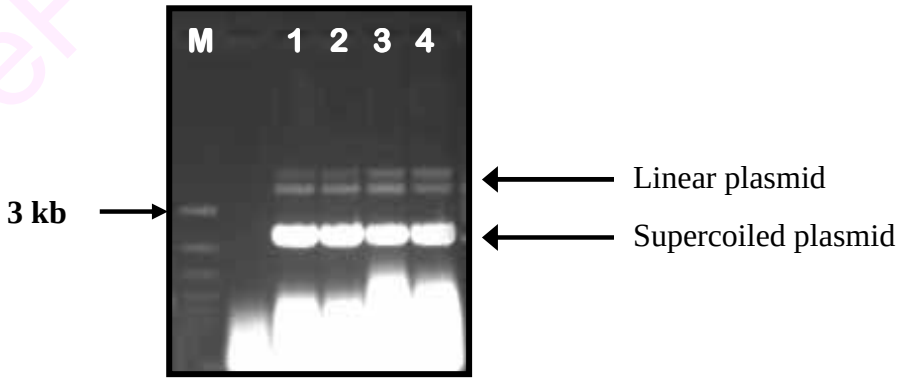


Figure 2.11 Plasmid pRT100 extracted from *E. coli*. Lane 1 to 4 is the plasmid pRT100 shows both supercoiled and linear plasmid. The super coiled plasmid shows the size less than 3.34 kb. But the actual size of the plasmid is 3.34 kb. M is the 3 kb marker.

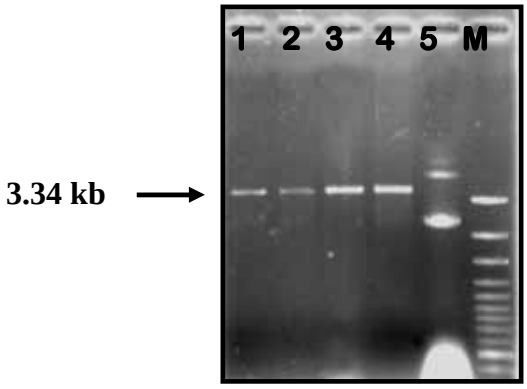


Figure 2.12 Plasmid pRT100 double digested with *XhoI* and *XbaI* and purified linear fragment. Lane 1 to 4 is the double digested, linear plasmid (3.34kb). Lane 5 is the undigested plasmid having super coiled plasmid shows the size less than 3.34 kb. M is the 3 kb marker.

These two double digested BKT gene and pRT100 were subjected for ligation and further transformation (as described in section 2.2.2.3 and 2.2.2.4) to the competent cells of *E. coli*.

2.2.2 Confirmation of cloning

2.2.2.1 Analysis by plasmid extraction

The cloned plasmid which has been transformed to *E. coli* was extracted and analyzed for the size of the cloned and wild pRT100. The cloned plasmid showed the size which is more than the wild pRT100. Approximately the size of the cloned plasmid is 5.1kb (Fig 2.14). Since size of the wild plasmid is 3.34 kb and the size of the amplicon is approximately 1.8 kb, the cloned plasmid showed the size of 5.1 kb.

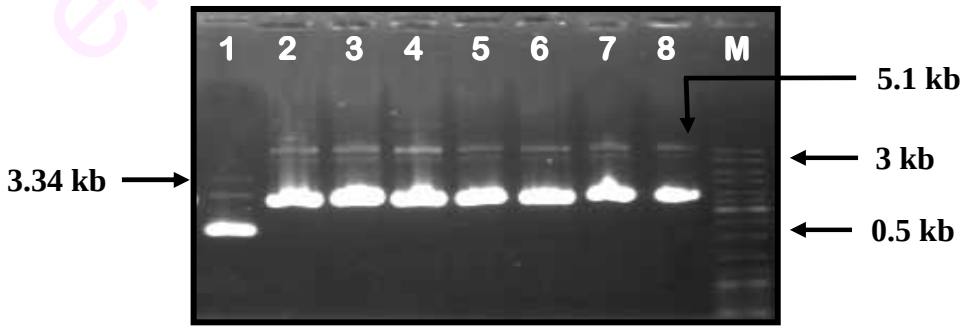


Figure 2.13 Gel photograph showing the cloned and wild plasmid pRT100. Lane 1 is the wild pRT100 which is 3.34 kb in size. Lanes 2 to 8 is the cloned pRT100 showing larger size (5.1 kb) than the wild pRT100. M is the 10 kb marker.

2.2.2.2 Confirmation by restriction digestion

To know the release of the insert in recombinant pRT100, the cloned pRT100 was subjected to restriction digestion with *HindIII* enzyme. It showed the fragments size of approximately 1.5 kb and 1.0 kb release. Whereas the control pRT100 showed the fragment size of 2.6 kb and 0.7 kb. The total length of the wild pRT100 is 3.34 kb. There are two *HindIII* site in the plasmid which are apart from 0.7 kb, possessing the CaMV 35S and the Poly A region. When this plasmid is digested with the *HindIII* enzyme, it released the 0.7 kb *HindIII* to *HindIII* insert and the vector backbone of 2.6 kb possessing the ampicillin resistance gene (Fig 2.15). When the cloned pRT100 was digested with the same enzyme the backbone of the cloned pRT100 was same (2.6 kb) as wild pRT100.

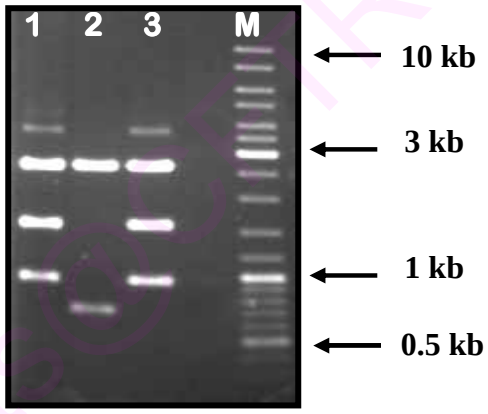


Figure 2.14 Gel photograph of the *HindIII* digested wild and cloned pRT100. Lane 1 and 3 are the cloned pRT100. Lane 2 is the wild pRT100 which is 3.34 kb in size. M is the 10 kb marker.

But the inserts released were differed. It showed two fragments as mentioned above (Figure 2.15, Lanes 1 and 3). These two released inserts indicating the presence of one more *HindIII* site inside the insert, which in inside the BKT gene.

2.2.2.3 Sequence result of the genomic BKT in cloned pRT100

The actual size of the BKT gene amplified from the primer BKT-A is using the cDNA is 891 bp. The sequence results showed the size of the genomic BKT in pRT100 is 1813 bp. This larger size indicating the presence of introns in the genomic BKT. It is observed that there were five introns in the sequence which are varied in size.

CTCGAGTGGGCGACACAGTATCACATGCCATCCGAGTCGTCAGACGCAGCTCGTCCTGCGTTGAA
GCACGCCTATAAACCTCCAGCATCTGACGCCAAGGGCATCACGATGGCGCTGACCATCATTGGCA
CCTGGACCGCAGTGTTTTTACACGCAATATTTCAAATCAGGCTACCGACATCCATGGACCAGCTT
CACTGGTTGCCTGTGTCCGAAGCCACAGCCCAGCTTTTGGGCGGAAGCAGCAGCCTATTGCACAT
CGCCGCAGTCTTCCTTGTACTTGAGTTCCTGTACACTGGTGGGACAGTGAGAGTATACTGCCTAT
CAGCGCATGCATTAGCTTTCCTGTTCTGCTCCCATAGCTCGCATTGCAAGCGCGTGTGTCCCTGT
GGACATGCGTAATCCGCTCTAAATTTTGTCATGCAGGTCTATTCATCACCACACATGACGCAATG
CATGGCACCATAGCTTTGAGGAACAGGCAGCTCAATGATCTCCTTGGCAACATCTGCATATCACT
GTACGCCTGGTTTGACTACAGCATGCTGCGTCGCAAGGTGAGGAAGCGTCTGACCTCCTTCTCTG
CGAGTTCAACACATTACAGCCGCTGAGACTGGTGCGCTGCGCTGCCTGTCCTTCGCAGCACTGGG
AGCACCACAACCATACTGGCGAAGTGGGGAAAGACCCTGACTTCCACAAGGGAAATCCCGGCCTT
GTCCCCTGGTTCGCCAGGTAAGGCTCATAAGTGGCTCTGTTGCGGTGCTGGTGGCCTATGCATCC
GTGACATCTGCAAGGAATACATCTTGCTGCACACAATCCCTCCAGATGTTCATGGCATATGTGGG
CAAAAAACAAGTGTGACAGGGTGTGGTGCCGCCTGCCGCGCAACTTCATGTCCAGCTACATGTCC
CTGTGGCAGTTTGCCCGGCTGGCATGGTGGGCAGTGGTGATGCAAATGCTGGGGGCGCCCATGGC
GAATCTCCTAGTCTTCATGGCTGCAGCCCCAATCTTGTCAGCATTCCGCCTCTTCTACTTCGGTG
TGTGCCTCTTGACCCCCAAACCTAACCCTGGACATGCACCAGTCAGCTTGCATGCTTTCCAAGCT
TTGTGCATGCTGAGCTTCTGGTGAGGACTGCAGTGCTCCGGCCAGCTTTCATGTAAACTACCGGT
GACATCTGCTAGTGCCAGGGCTGTGCACACCATGGTGATGGAGGTTTCGCACATCAGCAGCACCA
CAGGGTCATAGCCTTGTGTGTACAGTGTTTCTGCCCTGCCTGCTGATGCTTGTAGCGTCCTGACG
CCCCTGATACCCCAGTAGCCCAAATGGAGGTGGCAACTGGCCAATAGTGTGGTGCTCTGCTCAGC
TCACTTGTATGCTTGCGGTGCTGGTTTGCAGGCACTTACCTGCCACACAAGCCTGAGCCAGGCCC
TGCAGCAGGCTCTCAAGTGATGGCCTGGTTCAGGGCCAAGACAAGTGAGGCATCTGATGTGATGA
GTTTCCTGACATGCTACCACTTTGACCTGGGTGGGTGCTAACCCACACTGACAAAGCTGCAAACC
ACCTCTGCTGGCTGCGTGTAGTGCATCAGCAAGCATGGTGCATAGACCCAAGCTGCGTGGTAAGT
GTGTCCTGGTCCCAACCTGGCCTGGGATGGCATGCTGCATGTCACTTACCAGCTTTTGGCTGTGC
CCGGGCTGTCCTGTTGCTGCCTTGTAGCACTGGGAGCACCACAGGTGGCCCTTTGCCCCCTGGTG
GCAGCTGCCCCACTGCCGCCGCCTGTCTGAGCGTGGCCTGGTGCCTGCCGTGGCATGACCTGGTT
CTAGA

Figure 2.15 Gene sequence of the cloned BKT from pRT100. The exon regions were highlighted with yellow and the intron region were highlighted in blue colour. The start codon ATG and the restriction sites were indicated in red background.

The size of first, second, third, fourth and fifth introns are 128 bp, 86 bp, 156 bp, 359 bp and 193 bp respectively. The ATG region which is starting codon (metheonine) present at 18th position in the sequence. There were many restriction sites in the intron regions of the sequence. There were 5 restriction sites of *Sph*I, 3 restriction sites of *Pst*I and 1 *HindIII* site. The *HindIII* site present at the 1094 bp of the sequence. Since the *HindIII* site is single in number, it was chosen to release the insert (CaMV 35S + BKT + polyA) from the recombinant pRT100. This *HindIII* will digest the recombinant pRT100 in two fragments viz, 1095 bp and 718 bp. The 1095 bp fagment joins with

the CaMV 35S by *XhoI* to give 1534 bp and the another fragment 718 bp joins with poly A region by *XbaI* to give 990 bp (Fig 2.15).

The full sequence of the genomic BKT with introns has been given in the Figure 2.16. The highlighted region is the exon region of the BKT and the dark field is the intron region. The six bases presents in the starting and end of the sequence are the restriction sites *XhoI* and *XbaI*. The aligment of the genomic DNA and the cDNA of BKT (cloned) was compared with the available known BKT gene sequence ID D45881 and showed the 100% matching (Fig 2.17 and 2.18). The nucleotide sequence was also submitted to the NCBI and the accession number given is GQ214765

2.2.2.4 Alignment (DIALIGN format) of genomic BKT with D45881

The sequence alignment of cloned genomic BKT showed 99 % matching with the original known BKT gene sequence ID D45881 (Fig 2.17) and also the cDNA showed exact alignment with cDNA of BKT. There were five nucleotide bases interchange with other complementary base. The positions were highlighted with black background at 69, 258, 471, 1440, 1777 nucleotide bases in the genomic DNA of BKT.

```
Cloned BKT 1    ---------- ---------- ---------- ---------- ----------
D45881     1    cggggcaact caagaaattc aacagctgca agcgcgcccc agcctcacag

Cloned BKT 1    ---------- ---------- ---------- ---------- ----------
D45881    51    cgccaagtga gctatcgacg tggttgtgag cgctcgacgt ggtccactga

Cloned BKT 1    ---------- ---------- ---------- ---------- ----------
D45881   101    cgggcctgtg agcctctgcg ctccgtcctc tgccaaatct cgcgtcgggg

Cloned BKT 1    ---------- ---------- ---------- ---------- ----------
D45881   151    cctgcctaag tcgaagaatg cacgtcgcat cggcactaat ggtcgagcag

Cloned BKT 1    ---------- ---------- ---------- ---------- -----TGGGC
D45881   201    aaaggcagtg aggcagctgc ttccagccca gacgtcttga gagcgTGGGC
                                                                *****
                                                                *****
                                                                *****
                                                                *****
Cloned BKT 6    GACACAGTAT CACATGCCAT CCGAGTCGTC AGACGCAGCT CGTCCTGCGT
D45881   251    GACACAGTAT CACATGCCAT CCGAGTCGTC AGACGCAGCT CGTCCTGCGC
                **********  **********  **********  **********  **********
                **********  **********  **********  **********  **********
                **********  **********  **********  **********  **********
                **********  **********  **********  **********  **********

Cloned BKT56    TGAAGCACGC CTATAAACCT CCAGCATCTG ACGCCAAGGG CATCACGATG
D45881   301    TAAAGCACGC CTACAAACCT CCAGCATCTG ACGCCAAGGG CATCACGATG
                **********  ***  ******  **********  **********  **********
                **********  ***  ******  **********  **********  **********
                **********  ***  ******  **********  **********  **********
                **********  ***  ******  **********  **********  **********
                            ******  **********  **********  **********
```

```
ClonedBKT106    GCGCTGACCA TCATTGGCAC CTGGACCGCA GTGTTTTTAC ACGCAATATT
D45881    351   GCGCTGACCA TCATTGGCAC CTGGACCGCA GTGTTTTTAC ACGCAATATT

                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                ********** ********** ********** ********** ****
                ********** ********** ********** ********** ****

ClonedBKT156    TCAAATCAGG CTACCGACAT CCATGGACCA GCTTCACTGG TTGCCTGTGT
D45881    401   TCAAATCAGG CTACCGACAT CCATGGACCA GCTTCACTGG TTGCCTGTGT

                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                                       *** ********** **********
                                       *** ********** **********

ClonedBKT206    CCGAAGCCAC AGCCCAGCTT TTGGGCGGAA GCAGCAGCCT ATTGCACATC
D45881    451   CCGAAGCCAC AGCCCAGCTT TTGGGCGGAA GCAGCAGCCT ACTGCACATC

                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                ********** *****

ClonedBKT256    GCCGCAGTCT TCCTTGTACT TGAGTTCCTG TACACTGGTg ggacagtgag
D45881    501   GCtGCAGTCT TCATTGTACT TGAGTTCCTG TACACTGGT- ----------

                      ** ******* ********** ********** *********
                      ** ******* ********** ********** *********
                      ** ******* ********** ********** *********
                      ** ******* ********** ********** *********

ClonedBKT306    agtatactgc ctatcagcgc atgcattagc tttcctgttc tgctcccata
D45881    540   ---------- ---------- ---------- ---------- ----------

ClonedBKT356    gctcgcattg caagcgcgtg tgtccctgtg gacatgcgta atccgctcta
D45881    540   ---------- ---------- ---------- ---------- ----------

ClonedBKT406    aattttgtca tgcaggtCTA TTCATCACCA CACATGACGC AATGCATGGC
D45881    540   ---------- -------CTA TTCATCACCA CACATGACGC AATGCATGGC

                           *** ********** ********** **********
                           *** ********** ********** **********
                           *** ********** ********** **********

ClonedBKT456    ACCATAGCTT TGAGGaACAG GCAGCTCAAT GATCTCCTTG GCAACATCTG
D45881    573   ACCATAGCTT TGAGGcACAG GCAGCTCAAT GATCTCCTTG GCAACATCTG

                ********** ***** **** ********** ********** **********
                ********** ***** **** ********** ********** **********
                ********** ***** **** ********** ********** **********
                                 **** ********** ********** **********
                                 **** ********** ********** **********
ClonedBKT506    CATATCACTG TACGCCTGGT TTGACTACAG CATGCTGCGT CGCAAggtga
D45881    623   CATATCACTG TACGCCTGGT TTGACTACAG CATGCTGCAT CGCAA-----

                ********** ********** ********** ********** *****
                ********** ********** ********** ********** *****
                ********** ********** ********** ********** *****
                ********** ********** ********** ********** *****
                                                             *****

ClonedBKT556    ggaagcgtct gacctccttc tctgcgagtt caacacatta cagccgctga
D45881    668   ---------- ---------- ---------- ---------- ----------
```

```
ClonedBKT606   gactggtgcg ctgcgctgcc tgtccttcgc aGCACTGGGA GCACCACAAC
D45881   668   ---------- ---------- ---------- -GCACTGGGA GCACCACAAC
                                                 ********* **********
                                                 ********* **********
                                                 ********* **********
                                                 ********* **********

ClonedBKT656   CATACTGGCG AAGTGGGGAA AGACCCTGAC TTCCACAAGG GAAATCCCGG
D45881   687   CATACTGGCG AAGTGGGGAA AGACCCTGAC TTCCACAAGG GAAATCCCGG
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********

ClonedBKT706   CCTTGTCCCC TGGTTCGCCA Ggtaaggctc ataagtggct ctgttgcggt
D45881   737   CCTTGTCCCC TGGTTCGCCA G--------- ---------- ----------
               ********** ********** *
               ********** ********** *
               ********** ********** *
               ********** ********** *
ClonedBKT756   gctggtggcc tatgcatccg tgacatctgc aaggaataca tcttgctgca
D45881   758   ---------- ---------- ---------- ---------- ----------

ClonedBKT806   cacaatccct ccagatgttc atggcatatg tgggcaaaaa acaagtgtga
D45881   758   ---------- ---------- ---------- ---------- ----------

ClonedBKT856   cagggtgtgg tgccgcctgc cgcgcaaCTT CATGTCCAGC TACATGTCCC
D45881   758   ---------- ---------- -------CTT CATGTCCAGC TACATGTCCC
                                      *** ********** **********
                                      *** ********** **********
                                      *** ********** **********
                                      *** ********** **********
                                      *** ********** **********
ClonedBKT906   TGTGGCAGTT TGCCCGGCTG GCATGGTGGG CAGTGGTGAT GCAAATGCTG
D45881   781   TGTGGCAGTT TGCCCGGCTG GCATGGTGGG CAGTGGTGAT GCAAATGCTG
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** *****                             ***
               ********** *****                             ***

ClonedBKT956   GGGGCGCCCA TGGCGAATCT CCTAGTCTTC ATGGCTGCAG CCCCAATCTT
D45881   831   GGGGCGCCCA TGGCAAATCT CCTAGTCTTC ATGGCTGCAG CCCCAATCTT
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********

CloneBKT1006   GTCAGCATTC CGCCTCTTCT ACTTCggtgt gtgcctcttg acccccaaac
D45881   881   GTCAGCATTC CGCCTCTTCT ACTTC----- ---------- ----------
                       ********** ********** *****
                       ********** ********** *****
                       ********** ********** *****
                       ********** ********** *****
                       ********** ********** *****

CloneBKT1056   ctaaccctgg acatgcacca gtcagcttgc atgctttcca agctttgtgc
D45881   906   ---------- ---------- ---------- ---------- ----------

CloneBKT1106   atgctgagct tctggtgagg actgcagtgc tccggccagc tttcatgtaa
D45881   906   ---------- ---------- ---------- ---------- ----------
```

```
CloneBKT1156   actaccggtg acatctgcta gtgccagggc tgtgcacacc atggtgatgg
D45881   906   ---------- ---------- ---------- ---------- ----------

CloneBKT1206   aggtttcgca catcagcagc accacagggt catagccttg tgtgtacagt
D45881   906   ---------- ---------- ---------- ---------- ----------

CloneBKT1256   gtttctgccc tgcctgctga tgcttgtagc gtcctgacgc ccctgatacc
D45881   906   ---------- ---------- ---------- ---------- ----------

CloneBKT1306   ccagtagccc aaatggaggt ggcaactggc caatagtgtg gtgctctgct
D45881   906   ---------- ---------- ---------- ---------- ----------


CloneBKT1356   cagctcactt gtatgcttgc ggtgctggtt tgcaGGCACT TACCTGCCAC
D45881   906   ---------- ---------- ---------- ----GGCACT TACCTGCCAC
                                                  ****** **********
                                                  ****** **********
                                                  ****** **********

CloneBKT1406   ACAAGCCTGA GCCAGGCCCT GCAGCAGGCT CTCAaGTGAT GGCCTGGTTC
D45881   922   ACAAGCCTGA GCCAGGCCCT GCAGCAGGCT CTCAgGTGAT GGCCTGGTTC
               ********** ********** ********** **** ***** **********
               ********** ********** ********** **** ***** **********
               ********** ********** ********** **** ***** **********
                                                  ***** **********

CloneBKT1456   AGGGCCAAGA CAAGTGAGGC ATCTGATGTG ATGAGTTTCC TGACATGCTA
D45881   972   AGGGCCAAGA CAAGTGAGGC ATCTGATGTG ATGAGTTTCC TGACATGCTA
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
               ********** ********** ********** ********** **********
                                     ******** ********** **********
CloneBKT1506   CCACTTTGAC CTgggtgggt gctaacccac actgacaaag ctgcaaacca
D45881  1022   CCACTTTGAC CT-------- ---------- ---------- ----------
               ********** **
               ********** **
               ********** **
               ********** **
               ********** **

CloneBKT1556   cctctgctgg ctgcgtgtag tgcatcagca agcatggtgc atagacccaa
D45881  1034   ---------- ---------- ---------- ---------- ----------

CloneBKT1606   gctgcgtggt aagtgtgtcc tggtcccaac ctggcctggg atggcatgct
D45881  1034   ---------- ---------- ---------- ---------- ----------

CloneBKT1656   gcatgtcact taccagcttt tggctgtgcc cgggctgtcc tgttgctgcc
D45881  1034   ---------- ---------- ---------- ---------- ----------

CloneBKT1706   ttgtaGCACT GGGAGCACCA CAGGTGGCCC TTTGCCCCCT GGTGGCAGCT
D45881  1034   -----GCACT GGGAGCACCA CAGGTGGCCC TTTGCCCCCT GGTGGCAGCT
                    ***** ********** ********** ********** **********
                    ***** ********** ********** ********** **********
                    ***** ********** ********** ********** **********
                    ***** ********** ********** ********** **********
                                                  ****** **********

CloneBKT1756   GCCCCACTGC CGCCGCCTGT CtGAGCGTGG CCTGGTGCCT GCCGTGGCAT
D45881  1079   GCCCCACTGC CGCCGCCTGT CcGGGCGTGG CCTGGTGCCT GCCTTGGCAT
               ********** ********** * ******** ********** **********
               ********** ********** * ******** ********** **********
               ********** ********** * ******** ********** **********
               ********** ********** * ******** ********** **********
               ********** ********** *
```

```
CloneBKT1806   GACCTGGT-- ---------- ---------- ---------- ----------
D45881  1129   GACCTGGTcc ctccgctggt gacccagcgt ctgcacaaga gtgtcatgct

               ********
               ********
               ********
               ********
CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1179   acagggtgct gcggccagtg gcagcgcagt gcactctcag cctgtatggg

CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1229   gctaccgctg tgccactgag cactgggcat gccactgagc actgggcgtg

CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1279   ctactgagca atgggcgtgc tactgagcaa tgggcgtgct actgacaatg

CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1329   ggcgtgctac tggggtctgg cagtggctag gatggagttt gatgcattca

CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1379   gtagcggtgg ccaacgtcat gtggatggtg gaagtgctga ggggtttagg

CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1429   cagccggcat ttgagagggc taagttataa atcgcatgct gctcatgcgc

CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1479   acatatctgc acacagccag ggaaatccct tcgagagtga ttatgggaca

CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1529   cttgtattgg tttcgtgcta ttgttttatt cagcagcagt acttagtgag

CloneBKT1814   ---------- ---------- ---------- ---------- ----------
D45881  1579   ggtgagagca gggtggtgag agtggagtga gtgagtatga acctggtcag

Cloned BKT      1814   ---------- ---------- ---------- ----
D45881          1629   cgaggtgaac agcctgtaat gaatgactct gtct
```

Figure 2.16 Dialign output of the cloned BKT (genomic) with the gene sequence ID D45881

2.2.2.5 Alignment (DIALIGN format) of cloned cDNA with cDNA of D45881

The sequence alignment of cDNA obtained from cloned BKT showed 99 % matching with the known cDNA of BKT sequence ID D45881 (Fig 2.18). Here also the same five nucleotide bases interchange with other complementary base. The positions were highlighted with black background at 69, 268, 343, 416, 855 nucleotide bases in the genomic DNA of BKT.

```
Cloned BKT 1    TGGGCGACAC AGTATCACAT GCCATCCGAG TCGTCAGACG CAGCTCGTCC
D45881     1    TGGGCGACAC AGTATCACAT GCCATCCGAG TCGTCAGACG CAGCTCGTCC

                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********
                ********** ********** ********** ********** **********

Cloned BKT51    TGCGTTGAAG CACGCCTATA AACCTCCAGC ATCTGACGCC AAGGGCATCA
D45881    51    TGCGCTAAAG CACGCCTACA AACCTCCAGC ATCTGACGCC AAGGGCATCA

                ********** ******** * ********** ********** **********
                ********** ******** * ********** ********** **********
                ********** ******** * ********** ********** **********
```

```
                  ********** ******** * ********** ********** **********
                                       * ********** ********** **********

Cloned BKT101     CGATGGCGCT GACCATCATT GGCACCTGGA CCGCAGTGTT TTTACACGCA
D45881    101     CGATGGCGCT GACCATCATT GGCACCTGGA CCGCAGTGTT TTTACACGCA

                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********

Cloned BKT151     ATATTTCAAA TCAGGCTACC GACATCCATG GACCAGCTTC ACTGGTTGCC
D45881    151     ATATTTCAAA TCAGGCTACC GACATCCATG GACCAGCTTC ACTGGTTGCC

                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                                                           * **********

Cloned BKT201     TGTGTCCGAA GCCACAGCCC AGCTTTTGGG CGGAAGCAGC AGCCTATTGC
D45881    201     TGTGTCCGAA GCCACAGCCC AGCTTTTGGG CGGAAGCAGC AGCCTACTGC

                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** *******
Cloned BKT251     ACATCGCCGC AGTCTTCcTT GTACTTGAGT TCCTGTACAC TGGTCTATTC
D45881    251     ACATCGCTGC AGTCTTCaTT GTACTTGAGT TCCTGTACAC TGGTCTATTC

                  ********** ******* ** ********** ********** **********
                  ********** ******* ** ********** ********** **********
                  ********** ******* ** ********** ********** **********
                  ********** ******* ** ********** ********** **********

Cloned BKT301     ATCACCACAC ATGACGCAAT GCATGGCACC ATAGCTTTGA GGaACAGGCA
D45881    301     ATCACCACAC ATGACGCAAT GCATGGCACC ATAGCTTTGA GGcACAGGCA

                  ********** ********** ********** ********** ** *******
                  ********** ********** ********** ********** ** *******
                  ********** ********** ********** ********** ** *******
                  ********** ********** ********** ********** ** *******
                    ******** ********** ********** ********** **

Cloned BKT351     GCTCAATGAT CTCCTTGGCA ACATCTGCAT ATCACTGTAC GCCTGGTTTG
D45881    351     GCTCAATGAT CTCCTTGGCA ACATCTGCAT ATCACTGTAC GCCTGGTTTG

                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********

Cloned BKT401     ACTACAGCAT GCTGCgTCGC AAGCACTGGG AGCACCACAA CCATACTGGC
D45881    401     ACTACAGCAT GCTGCaTCGC AAGCACTGGG AGCACCACAA CCATACTGGC

                  ********** ***** **** ********** ********** **********
                  ********** ***** **** ********** ********** **********
                  ********** ***** **** ********** ********** **********
                  ********** ***** **** ********** ********** **********
                             ***** **** ********** ********** **********

Cloned BKT451     GAAGTGGGGA AAGACCCTGA CTTCCACAAG GGAAATCCCG GCCTTGTCCC
D45881    451     GAAGTGGGGA AAGACCCTGA CTTCCACAAG GGAAATCCCG GCCTTGTCCC

                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
                  ********** ********** ********** ********** **********
```

```
Cloned BKT501    CTGGTTCGCC AGCTTCATGT CCAGCTACAT GTCCCTGTGG CAGTTTGCCC
D45881    501    CTGGTTCGCC AGCTTCATGT CCAGCTACAT GTCCCTGTGG CAGTTTGCCC

                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
Cloned BKT551    GGCTGGCATG GTGGGCAGTG GTGATGCAAA TGCTGGGGGC GCCCATGGCG
D45881    551    GGCTGGCATG GTGGGCAGTG GTGATGCAAA TGCTGGGGGC GCCCATGGCA

                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
Cloned BKT601    AATCTCCTAG TCTTCATGGC TGCAGCCCCA ATCTTGTCAG CATTCCGCCT
D45881    601    AATCTCCTAG TCTTCATGGC TGCAGCCCCA ATCTTGTCAG CATTCCGCCT

                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
Cloned BKT651    CTTCTACTTC GGCACTTACC TGCCACACAA GCCTGAGCCA GGCCCTGCAG
D45881    651    CTTCTACTTC GGCACTTACC TGCCACACAA GCCTGAGCCA GGCCCTGCAG

                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** ******
Cloned BKT701    CAGGCTCTCA AGTGATGGCC TGGTTCAGGG CCAAGACAAG TGAGGCATCT
D45881    701    CAGGCTCTCA GGTGATGGCC TGGTTCAGGG CCAAGACAAG TGAGGCATCT

                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********

Cloned BKT751    GATGTGATGA GTTTCCTGAC ATGCTACCAC TTTGACCTGC ACTGGGAGCA
D45881    751    GATGTGATGA GTTTCCTGAC ATGCTACCAC TTTGACCTGC ACTGGGAGCA

                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                                       **** ********** **********

Cloned BKT801    CCACAGGTGG CCCTTTGCCC CCTGGTGGCA GCTGCCCCAC TGCCGCCGCC
D45881    801    CCACAGGTGG CCCTTTGCCC CCTGGTGGCA GCTGCCCCAC TGCCGCCGCC

                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********
                 ********** ********** ********** ********** **********

Cloned BKT851    TGTCtGAGCG TGGCCTGGTG CCTGCCGTGG CATGACCTGG T
D45881    851    TGTCcGGGCG TGGCCTGGTG CCTGCCTTGG CATGACCTGG T

                 **** ***** ********** ********** ********** *
                 **** ***** ********** ********** ********** *
                 **** ***** ********** ********** ********** *
                 **** ***** ********** ********** ********** *
                 ****
```

Figure 2.17 Dialign output of the cloned BKT (cDNA) with the cDNA gene sequence ID D45881

2.2.2.6 Alignment of aminoacid sequence with protein sequence of D45881

The aminoacid alignment of the BKT is given in the Figure 2.19. Sequence analysis revealed that though there there were some nucleotide polymorphism but no shift in reading frame of the sequence.

```
Cloned BKT 1   WATQYHMPSESSDAARPALKHAYKPPASDAKGITMALTIIGTWTAVFLHAIFQIRLPTSM  60
D45881     27  WATQYHMPSESSDAARPALKHAYKPPASDAKGITMALTIIGTWTAVFLHAIFQIRLPTSM  86

Cloned BKT61  DQLHWLPVSEATAQLLGGSSSLLHIAAVFLVLEFLYTGLFITTHDAMHGTIALRNRQLND  120
D45881    87  DQLHWLPVSEATAQLLGGSSSLLHIAAVFIVLEFLYTGLFITTHDAMHGTIALRHRQLND  146

Cloned BKT121 LLGNICISLYAWFDYSMLRRKHWEHHNHTGEVGKDPDFHKGNPGLVPWFASFMSSYMSLW  180
d45881   147  LLGNICISLYAWFDYSMLHRKHWEHHNHTGEVGKDPDFHKGNPGLVPWFASFMSSYMSLW  206

Cloned BKT181 QFARLAWWAVVMQMLGAPMANLLVFMAAAPILSAFRLFYFGTYLPHKPEPGPAAGSQVMA  240
D45881    207 QFARLAWWAVVMQMLGAPMANLLVFMAAAPILSAFRLFYFGTYLPHKPEPGPAAGSQVMA  266

Cloned BKT241 WFRAKTSEASDVMSFLTCYHFDLHWEHHRWPFAPWWQLPHCRRLSERGLVPAVA  294
D45881    267 WFRAKTSEASDVMSFLTCYHFDLHWEHHRWPFAPWWQLPHCRRLSGRGLVPALA  320
```

Figure 2.18 Dialign output of the aminoacid sequence of the cloned BKT with the protein sequence of BKT gene ID D45881

2.2.2.7 PCR analysis of the genomic BKT using different primers

The cloned BKT gene from the plasmid pRT100 was used for the PCR analysis using different combinations of primers. Different amplification size were observed for the different primers (Fig 2.20).

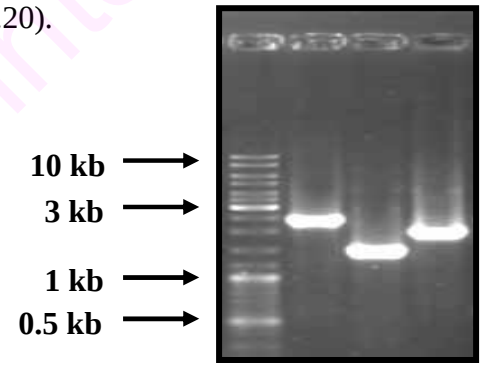


Figure 2.19 PCR amplification of the BKT gene from recombinant pRT100 using different primer combinations

There was an exactly 2.24 kb amplicon was observed (Fig 2.20, lane 1) when the primer CaMV 35S forward and BKT-A reverse was used. For the primer CaMV 35S forward and the internal primer BKT-C reverse was used, there was an amplification of 1.44 kb size was observed (Fig 2.20, lane 2). A 1.99 kb amplicon size was observed when the primer BKT-A forward and reverse primer poly A (Fig 2.20, lane 3) was used.

2.2.3 Sub-cloning of the cloned BKT from pRT100 to pCAMBIA1304

After the BKT gene was cloned to the pRT100 between the CaMV 35S promoter and the poly A, the entire BKT cassette has been subcloned to the binary vector pCAMBIA1304 which having the *hpt* as the selection gene and the GUS and GFP as the reporter gene. In this binary vector the BKT gene cassette has been cloned in the *HindIII* site of the MCS. The successful cloning of BKT in this binary vector was further analysed by transferring this cloned binary vector to the *E. coli* (DH5α) for the analysis of the size of the wild and recombinant plasmid. The plasmid isolated from the white colonies showed a 2.5 kb (i.e pCAMBIA1304-BKT) size larger than the wild pCAMBIA1304 (Fig 2.21, lanes 1 to 5). The wild pCAMBIA1304 showed only the 12.3 kb (Figure 2.21, lanes 6 and 7). After confirming recombinant binary vector is larger in size than the wild vector, it has further mobilized to the *Agrobacterium* (EHA 101). After 36 hrs of incubation, the transformed *Agrobacterium* observed as single colonies, but lesser in numbers.

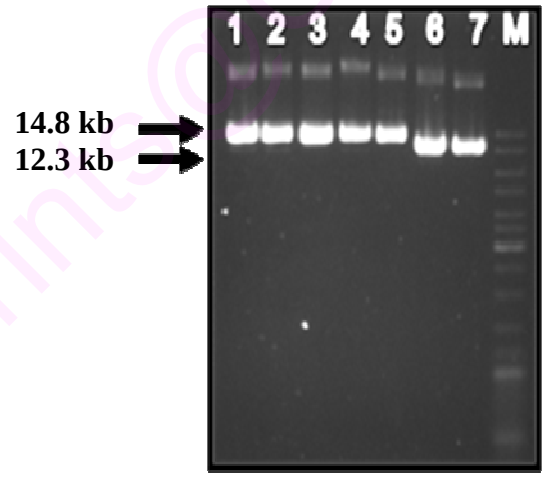


Figure 2.20. Plasmid extracted from the wild and recombinant binary vector pCAMBIA1304. Lanes 1 to 5 are the recombinant pCAMBIA1304. Lanes 6 and 7 are the wild pCAMBIA1304. M is the 10 kb marker

The colonies were isolated, cultured and the plasmids were extracted for further analysis like restriction digestion and PCR analysis. The recombinant plasmid pCAMBIA1304 has been referred as pCAMBIA1034-BKT.

2.2.3.1 Restriction digestion analysis cloned BKT in pCAMBIA-1304

The restriction analysis of the pCAMBIA1304-BKT and the wild pCAMBIA1304 with *HindIII* showed the confirmation of cloning in this binary vector.

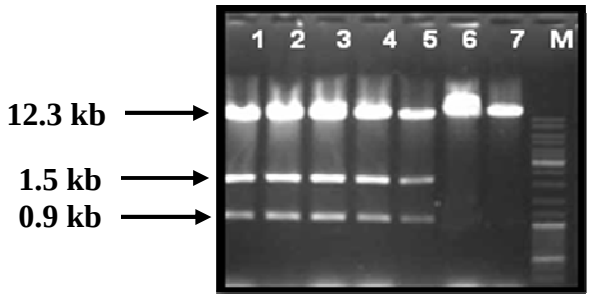


Figure 2.21 Restricted digested wild pCAMBIA1304 and cloned pCAMBIA1034-BKT with *HindIII*. Lanes 1 to 5 are the cloned pCAMBIA1304-BKT and lanes 6 and 7 are the wils pCAMBIA1304. M is the 10 kb marker

It showed the two inserts released from the recombinant plasmid which are 1534 bp and 990 bp size in length. Since there is the presence of *HindIII* site in the fourth intron region, these two inserts were released (Fig 2.22, lanes 1 to 5). Whereas in case of the wild pCAMBIA1304 there was no inserts released after the digestion. Only the linearized plasmid was observed in the gel (Fig 2.22, lanes 6 and 7).

2.3 Discussion

The result presented in this chapter clearly indicating, the cloning of BKT from the genomic DNA of *H. pluvialis* has been carried out successfully. This is the first report of cloning the BKT gene from the genomic DNA from *H. pluvialis* with CaMV 35S promoter and poly A region. Initially the genomic BKT was cloned to the T tailed vector, followed by pRT100 and mobilzed to the binary vector pCAMBIA1304. So far the cloning of the BKT has been made only from the cDNA of *H. pluvialis* (Kajiwara et al. 1995; Lotan and Hirschberg 1995), and also from the partial fragment of the cDNA of BKT for the expression studies (Meng et al. 2005; Huang et al. 2006a; Vidhyavathi 2008). Here we have reported the full gene cloning of BKT using the gene accession number D45881 (Kajiwara et al. 1995). After cloning of genomic BKT, it was confirmed by the plasmid extraction, restriction digestion, PCR analysis using the CaMV 35S forward and BKT-C reverse primers. Nucleotide and amino acid sequence analysis also revealed the reading frame of the BKT is exactly similar (99%) to the nucleotide and amino acid sequence of the known BKT gene accession number D45881.

For cloning studies, the primer BKT-A is used for the amplification of the BKT gene. The forward primer starts from the 264^{th} position (ATG) and ends before the 3' UTR which include the stop codon TGA of the BKT gene D45881. Since, the expression of the whole BKT enzyme is complete even if gene start from the 3^{rd} ATG (264^{th} nucleotide) of BKT gene, the amplification of the BKT was carried out from this third starting codon (Fraser et al. 1997; Kajiwara et al. 1995). Initially the amplified fragment of genomic BKT was cloned in between the *XhoI* and *XbaI* sites of the pRT100 (i.e in between CaMV 35S and poly A) and further the gene was mobilized with the promoter and poly A to the binary vector pCAMBIA1304 by digesting both the plasmid with the enzyme *HindIII*. Even though this enzyme released two fragments (Fig 2.15, lanes 1 and 3) of the BKT gene cassette from pRT100 (since the cloned BKT is having the one more *HindIII* site in the fourth intron), the partial restriction digestion method (at 37^0 C for 30 min), helped in releasing the whole gene cassette for the mobilization to the pCAMBIA1304.

The sequence analysis of the cloned BKT gene revealed that there were some nucleotide polymorphism when aligned and compared with the known BKT (D45881). But there were no change in the reading frame. The protein sequence synthesized from this sequence is also matching with the result of Kajiwara et al. (1995). The presence of six exons and five introns in the cloned BKT gene was observed. The varied size of exons and introns have been mentioned in the results section of this chapter. The cloned genomic BKT gives the exact amplification of 2.24 kb, 1.44 kb and 1.99 kb when the primers CaMV 35S forward and BKT-A reverse, CaMV 35S forward and BKT-C reverse and BKT-A forward and poly A reverse primers were used for the PCR analysis. These results confirmed the cloning of genomic BKT from *H. pluvialis* to the binary vector pCAMBIA1304 has been confirmed.

The promoter used for the cloning of BKT gene is CaMV 35S promoter and the stop codon is the poly A region of the nopaline synthase terminator gene. Since the suitable promoter for the expression of specific genes in *H. pluviailis* has not been studied so far, we have chosen this CaMV 35S which is a universal constitutive promoter (Odell et al. 1988). Steinbrenner and Sandmann 2006 also reported the phytoene desaturase (PDS) gene form *H. pluvialis* with control of SV 40 promoter which is not the gene specific promoter. Though Meng et al. (2005) identified the partial region of the promoter region for BKT in *H. pluvialis*, it has not been studied experimentally for its expression levels. Since the CaMV 35 is used to express selctable marker gene like *hpt* in *Chlamydomonas reinhardtii* (Kumar et al. 2004), reporter like *lac*Z gene in *H. pluvialis* (Teng et al 2002) this universal promoter has been chosen to clone the BKT gene.

Kajiwara et al. 1995; Lotan and Hirschberg 1995 isolated cDNAs from different strains of *H. pluvialis* for β -carotene ketolase. The predicted amino acid sequences of the cDNAs shared about 80% identity to the bacterial ketolase enzymes (Kajiwara et al., 1995; Lotan and Hirschberg, 1995; Misawa et al., 1995). In this study, the BKT produced from this *H. pluvialis* strain 19a showed 99.9% sequence similarity for the gene sequence of BKT D45881. Using this cloned BKT in the binary vector pCAMBIA1304, the whole gene cassette has been transferred to the *H. pluvialis* using

Agrobacterium mediated gene transfer method and the detail transformation protocol and its confirmation has been given in the next chapter.

There were non specific amplification of BKH gene when using the specific BKH primers designed from the known BKH gene accession number AF162276 and AY187011. The size of the amplicons from the cDNA for these primers should be more than 1.0 kb. But the amplification observed from the genomic DNA is lesser than 1.0 kb which is the non specific amplification. This might be due to the incompatibility of the genomic DNA of *H. pluvialis* strain (19a) to the BKH primers designed from the ID AF162276 and AY187011. This non specific amplification may also be due to mismatches in codon usage patterns (Schiedlmeier et al 1994), between the published BKH gene and the available gene in the *H. pluvialis* or changes in chromatin domain structure (Jakobiak et al. 2004; Babinger et al. 2001). Hence the cloning was proceeded with the BKT only.

The gene BKT is the crucial enzyme which is involved in the conversion of β-carotene to echinenone and then to canthaxanthin. Further the pathway proceeds with the conversion of canthaxanthin to astaxanthin by the use of BKH (Boussiba 2000; Grünewald et al. 2001; Hirschberg 2001). More over, BKT is the key enzyme which is limited to *H. pluvialis* and some other species like *Chlorella zofingiensis* (Ip and Chen 2005), *Scenedesmus vacuolatus* (Orosa et al. 2000), fungi like *Phaffia rhodozyma* (Jacobson et al. 2000) and bacterium like *Agrobacterium aurantiacum* (Yokoyama et al. 1995). Hence *H. pluvialis* is the alga which produces the higher amount of astaxanthin, and pathway for the astaxanthin production is well known, we have chosen *H. pluvialis* for the isolation and cloning of BKT. This cloned BKT will be further useful for transformation and the expression studies in the homologous (*H. pluvialis*) or heterologous host like *Dunaliella* sp, *Dacus* sp, *Lycopersicum* sp which are producing β-carotene to regulate the carotenoid biosynthesis.

Chapter III

Analysis of carotenoid profile in transformants

3.0 Introduction

Carotenoid pigments are synthesized in all of the photosynthetic bacteria, cyanobacteria, algae, higher plants, and also in some nonphotosynthetic bacteria, yeast, and fungi. Carotenoids have attracted strong attention due to their beneficial effects on human health as well as their usefulness in food colorants and animal feed because animals are unable to synthesize them de novo. In particular, dicyclic ketocarotenoids such as astaxanthin and canthaxanthin are high-value carotenoids used industrially as colorants and feed supplements (Meyers 1993).

Metabolic engineering, using a variety of carotenoid biosynthesis genes should be one of the most powerful methods to produce such ketocarotenoids for industrial and nutritional purposes because, organisms which are capable of synthesizing such ketocarotenoids are not common in nature. For example, astaxanthin and its dicyclic-carotenoid intermediates are synthesized in some marine eubacteria (Yokoyama et al. 1995), in the yeast *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) (Andrewes et al. 1976), in the green algae *Haematococcus pluvialis* (Boussiba and Vonshak 1991), and in the petal of Adonis plants (Renstrom et al. 1981). Isolating the astaxanthin biosynthetic genes like BKT, BKH, LCY etc from these organism were not well studied. To over express / regulate carotenoid biosynthesis in these organism, the genes has to be isolated, cloned and transferred to the homologous or heterologous host.

Cloning of most of the astaxanthin biosynthesis genes in *H. pluvialis* has now opened the door to genetically manipulating this pathway not only in algae, but also in other organisms. Production of natural astaxanthin by genetically engineered microorganisms has been reported by Misawa and Shimada (1998). The highest yield achieved in *E. coli* is 1.4 mg/g dry weight (Wang et al 1999). Other carotenoids, such as lycopene, which is synthesized in *E. coli*, reached a concentration of 0.5% of dry weight (Albrecht et al 1999). Mutants of the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) accumulate (3R, 3R) astaxanthin up to 0.5% of the dry weight (Chen et al 2003). But no studies were made to regulate the astaxanthin biosynthesis in *H. pluvialis* through genetic engineering and transformation. Here we are reporting the

Agrobacterium mediated transformation of the cloned BKT gene to the same *H. pluvialis* to regulate the carotenoid biosynthesis.

3.1 Materials and Methods

The culture and culture condition, glasswares, plasticwares, chemicals used were same as in the section 1.1.1 to 1.14 of chapter 1.

3.2 Methodolgy

3.2.1 Plasmid constructs and bacterial strains

The recombinant binary vector pCAMBIA1304-BKT having the extra fragment of BKT gene (1813 bp) was introduced into *Agrobacterium tumefaciens* strain EHA 101 (nopaline type). This plasmid harbours *hpt* (hygromycin phosphotransferase) as selection marker gene and GFP (green fluorescence protein) and *UidA* (β-glucuronidase) as reporter genes interrupted by an intron both driven by the CaMV 35S promoter and has been constructed from pCAMBIA 1301. Kanamycin 100 mgL^{-1} was used which is the bacterial selection marker while DH5α was the *E. coli* strain used.

3.2.3 *Agrobacterium* mediated genetic transformation

The *Agrobacterium* mediated genetic transformation of the cloned BKT in the binary vector pCAMBIA1304-BKT to *H. pluvialis* was followed the same procedure as given in the section 2.3.2 in chapter 1.

3.3 Confirmation of Transformation

The procedures used for the confirmation are GUS assay, GFP analysis, Scanning electron microscopy. These procedures are same as given in section 1.4.2 to 1.4.4 of chapter 1

3.3.1 Molecular confirmation

3.3.1.1 Genomic DNA isolation and detection by PCR

Genomic DNA from hygromycin resistant and non-transformed cells of *H. pluvialis* were extracted using a DNA extraction kit (Gene Elute-Plant Genomic extraction kit, Sigma-Aldrich chemicals, USA). The cloned BKT DNA in transformed *H. pluvialis* was amplified using the PCR thermal cycler (Eppendorf Mastercycler personal, Germany) with the combination of different primers. They were CaMV forward; 5’ ATGGTGGAGCACGACACTCT 3’ and BKT-C reverse; 5’ GTAGAAGAGGCGGAATGCTG 3’ and also with the BKT-C forward 5’ F-

TGGGCGACACAGTATCACAT 3' and poly A reverse 5' GCTCAACACATGAGCGAAAC 3' (synthesized by Sigma-Aldrich, USA). PCR was performed with 200ng of genomic DNA and 100 pmol of each of the primers (Sigma-Aldrich Chemicals, USA) using Taq polymerase (MBI Fermentas, Lithuania). The cycling parameters were: 4 min initial denaturation at 94^0C and 35 cycles involving 1 min denaturation at 94^0C, 1 min annealing at 55^0C and 1 min at 72^0C extension. The PCR products were separated on agarose gels (1.5%) using TAE buffer (Appendix) and stained with ethidium bromide (Appendix). Images of gels were recorded with a gel documentation system (Hero Lab, GmbH, Wiesloch, Germany).

3.3.1.2 Southern blotting analysis

The southern blotting procedures like restriction digestion of DNA, transformation of digested DNA to nylon membrane, purination, repurination, washing, prehybridization, post hybridization, blocking, detection etc were carried out by following the procedures in Sambrook et al. 2001 ; Ckezhk et al. 2004 (appendix). The probe used for the detection of T-DNA in the transformants is differed from the probe prepared in the section 2.5.2.1 in chapter 1.

3.3.1.2.1 Probe preparation and Labeling

For preparation of probe a portion of the BKT gene was amplified using the CaMV 35S forward; 5' ATGGTGGAGCACGACACTCT 3' and BKT-C reverse; 5' GTAGAAGAGGCGGAATGCTG 3' primers using the recombinant plasmid pCAMBIA1304-BKT DNA as template and then purified using a Qiagen PCR purification kit (Qiagen GmpH, Germany). The fragment was labeled with psoralen - biotin labeling kit (from Ambion Inc, Texas, USA), according to the method prescribed by the manufacturer. The BKT labeled probe was used for the detection of the T-DNA in the BKT transformants. The reagents, stock solutions, buffers etc for the southern blotting were described in detail in Appendix.

3.4 Growth measurement and pigment extraction from transformed *H. pluvialis*

3.4.1 Cell number

Algal cell number was determined by counting algal cells using Neubauer haemacytometer (Thoma neu, Germany) and expressed as number of cells/ml.

3.4.2 Dry weight

Known volume of culture was centrifuged at 1500 rpm for 10 min and the algal biomass was washed with distilled water and dried in a hot air oven (Sanyo, Electrical Biomedical Co. Ltd., Japan) at 60°C till constant weight was obtained. Biomass weight was expressed as gL^{-1}.

3.4.3 Induction to astaxanthin production under different stress condition

Two weeks old culture of *H. pluvialis* (100ml) was centrifuged at 1500 rpm for 5 min and inoculated in a fresh nutrient-limiting BBM medium (Table 2.1) at 1:5 ratio of inoculum supplemented with different stress conditions, individually and in combination. The cultures were kept in 150 ml flasks in triplicates with the continuous light intensity of 60 µmol m^{-2} s^{-1} under controlled temperature of 25±1°C. At the end of the experimental period cultures were harvested and analysed for biomass and carotenoid content. The different stress condition were culture without sodium chloride, sodium acetate and any other stress conditions were used as control. To induce astaxanthin production from non transformants and transformed *H. pluvialis*, the following stress conditions were applied after one week of inoculation. The different stress conditions are NaCl 0.25%, sodium acetate 4.4mM, CO_2 2%, cycloheximide 0. mgL^{-1}, NaCl 0.25% + sodium acetate 4.4mM, NaCl 0.25% + CO_2 2%, NaCl 0.25% + cycloheximide 0.3mgL^{-1}, sodium acetate 4.4mM + cycloheximide 0.3mgL^{-1}, NaCl 0.25% + sodium acetate 4.4mM + cycloheximide 0.3mgL^{-1} and NaCl 0.25% + CO_2 2% + cycloheximide 0.3mgL^{-1}.

3.4.4 Effect of inhibitors

The cultures were harvested by centrifugation at 1500 rpm for 5min and resuspended in nutrient sufficient (NS) media. The initial cell concentration was adjusted to 15×10^4 cells ml^{-1}. The carotenoid synthesis inhibitor diphenylamine (DPA, Sigma) and nicotine (Fluka) was studied. For DPA the stock was prepared in 70% (pH 9.0) and used at final concentration of 45µM. Nicotine was added to the cultures at a final concentration of 9.8mM. The photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, (DCMU Sigma) was added to the cultures at a final concentration of 20 µM, The transcriptional inhibitor cycloheximide was added at a final concentration of

0.3 µg L^{-1}After the addition of inhibitors cultures were exposed to continuous high light intensity of 60µmol m^{-2} s^{-1} for 2 days for secondary carotenoid induction.

3.4.5 Pigment extraction, carotenoid and astaxanthin estimation

For pigment analysis, an aliquot of NT and transformed culture was harvested by centrifugation at 1500 rpm for 10 min and freeze-dried. A known quantity of biomass was extracted with 90% acetone in a mortar and pestle using neutralized sand. The extraction was repeated till the pellet became colorless. The extracts were centrifuged at 5000 rpm for 5 min and the supernatants were pooled. Aliquot of extract is flushed with N_2 gas and stored at -20^{0}C preferably at -80^{0}C for spectrophotometric analysis as in section 2.6.4 of chapter 1 for carotenoid estimation, and another aliquot of extract was evaporated to dryness using N_2 gas and stored at -20^{0}C or -80^{0}C for HPLC analysis as in the section 2.6.5 of chapter 1 for astaxanthin and other carotenoids profile. All operations were carried out under dim light.

3.5 Expression analysis of carotenoid biosynthetic genes

3.5.1 RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

To one week grown culture of NT and transformed *H. pluvialis*, sodium chloride (0.25%) and sodium acetate (4.4 mM) and the combination of two were added to induce carotenoid formation. The cultures were incubated under continuous light intensity of 3.5 klux for 48 hours. Thereafter, 1 × 10^8 cells from each culture were harvested, frozen under liquid nitrogen and subsequently powdered. Then total RNA was extracted using RNAqueous® kit according to instruction manual (Ambion, Austin, TX). Possible contaminant genomic DNA in RNA extract was removed using TURBO DNA-free™ kit (Ambion, Austin, TX). The concentration of total RNA was determined spectrophotometrically at 260 nm. Integrity of RNA was checked by electrophoresis in formaldehyde denaturing gels stained with ethidium bromide. The gene specific primers for phytoene synthase (PSY), phytoene desaturase (PDS), lycopene cyclase (LCY), β-carotene ketolase (BKT) β-carotene ketolase (BKH) and were designed using Primer3 software (Rozen and Skaletsky, 2000) (Table 3.1) and synthesized (Sigma - Genosys, Bangalore, India). First-strand cDNAs were synthesized from 0.2 µg of total RNA in 20 µl final volume, using M-MuLV reverse transcriptase

and oligo-dT (18-mer) primer (Fermentas GmbH, Germany). PCR amplifications were performed using PCR mixture (15 µl) which contained 1 µl of RT reaction product as template, 1× PCR buffer (Bangalore Genei, Bangalore, India), 200 µM dNTPs (Fermentas GmbH, Germany), 1 unit (U) of Taq DNA polymerase (Sigma-Aldrich, USA), 0.1 µM of each primer depending on the gene. PCR was performed at initial denaturation at 94°C for 4 min, 30 cycles (1min at 94°C; 1 min at 60°C; 1 min at 72°C) and final elongation (10 min at 72°C) using a thermal cycler (Biorad Thermal cycler, Germany). The PCR products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%) and documented in a gel documentation system (Herolab GmbH Laborgerate, Germany). The size of the amplification products was estimated from 100 bp DNA ladder.

Table 3.1. Specific primers, annealing temperatures, and total numbers of amplification cycles used for RT-PCR

Primer	Primer sequence (5'–3')	Annealing temperature (°C)	Total number of amplification cycles	GenBank ID / reference	Amplicon size (bp)
PSY- forward	ATGTACCATCCCAAGGCAAG	60	30	AY835634	402
PSY- reverse	CTGGACCAGGCCTACGAC				
PDS- forward	TCCATGATCTTTGCCATGC	60	30	AY768691	462
PDS- reverse	CGGGAGTTGAACATGAGGTC				
LCY- forward	CTTCTTCTCCGCCTTCTTCA	60	30	AY182008	565
LCY- reverse	GCATCCTACCGCTCAAAGAA				
BKT- forward	CATCTCCTTGTACGCCTGGT	55	30	X86782	423
BKT- reverse	CAGTGCAGGTCGAAGTGGTA				
BKH- forward	CTACACCACAGCGGCAAGTA	55	30	AF162276	521
BKH- reverse	GCCTCACCTGATCCTACCAA				

3.6 Experimental design and data analysis

Each experiment was repeated twice with at least three replications. All the observations and calculations were made separately for each set of experiments. The significance ($p \leq 0.05$) of the variables studied was assessed by one-way analysis of variance (ANOVA) using Microsoft Excel XP®. The mean separations were performed by Duncan's Multiple Range Test (DMRT) for segregating means where the level of significance was set at $p \leq 0.05$

3.7 Results

The genetic transformation of selection and marker gene in *H. pluvialis* through *Agrobacterium* mediation has been carried out successfully (Kathiresan et al 2009) and it is elaborated in chapter 1. Further the cloned BKT gene from *H. pluvialis* was transformed to the same host through *Agrobacterium* mediated transformation. The cocultivation procedure, selection of resistance cells, confirmation studies were followed as like the procedure followed for the transformation of selection and reporter genes and the results of these studies has been presented here.

3.7.1 Growth in cocultivation medium

The cocultivation was performed in the solid TAP medium and were directly grown in the selection medium having hygromycin 10 mgL^{-1}. The NT cells also grown in the same selection medium. The NT cells showed no growth (Fig 3.1A) in the selection medium whereas good growth in the plates without hygromycin (like a thick mat on the plate) was observed (Fig 3.1B). The cocultivated cells directly plated on the plates having hygromycin at 10 mgL^{-1} showed resistance and the colonies were observed in the selection medium as a single colonies (Fig 3.1C).

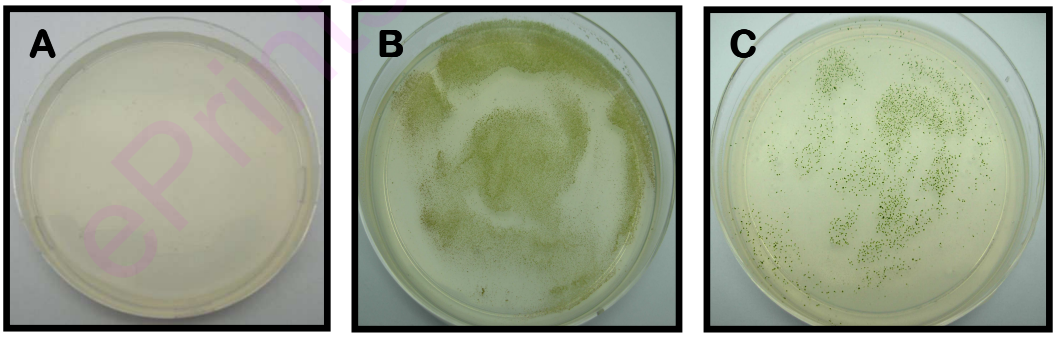


Figure 3.1 Growth of NT and BKT transformants in the TAP medium. A – NT cells in the selection medium shows no growth of the cells. B – NT cells plated on medium without hygromycin. C – BKT transformants on the selection medium shows a single resistant colonies

3.7.2 Confirmation of the transformants

3.7.2.1 Detection of reporter genes

3.7.2.1.1 GFP expression

The bright green fluorescence was characteristic of hygromycin resistant cells when observed under a fluorescence microscope. NT cells emitted only the

characteristic red fluorescence of chloroplasts (Fig 3.2A). For the transformants the fluorescence was distributed throughout the cell (Fig 3.2B).

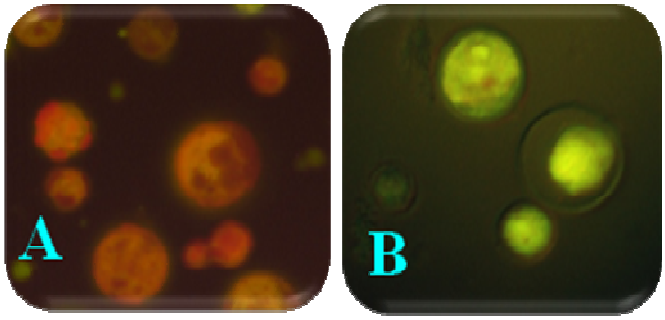


Figure 3.2 GFP observation in NT (A) and Transformed *H. pluvialis* (B).

3.7.2.1.2 GUS Assay

GUS assay was performed for both NT and transformed *H. pluvialis*. No positive GUS activity was detected in NT cells (Fig 3.3 A). Cells that were resistant to hygromycin were analyzed for GUS activity. The positive GUS activity was observed as blue colour in the hygromycin resistant cells (Fig 3.3 B).

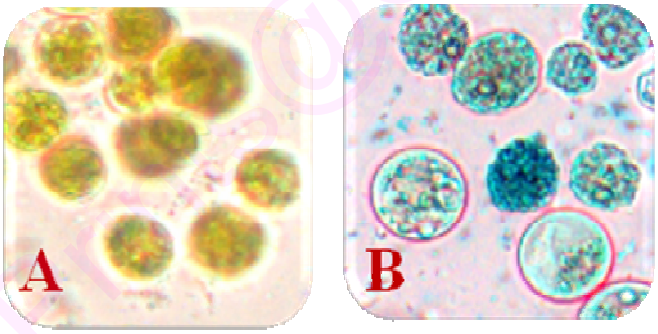


Figure 3.3 GUS assay for the NT (A) and Transformed *H. pluvialis* (B).Transformed *H. pluvialis* observed under 40X.

3.7.2.2 Scanning Electron Microscopy

Cells co-cultivated with *Agrobacterium* when observed under the SEM has shown numerous bacteria which are freely lying on the cell surface (Fig. 3.4 C). The cell surface showed typical pore-like openings, which are present only in cocultivated cells but not in, the NT cells. Hair like structures/protrusions (Fig. 3.4D) appeared on the cell surface of cocultivated cells. NT cells grown in the absence of *Agrobacterium* (Fig. 3.4 A,B) presented a smooth cell surface.

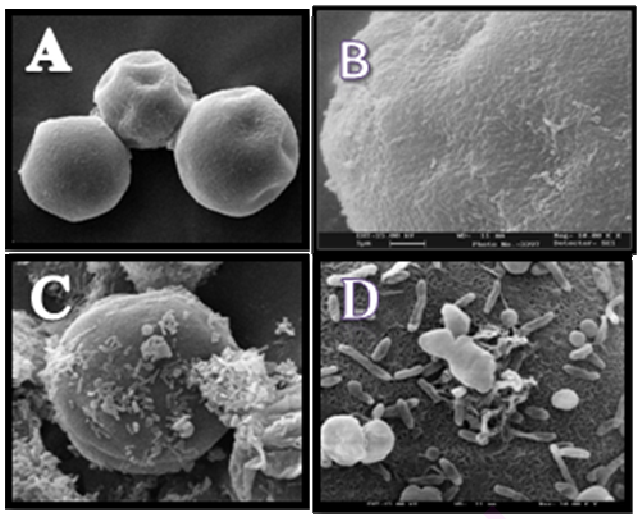


Figure 3.4 Scanning electron microscopic photograph of the NT and co-cultivated *H. pluvialis* with pCAMBIA 1304-BKT. A& B – *H. pluvialis* cells without co-cultivation showing a smooth cell surface. C - Cocultivated *H. pluvialis* showing a number of *Agrobacterium tumefaciens* adhering on the surface of the cells. D- Closer view of the cell surface of co-cultivated *H. pluvialis* showing rough surface.

3.7.2.3 PCR analysis for the BKT transformants

Amplification of the fragment of BKT gene in the transformed *H. pluvialis* was performed using different primers. Hence BKT gene was cloned in between CaMV promoter and Poly A tail, the primer for CaMV promoter and the combination of CaMV primers and the BKT primers were used for amplification. The size of the amplified fragments for each primer combinations are indicated in the table 3.2. For CaMV forward primer and BKT-C reverse primer the amplicon size is 1448 bp for both transformed *H. pluvialis* and the recombinant plasmid pCAMBIA1304-BKT (Fig 3.5, lane 1 and 2). The same CaMV forward primer with BKT-A reverse primer gives a larger fragment 2240 bp (Fig 3.5, lane 4 and 5). For the BKT-C forward and the poly A reverse primer the amplicon size is 1987 bp for both transformed *H. pluvialis* and the recombinant plasmid pCAMBIA1304-BKT (Fig 3.5, lane 7 and 8). The BKT-A forward and poly A reverse primer gives the amplification of 1996 bp (Fig 3.5, lane 10 and 11). No amplification was observed for the NT *H. pluvialis* using these primers (Fig 3.5, lanes 3, 6, 9 and 12).

Table 3.2 Amplicon size of the BKT from transformed *H. pluvialis* and recombinant pCAMBIA1304 using different combinations

Forward primer	Reverse Primer	Amplicon size (bp)
CaMV – Forward	BKT – C Reverse	1448
CaMV – Forward	BKT – A Reverse	2240
BKT – C Forward	Poly A – Reverse	1987
BKT – A Forward	Poly A – Reverse	1996

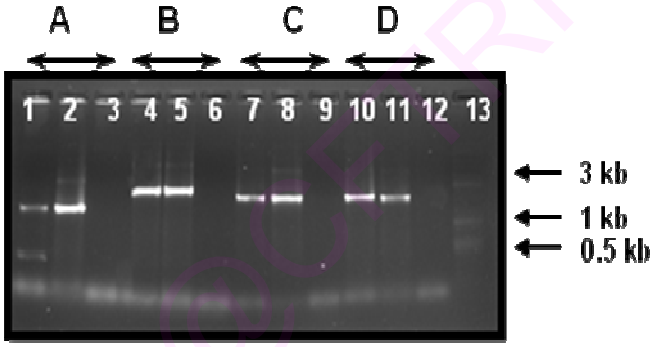


Figure 3.5 PCR Amplification of the BKT gene cloned in between CaMV promoter and poly A using different primer combinations. A, B, C and D are the amplicons from the primers CaMV 35S forward and BKT C reverse, CaMV 35S forward and BKT A reverse, BKT C forward and CaMV 35S reverse and BKT A forward and CaMV 35S reverse primers respectively. Lanes 1, 4, 7 and 10 are the amplification from transformed *H. pluvialis*. Lanes 2, 5, 8 and 11 are the amplification from recombinant pCAMBIA1304-BKT. Lanes 3, 6, 9 and 12 are the NT *H. pluvialis*.

3.7.2.4 Southern blotting analysis

The BKT transformed *H. pluvialis* and the recombinant plasmid pCAMBIA1304 were digested with the restriction enzyme *Hind*III. The enzyme *Hind*III, cut the BKT gene into two fragments. The inserts released from the recombinant plasmid are 1534 bp and 990 bp (Fig 3.6, Lanes 1 and 5). Whereas in case of the transformants, apart from these fragments some more different sized bands were observed in each of the samples digested with *Hind*III (Lanes 2 and 3). The size of these fragments are higher than the fragments obtained from the insert. In case of the

NT cells two reactive bands were observed. These bands are due to the presence of the host BKT gene in the NT cells. Whereas the transformed cells showed more than two bands. The difference in the banding pattern between the two samples (lane 2 and 3) is due to the DNA isolated from the two different resistant colonies. Since the place of integration of the T-DNA might be vary between the different colonies, the banding pattern digested fragments observed in each of the samples are also not similar.

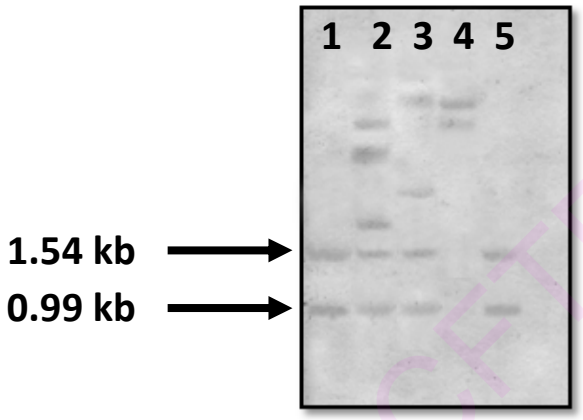


Figure 3.6 Southern blot analysis of the BKT transformed *H. pluvialis* and pCAMBIA1304-BKT. The DNA of the positive NT (lane 1 and 5), transformant (lane 2 and 3) and NT (lane 4) were digested with the restriction enzyme *Hind*III.

The different banding pattern obtained with DNA from BKT transformants as compared to plasmid DNA clearly indicates that integration of the BKT gene into the genomic DNA of *H. pluvialis* has occurred.

3.7.2.5 RT-PCR analysis of the transformants

RT-PCR was performed from the total RNA isolated from the NT and transformed *H. pluvialis*. The primers used for the amplification is cDNA of BKT is BKT A which gives 908 bp. There was a difference in intensity of the amplicons observed for transformants and the NT cells (Fig 3.7). The intensity of the amplicons were measured using the software Easywin and its corresponding area was plotted in the graph (Fig 3.8). All amplification obtained from the BKT transformed *H. pluvialis* is high in intensity (Lane 1 to 4). Whereas for the NT cells the amplification intensity is less. This shows the expression levels of BKT is 3.5 to 4 fold higher in transformants when compared to the NT cells.

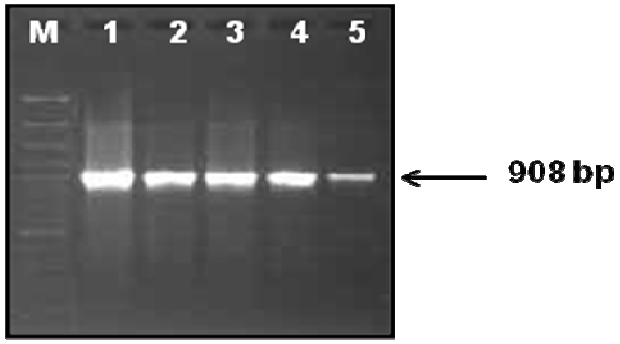


Figure 3.7 RT-PCR analysis for the *H. pluvialis*. Lanes 1 to 4 are the amplification from BKT transformants. Lane 5 is the amplification from NT cells. M is the marker

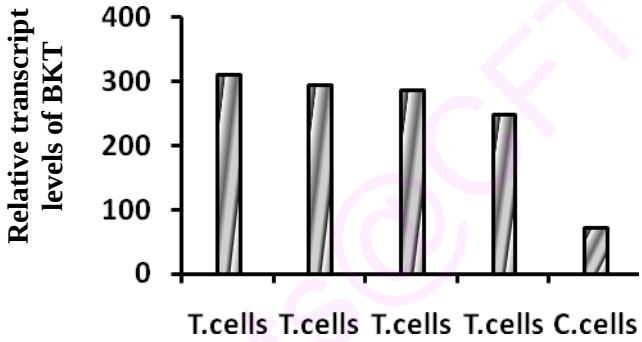


Figure 3.8 Amplification intensity of the BKT from the transformed cells (T. cells) and NT cells i.e. control cells (C. cells) of *H. pluvialis*

3.7.3 Carotenoid analysis under different stress conditions

3.7.3.1 Analysis of total chlorophyll and total carotenoids

The transformed and NT *H. pluvialis* cells were subjected to different stress induction and were analysed for the changes in composition of both carotenoids and chlorophylls through spectrophotometer. The spectrophotometric analysis revealed the total carotenoid content transformed cells (11.21 mg/g) is 10 fold higher than the NT cells (1.1 mg/g) in case of the NaCl 0.25% + sodium acetate 4.4mM treated cells. The changes in the total chlorophyll and total carotenoid due to the other stress induced culture of both NT and transformed *H. pluvialis* are indicated in table 3.3.

Table 3.3 Amount of total chlorophyll and total carotenoids in of both NT and transformed *H. pluvialis*

Sl.No	Total Chlorophyll (mg/g)		Total Carotenoids (mg/g)	
	Non Transformed	Transformed	Non Transformed	Transformed
A	0.1	0.89	0.7	5.12
B	1.06	1.64	4.51	9.55
C	1.7	1.66	0.73	7.73
D	1.3	1.77	0.61	7.19
E	0.2	0.38	0.12	1.76
F	**2.01**	**2.73**	**1.1**	**11.21**
G	0.2	0.7	1.1	3.76
H	0.3	1.33	1.7	6.5
I	0.5	0.91	2.2	3.81
J	0.11	0.4	0.56	1.74
K	0.2	0.5	0.19	2.18

A – Control (No stress), **B** - NaCl 0.25%, **C** - Sod.Acetate 4.4mM, **D**- CO_2 2%, **E** - Cycloheximide 0.3 mgL^{-1}, **F** - NaCl 0.25% + Sod.Acetate 4.4mM, **G** - NaCl 0.25% + CO_2 2%, **H** - NaCl 0.25% + Cycloheximide 0.3 mgL^{-1}, **I** - Sod.Acetate 4.4mM + Cycloheximide 0.3 mgL^{-1}, **J** - NaCl 0.25% + Sod.Acetate 4.4mM + Cycloheximide 0.3 mgL^{-1}, **K** - NaCl 0.25% + CO_2 2% + Cycloheximide 0.3 mgL^{-1}. Values are mean ±SD of three independent determinations

Under the stress condition NaCl 0.25%, sodium acetate 4.4mM, CO_2 2%, there was no major changes in the total chlorophyll in transformed cells when compare to the NT cells. But, the total carotenoids is more than 2 fold increase under NaCl 0.25% to 10 fold higher in substrate substituted with sodium acetate 4.4mM and CO_2 2%. The results of the combination of other stress condition are given in the Figure 3.9.

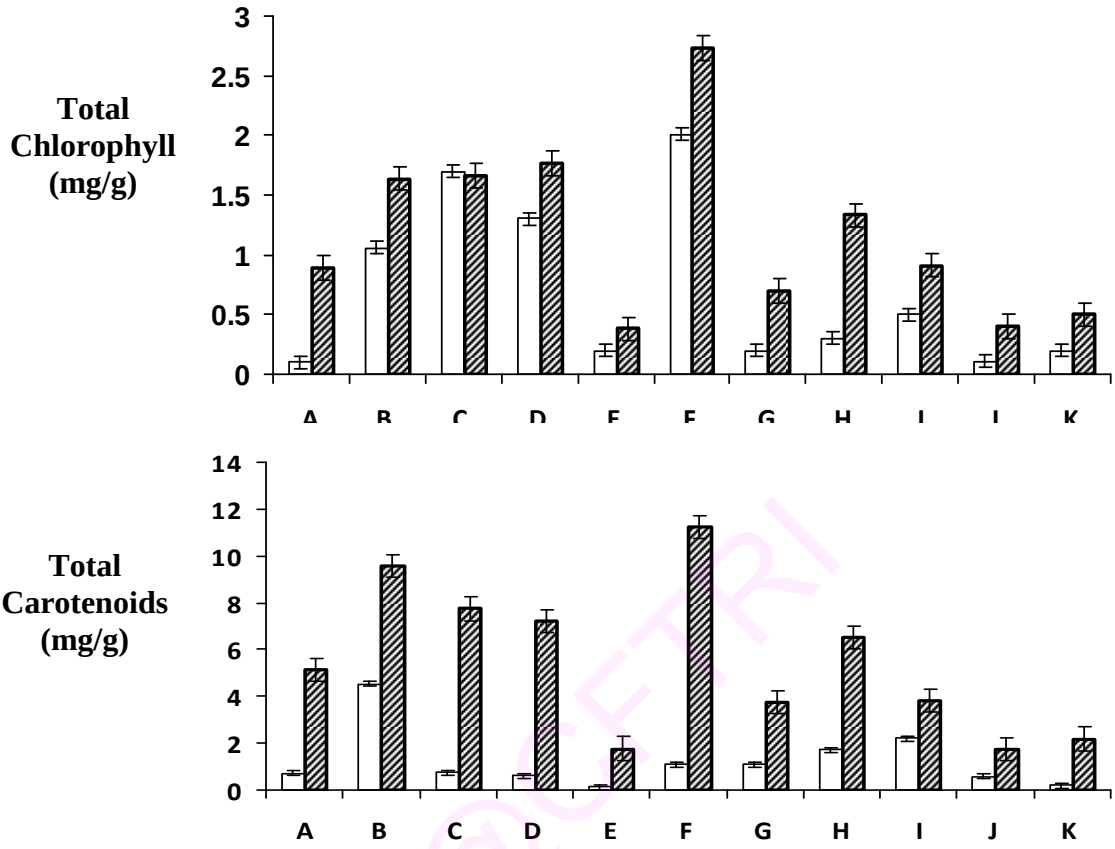


Figure 3.9 Effect of different stress condition on NT and Transformed cells A - Control, B - NaCl 0.25%, C - Sodium acetate 4.4mM, (D)- CO_2 2%, E - Cycloheximide 0.3 mgL^{-1}, F - NaCl 0.25% + Sodium acetate 4.4mM, G - NaCl 0.25% + CO_2 2%, H - NaCl 0.25% + Cycloheximide 0.3 mgL^{-1}, I - Sodium acetate 4.4mM + Cycloheximide 0.3 mgL^{-1}, J - NaCl 0.25% + Sodium acetate 4.4mM + Cycloheximide 0.3 mgL^{-1}, K - NaCl 0.25% + CO_2 2% + Cycloheximide 0.3 mgL^{-1}. Values are mean ±SD of three independent determinations.
□ NT cells, ▨ Transformed cells

3.7.3.2 Analysis of carotenoid profile in stress induced cultures

HPLC analyses revealed the occurrence of ketocarotenoids, astaxanthin and its esters, canthaxanthin and echinenone under different stress conditions. The HPLC analysis of the both NT and BKT transformed *H. pluvialis* showed the significant changes in the carotenoid profile. It was observed that the level of lutein, echinenone canthaxanthin and astaxanthin level (peaks 4, 5, 8 and 9 of Figure 3.10) were higher in

the transformed cells. The differences between the carotenoid profile of NT and transformants are given in Figure 3.10.

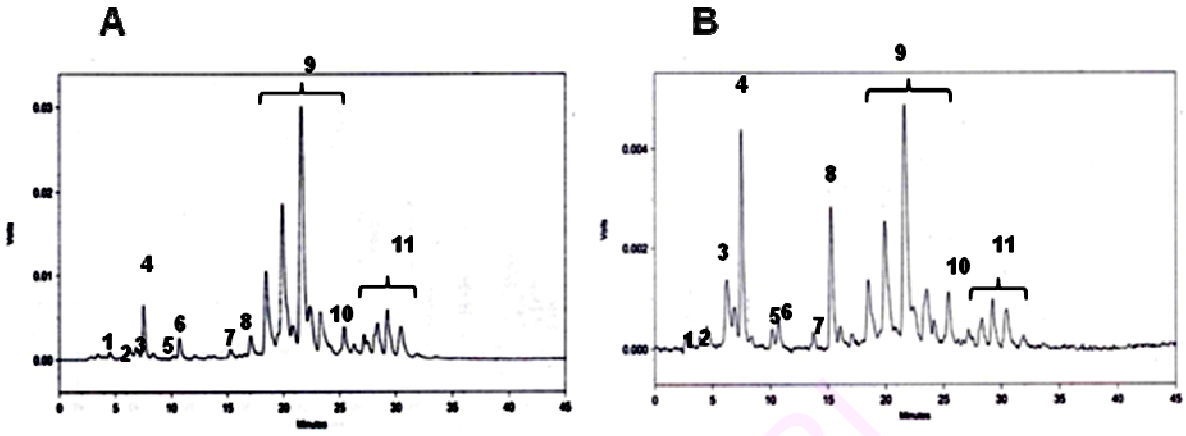


Figure 3.10 HPLC profile of the carotenoid extracts from NT (A) and BKT transformed *H. pluvialis* (B) Peaks were identified as (1) Neoxanthin, (2) Violaxanthin, (3) Free astaxanthin, (4) Lutein, (5) Canthaxanthin, (6) Chlorophyll b, (7) Chlorophyll b', (8) Echinenone, (9) Astaxanthin monoesters, (10) β-carotene and (11) Astaxanthin diesters.

In the preliminary study, it was revealed that exposure of *H. pluvialis* cells to different stress conditions increased over all carotenoid production by 3 to 5-fold in transformed cells over NT cells. The individual carotenoids like lutein, β-carotene, astaxanthin, echinenone, and canthaxanthin were analysed in the transformant and the NT cells which were cultured under different stress condition. There were significant increase in the individual carotenoid levels in the transformants under the stress condition studied. The changes in the individual carotenoids are given in the Figure 3.11.

3.7.3.2.1 Analysis of lutein content in the transformants

Higher lutein content was observed in the transformed cells under salinity stress - NaCl 0.25% (7.57 mg/g) and - NaCl 0.25%+ CO_2 2% (6.24 mg/g), while the non transformed (NT) cells produced only 1.18 and 0.9 mg/g of lutein respectively under the same stress condition (Fig 3.11). This is approximately 7 fold higher than the NT cells. The cells which are not exposed to stress (control) both for NT and transformed cells accumulated 0.74 and 4.59 mg/g of lutein. This shows that about 5 times higher lutein content is present in transformed cells. Under all other stress conditions studied the lutein content was found to be lesser than that in control cells.

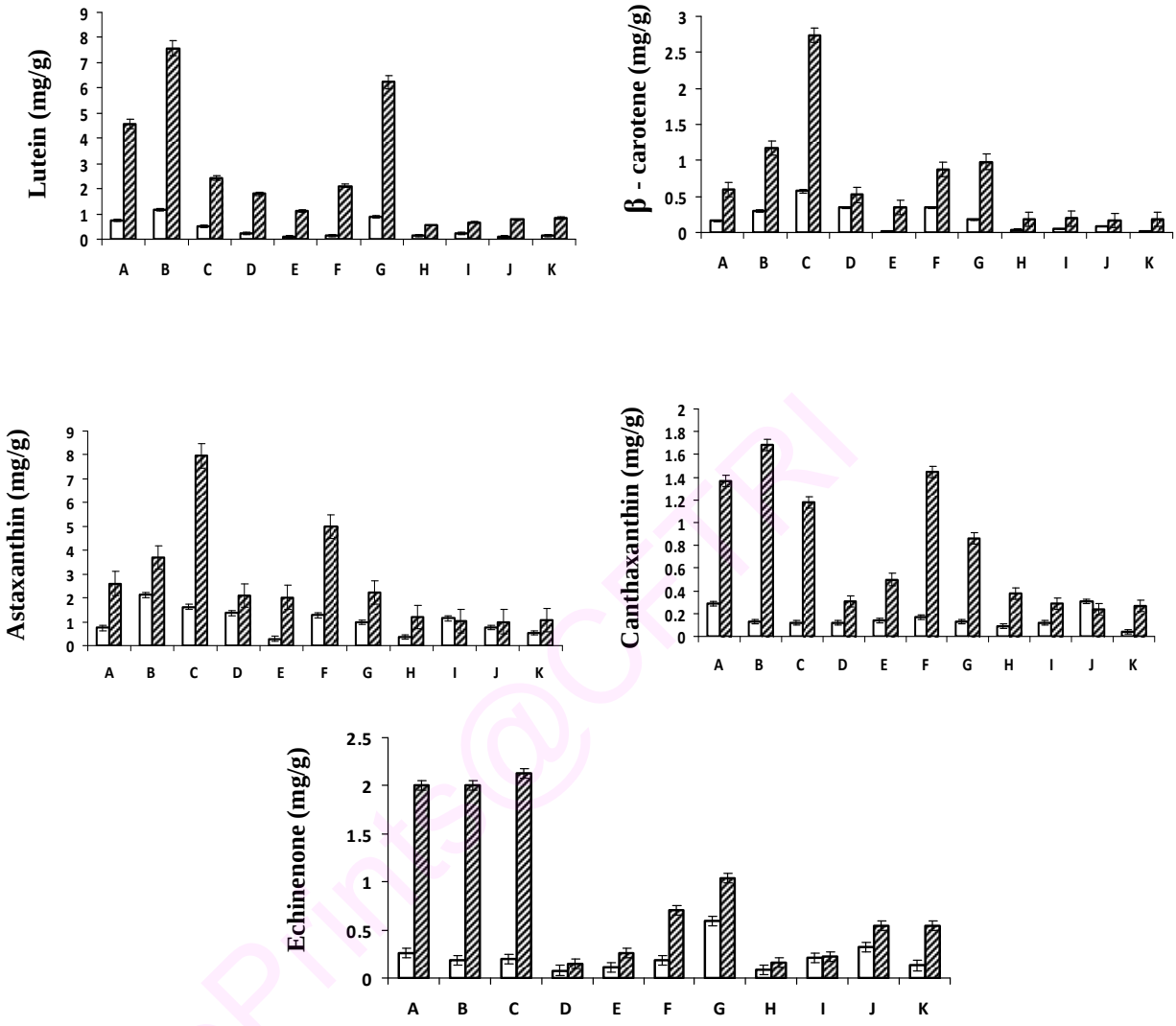


Figure 3.11 Effect of different stress in control and transformed cells for the production of carotenoids like lutein, β-carotene, astaxanthin, canthaxanthin, and echinenone. A – Control (untreated), B - NaCl 0.25%, C – Sodium acetate 4.4mM, D - CO_2 2%, E - Cycloheximide 0.3 mg/l, F - NaCl 0.25% + Sodium acetate 4.4mM, G - NaCl 0.25% + CO_2 2%, H - NaCl 0.25% + Cycloheximide 0.3 mg/l, I - Sodium acetate 4.4mM + Cycloheximide 0.3 mg/l, J - NaCl 0.25% + Sodium acetate 4.4mM + Cycloheximide 0.3 mg/l, K - NaCl 0.25% + CO_2 2% + Cycloheximide 0.3 mg/l. □ - Control cells ▨ - BKT-Transformed cells

3.7.3.2.2 Analysis of β-carotene content in the transformants

There was significant difference in the amount of β-carotene content in the transformed and NT cells under the individual treatment of NaCl 0.25% and sodium acetate 4.4mM (Fig 3.11). Under the NaCl 0.25% and sodium acetate 4.4mM

treatments the transformed cells produced 1.77 mg/g and 2.73 mg/g of β-carotene while NT cells accumulated 0.3 and 0.77 mg/g. The untreated NT and transformed cells gave 0.16 and 0.59 mg/g of β-carotene with 3.5 fold higher β-carotene in transformed cells than in the NT cells. Other stress conditions had no effect on β-carotene content and was lower than that in control cells.

3.7.3.2.3 Analysis of astaxanthin content in the transformants

Maximum astaxanthin content was observed in transformed culture treated with sodium acetate 4.4mM (7.96 mg/g) followed by sodium acetate 4.4mM + NaCl 0.25% treated cells (4.99 mg/g) (Fig 3.11). Under the same conditions the NT cells produced only 1.62 mg/g and 1.28 mg/g respectively. The NaCl 0.25% alone also resulted in increased astaxanthin content. The other stress treatments had no effect on astaxanthin content and the content in those treated cells was lesser than the control transformed cells.

3.7.3.2.4 Analysis of canthaxanthin content in the transformants

The control transformed cells (without stress) itself showed 1.37 mg/g of canthaxanthin production while the control NT cells showed 0.29 mg/g, with approximately 4 fold higher canthaxanthin in transformed cells. Among different stress condition, NaCl 0.25% showed maximum effect for the production of canthaxanthin. The canthaxanthin production in the stress induced transformed and NT cells, is 1.68, 1.18, 1.45 and 0.86 mg/g and 0.13. 0.12, 0.17 and 0.13 mg/g respectively in NaCl 0.25%, sodium acetate 4.4mM, NaCl 0.25% + sodium acetate 4.4mM and NaCl 0.25% + CO_2 2%. The remaining treatments have no effect on the canthaxanthin production when compared to the untreated control and transformed cells.

3.7.3.2.5 Analysis of echinenone content in the transformants

Echinenone is the intermediate in the biosynthetic pathway of astaxanthin. Under all the conditions the transformed cells showed increased levels of echinenone than the NT cells. The control transformed and NT cells produced 2.01 and 0.26 mg/g of echinenone production. But in NaCl 0.25% and the sodium acetate 4.4mM treated cultures, the level of echinenone production in transformants was 2.0 and 2.13 mg/g

while in NT cells it was 0.19 and 0.2 mg/g respectively. This increased echinenone production is approximately 10 fold in transformed cells when compared to NT cells. The other treatments showed lower echinenone content than the control cells.

Table 3.4 Effect of different inhibitors in the transformed and NT *H. pluvialis* for chlorophyll and carotenoid production

Sl.No	Total Chlorophyll (mg/g)		Total Carotenoids (mg/g)		β-carotene mg/g		Astaxanthin mg/g	
	NT	Trans formed	NT	Trans formed	NT	Trans formed	NT	Trans formed
Control	7.04	8.9	1.31	5.12	0.74	1.9	1.06	2.59
Cycloheximide	1.9	4.96	1.41	5.29	0.02	0.1	0.15	0.93
DCMU	4.09	4.2	4.22	4.25	0.26	0.08	1.18	1.3
DPA	2.65	10.83	6.99	13.91	0.27	0.47	1.67	3.27
Nicotine	3.5	2.83	6.48	3.38	0.17	0.04	0.88	0.96

3.7.4 Effect of inhibitors on NT cells and Transformed cells

3.7.4.1 Analysis of total chlorophyll and total carotenoids in inhibitor treated cells

The effect of carotenoid synthesis inhibitor like, diphenylamine (DPA) and nicotine, the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, (DCMU) and the translational inhibitor cycloheximide on pigment profile were studied in the NT and the transformed cells. The changes in the amount of chlorophyll and carotenoid contents were presented in the table 3.4 and in Figure 3.12.

The chlorophyll content in the untreated control and transformed culture is 7.04 and 8.9 mg/g. The chlorophyll content was significantly inhibited by nicotine (2.83 mg/g), DCMU (4.2 mg/g) and cycloheximide (4.9 mg/g). Chlorophyll content was not inhibited by DPA (10.83 mg/g) in transformants. There is a decrease in chlorophyll content in cycloheximide treated NT and transformed cells, however the extent of inhibition is less in transformed cells.

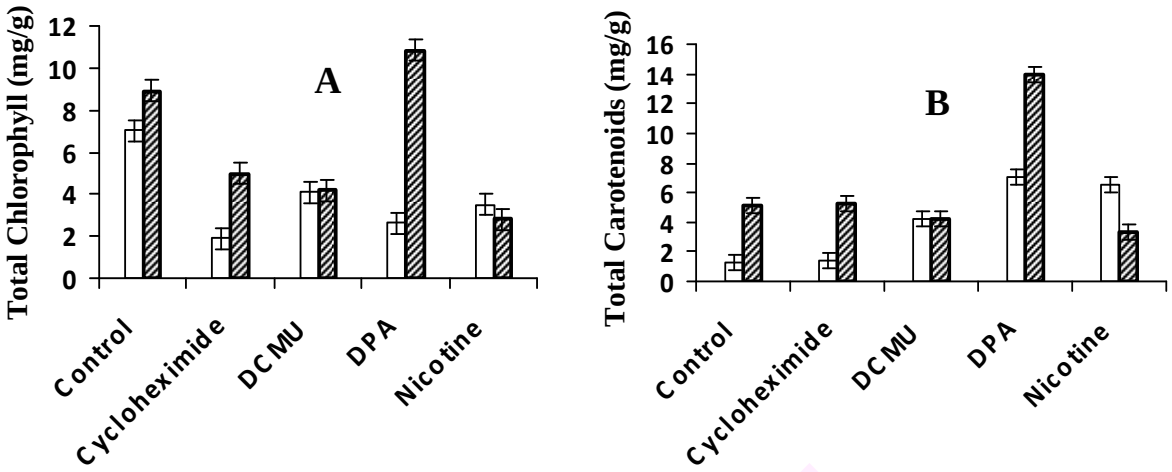


Figure 3.12 Effect of inhibitors on NT cells and Transformed cells
□ - NT cells ▨- BKT-Transformed cells

Nicotine inhibited carotenoid content in transformed cells (3.38 mg/g) significantly compared to other inhibitors in transformed cells. Overall the total carotenoid content is not effected by DCMU, cycloheximide and DPA. There is an increase in total carotenoid content in DPA treated transformed (13.91 mg/g) and NT cells (6.99 mg/g).

3.7.4.2 Analysis of β-carotene and astaxanthin in inhibitor treated cells

β-carotene production was inhibited by all the four inhibitors studied. the untreated cells showed 0.74 and 1.9 mg/g in NT and transformed cells (Fig. 3.13).

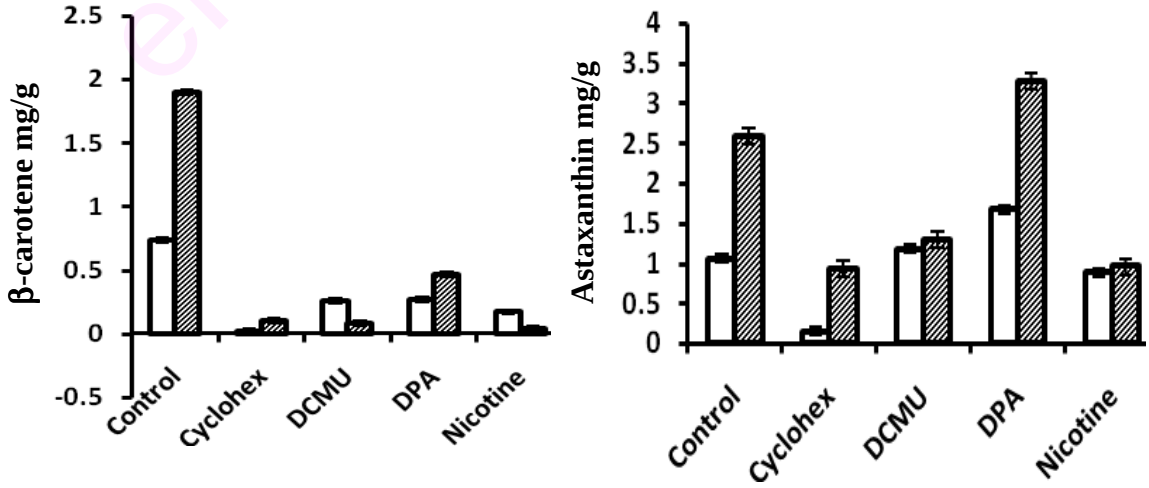


Figure 3.13 Effect of inhibitors on NT cells and Transformed cells for and β-carotene and astaxanthin. □- NT cells ▨- BKT-Transformed cells

β-carotene content was considerably inhibited by nicotine (0.04 mg/g) in transformed cells and cycloheximide (0.02 mg/g) in NT cells. Astaxanthin content decreased significantly under cycloheximide treatment both transformed and non transformed cells while nicotine and DCMU inhibited astaxanthin only in transformed cells only (Fig 3.13). Overall, cycloheximide exhibited higher inhibitory effect on pigments than other inhibitors. There was no inhibitory effect of DPA on astaxanthin production whereas the production of β-carotene was inhibited.

3.7.5 Expression analysis of carotenoid biosynthetic genes

The BKT transformants and NT cells were selected for carotenogenic genes expression studies under stress condition viz, NaCl, sodium acetate and combination of NaCl + sodium acetate. The expression levels of genes associated with general carotenogenesis and specific astaxanthin biosynthesis in transformants and NT cells were quantified by reverse transcription polymerase chain reaction (RT-PCR) and compared. These genes included phytoene synthase (PSY), the first committed step in the carotenoid pathway followed by phytoene desaturase (PDS), which converts phytoene to lycopene, lycopene cyclase (LCY), converts lycopene to β-carotene, BKT (specific to astaxanthin biosynthesis, which converts β-carotene to echinenone and to canthaxanthin), and BKH (which convert canthaxanthin to astaxanthin).

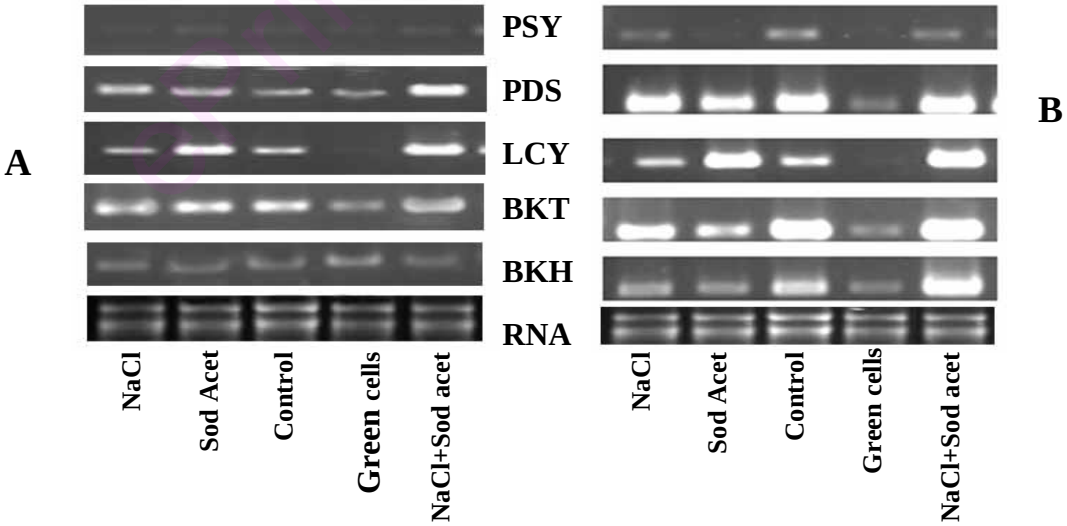


Figure 3.14 Expression of carotenoid biosynthetic genes in *H. pluvialis* under stress conditions. A – NT cells, B – Transformed cellsStress conditions used were nutrient deficiency and high light (control), combined with NaCl, sod acetate, and NaCl/ sod acetate.

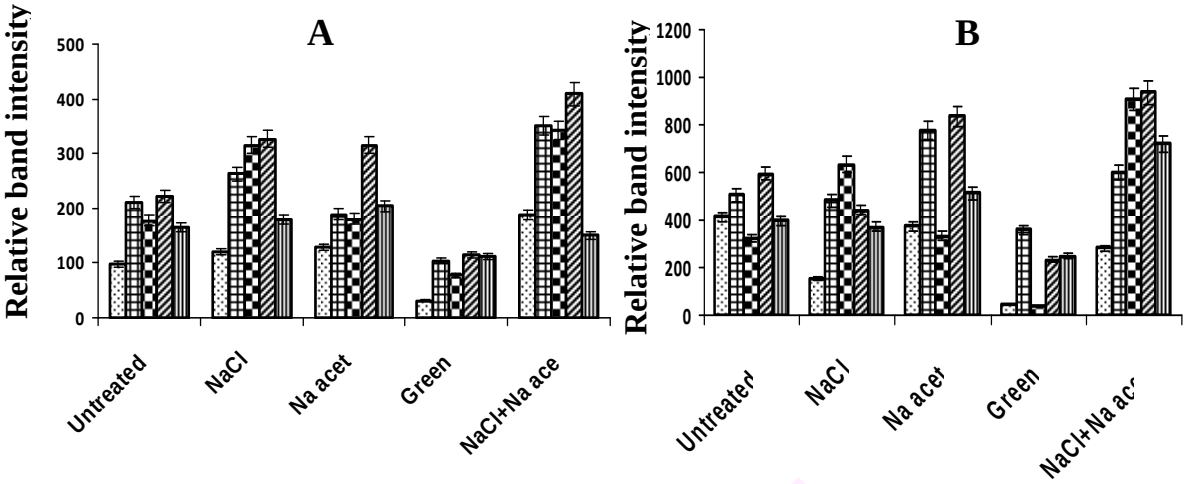


Figure 3.15 Expression of carotenoid biosynthetic genes in *H. pluvialis* under stress conditions. A – NT cells, B – Transformed cells. The graph prepared based on the relative band intensity of the each gene's transcripts levels. ▨ – PSY, ▦ - PDS, ▩ - LCY, ▨ - BKT, ▥ - BKH. Data shown are mean ±SD of three independent experiments expressed in PSY, PDS, LCY, BKT, and CHY genes of NaCl, sod acetate, control, Green cells and NaCl/ sod acetate added to *H. pluvialis*.

The transcript levels of these enzymes were analysed after 48hrs of stress induction in both transformed and NT cells. The transcript levels in stress induced culture was compared with the control cells and also with the green cells cultured under normal conditions. The transcript levels of all the genes were found to be higher in transformed cells than in control cells (Fig 3.14). The transformants differed from control green cells in the extent of increase in transcript levels. When compared to the green cells of transformants, the NaCl treated culture showed 3.4, 1.3, 15.8, 1.9 and 1.7 fold increase in levels of PSY, PDS, LCY, BKT and BKH respectively. The sodium acetate treated culture exhibited 12 fold increase in PSY, 7 fold increase in PDS and BKT, and 4 fold increase in LCY and BKH, when compare to the control green cells. The NaCl+sodium acetate showed higher level of expression for all the enzymes studied. It was that observed the expression levels of PSY, PDS, LCY, BKT and BKH were 8.8, 5.8, 11.7, 8.1 and 6.4 fold higher respectively when compared to the control green cells. But when compared to the transformed green cells it is 6.1, 1.7, 22.7, 4.0 and 2.9 fold higher respectively.

Between the transformants and non transformants higher the expression levels of PSY, PDS, LCY, BKT and BKH by 1.5, 1.7, 2.7, 2.3 and 4.8 fold was obtained in

NaCl + sodium acetate treated cells of transformants. Individually, the NaCl and sodium acetate treated cells showed the elevated transcript levels of PSY, PDS, LCY, BKT and BKH by 1.3, 1.8, 2.0 1.3 and 2.1 and 2.9, 4.1, 1.8, 2.6 and 2.5 fold higher respectively in transformants. Overall, the transcript levels of transformants were more in NT cells treated under the high light and salt stress conditions.

3.8 Discussion

The results presented in this chapter confirms the transformation of BKT gene from *H. pluvialis* to the same homologous host. This is the first report to transform the BKT gene to *H. pluvialis* through *Agrobacterium* mediated gene transfer to over express / regulate the carotenoid levels. So far, the gene transformation in *H. pluvialis* was restricted to the selectable marker and reporter genes (Teng et al 2002) and phytoene desaturase (PDS) (Steinbrenner and Sandmann 2006).

The successful *Agrobacterium* mediated gene transformation, reported in *H. pluvialis* for selectable marker and reporter genes has been published (Kathiresan et al. 2009). The transformation of BKT through *Agrobacterium* mediation was followed up using the same transformation procedure. Having the knowledge of the different cocultivation media studied for *H. pluvialis* and *Agrobacterium* (Kathiresan and Sarada 2009), the co-cultivation was directly performed in the TAP medium and the selection was made on the plates having hygromycin at 10 mgL^{-1}. The same medium was also used for the cocultivation of *Chlamydomonas reinhardtii* and *Agrobacterium* for the transformation of selectable marker and reporter genes (Kumar et al. 2004).

Since the transformation was performed by *Agrobacterium*, it took more than 8 weeks to get a clear single resistance colonies on the plates. This confirms the integration of T-DNA region of pCAMBIA1304-BKT (which is having the hygromycin phosphotransferase gene) into the nuclear genome of *H. pluvialis*. The genetic transformation of BKT gene in the homologous host was also further confirmed by GFP and GUS assay. Scanning electron microscopy also showed the changes in the cell wall pattern of the cocultivated cells as given in section 1.9 of chapter 1 (Kathiresan et al. 2009). PCR analysis for different primer combination gave the exact amplification size of the BKT gene and also the BKT amplification with CaMV 35S and poly A region. Southern blotting analysis of the transformed DNA showed that

there was a positvie hybridization of the CaMV 35S and BKT-C probe as like the positive control pCAMBIA1304-BKT DNA when digested with the enzyme *HindIII*. These hybridized bands were not observed for the NT cells. But there was some extra bands observed in the upper end of the membrane in the NT and also for transformed DNA digested with the *HindIII*. These extra bands might be due to the presence of endogenous BKT gene in the host. The transcript expression levels of the BKT was studied by RT-PCR. The expression level of the BKT in transformants was 3 to 4 fold higher than NT cells. Eventhough the cloning of BKT starts from the 3rd ATG (264th nucleotide) of D45881 the gene has been expressing in the homologous host. This result was reported by Fraser et al. 1997; Kajiwara et al. 1995, in the cDNA of the D 45881.

The chlorophyll and other carotenoids were analysed in the transformants grown under different stress condition. Among the substrates studied sodium acetate (4.4 mM) showed 4 fold increase in astaxanthin content, and 8 – 10 fold increase in intermediates like echinenone and canthaxanthin. The enhanced accumulation of these carotenoids might be due to the increase in copy number of the BKT in *H. pluvialis* as a result of integrated cloned BKT gene. This is in accordance with the results of cloned PDS gene in *H. pluvialis* (Steinbrenner and Sandmann 2006). Enhanced production of the total and other carotenoids in *H. pluvialis* was also reported by Vidhyavathi et al. (2008) under the addition of sodium acetate.

Among the inhibitors studied the caorotenoid accumulation was highly inhibited by nicotine followed by the cycloheximide in both transformed and nontransformed cells. The reduction in β-carotene and lutein contents (low among all inhibitors) by nicotine suggests lycopene β-cyclase (which is a key enzyme in conversion of lycopene to β-carotene and α-carotene) is affected by nicotine. Similar kind of accumulation of lycopene by nicotine was observed by Harker and Young (1995), Vidhyavathi et al. (2008) and Fazeli et al. (2009). The extent of inhibition in transformants was always found to be lower compared to parent (NT cells). This may be possibly due to higher transcript levels of all the major carotenoid genes observed in the transformants under stress and non stress conditions.

The levels of both canthaxanthin and echinenone in transformants were higher than the NT cells. Similar types of results were observed in homologous expression of PDS in *H. pluvialis* (Steinbrenner and Sandmann 2006) and heterologous expression of PDS in tobacco (Misawa et al 1994) where the levels of lutein and zeaxanthin were higher in transformants.

Summary & Conclusions

Astaxanthin (3,3'-dihydroxy - β,β'carotene - 4,4'- dione) is a keto carotenoid produced in limited number of organisms like *Agrobacterium auranticum, Phaffia rhodozyma, Adonis aestivalis* and *Viola tricolor* (plants) and *Haematococcus pluvialis* green alga and other organisms. The green alga *Haematococcus pluvialis* is well known for its ability to accumulate high amounts of astaxanthin (2-3% of dry weight) and it is one of the most promising source for production of natural astaxanthin. Astaxanthin has potential clinical application in human health, because of its higher antioxidant capacity compared to β-carotene and vitamin-E (α-tocopherol). The genes responsible for the enzymes involved in astaxanthin biosynthesis in *H. pluvialis* have been well documented. β-carotene ketolase (BKT), and β-carotene hydroxylase (BKH) are involved in the biosynthesis of β-carotene to astaxanthin in *H. pluvialis*. Several approaches were followed for increasing the productivity of the high value compounds like carotenoid in bacteria, fungi and plants through manipulation of cultural condition, mutation, metabolic engineering and recombinant technology. In contrast to the large number of genetically modified bacteria, yeast and even higher plants, only few species of microalgae have been successfully transformed with efficiency. Efficient genetic transformation system in microalgae is therefore necessary to enhance their potential utility. The recent developments in algal transformations suggest the possibility of using *Agrobacterium tumefaciens* for incorporating desired traits in microalgae. Therefore attempts were made to transform *H. pluvialis* with *Agrobacterium tumefaciens* for expression of marker and reporter genes. Subsequent studies were made to transform the genes responsible for the enzymes in astaxanthin biosynthesis. Hence the present study is undertaken to develope the *Agrobacterium* mediated transformation protocol in *H. pluvialis* and further cloning of β-carotene ketolase and hydroxylase genes for regulation of carotenoid production with the following objectives.

Objectives

1. Genetic transformation of *Haematococcus pluvialis* using selectable marker genes
2. Cloning of genes responsible for enzymes (β-carotene ketolase, and β-carotene hydroxylase) involved in carotenoid biosynthesis and their expression in *Haematococcus pluvialis*.

4.0 Summary of results

4.1 Genetic transformation of *Haematococcus pluvialis* using selectable marker genes

For the genetic transformation studies in *H. pluvialis*, different cocultivation medium were tested and TAP medium showed good growth for both alga and bacterium. Among the different antibiotics screened for the sensitivity of *H. pluvialis* hygromycin more than 2 mgL^{-1} showed lethal effect to the alga. Cefotaxime and augmentin showed no effect up to the concentration of 2000 mgL^{-1}. The first genetic transformation in *H. pluvialis* was achieved through *Agrobacterium* mediation using the selection and marker genes *hpt*, GFP and GUS from the binary vector pSK53. The transformation achieved here does not require any phenolics like actosyringone. GUS and GFP assay for the transformation showed positive reaction in transformants while the control cells did not show the positive reaction. Scanning electron microscopic study showed close association between *H.pluvialis* and *Agrobacterium*, difference in the cell wall pattern like rough surface and pores on the surface of cocultivated cells whereas smooth cell surface was observed in control cells. PCR analysis of *hpt*, and GUS primer gave 407bp and 515bp amplification for the transformed cell and the plasmids. But no amplification was observed for the control cells. Southern blotting analysis exhibited, difference in banding pattern of the restriction enzyme digested plasmid and the transformed DNA. While no reaction was observed for the control cells. The carotenoid profile of the transformed *H. pluvialis* showed no difference with the control cells.

4.2 Cloning of genes responsible for enzymes (β-carotene ketolase and β-carotene hydroxylase) and transformation to *H. pluvialis*

The amplification of β-carotene ketolase (BKT) was observed for different primers synthesized. No amplification was observed for the any of the BKH primers

synthesized. The 1.8 kb amplicon of BKT gene was cloned to a cloning vector pRT100 in between the CaMV 35S promoter and the poly A region. Restriction analysis and the PCR study showed the confirmation of the BKT gene in the cloned pRT100. Sequence analysis of cloned BKT and also amino acid analysis showed 99% similarity of the reported BKT gene accession number D45881 which the primer has been designed. Six exons and five introns were observed for the BKT gene cloned from the *H. pluvialis.* Even though there were few nucleotide polymorphism, there is no shift in the reading frame. The cloned fragment of BKT from the pRT100 was further cloned into the binary vector p1304 which is having the selection and marker genes *hpt,* GUS *and* GFP. Further the cloned binary vector was mobilized to the *E. coli* and *Agrobacterium.* Standardized protocol for *Agrobacterium* mediated genetic transformation was used to transform the cloned binary vector having BKT (pCAMBIA1304-BKT) gene to the *H. pluvialis.* Confirmation of cloned BKT to the *H. pluvialis* was studied by analyzing the GUS, GFP expression. PCR analysis for the CaMV 35S primers showed 2.5 kb of the BKT gene with the promoter and poly A region. For the BKT forward primer and the CaMV 35S reverse primer also the exact size of amplicon was observed. Southern blotting also showed the difference in the banding pattern of the enzyme digested plasmid and the transformed *H. pluvialis*. No bands were observed for the control cells.

Transcript level analysis of the BKT showed 3 to 4 fold higher expression of BKT in transformants than the control cells

4.3 Analysis of carotenoid profile in transformants

The transformed cells were subjected to different stress condition for the induction of secondary carotenoids. The total carotenoid was higher (2 fold) in NaCl 0.25% + sodium acetate (4.4 mM) treated cells than the control cells. Lutein content was higher in transformed cells under NaCl 0.25% (7.57 mg/g), and 3.5 fold increase β-carotene was observed in NaCl treated transformed cells when compared to the control cells. The intermediates like canthaxanthin and echinenone are the major carotenoids which are present more in the transformants than in the control *H. pluvialis.* Echinenone and canthaxanthin content were approximately 8 to 10 fold higher in sodium acetate 4.4mM and NaCl 0.25% treated cultures. The effect of different

inhibitors on the transformants indicated that, nicotine and cycloheximide inhibits the total chlorophyll and carotenoids significantly followed by DCMU. While no inhibition of chlorophyll, total carotenoids and astaxanthin content in the DPA treated transformed cells. The transcripts level of carotenoid biosynthetic enzymes were studied under high light with different salt stress condition. NaCl + sodium acetate treated culture in transformed cells showed higher level of expression for all the enzymes studied. It was also observed that the expression levels of PSY, PDS, LCY, BKT and BKH were 8.8, 5.8, 11.7, 8.1 and 6.4 fold higher respectively when compared to the control green cells. But when compared to the transformed green cells it is 6.1, 1.7, 22.7, 4.0 and 2.9 fold higher respectively.

4.4 Conclusions

Genetic engineering and transformation studies is one of the new technique in molecular biology to improve/regulate the carotenoid production in commercially important microalgae. In contrast to the large number of genetically modified bacteria, yeast, and even higher plants, only a few species of microalgae have been successfully transformed. However, protocols for the genetic transformation (*Agrobacterium*-mediated, electroporation, biolistic gun, etc.) are being developed and improved for a few green algal species such as *C. reinhardtii*, *V. carteri*, and *Chlorella* sp. However, the full potential of genetic transformation has not been realized for most algal species. The *Agrobacterium* mediated genetic transformation method described here is highly efficient in developing transgenic *H. pluvialis*, a commercially important microalga. This robust transformation method would also pave the way for manipulation of many important pathways relevant to food, pharmaceutical and nutraceutical industries. Further this result will be very much useful for the *Agrobacterium* mediated transformation studies in other green microalgae like *Dunaliella* sp, *Botryococcus* sp, *Chlorella* sp etc which are having high commercial and economic value.

. Metabolic engineering is generally carried out to improve the production of existing compounds, to mediate the degradation of compounds, or to produce new compounds by redirecting one or more enzymatic reaction. Approaches for achieving genetic/metabolic engineering include over expression of a single gene. Multiple gene

combinations or a transcription factor to establish single gene or multigenes control in the biosynthesis pathway for carotenoids, or use of RNAi/antisense knockout of a pathway in order to increase the content or change the composition of carotenoids. There is growing interest worldwide in manipulating carotenoid biosynthesis in carotenoid producing organisms. Cloning of most of the astaxanthin biosynthesis genes in *H. pluvialis* has now opened the door to genetically manipulating this pathway not only in algae, but also in other organisms. The cloning of the gene BKT which convert β-carotene to astaxanthin, biosynthesis in *H. pluvialis* was successfully carried out and it has been transformed to same homologous host through the standardized *Agrobacterium* mediated genetic transformation method. This cloned BKT will be further useful for transformation and the expression studies in the heterologous host like *Duanliella* sp, *Dacus* sp, *Lycopersicum* sp which are producing β-carotene to regulate the carotenoid biosynthesis.

Appendix

Acetosyringone

Acetosyringone stock of 10 mM was prepared by adding 98.1 mg in 50 ml of sterile dH_2O, filter sterilized and used. It takes two to three hours for completely dissolving the acetisyringone.

LB (Luria-Bertaini) medium

Tryptone	-	10 .0 g
Yeast extract	-	5.0 g
NaCl	-	5.0 g

Dissolved double distilled water and adjusted the volume to 1 liter. Adjusted the pH to 7.2 with 2 N NaOH and autoclaved at 121^0C for 20 min at 15 lbs pressure.

SOB (per liter)

Bacto-tryptone	20.0 g
Bacto-Yeast extract	5.0 g
Sodium chloride	0.6 g
Potassium chloride	0.19 g
Magnesium sulphate	10.0 mM (added from 1.0 M stock)
Magnesium chloride	10.0 mM (added from 1.0 M stock)

Autoclaved the first four components and the magnesium salt separately and then mixed to constitute the SOB medium

LB plates/slants

Added 15 gL^{-1} of agar to liquid LB medium and autoclaved. When the medium cools to 50 – 60 added appropriate antibiotics, mixed well and pour 20-25 ml of medium into 90mm sterile petri dishes/10 ml for slant in sterile test tubes. Allowed the media to harden in a laminar-flow hood. Stored at room temperature for 10 days or 40C for up to 2 months

Cellulase 0.1% and Pectinase 0.2%

The cellulase and pectinase powder (Sigma Aldrich) were weighed at 100 mg and 200 mg and dissolved in 1 ml of 0.02 M phosphate buffer. The enzyme solution were prepared every time freshly.

50X TAE (Tris-Acitic acid-EDTA)

Dissolved 242 g Tris-base in 800 ml of sterile distilled water. Add 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0). Adjusted the volume to 1 liter and store at room temperature. For preparation of 1X TAE for agarose gel preparation and running the agarose gel electrophoresis dissolve 1 ml of 50X TAE/49 ml of distilled water.

1 M Tris (pH 7.5 or 8.0)

Dissolved 121.1 g Tris-HCl in 800 ml of distilled water and adjusted the pH with 2 N HCl to desired pH. Adjusted the final volume to 1 liter, autoclaved and stored it at room temperature.

0.5 M EDTA (pH 8.0)

Dissolved 93.06 g Na_2 EDTA in 300ml distilled water and adjust the pH to 8.0 with 2 N NaOH solution. Adjusted the final volume to 500 ml and stored at room temperature.

Loading dye (6 x)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol

The above components were dissolved in Milli-Q water, dispensed in aliquots and stored at 4^0C.

Phosphate buffer

A. NaH_2PO_4 (0.2 M)

B. Na_2HPO_4 (0.2M)

Added 56.3 ml of A and 43.4 ml of B and make upto 200 ml. Adjust the pH to 6.7.

TE buffer

10mM Tris, pH 8.0

1 mM EDTA, pH 8.0

0.1 M IPTG stock solution

Dissolved 0.12 g of IPTG in 5.0 ml of deionized water. Filter-sterilized the solution and stored as aliquots at -20°C.

X-Gal stock solution

Dissolved 100 mg of X-Gal in 2.0 ml of N, N'-dimethylformamide (DMF). Stored the solution in microcentrifuge tube, wrapped in aluminium foil at -20°C.

Ethidium Bromide (Stock)

10 mg/ml in distilled water. Used at a working concentration of 0.5ug/ml. Store at 4 °C.

0.1 M $CaCl_2$ stock solution

Dissolved 1.47 g of $CaCl_2$ in 100 ml of deionized water. Sterilize the solution by filtration and store as 20 ml aliquots at -20°C.

Ampicillin/Kanamycin stock solution

Dissolved 100 mg ampicillin in 1.0 ml of deionized water. Sterilized by filtration. Stored at -20°C and used at a working concentration of 100 μg ml^{-1}

Plasmid Isolation solution - Alkaline lysis for plasmid DNA isolation (Sambrook et al., 1989)

1. Two ml of the overnight LB *E.coli* culture was transferred to an eppendorff tube and harvested the cells by centrifuging the cells at 12,000 rpm for 30 sec.
2. Tubes were kept on ice after discarding the supernatant.
3. Solution I (100 μl) was added to it and vortexed vigorously.
4. Solution II (200 μl) was added and inverted gently several times.
5. Solution III (2000 μl) was added icecold and vortexed gently.
6. The tubes were placed in ice for 5 min.
7. The tubes centrifuged at 12,000 rpm for 15 min at 4 C to precipitate the proteins.
8. Supernatant was transferred to a fresh tube.
9. Equal volume of phenol:chloroform mixture was added to it and thoroughly vortexed.
10. Two phases were separated by centrifuging at 12,000 rpm at 4 C for 10 min.
11. The upper aqueous phase was transferred to a fresh tube and equal volume of chloroform was added and again centrifuged at 12,000 rpm at 4 C for 10 min to remove the phenol traces.
11. The upper aqueous phase was transferred to a fresh tube and was filled with distilled ethanol.

12. The tube was kept at -20 C overnight and DNA precipitated at 4 C for 20 min at 12,000 rpm.
13. Supernatant discarded and the pellet was washed with 70% ethanol.
14. The pellet was completely air dried and re dissolved in 20 µl of TE buffer.

Solution I

50 mM glucose

25 mM Tris.Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution II

0.2 N NaOH (freshly diluted from a stock of 10 N)

1% SDS (freshly prepared from the stock of 10%)

Solution III

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Preparation of competant cell preparation

1. A loopful of *E.coli* DH5α cells taken from a LB-agar slant was inoculated into 2 ml of LB broth and was grown overnight at 37^{o}C in a shaker incubator set at 150 rpm.
2. The overnight grown culture was inoculated into 50 ml of SOB medium taken in a 250 ml conical flask.
3. The flask was incubated at 37^{o}C and 180 rpm in a shaker incubator till the O.D $_{600\ nm}$ of the cells reached 0.45-0.55.
4. The cells were kept on ice for 10-15 min.
5. The cells were pelleted by centrifugation at 2500 rpm for 10 min at 4^{o}C. The supernatant was discarded.
6. The cells were resuspended in 17 ml of TFB and left on ice for 10-15 min.
7. The cells were pelleted by centrifugation at 2500 rpm for 10 min at 4^{o}C. The supernatant was discarded.

8. The cells were resuspended gently in 4 ml of TFB and left on ice.
9. 140 µl of DMSO was added to the cells. The contents were mixed gently and the cells were left on ice for 5 min.
10. 315 µl of 1 M DTT was added to the cells. The contents were mixed gently and the cells were left on ice for 10 min.
11. 150 µl of DMSO was added to the cells. The contents were mixed gently and the cells were left on ice for 5 min.

Transformation of competent cells

1. DNA was added to competent cells as follows:

Sample	DNA (µl)	Competent cells (µl)
Control	-	200
Transformation control	2	200
Positive control	2	200
Ligated sample	2	200

2. Cells were incubated on ice for 30 min.
3. The cells were subjected to heat shock at 42^{o}C for 2 min followed by cooling on ice for 2 min.
4. 400 µl of SOC was added to the cells and the cells were grown at 37^{o}C for 45 min in a shaker incubator set at 150 rpm.

Selection of transformants

To select the transformants 100 µl of the transformation mixture was plated onto LB-agar plates containing 100 µg ml $^{-1}$ ampicillin / kanamycin, 0.5 mM IPTG and 80 µg ml $^{-1}$ X-Gal. The plates were incubated overnight at 37^{o}C for the colonies to grow. The white colonies were the transformed and the blue colonies were the control i.e positive control.

Restrciton Digestion

Materials required:

1. Restriction enzyme : Any
2. 10 X restriction enzyme buffer
3. BSA, acetylated, 1mg ml^{-1}

4. Nuclease-free deionized water.

The following were added in a micro centrifuge tube in the order stated:

Nuclease-free water	13.8 µl
Restriction enzyme 10 x buffer	2.0 µl
BSA, acetylated (1mg ml-1)	0.2 µl
DNA (Plasmid/genomic)	3.0 µl
Enzyme	1.0 µl
Final volume	20.0 µl

The reaction were kept at 37^0C for 4 hrs to 12 hrs. After the complete digestion the reaction was stopped by heating the mixture at 60 - 80^0C for 20 min.

Southern blotting protocol

Materials required

1. Probe DNA (Biotin-labeled amplicon)
2. Target DNA
3. positive control
4. Cesium chloride purifiied pBR322 plasmid DNA (negative control)
5. Nylon membrane (Ambion)
6. 20XSSC, 3M NaCl , 0.3M sodium citrate pH 7.2.
7. Blocking stock solution (10 X concentration): Blocking reagent - 10% (w/v) was dissolved in maleic acid buffer by constantly stirring on a heating block (65^0C). The solution was autoclaved and stored at 4^0C.
8. Blocking solution: The 1 X working solution was prepared by diluting the stock solution 1:10 in maleic acid buffer.
9. Hybridization buffer: 5X SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v)
10. SDS 1%, (1/10 volume of 10X blocking solution)
11. Post-hybridization washing buffer I : 2XSSC, 0.1% SDS
12. Post-hybridization washing buffer II : 0.1% SSC, 0.1% SDS
13. Alkaline phosphatase
14. Detection buffer : 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5

15. Color-substrate solution: Prepared freshly by adding 45 µl NBT-solution and 35 µl X-phosphate solution in 10 ml of detection buffer.

Nucleic acid labeling

It involve 2 procedure

I PCR purification

II Nucleicacid labeling

I - PCR purification

1. Did the PCR for the interested Plasmid (p 1301 / p SK 53) DNA (before gone for purification ran the gel and confirmed the amplicon is there or not)
2. Pooled all the PCR products into the separate Eppendorff tube
3. Added 5 vol. of PB buffer to 1 vol. of the PCR reaction mix (ex: 250 µl of buffer 50µl PCR reaction mix)
4. Place a mini elute column in a provided 2 ml collection tube
5. To bind DNA applied the sample to the minielute column and centrifuged for 1 min (transfered all the DNA sample by repeating the centrifugation)
6. Discarded the flow through liquid and transfered the remaining DNA to the column until the completion of DNA samples
7. To wash added 750µl of PE buffer to minielute column and centrifuged for 1 min
8. Discarded the flow through liquid and placed the minielute column back in the same tube. Centrifuged the column for additional 1 min at maximum speed
9. Placed a minielute column in a clean 1.5 ml microcentrifuge tube
10. To elute DNA, 10 µl of TE buffer 10mM (Tris HCl pH 8.5) or Sterile distilled water was added to the centre of the membrane. Allowed the column to stand for 1 min and then centrifuged for 1 min

Nucleic acid labeling

1. Placed a clean untreated 96 well Elisa plate on a ice bath
2. Denatured the eluted DNA sample by heat it to 100^0 C for 10 min and snap cool in ice
3. Added 1µl of Psorlen-Biotin to 10µl of nucleic acid solution in micro centrifuge tube. Mixed it and transfered the sample to one of the well in 96

plates. (Nucleic acid solution should have the final conc. of 0.5-50ng/µ/. The pH of the nucleic acid should 2.5 – 10)

4. Placed a 365nm UV light source on the plate directly for 45 min
5. Diluted the samples to 100µl by adding 89µl of TE buffer and the transfered the mixture to clean tube.
6. Added 200µl of water saturated n-butanol, shaked and vortexed it well and centrifuge it for 1 min at 7000g. Pipetted off the top n-butanol layer and repeated it.
7. Traces of n-butanol may be removed by extracting with two vol of water-saturated diethyl ether. Stored the biotin labeled nucleic acid at -20^0/-80^0 C.

Southern blotting Procedure

1. Ran the PCR for the hpt primers with template DNA
2. Loaded the PCR product to the gel (make 2 sets of gel- 1 for documentation and 1 for blotting)
3. After confirmed with the gel documentation loaded the DNA 20-25 µl /well with bromophenol blue (Run it for ¾ th of the gel)
4. Removed the gel and soaked it in 1.5M NaCl and 0.5 N NaOH
5. Then soaked it in 0.2 N HCl for 10 min (rinse the gel in water for few min)
6. Neutralized with neutralizing buffer for 30 min (1 M Tris & 1.5M NaCl)
7. Prepared the southern blotting tray filter paper and membrane ready
8. Placed the gel on the filter paper (3 no), which is on the wick. (The wick should be placed in the tray and fill it with 10x SSC buffer before placing the gel)
9. Placed the membrane (nylon) over the gel (membrane and filter paper should wet/rinsed with 10X SSC buffer before placing)
10. Again placed 3 filter paper over on it
11. Placed the stack of dry filter paper one above the above
12. Ran it for 24-36 hrs
13. Removed the gel and membrane separately and rinsed the membrane in transfer buffer

14. Immobilized the nucleic acid on the membrane by placing below the UV (2min in 365nm) to cross link the DNA
15. Placed the membrane in pre warmed hybridizing buffer (68^0 C) in the hybridizing bottle
16. Incubated the bottle (overnight) at 60-68^0 C in hybridizing buffer
17. After incubation discarded the buffer and take out the membrane (take care that the membrane should not dry)
18. Added fresh Hyb. buffer with diluted denatured probe quickly (probe should be diluted @ 1:10 with 10mM EDTA, heat denatured and snap cool and then incubate it at 90^0 C for 10 min) into the bottle.
19. Incubate the membrane at 63^0 C for 6 hrs with mild agitation (After incubation follow the post hybridization washing)
20. Washed the Memb. twice in 50 ml of post hyb buffer-I for 10 min
21. Again washed twice with 50 ml of post hyb buffer-II for 15 min at 68^0 C
22. Washed two times with Ambion wash buffer (1X) briefly
23. Then incubated the membrane in blocking buffer for 20 min. (use approx 3-5 l of blocking buffer
24. Take out the membrane and added fresh blocking buffer which having Strep-alkaline phosphatase @ 0.5µl /5 ml and incubated for 10 min
25. Washed the membrane three times (each wash 5 times) in 1X wash buffer
26. Incubated it in 1X detection buffer for 2 min
27. Then kept the membrane in freshly prepared detection buffer with substrate (1:10 BCIP/NBT substrate) for the colour development.

Hybridization

1. A 5 µl aliquot of biotin labelled probe was heat-denatured by incubating in boiling water for 10 min followed by snap cooling on ice.
2. Hybridization buffer was discarded from the bag.
3. 2 µl of denatured probe was added to 2 ml of prewarmed (68^0C) hybridization buffer.
4. Probe was added to membrane which was put in a polythene bag and sealed.

5. Membrane was incubated at 68^0C for 6 h with mild agitation in a hybridization oven.

Prehybridization

1. Membrane was air dried to allow absorption of the samples by the membrane.
2. Membrane was kept on a UV transilluminator for 2 min to allow crosslinking of single stranded DNA to the membrane.
3. Membrane was put in a polythene bag to which 15 ml prewarmed (68^0C) hybridization buffer was added.
4. The bag was sealed and incubated overnight at 68^0C in a hybridization oven

Post-hybridization washes

1. Membrane was washed twice in 50 ml of post hybridization washing buffer I for 5 min at room temperature.
2. Membrane was washed twice in 50 ml of post hybridization washing buffer II for 15 min at 68^0C under mild agitation.

Bibliography

Albrecht M, Misawa N, Sandmann G (1999) Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for the production of the carotenoids β-carotene and zeaxanthin. Biotechnol Lett 21:791–795.

Almgren K (1966) Ecology and distribution in Sweden of algae belonging to *Haematococcaceae*. I. Notes on nomenclature and history. Svensk Bot. Tidskr. 60(1): 49-73

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic Local Alignment Search Tool. J Mol Biol 215: 403-410.

Amar EC, Kiron V, Satoh S, Watanabe T (2001) Influence of various dietary synthetic carotenoids on bio-defense mechanisms in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacul Res 32:162–173.

Andrewes AG, Borch G, Liaaen-Jensen S, Snatzke G (1974) Animal carotenoids. IX. On the absolute configuration of astaxanthin and actinioerythrin. Acta Chem Scand B28: 730.

Apt KE, Behrens PW (1999) Commercial developments in micro algal biotechnology. J Phycol 35:215-26

Apt KE, Kroth-Pancic PG, Grossman AR (1996) Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. Molecul Gen Genet 252:572-579

Arad S, Cohen E, Ben-Amotz A (1993) Accumulation of canthaxanthin in *Chlorella emersonii*. Physiol Plant 87: 232–236.

Armstrong GA (1994) Eubacteria show their true colors: Genetics of carotenoid pigment biosynthesis from microbes to plants. J Bacteriol 176: 4795–4802.

Asami S, Yang Zhi-bo, Yamashita E, Otoze H (2001) Anti-stress composition. Patent US6265450.

Ball SG (2005) Eukaryotic microalgae genomics. The essence of being a plant. Plant Physiol 137, 397-39

Banerjee A, Sharma R, Yusuf C, Banerjee UC. 2002. *Botryococcus braunii*: A renewable source of hydrocarbons and other chemicals. Crit Rev Biotechnol, 22: 245-279.

Becher E, Albrecht R, Bernhard K, Leuenberger HGW, Meyer H, Muller R, Schuep W. Wanger HP (1981) Synthese von astaxanthin aus β-Jonon. I. Erschlissung der enantiomeren C_{15}-Wittigsalze durch chemische und mikrobiologische Racematspaltung von (±)-3-Acetoxy-4-oxo-β-jonon. Helv. Chem. Acta. 64:2419.

Becker EW, Venkataraman LV. (1982) Biotechnology and exploitation of algae-The Indian Approach. Deutsche Gesellschaft fűr Technische Zusemmenarbeit GmbH, Eschborn, F.D.R., 1-216.

Becker EW. (2007) Micro-algae as a source of protein. Biotechnol Adv, 25: 207-210.

Bell JG, McEvoy J, Webster JL (1998) Flesh lipid and carotenoid composition of scottish farmed atlantic salmon (*Salmo salar*). J Agric Food Chem 46:119–127.

Ben-Amotz A, Avron M. (1990) The biotechnology of cultivating the halotolerant algae *Dunaliella* for industrial products. Trends Biotechnol 8:121-126.

Bennedsen M, Wang X,Willen R (1999) Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunol Lett 70:185–189

Bernhard, K. (1991) Synthetic astaxanthin. The route of carotenoid from research to commercialization, In Carotenoids, chemistry and biology. Krinsky NI, Mathews-Roth MM, Taylor RF Eds., Plenum Press, New York, 337.

Berthold P, Schmitt R, Mages W (2002) An engineered Streptomyces hygroscopicus aph 7″ gene mediates dominant resistance against hygromycin B in *Chlamydomonas reinhardtii.* Protist 4:401–412.

Bhosale P, Bernstein PS (2005) Microbial xanthophylls. Appl Microbiol Biotechnol 68: 445–455.

Bidigare RR, Ondrusek ME, Kennicutt MC, Iturriaga R, Harvey HR, Hoham RW, Macko SA (1993) Evidence for a photoprotective function for secondary carotenoids of snow algae. J Phycol 29:427-434.

Bone RA, Landrum JT, Tarsis SL. (1985) Preliminary identification of the human macular pigment. Vision Res 25:1531-5.

Borlongan C (1996) Free radical damage and oxidative stress in Huntington's disease. J. Fla. Med. Assoc. 83: 335–341

Borowitzka MA (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. Journal of Biotechnology 70: 313-321.

Boussiba S (2000) Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response. Physiol Plant 108: 111-117.

Boussiba S, Bing W, Zarka A, Yuan JP, Chen F (1999) Changes in pigment profiles of *Haematococcus pluvialis* during exposure to environmental stresses. Biotechnol Lett 21: 601–604.

Boussiba S, Fan L, Vonshak A (1992) Enhancement and determinationof astaxanthin accumulation in green alga *Haematococcusplu*6*ilis*. In: Packer L (ed) Methods in Enzymology, Carotenoids Part A 213. Academic Press, London, London, pp 371–386.ISBN 0-12-182114-5

Boussiba S. and Vonshak A. 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Plant Cell Physiol. 32:1077–1082.

Bowen J, Soutar C, Serwata R (2002) Utilization of (3S, 3'S) astaxanthin acyl esters in pigmentation of rainbow trout (*Oncorhynchus mykiss*). Aquacul Nut 8:59–68.

Breitenbach J, Misawa N, Kajiwara S, Sanmann G (1996) Expression in E and properties of the carotene ketolase from *Haematococcu pluvialis*. FEMS Microbiol Lett 140:241-246.

Brinda BR, Sarada R, Sandesh KB, Ravishankar GA (2004) Accumulation of astaxanthin in flagellated cells of *Haematococcus pluvialis* – cultural and regulatory aspects. Curr Sci 87(9): 1290-1295

Britton, G. (1995) Structure and properties of carotenoids in relation to function. FASEB J 9:1551–1558.

Brown LE, Sprecher SL, Keller LR (1991) Introduction of exogenous DNA into *Chlamydomonas reinhardtii* by electroporation. Mol Cellular Biol 11:2328-2332.

Bundock P, Den Dulk-Ras A, Beijersbergen AGM, Hooykas PJJ (1995) Trans-kingdom gene transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J 14: 3206–3214.

Burchardt L, Balcerkiewicz S, Kokocinski M, Samardakiewicz S, Adamski Z (2006) Occurrence of *Haematococcus pluvialis* Flotow emend. Wille in a small artificial pool on the university campus of the collegium biologicum in Poznan (Poland). Biodiv Res Conserv 1-2: 163-166.

Burlew JS (1953) Algal Culture: From Laboratory to Pilot Plant, Publication No. 600, Carnegie Institution of Washington, Washington, DC, 357 pp

Buttle L, Crampton V, andWilliams P (2001) The effect of feed pigment type on flesh pigment deposition and colour in farmed Atlantic salmon, Salmosalar L. Aquacul Res 32:103–111.

Chan CX, Ho CL, Phang SM (2006) Trends in seaweed research. Trends in Pl Sci 11:165-166.

Chen F, Li H-B, Wong RNS, Ji B, Jiang Y (2005) Isolation and purification of the bioactive carotenoid zeaxanthin from the microalga *Microcystis aeruginosa* by high-speed counter-current chromatography. J Chromatography A 1064:183–186.

Chen Y, Li D, Lu W, Xing W, Hui B, Han Y (2003) Screening and characterization of astaxanthin-hyperproducing mutants of *Haematococcus pluvialis*. Biotechnol Lett 25: 527-529.

Chen Y, Wang Y, Sun Y, Zhang L, Li W (2001) Highly efficient expression of rabbit neutrophil peptide-1 gene in *Chlorella ellipsoidea* cells. Curr Genet 39:365–370.

Cheney D, Metz B, Stiller J (2001) *Agrobacterium*-mediated genetic transformation in the macroscopic marine red alga *Porphyra yezoensis*. J Phycol 37:11

Chew BP, Park JS, Wong MW, Wong TS (1999) A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. Anticancer Res 19:1849–1853.

Chien Y, Pan C, Hunter B (2003) The resistance to physical stresses by *Penaeus monodon* juveniles fed diets supplemented with astaxanthin. Aquacul 216:177–191.

Choubert G, Heinrich O (1993) Carotenoid pigments of the green algae *Haematococcus pluvialis*: Assay on rainbow trout, *Oncorhynchus mykiss*, pigmentation in comparison with synthetic astaxanthin and canthaxanthin. Aquacul 112:217–226.

Chow K-C, Tung WL (1999) Electrotransformation of *Chlorella vulgaris*. Plant Cell Reports 18:778-780.

Cohen SN, Chang ACY, Hsu L (1972) Non-chromosomal antibiotic resistance in Bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc.Natl. Acad. Sci. USA, 69: 2110-2114.

Coral G, Huberman A, De la Lanza G, Monroy-Ruiz J (1998) Muscle pigmentation of rainbow trout (*Oncorhynchus mykiss*) fed on oil extracted pigment from langostilla (*Pleuroncodes planipes*) compared with two commercial sources of astaxanthin. J Aquatic Food Product Technol 7:31–45.

Cordero B, Otero A, Patino M, Arredondo BO, Fabregas J (1996) Astaxanthin production from the green alga *Haematococcus pluvialis* with different stress conditions. Biotech Lett 18:213–8.

Cort´es, CR (1993) Aspectos generales de pigmentaciones para peces ycrust´aceos. Memorias del Primer Simposium Internacional de Nutrici´on yTecnolog´ıa de Alimentos para Acuacultura. Universidad Aut´onoma de Nuevo Le´on. pp. 345–353.

Cseke LJ, Kaufman PB, Podila GK, Tsai GJ (2004) Handbook of molecular and cellular methods in biology and medicine. Second edition, CRC press, USA.

Cunningham F, Jr X, Gantt E (2000) Identification of multi-gene families encoding isopentenyl diphosphate isomerase in plants by heterologous complementation in *Escherichia coli*. Plant Cell Physiol 41: 119-123.

Cunningham FX (2002) Regulation of carotenoid synthesis and accumulation in plants. Pure Appl Chem 74: 1409-1417.

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 49: 577-583.

Cyanotech (2002) Cyanotech reports two clinical studies of Bioastin. www.cyanotech.com/news/02/10-07-02.html. Presented at American College of Nutrition Meeting.

Darachai J, Piyatiratitivorakul S, Menasveta P (1999) Effect of astaxanthin on growth and survival of *Penaeus monodon* larvae. Oates CG. Ed. In: Proceedings of the 37th Kasetsart University Annual Conference. pp. 36–41.

Davis BH (1976) Carotenoids. In: Chemistry and biochemistry of plant pigments.Vol II. 2nd ed. Goodwin TW. Ed. Academic press, London, 38.

Davis JB, Weedon BCL (1960) Total synthesis of astacene. Proc. Chem. Soc. 182.

Dawson HN, Burlingame R, Cannons AC (1997) Stable transformation of *Chlorella*: rescue of nitrate reductase-deficient mutants with the nitrate reductase gene. Current Microbiol 35:356-362

Dayananda C, Sarada R, Bhattacharya S, Ravishankar GA (2005) Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii*. Process Biochem, 40:3125-3131.

De Groot MJA, Bundock P, Hooykas PJJ, Beijersbergen AGM (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol 16: 839–842

De la Noue J, De Pauw N (1988) The potential of microalgal biotechnology: A review of production and uses of microalgae. Biotechnol Adv 6:725-770.

De Rijk M (1997) Dietary antioxidants and Parkinson disease. The Rotterdam Study. Arch. Neurol 54, 762–765.

Debuchy R, Purton S, Rochaix JD (1989) The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and

for correlating the genetic and molecular maps of the ARG7 locus. EMBO J 8:2803-2809.

Del Campo JA, García-González M, Guerrero MG. (2007) Outdoor cultivation of microalgae for carotenoid production: Current state and perspectives. Appl Microbiol Biotechnol, 74:1163-1174.

Del Campo JA, Rodriguez H, Moreno J, Vargas MA, Rivas J, Guerrero MG (2001) Lutein production by *Muriellopsis* sp. in an outdoor tubular photobioreactor. J Biotechnol 85:289–295.

Del Campo JA, Rodriguez H, Moreno J, Vargas MA, Rivas J, Guerrero MG (2004) Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). Appl Microbiol Biotechnol 64:848–854.

Demmig-Adams B, Gilmore AM, Adams, WW (1996) In vivo functions of carotenoids in higher plants. FASEB J. 10: 403–412.

Di Mascio P, Devasagayam TPA, Kaiser S, Sies H. (1990) Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers. Biochem Soc 18:1054-1056.

Di Mascio P, Murphy ME, Sies H (1991) Antioxidant defense systems: the role of carotenoids, tocopherols and thiols. Am J Clin Nutr 53:194S–200S.

Dillen W, Clercq JD, Kapila J, Zambre M, Montagu MV, Angenon G (1997) The effect of temperature on *Agrobacterium tumefaciens* mediated gene transfer to plants. Plant J 12:1459–1463.

Disch A, Schwender J, Mueller C, Lichtenthaler HK, Rohmer M (1998) Distribution of the mevalonate and glyceraldehyde phosphate/pyruvate pathways for isoprenoid biosynthesis in unicellular algae and the cyanobacterium *Synechocystis* PCC 6714. Biochem J 333: 381-388.

Doetsch NA, Favreau MR, Kuscuoglu N, Thompson MD, Hallick RB (2001) Chloroplast transformation in *Euglena gracilis*: splicing of a group III twintron transcribed from a transgenic *psbK* operon. Current Genetics 39:49-60

Dolley TP, Moyle PR (2003) History and overview of the U.S. diatomite mining industry, with emphasis on the Western United States. In Bliss JD, Moyle PR, Long KR (Eds) Contributions to Industrial-Minerals Research: U.S. Geological Survey Bulletin 2209, pp E1-E8

Droop MR (1961) *Haematococcus pluvialis* and its allies; III: Organic nutrition. Rev Algol 5:247-259.

Dufosse L, Galaup P, Yaron A, Arad SM, Blanc P, Chidambara Murthy KN, Ravishankar GA. 2005. Microorganisms and micro algae as sources of pigments for food use: A scientific oddity or an industrial reality? Trends Food Sci Tech 16: 389-406.

Dunahay TG (1993) Transformation of *Chlamydomonas reinhardtii* with silicon carbide whiskers. BioTechniques 15:452-455.

Dunahay TG, Jarvis EE, Roessler PG (1995) Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. J Phycol 31:1004-1012

Dzelzkalns VA, Bogorad L (1986) Stable transformation of the cyanobacterium *Synechocystis* sp. PCC 6803 induced by UV irradiation. J Bacteriol 165, 964-971

Edge R, McGarvey DJ, Truscott TG (1997) The carotenoids as anti-oxidants - A review. J Photochem Photobiol B 41:189–200.

Egeland ES. (1993) Carotenoids in combs of capercaillie (*Tetrao urogallus*) fed defined diets. Poult Sci 72:747-51.

El-Sheekh MM (1999) Stable transformation of the intact cells of *Chlorella kessleri* with high velocity microprojectiles. Biologia Plantarum 42:209-216

Escudero J Hohn B (1997) Transfer and integration of T-DNA without cell injury in the host plant. Plant Cell 9:2135–2142.

Fabregas J, Dominguez A, Alvarez DG, Lamela T, Otero A (1998) Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies in *Haematococcus pluvialis*. Biotechnol Letters 20:623-626.

Fabregas, J., Dominguez, A., Regueiro, M., Maseda, A., and Otero, A. (2000). Optimization of culture medium for the continuous cultivation of the microalga *Haematococcus pluvialis*. Appl Microbiol Biotechnol 53, 530-535.

Facchinetti F (1998) Free radicals as mediators of neuronal injury. Cell Mol Neurobiol 18: 667–682.

Falciatore A, Casotti R, Leblanc C, Abrescia C, Bowler C (1999) Transformation of nonselectable reporter genes in marine diatoms. Marine Biotechnol 1:239-251

Fan L, Vonshak A, Zarka A, Boussiba S (1998) Does astaxanthin protect *Haematococcus* against light damage? Z Naturforsch 53: 93-100.

Farmer WR, Liao JC (2000) Improving lycopene production in *Escherichia coli* by engineering metabolic control. Nat Biotechnol 18:533–537.

Fayaz M, Namitha KK, Chidambara Murthy KN, Mahadeva Swamy M, Sarada R, Salma Khanam, Subbarao PV, Ravishankar GA. (2005) Chemical composition, iron bioavailability, and antioxidant activity of *Kappaphycus alvarezzi* (Doty). J Agric Food Chem 53: 792-797.

Fazeli MR, Tofighi H, Sobhani AM, Shahverdi AR, Sattari TN, Mirzaie S, Jamalifar H (2009) Nicotine inhibition of lycopene cyclase enhances accumulation of carotenoid intermediates by *Dunaliella salina* ccap 19/18 Eur J Phycol 44(2): 215–220

Ferrante R (1997) Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. J Neurochem 69: 2064–2074.

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281:237-240.

Fischer H, Robl I, Sumper M, Kröger N (1999) Targeting and covalent modification of cell wall and membrane proteins heterologously expressed in the diatom *Cylindrotheca fusiformis*. J Phycol 35:113-120

Fopppe FH (1971) Tables for the identification of carotenoid pigments. Chromatography Reviews. 14:133-136.

Franklin SE, Mayfield SP (2004) Prospects for molecular farming in the green alga *Chlamydomonas*. Current Opinion in Plant Biology 7:159-165.

Fraser PD, Miura Y, Misawa N (1997) In vitro characterization of astaxanthin biosynthetic enzymes. J Biol Chem 272:6128–6135.

Fraser PD, Shimada H, Misawa N (1998) Enzymatic confirmation of reactions involved in routes to astaxanthin formation, elucidated using a direct substrate in vitro assay. Eur J Biochem 252: 229–236.

Fujii K, Nakajima H, Anno Y (2008) Potential of *Monoraphidium* sp. GK12 for energy-saving astaxanthin production. J Chem Technol Biotechnol 83: 1578 – 1584.

Gabaudan J (1996) Dietary astaxanthin improves production yield in shrimp farming. Fish Chimes 16:37–39.

Gan S-Y, Qin S, Othman RY, Yu D, Phang S-M (2003) Transient expression of *lacZ* in particle bombarded *Gracilaria changii* (Gracilariales, Rhodophyta). J App Phycol 15:345-349

Garcia-Gonzalez M, Moreno J, Manzano JC, Florencio FJ, Guerrero MG (2005) Production of *Dunaliella salina* biomass rich in 9-cis-β-carotene and lutein in a closed tubular photobioreactor. J Biotechnol 115: 81-90.

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Ann Rev Plant Physiol Plant Mol Biol 51: 223–256.

Geng D, Wang Y, Wang P, Li W, Sun Y (2003) Stable expression of hepatitis B surface antigen gene in *Dunaliella salina* (Chlorophyta). J Appl Phycol 15:451-456.

Gentles A, Haard NF (1991) Pigmentation of rainbow trout with enzyme treated and spray dried *Phaffia rhodozyma*. The Progressive Fish Culturist 53:1–6.

Gerster H (1991) Review: Antioxidant protection of the ageing macula. Age ageing 20:60-9.

Golstein JL, Brown MS (1977) Low DL pathway and its relation to atherosclerosis. Annu Rev Biochem 46:897–930.

Goodwin TW, Jamikorn M (1954) Studies in Carotenogenesis 11.Carotenoid synthesis in the alga *Haematococcus plu*6*ialis*.Biochem J 57: 376–381

Goodwin, T.W. 1980. The Biochemistry of Carotenoids. Chapman- Hall, New York.

Goto S, Kogure K, Abe K, Kimata Y, Kitahama K, Yamashita E, Terada H (2001) Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. Biochim Biophys Acta 1512:251-258.

Gouveia L, Choubert G, Pereira N (2002) Pigmentation of gilthead seabream, *Sparus aurata* (L. 1875) using *Chlorella vulgaris* (Chlorophyta,Volvocales) microalga. Aquacul Res 33:987–993.

Grant WB (1997) Dietary links to Alzheimer's disease. J Alzheimers Dis 2:42–55.

Grossman AR (2005) Paths toward algal genomics. Plant Physiol 137:410-427.

Grünewald K, Eckert M, Hirschberg J, Hagen C (2000) Phytoene desaturase is localized exclusively in the chloroplast and up-regulated at the mRNA level during accumulation of secondary carotenoids in *Haematococcus pluvialis* (Volvocales, Chlorophyceae). Plant Physiol 122: 1261-1268.

Grünewald K, Hagen C (2001) β-carotene is the intermediate exported from the chloroplast during accumulation of secondary carotenoids in *Haematococcus pluvialis.* J Appl Phycol 13: 89-93.

Grünewald K, Hirschberg J, Hagen C (2001) Ketocarotenoid biosynthesis outside of plastids in the unicellular green alga *Haematococcus pluvialis*. J Biol Chem 276: 6023-6029.

Grung M, D'souza FM, Borrowitzka MA, Liaaen-Jensen S (1992) Algal carotenoids secondary carotenoids: *Haematococcus pluvialis* aplanospores as a source of (3S, 3'S) astaxanthin esters. J Appl Phycol 4: 165-171.

Guerin M, Huntley ME, Olaizola M (2003). *Haematococcus* astaxanthin: applications for human health and nutrition. Trends in Biotechnol 21:210-216.

Guyen VC, Kenmotsu M, Arai H, Yamashita E (1998) Astaxanthin containing food or drink. Patent abstract JP10276721.

Haematococcus pluvialis. Ph.D thesis, Pp-165

Hagen C, Braune W, Bjorn LO (1994) Funtional aspects of secondary carotenoids in *Haematococcus lacustris* (Volvocales). III. Action as a 'sunshade'. J Phycol 30: 241–248.

Hagen C, Gruenewald K, Xylaender M, Rothe E (2001) Effect of cultivation parameters on growth and pigment biosynthesis in flagellated cells of *Haematococcus pluvialis*. J Appl Phycol 13: 79-87.

Hall LM, Taylor KB, Jones DD, (1993) Expression of a foreign gene in *Chlamydomonas reinhardtii*. Gene 24:75–81.

Hallmann A (2007) Algal transgenics and biotechnology. Trangenic Plant J 1: 81-98.

Hamilton CM, Frary A, Lewis C,Tanksley S (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc Natl Acad Sci USA 93:9975–9979.

Hanagata N, Dubinsky Z (1999) Secondary carotenoid accumulation in *Scenedesmus komarekii* (Chlorophyceae, Chlorophyta). J Phycol 35: 960 – 966.

Harker M, Tsavalos AJ, Young AJ (1996) Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis*. Bioresource Technol 55: 207-214.

Harker M, Young AJ (1995) Inhibition of astaxanthin synthesis in the green alga *Haematococcus pluvialis*. Eur J Phycol 30: 179–87.

Harker M, Young AJ. Inhibition of astaxanthin synthesis in the green alga 418 *Haematococcus pluvialis*. Eur J Phycol 1995;30:179–87.

Harris EH (1989) The *Chlamydomonas* source book: A comprehensive guide to biology and laboratory use. USA Academic press, San Diego, CA.

Hata N, Ogbonna JC, HasegawaY, Taroda H, Tanaka H (2001) Production of astaxanthin by *Haematococcus pluvialis* in a sequential heterotrophic photoautotrophic culture. *Journal of Applied Phycology* 13:395-402.

Havaux M, Niyogi KK (1999) The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. Proc. Natl. Acad. Sci. 96:8762–8767.

Hiei Y, Ohta S, Komari T, Komashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271–282.

Higuera-Ciapara I, Felix-valenzuela, Goycoolea FM. (2006) Astaxanthin: A review of its Chemistry and applications. Crit Rev Food Sci Nutr, 46: 185-196.

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

Hirschberg J, Cohen M, Harker M, Lotan T, Mann V, Pecker I (1997) Molecular genetics of the carotenoid biosynthesis pathway in plants and algae. Pure Appl Chem 69: 2151-2158.

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179-180

Hoshino T, Fujii R,Nakahara T (1994) Overproduction of carotenoids in *Thermus thermophilus*. J Ferment Bioeng 77:423–424.

Hu G (1993) DNA polymerase – catalyzed addition of non-templated extra nucleotides to the 3' end of a DNA fragment. DNA Cell Biol 12: 763-770.

Huang JC, Chen F, Sandmann G (2006a) Stress-related differential expression of multiple β-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis* J Biotechnol 122**:**176-185.

Huang JC, Wang Y, Sandmann G, Chen F (2006b) Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta). Appl Microbiol Biotechnol 71: 473-479.

Huang X, Weber JC, Hinson TK, Mathieson AC, Minocha SC (1996) Transient expression of the GUS reporter gene in the protoplasts and partially digested cells of *Ulva lactuca*. Botanica Marina 39:467-474.

Ikan R (1991) Natural products: A laboratory guide. Academic press, San diego, CA, 105.

Ip PF, Chen F (2005) Production of astaxanthin by the green microalga *Chlorella*

zofingiensis in the dark. Process Biochem 40: 733–738.

Iwamoto T, Hosoda K, Hirano R (2000) Inhibition of low-density lipoprotein oxidation by astaxanthin. J Atherosclerosis Thrombosis 7:216–222.

Jacobson GK, Jolly SO, Sedmark JJ, Skatrud TJ, Wasileski JM (2000)Astaxanthin over-producing strains of *Phaffia rhodozyma.* Method for their cultivation and their use in animal feeds. US Patent 6,015,684.

Jacques P (1999) The potential preventive effects of vitamins for cataract and agerelated macular degeneration. Int J Vitam Nutr Res 69:198-205.

Jarvis EE, Brown LM (1991) Transient expression of firefly luciferase in protoplasts of the green alga *Chlorella ellipsoidea*. Curr Genetics 19:317-321.

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: Beta –glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907.

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907.

Jefferson RA, Kilian A, Keese PK (1998) Microbial genes for secreted betaglucuronidases,gene products and uses thereof. patent PCT (PCT/US98/19217).09th September 1998.

Jialal I, Fuller CJ (1995) Effect of vitamin E, vitamin C and beta carotene on LDL oxidation and atherosclerosis. The Canadian J of Cardiol Suppl G:97G–103G.

Jin E, Lee CG, Polle JEW (2006) Secondary carotenoid accumulation in *Haematococcus* (Chlorophyceae): biosynthesis, regulation, and biotechnology. J Microbiol Biotechnol 16: 821-831.

Jin ES, Feth B, Melis A (2003) A mutant of the green alga *Dunaliella salina* constitutively accumulates zeaxanthin under all growth conditions. Biotechnol Bioeng 81: 115– 124.

Johnson EA, An GH (1991) Astaxanthin from Microbial Sources. Critical Rev Biotechnol 11(4):297-326

Johnson EA, Schroeder WA (1995) Microbial carotenoids. In: Advances in Biochemical Engineering and biotechnology Ed Feichter A: Springer- Verlag, Berlin, Heidelberg 53: 119-178.

Jorgensen K, Skibsted L (1993) Carotenoid scavenging radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. Z Lebensm Unters Forsch 196: 423–429.

Jyonouchi H, Sun S, Gross M (1995) Effect of carotenoids on in vitro immunoglobulin production by human peripheral blood mononuclear cells: Astaxanthin, a carotenoid without vitamin A activity, enhances in vitro immunoglobulin production in response to a T-dependent stimulant and antigen. Nutr Cancer 23:171-83.

Jyonouchi H, Sun S, Gross M (1995) Effect of carotenoids on in vitro immunoglobulin production by human peripheral blood mononuclear cells: astaxanthin, a carotenoid without vitamin A activity, enhances in vitro immunoglobulin production in response to a T-dependent stimulant and antigen. Nutrition and cancer 23:171–183.

Jyonouchi H, Sun S, Iijima K, Gross MD (2000) Antitumor activity of astaxanthin and its mode of action. Nutr Cancer 36:59–65.

Jyonouchi H, Sun S, Mizokami M, Gross M (1996) Effects of various carotenoids on cloned, effector-stage T-helper cell activity. Nutr Cancer 26:313–324.

Jyonouchi H, Zhang L, Gross M, Tomita Y (1994) Immunomodulating actions of carotenoids: enhancement of in vivo and in vitro antibody production to T-dependent antigens. Nutr Cancer 21:47–58.

Kajiwara S, Fraser PD, Kondo K, Misawa N (1997) Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli.* Biochem J 324:421–426.

Kajiwara S, Kakizono T, Saito T, Kondo K, Ohtani T, Nishio N, Nagai S, Misawa N (1995) Isolation and functional identification of a novel cDNA for astaxanthin

biosynthesis from *Haematococcus pluvialis*, and astaxanthin synthesis in *Escherichia coli*. Plant Mol Biol 29:343-352.

Kakizono T, Kobayashi M, Nagai S (1992) Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, *Haematococcus pluvialis.* J Ferment Bioeng 74: 403-405.

Kang CD, Lee JS, Park TH, Sim SJ (2005) Comparison of heterotrophic and photoautotrophic induction on astaxanthin production by *Haematococcus pluvialis*. Appl Microbiol Biotechnol 68:237–241.

Kang JO, Kim SJ, Kim H (2001) Effect of astaxanthin on the hepatotoxicity, lipid peroxidation and antioxidative enzymes in the liver of CCl4 treated rats. Methods Findings in Exp Clin Pharmacol 23:79–84.

Kanz T, Bold HC (1969) Physiological Studies. Morphological and taxonomic investigations of *Nostoc* and *Anabaena* in culture. Univiversity of Texas publication Number 6924, Univinersity of Texas, Austin,TX.

Kathiresan S, Chandrashekar A, Ravishankar GA, Sarada R (2009) *Agrobacterium*-mediated transformation in the green alga *Haematococcus pluvialis* (chlorophyceae, volvocales) J Phycol 45:642-649

Kathiresan S, Sarada R (2009) Towards genetic improvement of commercially important microalga *Haematococcus pluvialis* for biotech applications. J Appl Phycol DOI: 10.1007/s10811-009-9414-0

Kathiresan S, Sarada R, Sila Bhattacharya, Ravishankar GA (2007) Culture media optimization for growth and phycoerythrin production from *Porphyridium purpureum*. Biotechnol Bioeng 96: 456-463.

Kawata Y, Yano S, Kojima H, Toyomizu M (2004) Transformation of *Spirulina platensis* strain C1 (*Arthrospira* sp. PCC9438) with Tn5 transposase transposon DNA-cation liposome complex. Marine Biotechnol (NY) 6:355-363

Kindle KL (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc Nat Acad Sci USA 87:1228-1232

Kindle KL, Schnell RA, Fernandez E, Lefebvre PA (1989) Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. J Cell Biol 10: 2589-2601.

Kobayashi M, Kakizono T, Nagai S (1993) Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. Appl Envir Microbiol 59: 867-873.

Kobayashi M, Kakizono T, Nishio N, Nagai S, Kurimura Y, Tsuji Y (1997) Antioxidant role of astaxanthin in the green alga *Haematococcus pluvialis*. Appl Microbiol Biotechnol 48: 351-356.

Kobayashi M, Kakizono T, Yamaguchi K, Nishio N, Nagai S (1992) Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. J Ferment Bioeng 74: 17-20.

Kobayashi M, Sakamoto Y (1999) Singlet oxygen quenching ability of astaxanthin esters from the green algae *Haematococcus pluvialis*. Biotechnol Lett 21:265–269.

Koksharova OA, Wolk CP (2002) Genetic tools for cyanobacteria. Appl Microbiol Biotechnol 58:123-137

Krinsky NI, Johnson EJ (2005) Carotenoid actions and their relation to health and disease. Mol Aspects Med 26: 459-516.

Kübler JE, Minocha SC, Mathieson AC (1993) Transient expression of the GUS reporter gene in *Porphyra miniata* (Rhodophyta). J Marine Biotechnol 1:165-169

Kumar SV, Misquitta RW, Reddy VS, Rao BJ, Rajam MV (2004) Genetic transformation of the green alga *Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. Plant Science 166:731-738

Kumria R, Waie B, Rajam MV (2001) Plant regeneration from transformed embryogenic callus of an elite indica rice via *Agrobacterium.* Plant Cell Tissue Org Cult 67:63–71.

Kunn R, Sorenson NA (1983) Uber astaxanthin and ovoerdin. Ber Dtsch Chem Ges 71: 1879.

Kurashige M, Okimasu E, Inoue M, Utsumi K. (1990) Inhibition of oxidative injury of biological membranes by astaxanthin. Physiol Chem Phys Med NMR 22:27-38.

Kurihara H, Koda H, Asami S (2002) Contribution of the antioxidative property of astaxanthin to its protective effect on the promotion of cancer metastasis in mice treated with restraint stress. Life Sci 70:2509–2520.

Kurtzman AM, Cheney DP (1991) Direct gene transfer and transient expression in a marine red alga using the biolistic method. J Phycol 27 (Suppl.) 42

Ladygin VG (2004) Efficient transformation of mutant cells of Chlamydomonas reinhardtii by electroporation. Process Biochem 39:1685–1691.

Ladygin VG. (2000) Biosynthesis of carotenoids in the chloroplasts of algae and higher plants. Russ J Plant Physiol 47: 796-814.

Landrum JT, Bone RA, Moore LL, Gomez CM (1999) Analysis of zeaxanthin distribution within individual human retinas. Method Enzymol 299:457-467.

Lapidot M, Raveh D, Sivan A, Arad SM, Shapira M (2002) Stable chloroplast transformation of the unicellular red alga *Porphyridium* species. Plant Physiol 129, 7-12

Lawlor SM, O'Brien NM (1995) Astaxanthin: antioxidant effects in chicken embryo fibroblasts. Nutr Res 15:1695-1704.

Lee JH, Lee SJ, Choi YH, Chung KT, Jeong YK, Choi BT (2006) Effects of mycelial culture of *Phellinus linteus* on ethanol-induced gastric ulcer in rats. Phytotherapy Res 20: 396–402.

Lee KS (1986) Evaluation of crawfish astaxanthin as a natural red pigmenter of egg yolk. Poultry Sci 65:177–178.

León-Banares R, González-Ballester D, Galván A, Fernández E (2004) Transgenic microalgae as green cell-factories. Trends in Biotechnol 22:45-52.

Lers A, Biener Y, Zamir A (1990) Photoinduction of massive β-carotene accumulation by the alga *Dunaliella bardawil.* Plant Physiol 93: 389-395.

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Meth Enzymol 148:350-382.

Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 50: 47-65.

Lichtenthaler HK, Rohmer M, Schwender J (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiol Plant 101: 643–652.

Lignell A, Bottiger P (2001) Use of xanthophylls, astaxanthin for treatment of autoimmune diseases, chronic viral and intracellular bacterial infections. Patent WO01/24787 A1.

Lim B, Nagao A, Terao J, Tanaka K, Suzuki T, Takama K (1992) Antioxidant Copyright 2002 by Mera Pharmaceuticals, Inc. All rights reserved. 15 activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. Biochim Biophys Acta 1126:178-184.

Lin JJ, Ma J, Garcia-Assad N, Kuo J (1996) Hygromycin as an efficient antibiotic for the selection of transgenic plants. Focus 18:47–49.

Linden H, (1999) Carotenoid hydroxylase from *Haematococcus pluvialsis*: cDNA sequence, regulation and functional complementation. Biochem Biophys Acta 1446: 203-212.

Lorenz RT (1998) A review of the carotenoid, astaxanthin, as a pigment source and vitamin for cultured Penaeus prawn. http://www. cyanotech. com/pdfs/axbul51.pdf.

Lorenz RT (1999) A Technical Review of *Haematococcus* Algae. NatuRose^{TM} Technical Bulletin 60.

Lorenz RT (2002) Method for retarding and preventing sunburn by UV light. Patent US 6433025.

Lorenz RT, Cysewski GR (2000) Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends Biotechnol 18: 160-167.

Lotan T, Hirschberg J (1995) Cloning and expression in Escherichia coli of the gene encoding β-C-4-oxygenase, that converts β-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. FEBS Letters 364: 125-128

Lumbreras V, Stevens DR, Purton S (1998) Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. Plant J 14:441–447.

Lyons NM, O'Brien NM (2002) Modulatory effects of an algal extract containing astaxanthin on UVA-irradiated cells in culture. J Dermatol Sci 30:73–84.

Maher TJ (2000) www.nhir.com/tests/astaxanthin.pdf.

Mann V, Harker M, Pecker I, Hirschberg J (2000) Metabolic engineering of astaxanthin production in tobacco flowers. Nat Biotechnol 18: 888–892.

Margalith PZ (1999) Production of ketocarotenoids by microalgae. Appl Microbiol Biotechnol 51: 431-438.

Martin G, Mark E, Huntley, Miguel O (2003) *Haematococcus* astaxanthin: applications for human health and nutrition. Trends in Biotechnol 21:210-216

Maruyama M, Horakova´ I, Honda H, Xing X, Shiragami N, Unno H (1994) Introduction of foreign DNA into *Chlorella saccharophila* by electroporation. Biotechnol Techn. 8:821–826.

Mastuno T, Hirao S (1989) Marine carotenoids. In: Marine bioenic lipids, fats and oils. Ed. Ackman RG, CRC press, Boca Raton, FL, 11: 251.

McCoy M (1999) Astaxanthin market a hard one to crack. Chem Engin News. 5: 15–17.

McHugh DJ (2003) A guide to the seaweed industry. FAO Fisheries Technical Papers 441, 1-105.

Meng CX, Teng CY, Jiang P, Qin S, Tseng CK (2005) Cloning and Characterization of β-Carotene Ketolase Gene Promoter in *Haematococcus pluvialis*. Acta Biochim Biophys Sin 37: 270–275.

Mera Pharmaceuticals (1999) *Haematococcus pluvialis* and astaxanthin safety for human consumption. Technical Report TR.3005.001 available at http://www.astafactor.com/techreports/tr3005-001.htm

Meyer P (1995) Gene silencing in higher plants and related phenomena in other eukaryotes. Curr Top Microbiol Immunol 197:15–28.

Meyers SP, Bligh D (1981) Characterization of astaxanthin pigments from heat processed crawfish waste. J Agri Food Chem 3:505–508.

Meyers SP, Chen HM (1982) Astaxanthin and its role in fish culture. In: Proceeding of the warmwater fish culture. pp. 153–165. Stickney, R.R., and Meyers, P.S. Eds. Louisiana State University.

Meyers SP. (1993) The biological role of astaxanthin in salmonids and other aquatic species. Proceedings of the First International Symposium on Natural Colors, Amherst, Mass. F.J. Francis, ed. The Hereld Organization, S.I.C. Publishing Co., Hampden CT.

Miao F, Lu D, Li Y, Zeng M (2006) Characterization of astaxanthin esters in *Haematococcus pluvialis* by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. Analytical Biochem 352: 176–181.

Miki W (1991) Biological functions and activities of animal carotenoids. Pure and Appl Chem 63:141–146.

Miki W, Hosoda K, Kondo K, Itakura H (1998) Astaxanthin containing drink. Patent abstract JP10155459.

Miki W, Yamaguchi K, Konosu S, (1982) Comparison of carotenoids in the ovaries of marine fish and shellfish. Comp Biochem Physiol B 71:7-11.

Minoda A, Sakagami R, Yagisawa F, Kuroiwa T, Tanaka K (2004) Improvement of culture conditions and evidence for nuclear transformation by homologous recombination in a red alga, *Cyanidioschyzon merolae* 10D.Plant and Cell Physiol 45:667-671.

Misawa N, Shimada H (1998) Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. J Biotechnol 59:169–181.

Miura Y, Kondo K, Saito T, Shimada H, Fraser PD, Misawa N (1998) Production of the carotenoids lycopene, β- astaxanthin in the food yeast *Candida utilis*. Appl Env Microbiol 64:1226–1229.

Montsant A, Maheswari U, Bowler C, Lopez PJ (2005) Diatomics: toward diatom functional genomics. J Nanosci Nanotechnol 5:5-14.

Moore RE, Patterson M, Carmichael WW, (1988) New Pharmaceuticals from cultured blue green alga. In: Fautin DG, Ed. Biomedical importance of marine organisms. California Academy of Sciences, San Francisco. 143-150.

Naguib YMA (2000) Antioxidant acitivities of astaxanthin and related carotenoids. J Agric Food Chem 48:150-154.

Nakajima H (1995) Stabilized powder of *Phaffia* coloring matter oil containing astaxanthin as main component and its production. Patent JP7099924.

Nakano T, Tosa M, Takeuchi M (1995) Improvement of biochemical features in fish health by red yeast and synthetic astaxanthin. J Agric Food Chem 43:1570–1573.

Neils HJ, Leenheer AP (1991) Microbial sources of carotenoid pigments uses in foods and feeds. J Appl Bacteriol 70:181–191.

Nguyen TA, Jefferson RA (2001) Binary vector pCAMBIA 1305.2 (NCBI database Accession number: AF354046, 16th April 2001).

No HK, Storebakken T (1991) Pigmentation of rainbow trout with astaxanthin at different water temperatures. Aquacul 97:203–216.

O'Connor I, O'Brien N (1998) Modulation of UV-A light-induced oxidative stress by beta-carotene, lutein and astaxanthin in cultured fibroblasts. J Dermatol Sci 16: 226-230.

Odell JT, Knowlton S, Lin W, Mauvai CJ (1988) Properties of anisolated transcription stimulating sequence derived from the cauliflower mosaic virus 35S promoter. Plant Mol Biol 10:263–272

Olaizola M (2000) Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000-liter outdoor photobioreactors. J of Appl Phycol 12:499-506.

Olaizola M (2003) Commercial development of microalgal biotechnology: from the test tube to the marketplace. Biomol Engin 20:459-466.

Orosa M, Franqueira D, Cid A, Abalde J (2001) Carotenoid accumulation in *Haematococcus pluvialis* in mixotrophic growth. Biotechnol Lett 23:373–378.

Orosa M, Valero JF, Herrero C, Abalde J (2001a) Comparison of the accumulation of astaxanthin in *Haematococcus pluvialis* and other green microalgae under N-starvation and high light conditions. Biotech Lett 23: 1079-1085.

Ortiz JPA, Reggiardo MI, Ravizzini RA, Altabe SG, Cervigni GDL, Spitteler MA, Morata MM, Elias FE, Vellegos RH (1996) Hygromycin resistance as an efficient selectable marker for wheat stable transformation. Plant Cell Rep 15:877–881.

Osterlie M, Bjerkeng B, Liaaen-Jensen S (1999) Accumulation of astaxanthin all E, 9z and 13z geometrical isomers and 3 and 3' optical isomers in rainbow trout (*Oncorhynchus mykiss*) is selective. J Nutr 2:391–398.

Pearson, W.R., and Lipman, D.J. 1988. Improved tools for biological sequence analysis. *Proc. Natl. Acad. Sci. USA*, 85: 2444-2448

Peebles F (1909a) The formation and behavior of the microzooids of *Haematococcus pluvialis*. Science 21: 380.

Peebles F (1909b) The life history of *Sphaerella lacustris* (*Haematococcus pluvialis*) with reference to the nature and behavior of the zoospores. Centralbl Bakt Abt 2: 511-521.

Pocock MA (1937) Studies in South African Volvocales. 1. A new *Sphaerella (Haematococcus*). Proc Linn Soc London 149: 55-58.

Pocock MA (1961) *Haematococcus* in southern Africa. Trans. Royal Soc. South Africa 36: 5-59.

Proctor VW (1957a). Some controlling factors in the distribution of *Haematococcus pluvialis*. Ecol. 38:457-462.

Pulz O (2001) Photobioreactors: production systems for phototrophic microorganisms. Appl Microbiol Biotechnol 57:287-293.

Pulz O, Gross W (2004) Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol 65:635-648.

Putnam M (1991) A review of the nature, function, variability and supply of pigments in salmonid fish. In: Aquaculture and the environment. pp. 245–263. De Pauw N, Joyce J. Eds. European Aquaculture Society. Special Publication No. 16. Gent, Belgium.

Qin S, Jiang P, Tseng C (2005) Transforming kelp into a marine bioreactor. Trends Biotechnol 23:264-268.

Qin S, Sun G-Q, Jiang P, Zou L-H, Wu Y, Tseng C-K (1999) Review of genetic engineering of *Laminaria japonica* (Laminariales, Phaeophyta) in China. Hydrobiologia 398-399:469-472

Qin S, Yu D-Z, Jiang P, Teng C-Y, Zeng C-K (2003) Stable expression of *lacZ* reporter gene in seaweed *Undaria pinnatifida*. High Technol Letters 13:87-89

Renstrom B, Liaaen-Jensen S (1981) Fatty acid composition of some esterified carotenols. Comp Biochem Physiol B 69: 625-627.

Rodríguez-Concepción M, Boronat A (2002) Elucidation of the Methylerythritol Phosphate Pathway for Isoprenoid Biosynthesis in Bacteria and Plastids. A Metabolic Milestone Achieved through Genomics. Plant Physiol 130: 1079–1089.

Rohmer M (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep 16: 565–574.

Rozen S, Skaletsky J (2000) Primer3 on the www for general users and for biologist programmers. *In*: *Bioinformatics methods and protocols,* Methods in molecular biology (Vol. 132). (S. Misener and S.A. Krawetz. ed). Humana Press, Totowa, New Jersy, pp: 365-386.

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press

Sandesh KB (2007) Biotechnological production of microalgal carotenoids with reference to astaxanthin and evaluation of its biological activity. Ph. D thesis, Pp – 176

Sandmann G, Albrecht M, Schnurr G, Kno¨rzer O, Bo¨ger P (1999) The biotechnological potential and design of novel carotenoids by gene combination in *Escherichia coli*. TIBTECH 17:233–237.

Sarada R, Bhattacharya S, Ravishankar G A (2002a) A response surface approach for the production of natural pigment astaxanthin from green alga, *Haematococcus pluvialis*: Effect of sodium acetate, culture age, and sodium chloride. Food Biotech 16: 107-120.

Sarada R, Bhattacharya S, Ravishankar GA (2002) Optimization of culture conditions for growth of the green alga *Haematococcus pluvialis*. World Journal of Microbiol Biotechnol 18:517–521.

Sarada R, Tripathi U, Ravishankar GA (2002b) Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions. Process Biochem 37: 623-627.

Schell J, Herrera-Estrella L, Zambryski P, DeBlock M, Joos H, Willmitzer L, Eches P, Rosahl S, Van Montagu M (1984) Genetic engineering of plants. In: Schell JS, Starlinger P (eds) The impactof gene transfer technique in eukaryotic cell biology. Springer, Berlin

Schiedlmeier B, Schmitt R, Muller W, Kirk MM, Gruber H, Mages W, Kirk DL (1994) Nuclear transformation of Volvox carteri. Proc. Natl.Acad. Sci. USA 91:5080–5084.

Schmidt-Dannert C (2000) Engineering novel carotenoid in microorganisms. Curr Opin Biotechnol 11:255–261.

Schmidt-Dannert C, Umeno D, Arnold FH (2000) Molecular breeding of carotenoid biosynthetic pathways. Nat Biotechnol 18:750–753.

Schoefs B, Rmiki N, Rachadi J, Lemoine Y (2001) Astaxanthin accumulation in *Haematococcus* requires a cytochrome P450 hydroxylase and an active synthesis of fatty acids. FEBS Lett 5003: 125-128.

Schwender J, Gemuenden C, Lichtenthaler HK (2001) Chlorophyta exclusively use the 1-deoxyxylulose-5- phosphate/2-C-methylerythritol 4-phosphate pathway for the biosynthesis of isoprenoids. Planta 212: 416-423.

Shahidi F, Synowiecki J (1991) Isolation and Characterization of nutrients and value-added products from snow crab (*Chinoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. J Agric Food Chem 39:1527–1532.

Sharp PA, Sugden B, Sambrook J (1973) Detection of two restriction endonuclease activities in *Haemophilus influenzae* using analytical agarose ethidium bromide electrophoresis. Biochemistry 12: 3055-3063

Shi XM, Liu HJ, Zhang XW, Chen F (1999) Production of biomass and lutein by *Chlorella protothecoides* at various glucose concentrations in heterotrophic cultures. Process Biochem 34: 341–347.

Shimidzu N, Goto M, Miki W (1996) Carotenoids as singlet oxygen quenchers in marine organisms. Fisheries Sci 62:134-137.

Siefermann-Harms D (1987) The light-harvesting and protective functions of carotenoids in photosynthetic membranes. Physiol Plant 69:561–568.

Simpson KL, Katayama T, Chichester CO (1981) Carotenoids from microorganisms. In "Carotenoids as colorants and vitamin A precursors" J. C.Bauernfeld, ed. Academic Press, London, pp. 463-480.

Sivonen K, Kononen K, Esala AL, Niemela SI (1989) Toxicity and isolation of cyanobacterium *Nodularia spunigenei* from the southern baltic sea in 1986. Hydrobiologia 185: 3-8.

Smith BE, Hardy RW, Torrisen OJ (1992) Synthetic astaxanthin deposition on pan-size coho salmon (*Oncorhynchus kisutch*). Aquacul 104:105–119.

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101:87-96.

Stachel SE, Messens E, VanMontagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature 318:624–629.

Steinbrenner J, Linden H (2001) Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. Plant Physiol 125: 810-817.

Steinbrenner J, Sandmann G (2006) Transformation of the Green Alga *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis. Applied Environ Microbiol 72:7477–7484

Storebakken T, No HK (1992) Pigmentation of rainbow trout. Aquacul 100:209–229.

Straub O (1976) Key to carotenoids: List of natural carotenoids, Birkhauser-Verlag, Basel.

Sun Z, Gantt E, Cunningham FX (1996) Cloning and functional analysis of the β-carotene hydroxylase of *Arabidopsis thaliana*. J Biol Chem 271: 24349-24352.

Sun Z, Cunningham FX Gantt E (1998) Differential expression of two isopentenyl pyrophosphate isomerases and enhanced carotenoid accumulation in a unicellular chlorophyte. Proc Natl Acad Sci USA 95: 11482-11488.

Suseela MR, Toppo K (2006) *Haematococcus pluvialis* – A green alga, richest natural source of astaxanthin. Curr Sci 90: 1602–1603.

Tanaka T, Kawamori T, Ohnishi M (1995b) Suppression of azoxymethane–induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. Carcinogenesis 16:2957–2963.

Tanaka T, Makita H, Ohnishi M (1995a) Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. Cancer Res 55:4059–4064.

Tanaka T, Morishita Y, Suzui M (1994) Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. Carcinogenesis 15:15–19.

Tanaka Y, Matsuguchi H, Katayama T (1976) The biosynthesis of astaxanthin-XVI. The carotenoids in crustacea. Comp Biochem Physiol 54B:391–393.

Tao L, Wilczek J, Odom JM, Cheng Q (2006) Engineering a β -carotene ketolase for astaxanthin production. MetabolIC Engineering 8:523–531

Ten Lohuis MR, Miller DJ (1998) Genetic transformation of dinoflagellates (*Amphidinium* and *Symbiodinium*): expression of GUS in microalgae using heterologous promoter constructs. Plant J 13:427-435

Teng C, Qin S, Liu J, Yu D, Liang C, Tseng C (2002) Transient expression of *lacZ* in bombarded unicellular green alga *Haematococcus pluvialis*. J Appl Phycol 14:497–500.

Teng CY, Qin S, Tseng CK (2003) Genomic sequences of carotenoid hydroxylase from *Haematococcus pluvialis.* Unpublished. NCBI gene sequence ID AY187011 (gi:28911948).

Terao J (1989) Antioxidant activity of betacarotene- related carotenoids in solution. Lipids 24:659-61.

Thiel T, Poo H (1989) Transformation of a filamentous cyanobacterium by electroporation. J Bacteriol 171:5743-5746

Tjahjono AE, Hayama Y, Kakizono T, Terada Y, Nishio N, Nagai S (1994) Hyperaccumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. Biotechnol Lett 16:133–8.

Topfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acid Res 15:5890

Torrisen OJ (1989) Pigmentation of salmonids: interactions of astaxanthin and canthaxanthin on pigment deposition in rainbow trout. Aquacul 79:363–374.

Toyomizu M, Suzuki K, Kawata1 Y, Kojima1 H, Akiba Y (2001) Effective transformation of the cyanobacterium *Spirulina platensis* using electroporation. J Appl Phycol 13: 209–214.

Tseng CK (2001) Algal biotechnology industries and research activities in China. J Appl Phycol 13:375-80.

Tso MOM, Lam TT (1996) Method of retarding and ameliorating central nervous system and eye damage. US Patent #5527533.

Tsubokura A, Yoneda H, Mizuta H (1999) *Paracoccus carotinifaciens* sp. nov., new aerobic Gram-negative astaxanthin-producing bacterium. International J Sys Bacteriol 49, 277–282.

Tsubokura A, Yoneda H, Mizuta H (1999) *Paracoccus carotinifaciens* sp. nov., a new aerobic Gram-negative astaxanthin-producing bacterium. Inter J Syst Bacteriol 49: 277–282.

Turujman SA (1997) Rapid liquid chromatographic method to distinguish wild salmon from aquacultured salmon fed synthetic astaxanthin. J. AOAC Int. 80:622–632.

Tzfira T, Citovsky V (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol 12:121–129.

Uchiumi K (1990) Astaxanthin containing composition. Patent abstract JP2049091.

Ullmann A, Jacob F, Monod J (1967) Characterization by in vitro complementation of a peptide corresponding to an operator-proximal segment of the beta-galactosidase structural gene of *Escherichia coli*. J Mol Biol 24: 339-343.

Urich K (1994) Comparative Animal Biochemistry. Springer Verlag. Germany.

Usha Tripathi, Sarada R, Ravishankar GA (1999) Production of astaxanthin in *Haematococcus pluvialis* cultured in various media. Biores Technol 68:197–9.

Vidhyavathi (2008) Molecular and Biochemical Studies of astaxanthin biosynthesis in

Vidhyavathi R, Venkatachalam L, Sarada R, Ravishankar GA (2008) Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. J Exp Bot 59: 1409–1418.

Vioque A (2007) Transformation of cyanobacteria. In: León R, Gaván A, Fernández E Eds Transgenic Microalgae as Green Cell Factories, Landes Bioscience, Austin, Texas.

Wadstrom T, Alejung P (2001) Oral preparation for the prophylactic and therapeutic treatment of *Helicobacter* sp. infection. Patent US6262316.

Walker TL, Collet C, Purton S (2005a) Algal transgenics in the genomic era. J Phycol 41: 1077-1093.

Walker TL, Purton S, Becker DK, Collet C (2005b) Microalgae as bioreactors. Plant Cell Rep 24:629-64.

Wang CW, Oh MK, Liao JC (1999) Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli.* Biotechnol Bioeng 62:235–241.

Wang X, Willen R, Wadstrom T (2000) Astaxanthin rich algal meal and vitamin C inhibit *Helicobacter pylori* infection in BALB/cA mice. Antimicrobial Agents Chemother 44:2452–2457.

Yamada S, Tanaka Y, Sameshima M, Ito Y (1990) Pigmentation of prawn *Panaeus japonicus* with carotenoids. Aquacul 87:323–330.

Yan Y, Zhu Y, Jiang JG, Song DL (2005) Cloning and sequence analysis of the phytoene synthase gene from a unicellular chlorophyte, *Dunaliella salina*. J Agric Food Chem 53: 1466-1469.

Yokoyama A, Adachi K, Shizuri Y (1995) New carotenoid glucosides, astaxanthin glucoside and adonixanthin glucoside, isolated from the astaxanthin-producing marine bacterium, *Agrobacterium aurantiacum*. Journal of Natural Products 58:1929-1933.

Yuan JP, Chen F (1999) Hydrolysis kinetics of astaxanthin esters and stability of astaxanthin of *Haematococcus pluvialis* during saponification. J Agric Food Chem 47: 31-35.

Yuan JP, Xian DG, Chen F (1997) Separation and analysis of carotenoids and chlorophylls in *Haematococcus lacustris* by high performance liquid

chromatography photodiode array detection. J Agri Food Chem 45:1952–1956.

Zambre M, Terryn N, Clercq JD, Buck SD, Dillen W, Montagu M, Straeten DVD, Angenon G (2003) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta 216:580–586.

Zaslavskaia LA, Lippmeier JC, Kroth PG, Grossman AR, Apt KE (2000) Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. J Phycol 36:379-386

Zaslavskaia LA, Lippmeier JC, Shih C, Ehrhardt D, Grossman AR, Apt KE (2001) Trophic conversion of an obligate photoautotrophic organism through metabolic engineering. Science 292:2073-2075

Zhang DH, Lee YK, Ng ML, Phang SM (1997) Composition and accumulation of secondary carotenoids in *Chlorococcum* sp. J Appl Phycol 9:147–155.

Zhu YH, Jiang JG, Yan Y, Chen XW (2005) Isolation and characterization of phytoene desaturase cDNA involved in the β-carotene biosynthetic pathway in *Dunaliella salina*. J Agric Food Chem 53: 5593-5597.

List of Publications

Papers Published

Kathiresan S, R. Sarada, Sila Bhattacharya and G.A. Ravishankar 2007. Culture media optimization for growth and phycoerythrin production from *Porphyridium purpureum* Biotechnology and Bioengineering 96: 456-463 (Impact Factor – 3.0)

Kathiresan, S., Sarada, R., Arun Chandrashekar and Ravishankar G.A 2009. *Agrobacterium* mediated transformation in green alga *Haematococcus pluvialis*. Journal of Phycology 45:642–649 (Impact factor 2.82)

Kathiresan, S and Sarada R 2009. Towards genetic improvement of commercially important microalga *Haematococcus pluvialis* for biotech applications. Journal of Applied Phycology (DOI: 10.1007/s10811-009-9414-0) (Impact factor 0.8)

Papers Under Preparation

Kathiresan S, R. Sarada, Arun Chandrashekar and G.A. Ravishankar. Cloning and genetic transformation of β-carotene ketolase for carotenoid biosynthesis in *Haematococcus pluvialis.*

Kathiresan S, Sarada R and Ravishankar G.A. Carotenoid profile in the Agrobacterium mediated transformed *Heamatococcus pluvialis.*

Kathiresan S, Sarada R and Ravishankar G.A. Production potential of Total lipids and Polyunsaturated Fatty acids - Eicosapentaenoic acid and Docosahexaenoic acid from *Porphyridium purpureum* through culture media optimization.

Patents

Kathiresan S, Sarada R, Sila Bhattacharya, and Ravishankar GA. An improved culture medium useful for enhancement of Phycobiliproteins in *Porphyridium* spp. 335/DEL/06.

Presentation of research papers

Kathiresan, S and Sarada, R., 2008. Towards genetic improvement of commercially important microalga *Haematococcus pluvialis* for biotech applications. In

11th International Conference on Applied Phycology held on 21-27 June – 2008 at National University of Ireland, Galway, Ireland. P271

Kathiresan S, Sarada R, Sila Bhattacharya, and Ravishankar GA 2008. Effect of media constituents on growth and polyunsaturated fatty acids (PUFA) production from *Porphyridium purpureum*. Presented in 95th Indian Science Congress held during January 3-7, 2008 in Visakhapatnam, India. P2-40

Kathiresan, S and Ramappa H.K (2007). Detection of Sugarcane Yellow Leaf Virus (ScYLV) in Yellow Leaf Disease affected Sugarcane. Presented in 8th Agricultural Science Congress – 2007, organized by National Academy of Agricultural Sciences, New Delhi, held during February 15 – 17 in Ciombatore, India P-101.

Kathiresan, S and Sarada R. 2004. Phycobiliproteins, A Natural colourants from micro alga *Porphyridium* spp. In: 16th Indian Convention of Food Scientists and Technologists-2004 (December 9 and 10) Association of Food Scientists and Technologists, CFTRI, Mysore, India