ELUCIDATION OF THE EXPRESSION AND ROLE OF CAROTENOGENIC GENES IN ASTAXANTHIN PRODUCTION IN A HYPER-PRODUCING MUTANT OF Xanthophyllomyces dendrorhous

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by

ANG FONG SIM

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

℃	Degree Celcius
%	Percent
™	Trademark
>	More Than
-	Negative
~	Approximately
0.5×	0.5 Time
×*g*	Gravitational Force
µg	Microgram (s)
µM	Micromolar (s)
µL	Microlitre (s)
A_{260}	Absorbance at 260 nm Wavelength
A_{280}	Absorbance at 280 nm Wavelength
AcaT	Acetyl-CoA Acetyltransferase,
A:T	Adenosine : Thymine
ATCC	American Type Culture Collection
ATM	Ataxia Relangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related
Au	Absorbance Unit (s)
BLAST	Basic Local Alignment Search Tool
bp	Base Pair (s)
C	Carbon
C:G	Cytosine : Guanine
cDNA	Complementary DNA
CPR	Cytochrome P450 Reductase
Ct	Cycle Threshold
Da	Dalton (s)
DCW	Dry Cell Weight
ddH_2O	Double Distilled Water

DMAPP	Dimethylallyl Pyrophosphate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleoside Triphosphates
dsDNase	Double-Strand Specific DNase
dT	Deoxythymine
EDTA	Ethylenediaminetetraacetic Acid
EMS	Ethyl Methanesulfonate
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
g	Gram (s)
GFP	Green Fluorescent Protein
GGPP	Geranylgeranyl Pyrophosphate
h	Hour (s)
H_2O	Water
HCl	Hydrogen Chloride
HDCO	3-hydroxy-4-ketotorulene
HDL	High Density Lipoprotein
HmgS	3-Hydroxy-3-Methylglutaryl Coenzyme-A Synthase
HmgR	3-Hydroxy-3-Methylglutaryl Coenzyme-A Reductase
HPLC	High Performance Liquid Chromatography
H. pluvialis	*Haematococcus pluvialis*
H. pylori	*Helicobacter pylori*
IPP	Isopentenyl-Pyrophosphate
kb	Kilobase (s)
kDa	Kilodalton (s)
L	Litre (s)
LDL	Low Density Lipoprotein
M	Molar (s)
MeOH	Methanol
mg	Milligram (s)

mg/g	Milligram per Gram (s)
mg/mL	Milligram (s) per Millilitre (s)
$MgCl_2$	Magnesium Chloride
Mig1	MADS-box Transcription Factor Mig1
min	Minute (s)
mL	Millilitre (s)
mM	Millimolar (s)
mm	Millimeter (s)
MNNG	N-Methyl-N′-Nitro-N-Nitrosoguanidine
mRNA	Messenger RNA
NaCl	Sodium Chloride
NAD^+	Nicotinamide Adenine Dinucleotide
NADPH	Dihydronicotinamide-adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
ng	Nanogram (s)
ng/µL	Nanogram (s) per Microlitre (s)
nm	Nanometer (s)
NTC	No Template Control
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
P. rhodozyma	*Phaffia rhodozyma*
pmol	Picomole (s)
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per Minute
RT	Reverse Transcription
SD	Standard Deviation

sec	Second (s)
TBE	Tris-Borate-EDTA
TCA	Tri-Carboxylic Acid
U	Unit (s)
UHQ	Ultra-high Q
USA	United States of America
UV	Ultraviolet
UVA	Ultraviolet A
UV-vis	Ultraviolet-visible
V	Volt (s)
v/v	Volume per Volume
w/v	Weight per Volume
X. dendrorhous	*Xanthophyllomyces dendrorhous*
YM	Yeast Malt
µg	Microgram
µg/g	Microgram per Gram
µg/mL	Microgram per Millilitre
µL	Microlitre
µm	Micrometer
µM	Micromolar
A (amino acid)	Alanine
D (amino acid)	Aspartic Acid/ Aspartat
E (amino acid)	Glutamic Acid/ Glutamate
H (amino acid)	Histidine
K (amino acid)	Lysine
P (amino acid)	Proline
Q (amino acid)	Glutamine
R (amino acid)	Arginine
S (amino acid)	Serine
V (amino acid)	Valine

ELUSIDASI PENGEKSPRESAN DAN PERANAN GEN-GEN KAROTENOGENIK DALAM PENGHASILAN ASTAXANTHIN OLEH MUTAN PENGHASIL HIPER *Xanthophyllomyces dendrorhous*

ABSTRAK

Astaxanthin merupakan satu pigmen xantofil merah yang digunakan secara komersil dalam akuakultur sebagai aditif makanan haiwan dan dalam industri farmaseutikal berdasarkan sifat antioksidannya yang kuat. Astaxanthin sintetik bukan sahaja dibataskan oleh ciri-cirinya, malah keperluan proses penghasilan dan juga peraturan keselamatan yang ketat terhadap bahan kimia sintetik sebagai aditif makanan. Ini telah mendorong penghasilan astaxanthin semulajadi secara mikrobial. *Xanthophyllomyces dendrorhous* mempunyai ciri-ciri yang diingini dan nilai komersial sebagai sumber diet bagi astaxanthin semulajadi, tetapi ia mempunyai kandungan astaxanthin yang rendah secara relatif. Pelbagai cara telah digunakan untuk meningkatkan hasil astaxanthin termasuk pengoptimuman keadaan pertumbuhan, mutagenesis secara rawak dan kejuruteraan genetik. Tujuan kajian ini adalah untuk menjana satu mutan penghasil hiper *X. dendrorhous* DSM 5626 melalui mutagenesis kimia menggunakan etil metansulfonat (EMS) dan N-metil-N'-nitro-N-nitrosoguanidin (MNNG). Tahap ekspresi dan perubahan jujukan nukleotida bagi gen-gen karotenogenik dalam mutan dikaji dengan Real-Time PCR dan PCR masing-masing, dan dikaitkan dengan penghasilan karotenoid. Satu mutan dengan kebolehan penghasilan astaxanthin yang lebih tinggi berjaya dipencilkan daripada mutagenesis MNNG melalui pengskrinan β-ionone, dengan jumlah

kandungan karotenoid sebanyak 602.36 μg/g berbanding dengan 285.71 μg/g bagi strain jenis liar, iaitu peningkatan sebanyak 110.83%. Berbanding EMS, rawatan dengan MNNG menghasilkan kadar kematian yang lebih tinggi dalam sel-sel penerima dan lebih efektif dalam menginduksikan mutan berpigmen dengan warna merah gelap dan saiz koloni yang lebih besar menunjukkan kandungan astaxanthin yang lebih tinggi dan pertumbuhan sel yang lebih baik, dengan kadar mutasi terbalik yang lebih rendah. Tiada mutasi terbalik diperhatikan dalam mutan M34 dalam kajian kestabilan dan M34 didapati stabil dalam pertumbuhan sel dan penghasilan astaxanthin sepanjang seluruh tempoh penyelidikan ini. Dalam HPLC fasa terbalik, puncak utama bagi ekstrak M34 telah dikenalpasti sebagai astaxanthin berdasarkan masa retensi dan spektrum penyerapan bebanding dengan standard sahih astaxanthin. Keputusan pengekspresan gen menggunakan Real-Time PCR menunjukkan bahawa kedua-dua gen *crtE* and *crtS* memberikan pengekspresan yang lebih tinggi berbanding dengan strain jenis liar sepanjang kitaran penghasilan, yang paling tinggi sebanyak 1.3 and 3.8 kali ganda masing-masing. Sementara gen-gen *idi, crtYB, crtI* and *crtR* tidak menunjukkan korelasi bermakna antara tahap transkripsi mRNA dengan penghasilan karotenoid. Corak pengekspresan gen adalah serupa apabila M34 dikultur dengan sama ada glukosa atau sukrosa sebagai sumber karbon untuk kesemua enam gen-gen *crt*. Selain itu, kesemua enam gen-gen karotenogenik mempamerkan sejumlah 38 perubahan nukleotida selepas mutasi. Walaupun kebanyakan perubahan nukleotida membawa kepada mutasi senyap, satu mutasi *missense* didapati di gen *idi* (E→A) dan gen *crtI* (H→Q), manakala dua mutasi *missense* dijumpai dalam gen *crtE* (K→R; S→D) dan gen *crtR* (S→V; Q→P). Kajian ini akan membantu dalam memahami peranan gen-gen carotenogenik dalam penambahbaikan penghasilan karotenoid.

ELUCIDATION OF THE EXPRESSION AND ROLE OF CAROTENOGENIC GENES IN ASTAXANTHIN PRODUCTION IN A HYPER-PRODUCING MUTANT OF *Xanthophyllomyces dendrorhous*

ABSTRACT

Astaxanthin is a red xanthophyll pigment which is commercially used in aquaculture as feed additive and in pharmaceutical industries due to its strong antioxidant properties. Synthetic astaxantin is limited by its properties, production requirements and also the strict regulations concerning the safety of synthetic chemicals as food additives. This has led to the alternative microbial production of natural astaxanthin. *Xanthophyllomyces dendrorhous* has desirable properties and commercial value as a dietary source of natural astaxanthin, but it has relatively low content of astaxanthin. Various methods had been applied to improve the astaxanthin yield including optimization of culture conditions, random mutagenesis and genetic engineering. The aim of this study was to generate a stable astaxanthin hyperproducing mutant of *X. dendrorhous* DSM 5626 through chemical mutagenesis using ethyl methanesulfonate (EMS) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). The expressions levels and changes of nucleotide sequence of carotenogenic biosynthesis genes were studied by Real-Time PCR and PCR resepectively, and correlated to the carotenoid production. A hyperproducing mutant, M34, was successfully isolated from MNNG mutagenesis through β-ionone screening, with a higher total carotenoid content of 602.36 μg/g compared to 285.71 μg/g in wild type strain, which was a 110.83% increment. Compared to EMS, MNNG treatment led to higher

lethality in recipient cells and was more effective in inducing the pigmented mutants with intense red colour and bigger colony indicating higher astaxanthin content and cell growth, with lower rate of reverse mutation. No reversion was observed in M34 mutant during stability test and M34 mutant was found to be stable in its cell growth and astaxanthin production throughout the whole research period. In reversed-phase HPLC, the major peak in M34 mutant extract was identified as astaxanthin based on the retention time and absorption spectrum compared to authentic astaxanthin standard. Gene expression results by Real-Time PCR showed that both *crtE* and *crtS* genes showed significantly higher expressions relative to wild type throughout the production cycle, the highest being 1.3 and 3.8 folds, respectively. Meanwhile *idi*, *crtYB*, *crtI* and *crtR* genes did not show meaningful correlation between their mRNA transcription levels and carotenoid production. The gene expression patterns were similar when M34 mutant was cultured with either glucose or sucrose as carbon source for all six *crt* genes. Besides, all six carotenogenic genes exhibited a total of 38 nucleotide changes after mutation. Although most of the nucleotide changes led to silent mutations, there was a missense mutation found in *idi* gene (E→A) and *crtI* gene (H→Q), while two missense mutations occurred in *crtE* (K→R; S→D) and *crtR* genes (S→V; Q→P). This study will help in understanding the role of the carotenogenic genes in further improvement of carotenoid production.

CHAPTER 1

INTRODUCTION

1.1 Background

Astaxanthin, also known as 3,3'-dihydroxy-β,β-carotene-4,4'-dione, is a ketocarotenoid synthesized by several species of plants and microorganisms including algae, bacteria, and fungi. It is a valuable red xanthophyll pigment with a high biotechnological interest, predominantly in the aquaculture, poultry industry and cosmetology, as a pigmentation source and in the food and pharmaceutical industries mainly for its outstanding antioxidant properties and other beneficial effects on human health (Ranga Rao *et al.*, 2014).

Astaxanthin is one of the most important pigments in the nature as it is responsible for the orange-red colour in microorganisms and marine species such as salmon, trout, lobster, shrimp and other seafood besides bird species including flamingo and quail (Chimsung *et al.*, 2014). However, these marine species cannot produced the astaxanthin *de novo*, but obtain it from their diet (Bon *et al.*, 1997; Kamath *et al.*, 2008). Thus, astaxanthin is conventionally used as feed additives for aquaculture and poultry industry. In aquaculture and poultry industry, it is used as a colouring agent to increase the organoleptic value and characteristics preferred by the consumer especially in lobster, shrimp, salmon, fish eggs and egg yolk, since the bright colour could be more attractive. It was later found that astaxanthin possesses strong antioxidant properties, which has been reported to outclass other carotenoids, including β-carotene and α-tocopherol (Lauritano & Ianora, 2016). Many studies have shown that astaxanthin is capable in promoting human health through

the prevention and treatment of various diseases including cancers, inflammatory diseases, diabetes, cardiovascular diseases, gastrointestinal diseases, neurodegenerative diseases, eye diseases and skin diseases. Consequently, astaxanthin is used as an ingredient in nutraceutical, medicinal and food products (Rodríguez-Sáiz *et al.*, 2010; Tanaka *et al.*, 2012). Due to its biological functions in various aspects, astaxanthin obtains significant economic value and a growing global commercial market, thus attracts the interest and attention from researchers and industries to put in more efforts in astaxanthin-related research.

Generally, astaxanthin production has been performed through chemical synthesis and this synthetic astaxanthin covers most of the world markets (Britton *et al.*, 1996). Compared to natural astaxanthin, the chemical synthesis is recognized as the lowest cost production process of astaxanthin, which very likely to be its advantage to remain as the primary pigment source as animal feed (Schmidt *et al.*, 2011). However, the production processes rely on total synthesis approaches, involving a rather long series of chemical steps ending with a C20+C20 reaction or a C19+C2+C19 reaction. Chemical synthesis of astaxanthin is dependent on petrochemicals as raw material which is not environmental friendly and sustainable. Synthetic carotenoids cannot be absorbed effectively by the body compared with other natural sources due to difference in isomer content, where astaxanthin from natural sources has optically pure chirality, while synthetic astxanthin consists of a mixture of enantiomers (3R,3'R and 3S,3'S) and mesoform (3R,3'S and 3S,3'R) (Lorenz & Cysewski, 2000). Furthermore, the chemical product cannot be labeled as a natural product, and thus does not penetrate the higher value and fastgrowing market segments. The consumption of synthetic pigments at high dose ranges has eventually been warned

by Food and Drug Administration since it might cause precarious effects to human health (Kläui & Bauernfeind, 1981). Only the astaxanthin obtained from algal and yeast are approved for human consumption, while the synthetic form is used only for animal feed purpose. This may be due to the insecure usage of petrochemicals for astaxanthin production since it was reported to be cancer-causing (Newsome, 1986). All these coupled with increasingly stringent regulations regarding the safety of chemicals to be used as food additives results in the preference for natural carotenoid products over synthetic pigments. However, there are also limitations for the production of astaxanthin by biological systems in industries such as the low astaxanthin yields in wild type strains and extraction methods which are costly compared to synthetic astaxanthin (Johnson & Schroeder, 1996; Canizares-Villanueva *et al*., 1998). Table 1.1 shows the sustainability comparison of different astaxanthin production methods.

Table 1.1 Sustainability comparison of different astaxanthin production methods (Nguyen, 2013)

Per kg of Astaxanthin	Economical				Environmental		Societal		
Production Method	Raw Materials Cost ($)	Land Usage (sq. km)	Energy Usage (kWh)	Energy Cost ($)	Waste Water *	Emissions (Air)*	Cost($)	Human Consumption?	ORAC
Chemical Synthesis	40	10	170	26	0	2	2,000	No	33%
Yeast Fermentation	140	20	1,062	160	9	5	2,500	Most	66%
Algal Synthesis	164	25	796	120	2	9	>7,000	Yes	100%

The concerns on chemical additives in foods and feed from the public and government have triggered research on natural sources to produce astaxanthin by biotechnology (Echavarri-Erasun & Johnson, 2004). There are only a few microorganisms reported to have the ability to synthesize astaxanthin, including the basidiomycetous yeast, *Phaffia*

rhodozyma; the green alga, *Haematococcus pluvialis* and the bacteria, *Agrobacterium aurantiacum, Paracoccus* sp., *Chlorococcum, Chlorella zofingiensis, Brevibaterium* and *Mycobaterium lacticola* (Goswami *et al.,* 2010). The heterobasidiomycetous yeast, *X. dendrorhous* is currently known as one of the most potential microorganism for synthesis of astaxanthin at industrial scale besides *H. pluvialis* (Ranga Rao *et al.,* 2010).

Currently the *H. pluvialis* is the primary contributor to astaxanthin market for human consumption (Kidd, 2011). However, *H. pluvialis* has limitations in its own characteristics, such as its tough cell wall that traps the astaxanthin, complicating the extraction process and yield of production. At the same time, disadvantages such as long growing period, low cell densities, ease of contamination by other bacteria and even protozoa and finally susceptibility to adverse weather conditions, have magnified the weakness of the algae, especially during the scale up for mass industrial production (Retamales *et al.*, 1998; Ukibe *et al.*, 2008; Rodr ́guez-S á̧z *et al.*, 2010).

Due to the shortcomings of the algae, researchers see the potential of *X. dendrorhous* to replace *H. pluvialis* as the main source, as the yeast produces astaxanthin as its principle carotenoid while having high metabolism and high cell densities can be achieved in fermenters. Besides rapid breeding, short growth cycle, and fully developed fermentation process (Bhatt *et al.*, 2013), *X. dendrorhous* is also able to grow on molasses, enzymatic wood hydrolysates, corn wet-milling co-products, corn syrup, grape juice and date juice, which were by-products from agricultural activities (An *et al.,* 2001; Ramirez *et al.*, 2006; Zheng *et al.*, 2006). This gives the yeast more advantages compared to *H. pluvialis*.

However, the disadvantage of *X. dendrorhous* compared to *H. pluvialis* is its rather low yield of astaxanthin in wild type strains with specific production at 200–400 mg/g of dry yeast. The commercialized grown alga cells contains astaxanthin up to 3% of their dry cell mass, while astaxanthin in wild type strains of *X. dendrorhous* is below 0.1% (Johnson & Schroeder, 1996). Various methods had been attempted to improve the yield of astaxanthin in *X. dendrorhous*, including optimization of culture condition such as modifying the concentration of glucose or carbon/nitrogen ratio, the use of low cost raw materials, temperature, pH, oxygen content, selective addition of chemical precursors such as mevalonic acid, ethanol, acetic acid and lycopene, illumination with white and UV light, strain improvement through genetic engineering and random mutagenesis (Schmidt *et al.*, 2011). This will help to make *X. dendrorhous* more competitive in becoming the main source of astaxanthin for large scale production in industrial processes.

1.2 Significance of the Study

Astaxanthin is a valuable red xanthophyll pigment with a high biotechnological interest, mainly in the aquaculture, poultry industry and cosmetology, as a pigmentation source and as an antioxidant agent in the food and pharmaceutical industries due to its potent antioxidant properties and other beneficial effects on human health. This contributes to its high market value and growing demand. Traditionally this colourant and anti-oxidant in the market has been produced by chemical synthesis using petroleum-derived raw materials. Chemical synthesis of astaxanthin is disadvantaged by its dependence on petrochemical which is not environmental friendly and sustainable as raw material, other than involving a rather long series of chemical steps in the synthesis reaction. Apart from

that, strict regulations regarding the safety of synthetic chemical as food additives have been implemented from time to time and the poor absorption of synthetic astaxanthin compared with that from biological sources may give preference on natural astaxanthin products over synthetic pigments.

The current growing demand for natural pigments favours the use of natural sources. Thus, *H. pluvialis* and *X. dendrorhous* become best choices as the main source for astaxanthin.

However, *H. pluvialis* has its limitation during mass production such as its tough cell wall complicating the extraction process and yields. It is also disadvantaged by slow growing rate, low cell densities, ease of contamination by other bacteria and protozoa, and vulnerability to the unfavorable weather conditions. As an alternative, *X. dendrorhous* produces astaxanthin as the principle carotenoid, while having high metabolism, short growth cycle, high cell density and mature fermentation process as well as being able to grow on by-products from agriculture. This gives the yeast more advantages compared to *H. pluvialis* but it is limited by its rather low yield of astaxanthin in wild type strains with specific production at 200–400mg/g of dry yeast. Various approaches have been attempted to increase the astaxanthin production of *X. dendrorhous* including optimization of fermentation methodologies, application of chemical stimulants, genetic engineering and mutagenesis.

Despite the rapid developments in the field of carotenoid biosynthesis, there are only few reports on the genes or enzymes that mediate astaxanthin metabolism, especially the

carotenogenic genes. It is of utmost importance to generate knowledge on the astaxanthin biosynthetic pathway at the level of biosynthetic genes and understand the role of these carotenogenic genes in improving and/or modifying astaxanthin biosynthetic pathway. Therefore, it is our interest to obtain a hyperproducing *X. dendrorhous* mutant by chemical mutagenesis, determine the possible mutations in its carotenogenic genes involved in the biosynthesis of astaxanthin and their transcription levels in the mutant and subsequently the possible influence of carbon sources on their transcription levels. By characterizing the possible alteration in these carotenogenic genes in a mutant and elucidating the transcription levels of these carotenogenic genes in relationship to astaxanthin biosynthesis, we aim to identify gene targets that can be genetically manipulated to improve astaxanthin production. The effect of different carbon sources on the mRNA expression of the carotenogenic genes was studied. Maximum carotenoid biosynthesis may be achieved through over-expression of *crt* genes in combination with advantageous cultivation conditions. The mutant *crt* genes can be used to develop new strains of *X. dendrorhous* with enhanced astaxanthin content or for the production of new xanthophylls. The findings may allow a rational amplification of astaxanthin production, either in *X. dendrorhous* or in alternative microbial systems for large scale carotenoid production. This all-round genetic approach constitutes the crucial steps towards the development of *X. dendrorhous* as a cell factory for carotenoid production which may be competitive economically with chemical synthesis.

1.3 Objectives

The aim of this study is to understand and identify the role of carotenogenic genes involved in the production of astaxanthin in a hyperproducing mutant of *X. dendrorhous* and the objectives of the study are:

1. To generate a *X. dendrorhous* mutant with higher astaxanthin production capacity by chemical mutagenesis.
2. To elucidate the transcription levels of the carotenogenic genes in relationship to astaxanthin biosynthesis.
3. To study the effect of mutagenesis on the nucleotide sequences of carotenogenic genes in the mutant.

CHAPTER 2

LITERATURE REVIEW

2.1 Carotenoids

2.1.1 Background

Carotenoids are lipid-soluble pigments with colours, usually in orange, red or yellow colour that are able to absorb light in the range of 300-600 nm of wavelength. They are important natural pigments occur in nature throughout the photosynthetic systems of plants and phototrophic microorganisms including algae, yeast, cyanobacteria and fungi, which are the main sources for isolation and production of carotenoids at big scale (Blanco *et al.*, 2007; Nelis & DeLeenheer, 1991; Bourgaud *et al.*, 2001; Olaizola, 2003). Animals can only obtain carotenoids from their diet since they cannot synthesize carotenoids *de novo* (Britton *et al.*, 2009).

Carotenoids consist of isoprene residues and a C40 hydrocarbon polyene chain of conjugated double bonds (Sandmann & Misawa, 2002; Alcaíno *et al.*, 2016a). The polyene system not only gives carotenoids their distinctive molecular structure, but also their chemical properties and light-absorption characteristics. The number of conjugated double bonds and functional groups present in the structure of the carotenoids are contributing to their light absorption ability. The presence of double bonds in the polyene chain allows the existence of two configurations in carotenoids; as geometric isomers cis or trans, where trans-isomers are more stable than the cis-isomers in the aspect of

thermodynamic. Thus, most carotenoids in nature are found typically in all trans-isomers (Britton, 1995; Miller *et al.*, 2014). Structurally and functionally, carotenoids can be classified into carotenes (hydrocarbon derivative) and xanthophylls (oxygenated derivative by addition of oxygen-containing functional groups to obtain cyclic or acyclic-xanthophylls). In xanthophyll, oxygen can be present as OH groups, which can be found in zeaxanthin, or as oxy-groups, which are found in cantaxanthin, or combination of both such as in astaxanthin. β-carotene is an example of carotene carotenoid and astaxanthin is a xanthopyll carotenoid (Schwab, 2011). Phytoene is produced as the first carotenoid in the carotenoid biosynthesis pathway, formed by condensation of two geranyl-geranyl diphosphate (GGDP) molecules (C20 hydrocarbon), which results in a basic symmetrical acyclic C40 hydrocarbon backbone structure. This carotenoid is important as the precursor to undergo enzyme-mediated biochemical reactions for the synthesis of other natural carotenoids.

The presence of polyene system in carotenoids contributes to its distinctive characteristics in molecular structure, chemical properties and ability to absorb light. The molecule of carotenoid provides antioxidant properties when it introduces a ring structure joined to its carbon chain (DellaPenna & Pogson, 2006). Other than their significant antioxidant activities and important role in inhibiting the onset of chronic diseases such as cardiovascular disease, diabetes and age related macular degeneration, carotenoids are involved in numerous other biological functions including vitamin A synthesis, gap junction communication, immune system modulation and antitumor activity (Nishino *et al.*, 2002; Minatel *et al.*, 2017). The discoveries of beneficial properties associated to these

bioactive phytonutrients have brought great interest in production of carotenoids in diverse structures for various applications. The interest in carotenoids has caused in the global carotenoid market value to increase considerably from $1.5 billion in 2014 to an expected $1.8 billion in 2019, with a compound annual growth rate (CAGR) of 3.9% (BCC Research, 2015).

Approximately 700 naturally occurring carotenoids are identified and over 600 different chemical structures have been described, but just a few of them are of economical importance and utilized by industry commercially as colourants or health and dietary supplements (Takaichi *et al.*, 1996; Amorim-Carrilho *et al.*, 2014; Arvayo-Enriquez *et al.*, 2013; Merhan, 2017). Only a few carotenoids can be produced commercially, such as β-carotene, lycopene, astaxanthin, canthaxanthin, capsanthin, lutein, β-apo-8-carotenal, β-apo-8-carotenal-ester, through processes such as chemical synthesis and fermentation (Johnson & Schroeder, 1996). Among them, astaxanthin is the most valuable carotenoid to be studied due to its outstanding antioxidant and pigmentation properties.

2.1.2 Astaxanthin

2.1.2(a) Structures and Chemical Forms of Astaxanthin

Astaxanthin is a carotenoid with a chemical formula $C_{40}H_{52}O_4$, with a molecular mass of 596 Da (Goswami *et al.*, 2010). It is a xanthophyll because of the presence of oxygen atoms besides carbon and hydrogen atoms. Astaxanthin is composed of eight isoprenoid units in the polyene chain, with two terminal rings that contain oxygen (Ducrey Sanpietro

& Kula, 1998; Alcaino *et al.*, 2014). It is derived from a central phytoene "backbone" of 40 carbon atoms, which is linked by single and double bonds alternatively, which is terminated on either end by ionone rings. At the 3, 3' positions of the β-ionone ring, there are two asymmetric carbons with the presence of hydroxyl group (-OH) on either end of the molecule (Figure 2. 1).

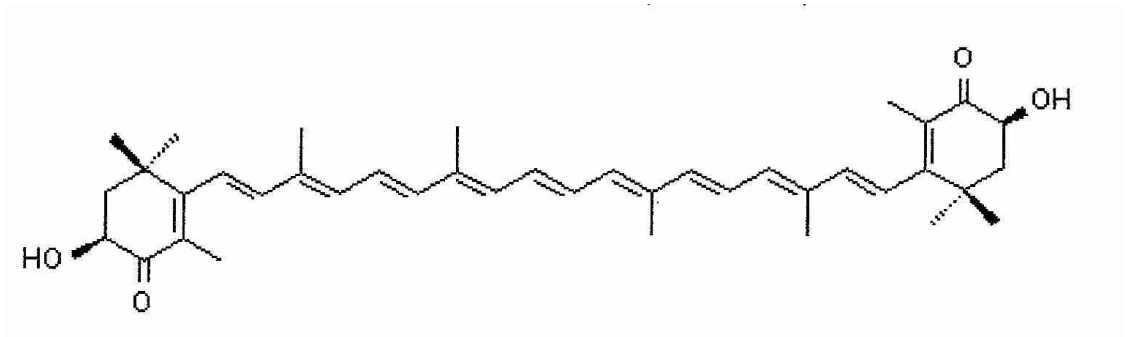

Figure 2.1 Chemical structure of astaxanthin (Urich, 1994)

This structure plays a role in the transfer and dissipation of energy, and at the same time contributes to the characteristic colours of the carotenoids. The presence of oxygen-containing functional groups on these rings not only decides the classification of astaxanthin as a xanthophyll, but also allows astaxanthin to be esterified by the presence of these hydroxyl and keto groups and contribute to its higher antioxidant activity and its high polarity compared to other related carotenoids (Guerin *et al.*, 2003; Kroll, 2006). The arrangement of thirteen conjugated double bonds in alternating single-double bonds contribute to strong antioxidant properties for scavenging activity of reactive oxygen species (ROS) and neutralizing the free radicals (Miki, 1991; Vershinin, 1999; Gomez Gomez & Estebanez, 2016; Haq *et al.*, 2016).

In nature, astaxanthin exists in different isomeric forms which can be categorized accordingly to geometric isomers (cis/trans), stereoisomers (R/S), and free or esterified forms (Higuera-Ciapara *et al.*, 2006). Each double bond found in the polyene chain in the astaxanthin molecule allow the existence of the molecule in two configurations: cis or trans, which belong to geometrical isomers as shown in Figure 2.2.

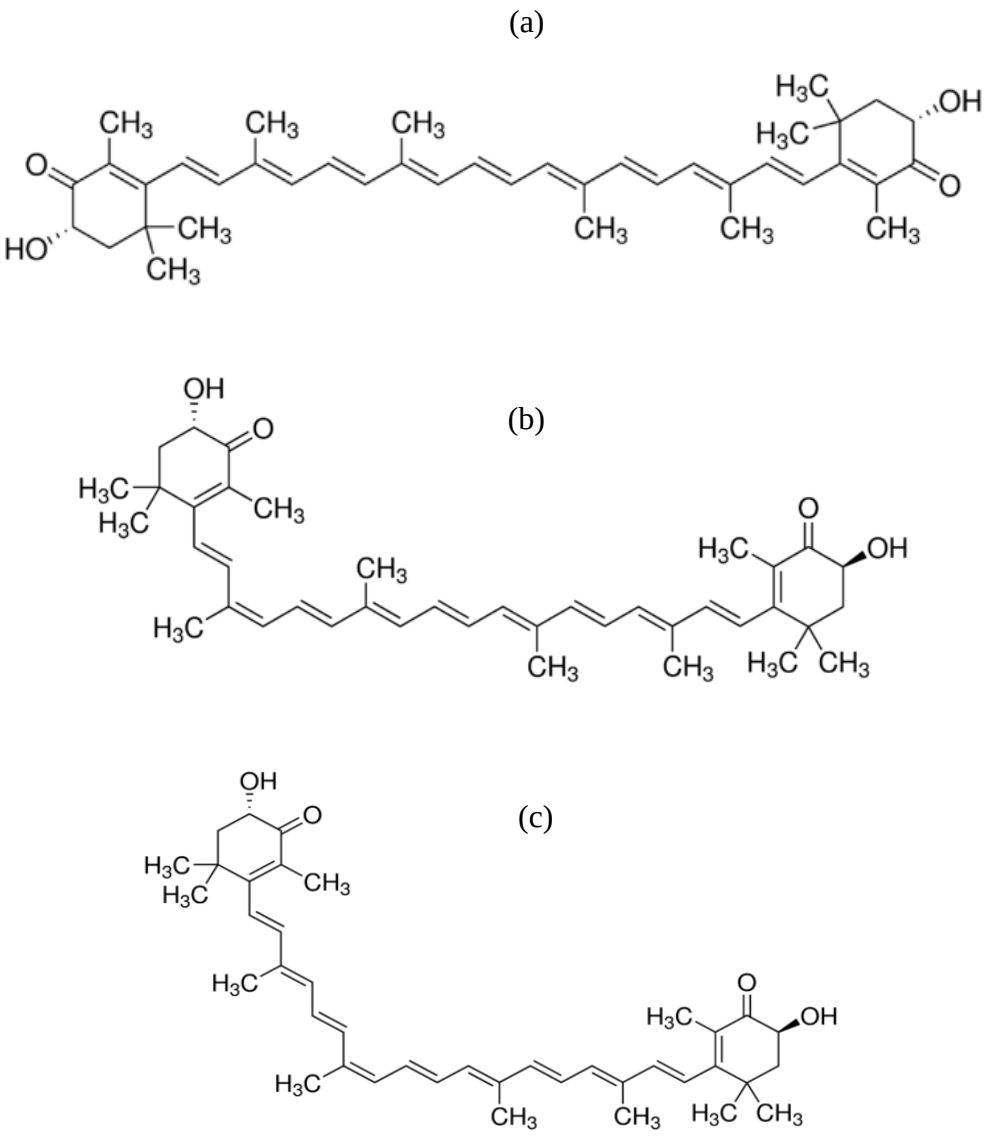

Figure 2.2 Geometrical isomers of astaxanthin. (a) All-trans-astaxanthin; (b) 9-cis-astaxanthin; (c) 13-cis-astaxanthin (Wang *et al.*, 2008)

Majority of natural carotenoids are principally all trans-isomers. Trans-isomer of astaxanthin is the predominant geometric isomer found in *H. pluvialis* (Yuan & Chen, 1998). Because of the steric reason, the isomerization of trans-astaxanthin into cis-trans mixtures, especially 9-cis and 13-cis, usually cannot be prevented. Among the geometric isomers, it was found that astaxanthin isomer in cis form, especially 9-cis astaxanthin, has a higher antioxidant capability than that of the all-trans isomer, which can be arranged in the order of 9-cis > 13-cis > all-trans (Liu & Osawa, 2007). In addition, compared to trans-astaxanthin that consists of shorter chain length, the cis-astaxanthin is able to accumulate especially in blood plasma (Bohn, 2008).

Isomers of astaxanthin molecule may present in three optical stereo configurations: two enantiomers (3R, 3′ R and 3S, 3′S) and a meso form (3R, 3′ S), as each astaxanthin molecule consists of two chiral centers in C-3 and C-3′, as shown in Figure 2.3.

The 3S, 3′ S is the most abundant form in nature. >98% of astaxanthin produced by *X. dendrorhous* is in (3R,3'R) form and microalgae *H. pluvialis* synthesizes only (3S, 3'S) isoform (Hussein *et al.*, 2006). Meanwhile synthetic astaxanthin like Carophyll Pink (La Roche) consists of a racemic mixture with the two enantiomers (3R,3'R) and (3S, 3'S) equally at 25% and 50% of the meso form.

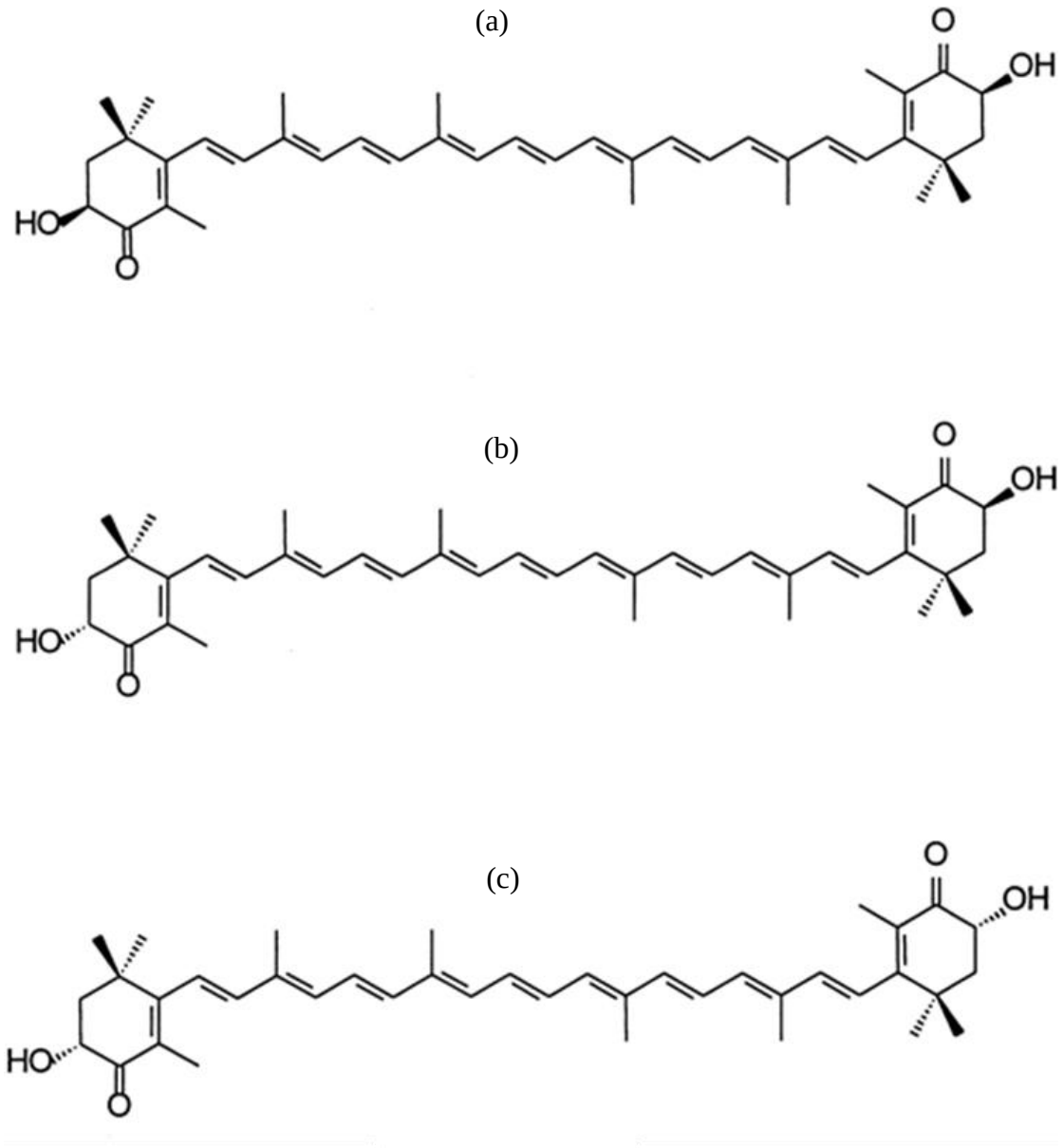

Figure 2.3 Optical stereoisomers of astaxanthin. (a) (3S,3'S)-all-trans-astaxanthin; (b) (3R,3'S)-all-trans-astaxanthin; (c) (3R,3'R)-all-trans-astaxanthin (Osterlie *et al.*, 1999)

Astaxanthin exists in three forms: free, monoester and diester (Torrissen *et al.*, 1989). Free astaxanthin particularly undergoes oxidation easily. Thereupon, in its natural state, astaxanthin is usually either conjugated with proteins or converted into mono- or diester forms by chemically bound to one or two fatty acids. On each of the terminal ring of

astaxanthin, there is a free (unreacted) hydroxyl (OH) groups. These allow astaxanthin molecule to exist in free form, or can be esterified to form an ester. Addition of a fatty acid to form an ester leads to higher hydrophobicity on the esterified end of the molecule, where the strength of hydrophobicity (difficulty in dissolving in water) in different form can be observed in the sequence of diesters>monoesters>free. Astaxanthin from different organisms exhibited different compositions of the free form and esterified forms as shown in Figure 2.4.

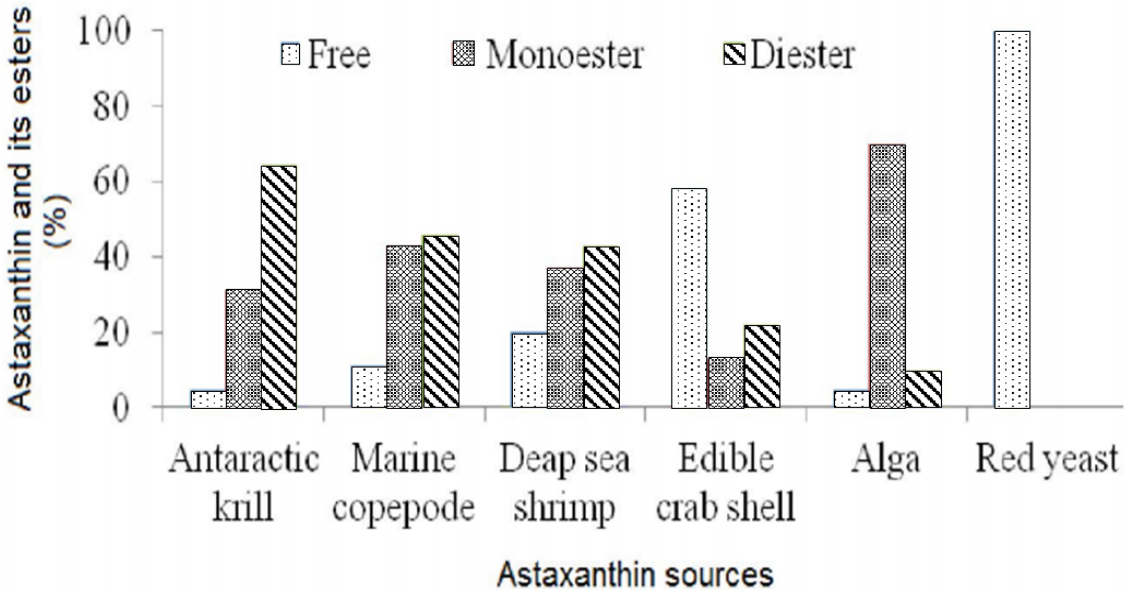

Figure 2.4 Astaxanthin and its esters from various sources (Lorenz, 1999)

Although xanthophyll esters seem to be of low bioavailability, but contradicting findings were also reported. Sugawara *et al.* (2009) suggested that the presence of enzymatic activity after intestinal absorption led to the esterification of consumed xanthophylls and followed by the incorporation of xanthophyll esters into the lipid core in chylomicron before being carried into various tissues of the body. The esterification of xanthophylls

into highly nonpolar products could also contribute in the protection of intestinal cells from the cytotoxic effects. Thus, the accumulation of astaxanthin in *H. pluvialis* in ester form might be an added advantage to provide positive influence to the higher bioavailability of astaxanthin (Ranga Rao *et al.*, 2010).

2.1.2(b) Analysis of Astaxanthin

Multiple techniques, including spectrophotometry, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR), have been developed to analyze and characterize the carotenoids profile, along with the chemical structures, for various specific species of organisms. Among them, UV spectrophotometry and HPLC are commonly employed for routine analysis of bulk samples.

Spectrometric analysis of *X. dendrorhous* extracts showed that it has a characteristic UV–Vis spectrum. The spectrum showed a straight peak with a maximum absorbance at 309-310 nm, and a distinctive broad spectrum band with no obvious absorption peaks and a maximum at 474-477 nm, corresponding to mycosporine-glutaminol-glucoside (MGG) and astaxanthin respectively (Figure 2.5) (Tognetti *et al.*, 2013).

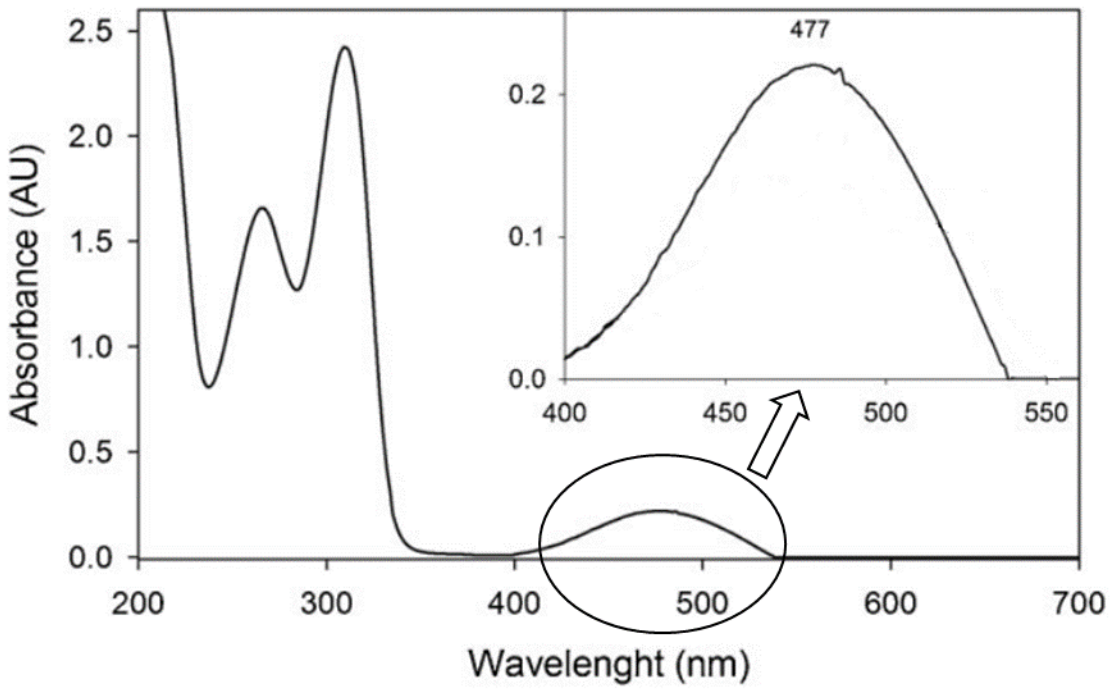

Figure 2.5 UV-visible spectrum of *X. dendrorhous* carotenoid extracts; AU: absorbance unit (modified from Tognetti *et al.*, 2013)

High-performance liquid chromatography is a very sensitive analysis and can elucidate the composition and quantity of carotenoids in an extract or sample. In most cases, reversed-phase high-performance liquid chromatography (RP-HPLC) is used for separation of carotenoids. RP-HPLC separates the molecules based on the hydrophobicity of molecules, in its interaction with a hydrophobic matrix. In an aqueous buffer, molecules are bound to the hydrophobic matrix, which plays the role as stationary phase, and eluted from the matrix by mobile phase which is composed of a gradient of organic solvent (non-polar). The hydrophobic matrix usually built up by attachment of spherical silica beads (3-5 micron) with linear octadecane groups (C_{18}) to the surface through formation of covalent bonds. The surface area of the beads which is available for binding are usually increase by the presence of the porous. The C_{18} groups are very hydrophobic but in highly

polar solvent such as water can bind to polar molecules such as charged peptides. The name "reversed phase" is originated from the opposite technique of "normal phase" chromatography which involves the separation of molecules through their interaction with a polar matrix (silica beads without octadecane groups attached) in the presence of a non-polar solvent.

Two conditions can be applied during the elution process, either isocratic conditions where the organic solvent has constant concentration throughout the experiment, or by gradient elution where there is an increment in the amount of organic solvent over a period of time. Hence, the solutes are eluted in the order of low to high molecular hydrophobicity strength (Aguilar, 2004). RP-HPLC is an excellent technique especially for the analysis of proteins and peptides with the advantages of its forceful resolution that can be achieved under a wide range of conditions in chromatographic for very closely related molecules as well as structurally different molecules. In addition, the experiment could be done with the manipulation of chromatographic selectivity through the modification in mobile phase characteristics. Moreover, it also has high recoveries that lead to high productivity. RP-HPLC also has sorbent materials which are stable under a wide range of mobile phase conditions that leads to excellent reproducibility for repeated separations carried out over a long period of time (Aguilar & Hearn, 1996; Mant & Hodges, 1996).

Absorbance spectra of carotenoids are important criteria for their identification. The properties of spectra of each carotenoids are determined by the basic structure of carotenoids and some of the substituents (Britton, 1995). For astaxanthin, the spectrum is

not affected by 3-OH groups. On the other hand, a 4-keto group in conjugation with the polyene chain causes a shifting of the bell-shaped spectrum towards higher absorbance wavelength. The presence of second keto group at position 4' resulted in a maximum absorbance of astaxanthin in the range of 470-477 nm in various reports. In *X. dendrorhous*, most of the carotenoids have a 3-HO-4-keto-β-ionone end group. Increment in the polarity will lead to decrease of the mobility of a compound in absorption chromatography on stationary phases like silica or reduce the retention time in reversed-phase HPLC. Astaxanthin normally appears first in HPLC chromatograms since it is a xanthophyll with high polarity. During RP-HPLC process, the expected order of elution is not affected by the presence of the hydrogen bond between the HO and keto group. Carotenoids like astaxanthin exist in different isomeric forms as discussed in Section 2.1.2(a). Majority of astaxanthin from *X. dendrorhous* is all-trans form, but there are also small amounts of 9-cis and 13-cis isomers. These cis forms have a slightly different optical spectrum, where the main maximum absorbance of 9-cis and 13-cis is shifted by 5 nm compared to all-trans forms (Figure 2.6) (Visser *et al.*, 2005). Other than these geometrical isomers, astaxanthin occurs in three optical isomers: 3R, 3'R, 3S, 3'S and 3R, 3'S but the optical absorbance is not affected by this chirality.

For carotenoid profiling, a sample can be compared to authentic chemical standards or a control strain in terms of retention time and absorbance spectrum. The quantification of carotenoids can be determined by integration of the peak areas in HPLC chromatogram.

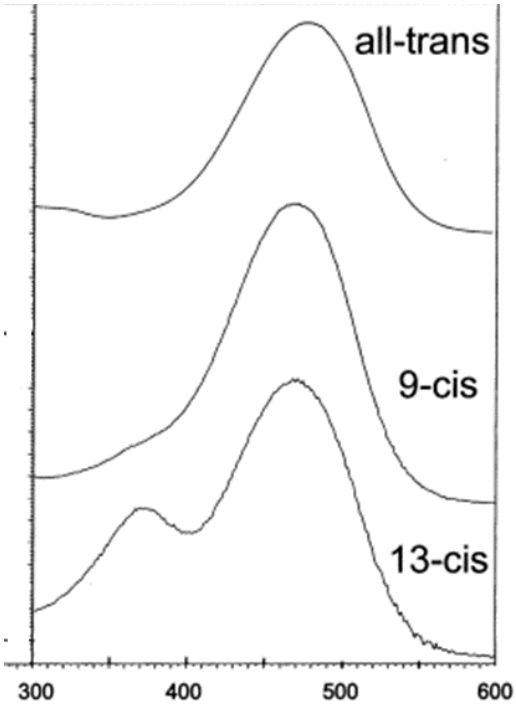

Figure 2.6 Spectra of astaxanthin geometrical isomers (Visser *et al.*, 2005)

2.1.2(c) Biological Properties of Astaxanthin and Its Health Benefits

Astaxanthin is a high-value ketocarotenoid mainly synthesized by several species of microalgae, plants, bacteria, and fungi. In *X. dendrorhous* and *H. pluvialis*, astaxanthin is built up through the stimulation of the environmental stress, and protects cellular DNA from photodynamic damage (Hagen *et al.*, 1993). In general, animals do not have the ability to produce carotenoids on their own and have to obtain these compounds from their diets. Astaxanthin is responsible for the colour of the flesh for large amount of marine species through consumption of microorganisms that synthesize the pigment (Gu *et al.*, 1997; Olaizola, 2003; Schroeder & Johnson, 1995a; Alvarez *et al.*, 2006). It plays a conventional role in poultry and aquaculture industry as the useful feed additive in providing characteristic pigmentation of the tissues that influences organoleptic values

and consumer preferences. It is also widely used as colourants in the food industry to ensure proper pigmentation and enhance acceptability of many foods. Astaxanthin has been approved by the United States Food and Drug Administration (USFDA) to be added in to animal and fish feed for colouration purpose (Pashkow *et al.*, 2008), while the European Commission has considered natural astaxanthin as a safe food dye (Roche, 1987).

Other than foods and feeds, the use of astaxanthin in nutritional supplement has been growing fast in nutraceuticals and pharmaceuticals as it was discovered that the consumption of astaxanthin can reduce or prevent risk of various diseases in humans and animals (Kidd, 2011; Yang *et al.*, 2013; Yuan *et al.*, 2011; Dhankhar *et al.*, 2012). The oxidative molecules, such as free radicals, including hydroxyls and peroxides, and reactive oxygen species are generated during aerobic metabolism in organisms. Excess quantities of such compounds might cause oxidative damage which lead to various human diseases. Human body can generate superoxide dismutase catalase and peroxidase as their own enzymatic antioxidants to control and reduce these oxidative effects. But these compounds are not enough most of the time, thus extra consumption of antioxidants is required. Astaxanthin plays the role as a strong antioxidant by reacting with free radicals through donation of electrons to convert them to more stable products and suspends free radical chain reaction in living organisms (Ranga Rao *et al.*, 2010). Astaxanthin shows strong scavenging activity on free radicals and protects LDL cholesterol, cell membranes, cells and tissues from lipid peroxidation and oxidative damage. It is a strong antioxidant that protects the phospholipid cell membrane and other lipid components by removing singlet

oxygen and inhibiting free radicals, thus shields the plants and organisms from oxidative damage caused by active oxygen species (Schroeder & Johnson, 1995b). Many studies have revealed that astaxanthin has powerful antioxidant activity, which may be ten times stronger than that of other carotenoids such as β-carotene, lutein, zeaxanthin, canthaxanthin, and lipophilic antioxidants like vitamin E (α-tocopherol) in scavenging of singlet oxygen ($^1O_2$) and peroxyl radicals (H_2O_2). The unique structure of astaxanthin allow it to stay both inside and outside of the cell membrane and terminate free radical chain reaction on both sides of the membrane, which gives better protection when compared to vitamin C and β-carotene (McNulty *et al.*, 2007; Lauritano & Ianora, 2016).

Besides mitigating the damaging effects of oxidative stress, astaxanthin modulates stimulation of inflammation and immune response. The antioxidation properties of astaxanthin make it among the bioactive phytochemicals credited for reducing the risks of human diseases such as cancer, cardiovascular diseases and diabetes besides protection against UVA-induced oxidative stress (Maldonade *et al.*, 2008).

Clinical and epidemiological data showed that there might be protection effect against cardiovascular disease through consumption of dietary antioxidants. In human body, high level of Low Density Lipoprotein (LDL), the bad cholesterol, may lead to development of cardiovascular diseases (Maher, 2000). Astaxanthin is carried by VLDL, LDL and HDL in the human blood and it can improve heart health by altering levels of LDL and HDL cholesterol in bloods (Guerin *et al.*, 2003). Astaxanthin supplementation prevented LDL-cholesterol from stimulation of *in vitro* oxidation and induced the increment of HDL in

blood levels, the form of blood cholesterol negatively correlated with coronary heart disease (Iwamoto *et al.*, 2000). Other than that, astaxanthin may reduce the risk of cardiovascular disease by lowering elevated blood pressure, inhibiting oxidation of LDL, stabilizing atherosclerotic plaque, lessening the risk of myocardial infarction and preventing atherosclerosis. The possibility of involvement of astaxanthin in nitric oxide-related mechanism and modulation of the blood fluidity might contribute to antihypertensive effect in human. It can also benefit the heart health by reducing inflammation most likely correlated with the increasing risk of coronary heart disease (Tracy, 1999). Astaxanthin has the potential to become therapeutic agent against atherosclerotic cardiovascular disease as it is a potent antioxidant agent against inflammation and oxidative stress, which are the pathophysiological properties of atherosclerotic cardiovascular disease (Fassett & Coombes, 2011).

Antioxidant compounds such as astaxanthin are capable to reduce the risk of mutagenesis and carcinogenesis by prohibiting cell damage caused by oxidation. Besides having higher antitumor activity when compared to other carotenoids such as β-carotene and cantaxanthin (Chew & Park, 2004), it also acts as an inhibitor for the development of breast and prostate cancer cells, fibrosarcoma and embryonic fibroblasts (Palozza *et al.*, 2009), cell death, cell proliferation and chemically induced mammary tumors (Jyonouchi *et al.*, 2000; Prabhu *et al.*, 2009; Nakao *et al.*, 2010). Moaka *et al.* (2012) found that Epstein-Barr virus and carcinogenesis in mouse skin-papillomas were effectively prohibited after astaxanthin treatment. Astaxanthin may unleash anti-tumor activities through the promotion of immune response by preventing stress-stimulated lipid