CHARACTERIZATION OF ASTAXANTHIN-RICH *XANTHOPHYLLOMYCES DENDRORHOUS* EXTRACT FROM A HYPERPRODUCING MUTANT AND ITS EFFECTS ON BREAST CANCER CELLS

KHAW SHIN YUAN

UNIVERSITI SAINS MALAYSIA

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by

KHAW SHIN YUAN

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Praises to LORD for the great gifts He gave me.

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LIST OF SYMBOLS

°C	Degree celcius
%	Percentage
±	Plus–minus sign
µl	Microliter
µg	Microgram
ml	Milliliter
nm	Nanometer
g	Gram
U	Unit
mmol	Millimole
kg	Kilogram
β	Beta
mm	Millimeter
L	Liter
µM	Micromolar

LIST OF ABBREVIATIONS

XD	*Xanthophyllomyces dendrorhous*
DW	Dry cell weight
IPPT	Advanced Medical and Dental Institute
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
TPC	Total Phenolic Content
DPPH	1,1-diphenyl-2picrylhydrazyl
ROS	Reactive Oxygen Species
DMSO	Dimethyl sulfoxide
ATCC	American Type Culture Collection
FBS	Fetal bovine serum
MTT	Methylthiazolydiphenyltetrazolium bromide
DMEM	Dulbecco's Modified Eagle Medium
CO_2	Carbon dioxide
IC_{50}	Half maximal inhibitory concentration
PBS	Phosphate-buffered saline
FITC	Fluorescein-5-isothiocyanate
AX	Astaxanthin
MNNG	N-methyl-N’-nitro-N-nitrosoguanidine
Rf	Retention factor
GAE	Gallic acid equivalents
ECM	Extracellular Matrix
USM	Universiti Sains Malaysia
UV	Ultraviolet
SD	Standard deviation
DAD	Diode-array detector
NCI	National Cancer Institute
NOK	Human oral keratinocytes
HUVEC	Human umbilical vein endothelial cells
2D	Two dimensions
3D	Three dimensions

DNA	Deoxyribonucleic acid
2-ME	2-methoxyestradiol
BCT	Breast conserving therapy
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
UHQ	Ultrapure

PERINCIAN EKTRAK *XANTHOPHYLLOMYCES DENDRORHOUS* YANG KAYA ASTAXANTHIN DARIPADA MUTAN HIPERPRODUKSI DAN KESANNYA KE ATAS SEL KANSER PAYUDARA

ABSTRAK

Astaxanthin merupakan karotenoid dengan pelbagai faedah kesihatan termasuk sifat antioksidan dan antikanser. Satu mutan penghasil hiper astxanthin M34 telah dijanakan memandangkan minat yang semakin meningkat terhadap pigmen semula jadi. Kajian ini bertujuan untuk mengkaji ciri-ciri ekstrak *X. dendrorhous* yang kaya astaxanthin dan kesannya terhadap kanser sel MCF-7 dan MDA-MB-231. Analisis spektrometri, TLC dan HPLC membuktikan bahawa mutan M34 menghasilkan astaxanthin dalam kuantiti dan ketulenan yang lebih tinggi berbanding jenis liar. Ekstrak astaxanthin mutan menunjukkan aktiviti pemuliharaan radikal yang lebih tinggi berbanding dengan ekstrak astaxanthin jenis liar dalam ujian DPPH. Ujian MTT membuktikan bahawa ekstrak astaxanthin jenis liar dan mutan tidak toksik terhadap sel bukan barah MCF-10A. Ekstrak astaxanthin jenis liar dan mutan menunjukkan kesan penyekatan pertumbuhan secara bergantung kepada dos pada sel kanser payudara MCF-7 dan MDA-MB-231. Ekstrak astaxanthin mutan menunjukkan IC_{50} lebih rendah daripada ekstrak jenis liar dan lebih berkesan untuk menghalang kedua-dua jenis sel MCF-7 dan MDA-MB-231. Nilai IC_{50} ekstrak astaxanthin jenis liar dan mutan tidak melebihi had atas yang ditetapkan pada 30 µg/ml. Pemerhatian morfologi menunjukkan bahawa sel MCF-7 dan MDA-MB-231 kehilangan bentuk asalnya setelah 24 jam rawatan ekstrak astaxanthin jenis liar atau mutan. Penyusutan sel, kondensasi nuklear dan peleburan membran dilihat berkembang mengikut jangka masa rawatan dengan ekstrak astaxanthin jenis liar dan mutan dalam sel MCF-7 dan

MDA-MB-231. Penilaian apoptosis Annexin-V FITC/PI menunjukkan induksi apoptosis oleh ekstrak astaxanthin jenis liar dan mutan pada kedua-dua jenis sel MCF-7 dan MDA-MB-231. Kesan apoptosis ekstrak astaxanthin menunjukkan kebergantungan pada jenis sel di mana peratusan apoptosis sel MCF-7 jauh lebih tinggi daripada sel MDA-MB-231 yang dirawat dengan ekstrak yang sama. Rencatan kitaran sel diperhatikan pada fasa S dan fasa G2/M untuk sel MCF-7 yang dirawat dengan ekstrak astaxanthin liar dan pada fasa G2/M untuk ekstrak mutan. Dalam sel MDA-MB-231, rencatan kitaran sel pada fasa S mungkin merupakan mekanisme utama untuk penyekatan pertumbuhan sel yang diakibatkan oleh ekstrak astaxanthin jenis liar dan mutan. Kira-kira 2 kali ganda ROS terkumpul di kedua-dua jenis sel MCF-7 dan MDA-MB-231 setelah rawatan dengan ekstrak astaxanthin jenis liar dan mutan pada IC_{50} berbanding dengan kawalan yang tidak dirawat. Ekstrak mutan menunjukkan perencatan yang lebih baik terhadap penghijrahan kedua-dua jenis sel MCF-7 dan MDA-MB-231. Kedua-dua ekstrak astaxanthin jenis liar dan mutan memberikan perencatan yang lebih baik pada sel MCF-7 berbanding sel MDA-MB-231. Ekstrak astaxanthin *X. dendrorhous*, terutamanya ekstrak mutan, terbukti mempunyai potensi antikanser.

CHARACTERIZATION OF ASTAXANTHIN-RICH *XANTHOPHYLLOMYCES DENDRORHOUS* EXTRACT FROM A HYPERPRODUCING MUTANT AND ITS EFFECTS ON BREAST CANCER CELLS

ABSTRACT

Astaxanthin is a carotenoid with multiple health benefits including antioxidant and anticancer properties. An astaxanthin-hyperproducing *X. dendrorhous* mutant was generated due to the growing preference for natural pigment. This work aimed to study the astaxanthin-rich *X. dendrorhous* extracts and its effect on MCF-7 and MDA-MB-231 cells. Spectrometric, TLC and HPLC analysis evidenced that mutant M34 produced astaxanthin in higher quantity and purity than the wild type. Mutant astaxanthin extract showed a higher scavenging activity compared to wild type astaxanthin extract in DPPH assay. MTT assays proved that wild type and M34 astaxanthin extracts were not toxic towards the non-cancer origin MCF-10A cells. Wild type and mutant astaxanthin extracts exhibit growth-inhibitory effect in dose-dependent manner in MCF-7 and MDA-MB-231 cells. Mutant astaxanthin extract showed a lower IC_{50} than wild type extract and was more effective in inhibiting both MCF-7 and MDA-MB-231 cells. The IC_{50} values of wild type and mutant astaxanthin extracts did not exceed the required upper limit of 30 µg/ml. Morphological observation showed that MCF-7 and MDA-MB-231 cells lost their original shape after 24 hours treatments of wild type or mutant astaxanthin extract. Cell shrinkage, nuclear condensation and membrane blebbing were seen developed time dependently upon the treatments with wild type and mutant astaxanthin extracts in MCF-7 and MDA-MB-231 cells. Annexin-V FITC/PI apoptosis assessment signified the induction of

apoptosis by wild type and mutant astaxanthin extracts in both MCF-7 and MDA-MB-231 cell lines. The apoptotic effects were exhibited in a cell-type dependent manner whereby the percentages of apoptotic MCF-7 cells were significantly higher than MDA-MB-231 cells treated with the same extracts. Cell cycle arrest was observed at the S phase and G2/M phase for MCF-7 cells treated with wild type astaxanthin extract and at G2/M phase for mutant extract. In MDA-MB-231 cell line, cell cycle arrest at S phase may be the major mechanism for the observed cell growth inhibition by both astaxanthin extracts. Approximately 2 fold of ROS accumulated in both MCF-7 and MDA-MB-231 cell lines after treatment with wild type and mutant astaxanthin extracts at IC_{50} in comparison to the untreated control. Mutant extract showed a slightly better inhibition on migration in both MCF-7 and MDA-MB-231 cell lines. Both wild type and mutant astaxanthin extracts gave a better inhibition on MCF-7 cells compared to MDA-MB-231 cells. *X. dendrorhous* astaxanthin extracts, especially the mutant extract, were shown to possess anticancer potential.

CHAPTER 1

INTRODUCTION

1.1 Overview and rational of study

Breast cancer is the most frequently diagnosed cancer among women and the leading cause of cancer death (Bray *et al.*, 2018). According to Hashim *et al*. (2016), breast cancer cases in Malaysia shows a trend of increment in mortality rates per year even though it is the lowest among South-East Asia countries. It is a heterogenous disorder with a wide range of molecular characteristics and therapeutic responses observed among patients (Turashvili & Brogi, 2017). Thus, a standard treatment approach is not suitable and breast cancer therapy is going through constant change and improvements. Currently, treatment for breast cancer varies depending on the patient age and tumour characteristics. The common treatments for breast cancer are surgery, radiation and chemotherapy. Adjuvant therapy is one of the new treatments for breast cancer which includes monoclonal antibodies and hormone blocking therapy (Bange *et al*., 2001). Despite significant progress in understanding the disease pathogenesis, clinical issues such as the non-selective sacrifice of normal vs. tumor cells still remain. Chemotherapeutic agents cause serious DNA damage across both normal and cancer cells, as well as adverse reactions such as digestive issues, leukopenia and hair loss. Moreover, drug resistance in particular contributes to the recurrence of the breast cancer after surgery followed by secondary tumor formation and remote metastasis, and limits chemotherapy efficacy (D'Alterio *et al*., 2020). Despite the improvements, current cancer drugs or treatments often induce adverse long term and side effects in which causing patient discomfort. Hence, there is an urgent need for alternative approaches to improve cancer therapy tolerance and

minimize side effects. Natural compounds with anticancer properties that help in chemosensitisation of tumours without increasing unwanted nexus effects or reduce the adverse effects due to reduced dosage of treatment have attracted attention from researchers.

Throughout the years, researchers found out that many natural products from microorganism, algae and plants contain high value compounds in helping cancer prevention and therapy. Natural sources account for roughly 60% of the medications approved for cancer treatment. Astaxanthin is a fat-soluble orange-red pigment that belongs to the xanthophyll subclass of the carotenoids (Zhang & Wang, 2015). It is a highly antioxidative pigment due to the presence of keto (C=O) and hydroxyl (OH) moieties on each ionone ring (Song *et al*., 2014). It showed strong antioxidant, immunomoduling, anti-inflammatory and enzyme inducing properties, all suggesting that this carotenoid can play a potential role in cancer prevention and cancer treatment (Hussein *et al*., 2006; Park *et al*., 2010a).

Almost 95% of astaxanthin preparations available in the market is a synthetic form derived from the petrochemical sources which is not environmental friendly and less stable than the natural one (Lorenz & Cysewski, 2000). The pursuit for natural sources of astaxanthin with industrialization potential has been sparked by rising demand for natural products and tight regulations on synthetic chemicals. Only a few microbial resources may compete with synthetic astaxanthin economically: the microalgae *Haematococcus pluvialis* and the red yeast *X. dendrorhous*, where astaxanthin contributes about 83-87% of total carotenoids generated (Capelli *et al*., 2013). However, so far, these products only take up a small fraction of the market due to their limited production and low level of pigment production in wild type strains.

Therefore, an astaxanthin-overproducing *X. dendrorhous* mutant M34 developed in our laboratory will be used in this study to overcome the problem.

Given the current prevalence of breast cancer and the need to discover and develop new pharmaceutical drugs, particularly those derived from natural sources, this study aims to characterize the astaxanthin extracts from wild type *X. dendrorhous* and its overproducing mutant and study the *in vitro* anticancer effects of the extracts on breast cancer cell lines. There is no related studies reported on the mutant *X. dendrorhous* extract and it is hypothesized that the extract from the astaxanthin-overproducing *X. dendrorhous* mutant would be able to show a better antiproliferation effect on breast cancer cells compared to the wild type due to its higher astaxanthin content. This study will provide an understanding for exploring the chemoprotective and therapeutic potentials of astaxanthin for the prevention and treatment of pathological conditions of breast cancer in further research.

1.2 Research Objectives

1. To profile and characterize the astaxanthin extracts from wild type and mutant *X. dendrorhous*.

2. To evaluate the antioxidant and cytotoxicity activities of the *X. dendrorhous* astaxanthin extracts in a concentration dependent manner.

3. To study the anticancer effects of the *X. dendrorhous* astaxanthin extracts *in vitro* on breast cancer cells (MCF-7 and MDA-MB-231 cells).

CHAPTER 2

LITERATURE REVIEW

2.1 *Xanthophyllomyces dendrorhous*

X. dendrorhous (previously known as *Phaffia rodozyma*) is a basidiomycetous yeast that first isolated by Herman Phaff in the 1970s in Japan and Alaska. It was mostly found in limited geographical areas such as Russia, Finland and the United States (Johnson, 2003). The vegetative cells of *X. dendrorhous* reproduce by budding. The cell wall ultrastructure and the budding process, the urease activity and production of cell surface-associated amyloid compounds, co-enzyme Q-10 system, the capacity to synthesize carotenoid pigments and starch-like compounds indicate the basidiomycetous nature of *X. dendrorhous* (Golubev, 1995; Johnson & An, 1991). The cell wall polysaccharides contain β-(1,3) and β-(1,6)-glucan and/or a small amount of chitin and an acidic polysaccharide-based capsule surrounds the cells as well. (Kucsera *et al*., 2000). It is a psychrophilic yeast with a growth temperature range of 0°C to 27°C, with an ideal temperature ranging between 18°C and 22°C depending on the strain (Schmidt *et al*., 2011). Individual spores from genetically marked strains revealed evidence of reproduction and mating between two cells under unfavourable conditions.

X. dendrorhous is one of the important industrial yeasts as it is able to synthesize natural pigments such as astaxanthin, beta-carotene and canthaxanthin in which astaxanthin makes up 83-87% of the total pigment mixture (Martínez-Cámara *et al*., 2021). It is believed that *crtYB, crtI, crtS* and *crtR* functional genes played an important role in the synthesis of the astaxanthin in *X. dendrorhous* (Barredo *et al*., 2017) as shown in Figure 2.1. Extensive studies extended to the phylogenetic investigation of

X. dendrorhous based on the acetyl-CoA derived pathways was reported by Sharma *et al*. (2015) in order to provide insights of the genomic information (Figure 2.2).

The oxygenated β-carotene derivative astaxanthin is widely used in the aquaculture, food, pharmaceutical and cosmetic industries due its outstanding colouring, antioxidative and health-promoting properties (Dhankhar *et al*., 2012). Currently, astaxanthin is produced commercially by either chemical synthesis or microbial fermentation. However, the market has been primarily dominated by the synthetic production using petroleum-based feedstocks. Nevertheless, the use of chemically synthesized compounds as food and feed additives has been subjected to strict restrictions in recent years. This is due to rising anxiety on the safety and biological function of the artificial pigments on human health besides the harmful effect on the environment. Natural products or those containing natural additives acquired from plant or microbial processes are often preferred by consumers. The growing demand for products from natural sources may open up the market to astaxanthin produced by biological processes. Other advantages of microbial pigment are cheaper production, easier extractions, high production yield, low environment variables and biodegradability (Charalampia *et al*., 2017). Thus, economically viable alternative bioprocesses for natural astaxanthin production attracts much research interest. An enhanced astaxanthin production by microbial fermentation is necessary to compete with the cost effective synthetic astaxanthin.

Several natural sources of astaxanthin have been explored owing to its growing demand for novel applications in the food, feed, pharmaceutical and cosmetic industries. Natural supplies of astaxanthin include shrimp, crawfish by-products, crustacean wastewaters and microbial cultivation (Amado *et al*., 2015). The production of astaxanthin by several yeast species belonging to the genera

Rhodotorula and *Phaffia*, by bacteria *Brevibacterium sp*. and by the microalgae *Chromochloris zofingiensis*, *Chlorococcum sp*. and *H. pluvialis* has served as potential pigment sources (Ambati *et al*., 2014). However, currently there are only two microbial sources of astaxanthin that can rival synthetic astaxanthin economically and mainly serve niche applications (Martínez-Cámara *et al*., 2021). The green microalga *H. pluvialis* and the red yeast *X. dendrorhous* are currently the main microorganisms used for astaxanthin production at the commercial scale. Despite its high content of astaxanthin, the use of *H. pluvialis* for astaxanthin production retains several limitations due to expensive cultivation technique and its low cell concentration and slow growing rate. Microalgae requires more than 10 days for cultivation compared to a short cultivation time of 3 to 4 days for *X. dendrorhous*. *X. dendrorhous* is preferred because of the higher growth rates, high biomass productivity and easier cultivation conditions that might reduce the production time and cost at an industrial scale (Mata-Gomez *et al*., 2018). It is particularly interesting because it can develop and synthesise astaxanthin using a variety of sugars such as glucose, xylose and arabinose (Dominguez-Bocanegra *et al*., 2007). Thus, *X. dendrorhous* may use fermentable sugars obtained from hydrolysis of lignocellulosic biomass from agro-byproducts as carbon sources for astaxanthin synthesis. Yet, the low concentration of astaxanthin in the wild type strains of *X. dendrorhous* is not cost efficient for the industrialization of this organism. The improvement of astaxanthin production in *X. dendrorhous* to increase the yield and diminish the production costs have been the main aim of the research performed on this yeast.

The optimization of the pigment production can be done through modern biotechnology approaches. New strains of *X. dendrorhous* have been isolated from the environment, and overproducing mutants have been selected using traditional methods

of random mutation and screening, as well as appropriate metabolic engineering (Liu *et al.*, 2008). Other studies have focused on growth conditions that would enhance carotenoid production in the red yeast. To achieve maximum astaxanthin productivity, low-cost raw materials from agricultural and industrial sources were used to minimise production costs and improve industrial competitiveness. There had been reports on cane molasses, corn wet-milling products, juices and wastes of vegetable and fruit, hydrolyzed peat etc., which were frequently used as additional carbon sources to enrich the substrates thus to cut costs by using less expensive substrates (Yang *et al*., 2011). Various food and agriculture by-products were explored as economical substrates for the production of astaxanthin by *X. dendrorhous*. This includes the use of a low-cost formulated medium derived from mussel-processing wastewater (Amado *et al*., 2015) as well as 6-benzylaminopurine (a phytohormone) as efficient substrates for enhanced cell biomass and astaxanthin aggregation in *X. dendrorhous* (Pan *et al*., 2020). Fermentation parameters have been thoroughly investigated at both the shake flask and fermenter scales to attain maximum astaxanthin yield (Rodriguez-Saiz *et al.*, 2010). The effects of important factors such as pH, temperature, percentage of inoculum, carbon and nitrogen concentrations on astaxanthin production were analysed using factorial design and response surface methodology (Ramirez *et al*., 2000). According to Marcoleta *et al*. (2011), ethanol is a powerful inducer of astaxanthin production, while glucose is a repressor. Astaxanthin content increased when citrate and glutamate, two precursors of carotenogenesis, were added to the cultures (de la Fuente *et al*., 2010). Other than that, mutagenesis using UV light and chemical mutagen N-metil-N'-nitro-N-nitrosoguanidin (MNNG) and ethyl methanesulfonate (EMS) have been used in some studies previously to obtain mutants of *X. dendrorhous* with hyperproducing properties. Rubinstein *et al.* (1998) reported the isolation of 15

mutants through a mutagenic treatment with MNNG. Similarly, a stable astaxanthin-hyperproducing mutant M34 was obtained through MNNG mutagenesis and screening on β-ionone containing YM agar plates in our lab. β-Ionone appears to be responsible in suppressing formation of astaxanthin at the β-carotene stage and was used to select mutants which can produce excess astaxanthin (Lewis *et al*., 1990). Figure 2.3 and Figure 2.4 shows the agar and broth cultures of wild type and mutant strains of *X. dendrorhous* obtained in our lab, respectively.

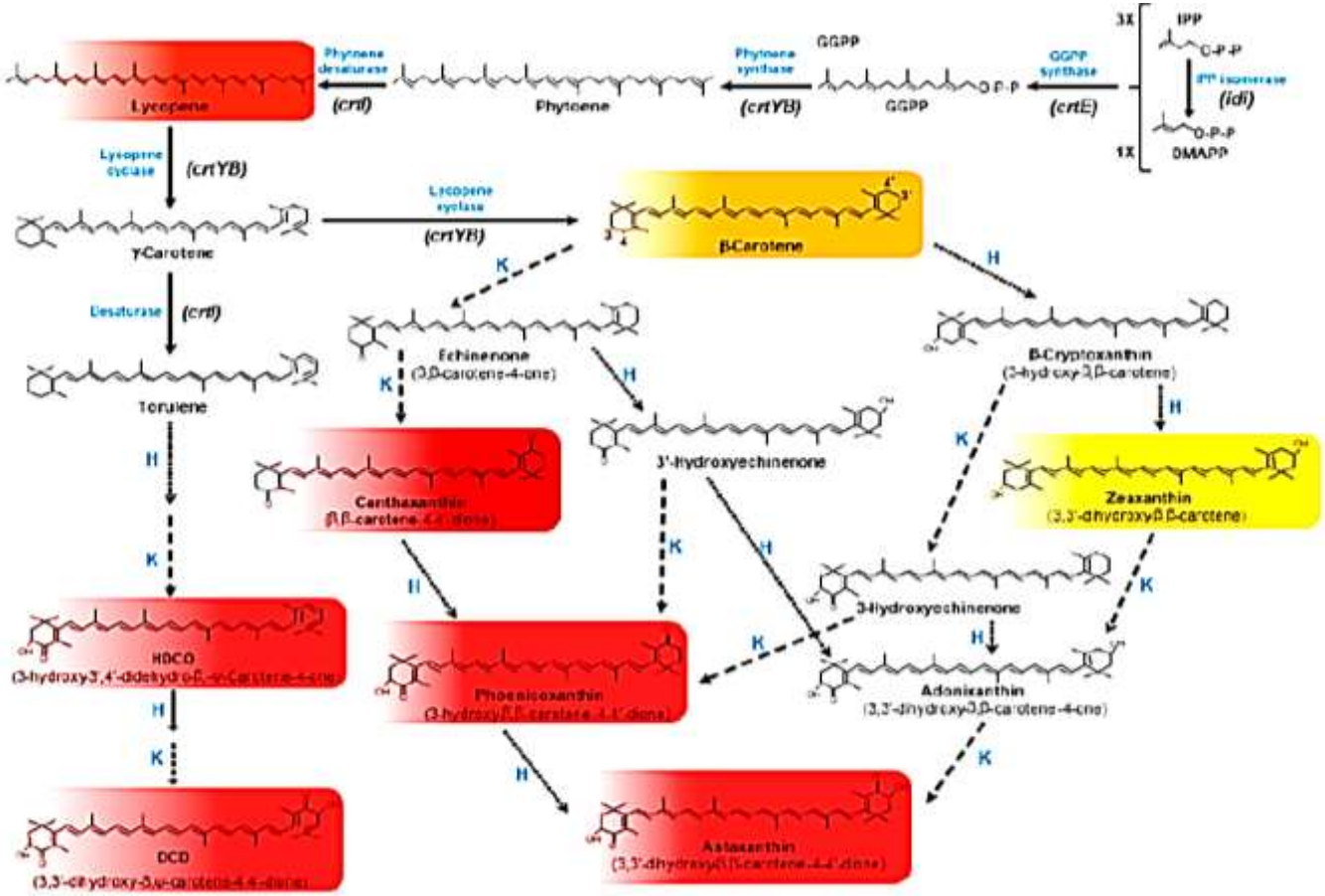

Figure 2.1 Biosynthesis pathway in X. dendrorhous (adapted from Barredo *et al*., 2017)

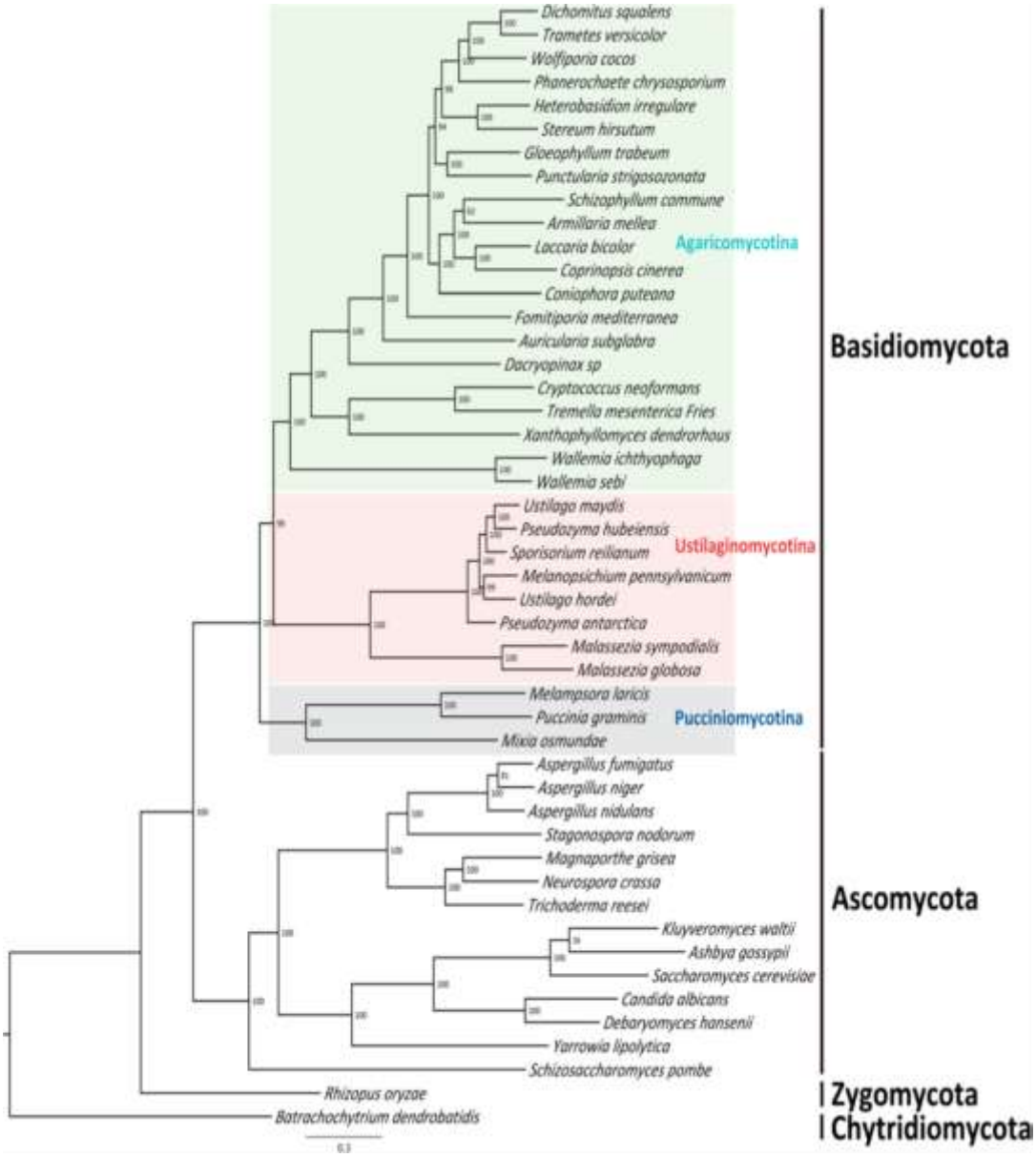

Figure 2.2 Phylogenetic tree of *X. dendrorhous* (adapted from Sharma *et al*., 2015)

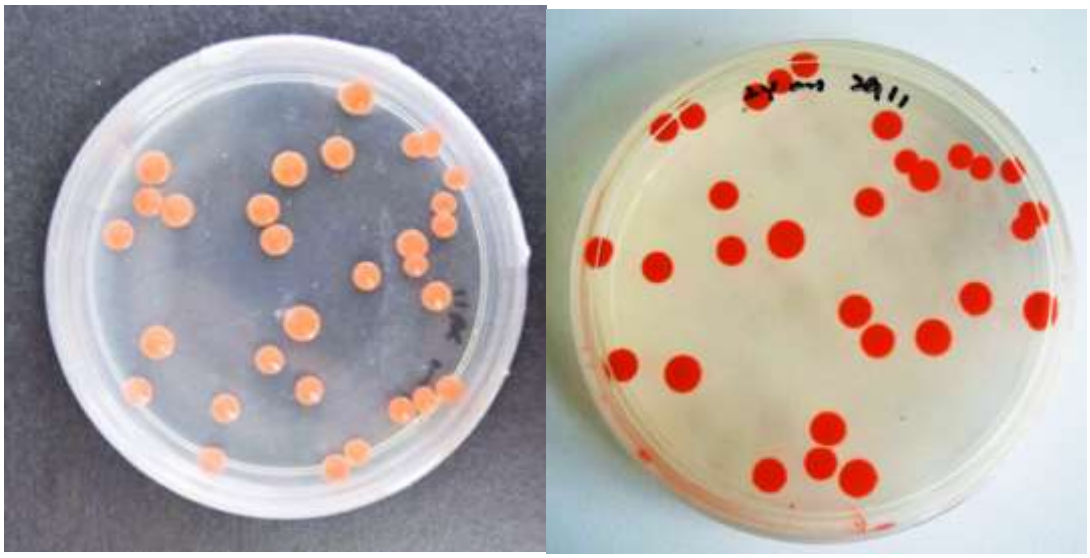

Figure 2.3 Wild type *X. dendrorhous* (left) and mutant strain of *X. dendrorhous* M34 (right) on YM agar

Figure 2.4 *X. dendrorhous* cultures in YM medium: wild type (left), yellow mutant (centre) and red mutant M34 (right).

2.2 Astaxanthin

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione, chemical formula $C_{40}H_{52}O_4$; molar mass = 596.84 g/mol) is an orange-red pigment that belongs to the family of the xanthophylls, the oxygenated derivatives of carotenoids whose synthesis derives from lycopene. The astaxanthin molecule consists of two terminal oxygenated ionone rings joined by a polyene chain of conjugated double bonds (Figure 2.5). Its molecular structure, chemical properties and light-absortion characteristics are all influenced by the polyene framework. The conjugated double bonds at the centre of the compound give the pigment its red colour. This conjugated double bond functions as a powerful antioxidant in living organisms by contributing electrons and interacting with free radicals to transform them to a more stable substance and stop the chain reaction of free radicals (Guerin *et al*., 2003). Astaxanthin structure has two asymmetric carbons situated at the 3,3'-position of the β-ionone ring, and both a hydroxyl (–OH) and a carbonyl (C=O) group on either end of the molecule (Higuera-Ciapara *et al*., 2007)

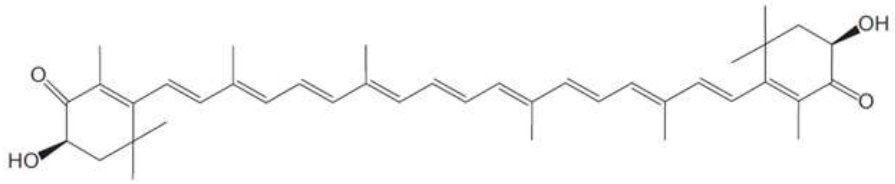

Figure 2.5 Molecular structure of astaxanthin (adapted from Oehlenschlager & Ostermeyer, 2016)

Astaxanthin prevails in stereoisomers, geometric isomers, free and esterified forms in natural sources. Based on the configuration of the double bonds in the polyene chain, astaxanthin occurs as cis and trans (Z and E) geometrical isomers (Figure 2.6). Thermodynamically, trans isomers are more stable than cis-isomers. Carotenoids found in nature are mainly all trans isomers (Britton, 1991). The dominant isomer is all-trans astaxanthin, although at least two cis-isomers (9-cis and 13-cis) are also found in synthetic preparations and in nature, depending on the host species and parts of the body (Osterlie *et al*., 1999). Buttle *et al*. (2001) found that the absorption of astaxanthin is related to the optical and symmetry isomerism of astaxanthin on the various tissues of salmonids. Trans isomers tend to preferentially accumulate in the muscle and plasma, while cis ones do so in the liver. Since each ionone ring has two chiral centres at carbon 3 and 3', astaxanthin can exist in three stereoisomers: a meso form (3R,3'S) and two enantiomers (3S,3'S and 3R,3'R)(Brotosudarmo *et al*., 2020)(Figure 2.7). The 3S,3’S stereoisomer is the most abundant in nature among the three optical or configurational isomers. *X. dendrorhous* synthesizes the (3R,3'R)-isomer and *H. pluvialis* produces (3S,3'S)-isomer. Synthetic astaxanthin comprises a racemic mixture of the two enantiomers (3S,3’S)(3R,3’R) and the meso form (3R,3’S)(Turujuman *et al*., 1997). Other than that, astaxanthin exists in a free (non-esterified) form, with the hydroxyl group not esterified, and in a chemical complex with proteins or lipoprotein. Meanwhile, esterification makes astaxanthin more stable to oxidation and increases its solubility in the cell (Udayan *et al*., 2017). The hydroxyl group on one or both rings can bind to different fatty acids, such as linoleic, estearic, oleic or palmitic acid, to form monoester or diester, accordingly. Studies showed that the difference between esterified and non-esterified forms influenced the antioxidant properties (Perez-Galvez & Minguez-Mosquera, 2002). The esterification process was affected by the growth

condition and eventually influence the liposolubility directly (Camara & Brangeon, 1981).

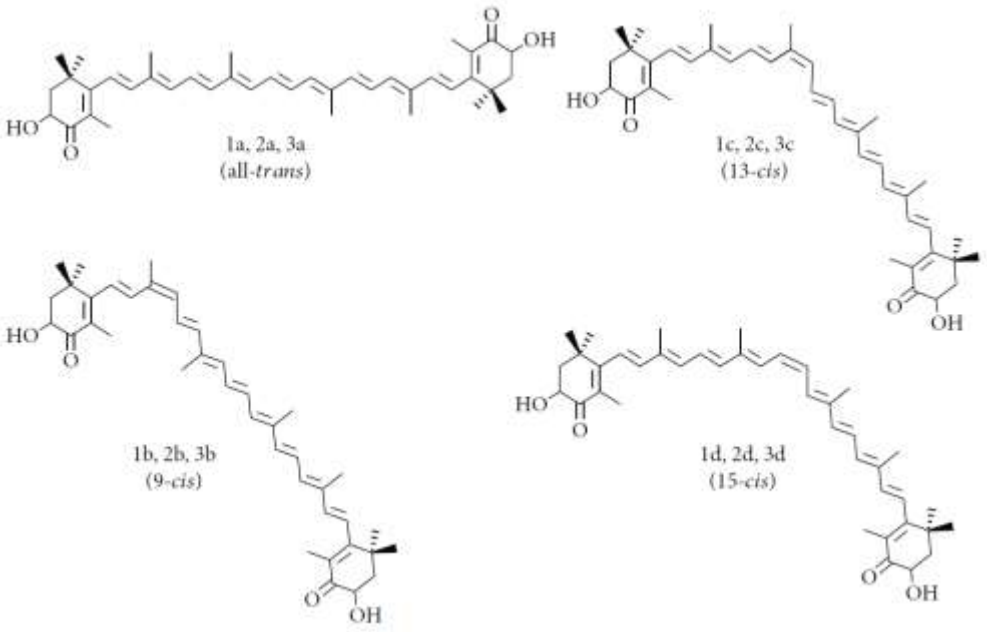

Figure 2.6 Structure of all-trans, 9-cis, 13-cis and 15-cis-astaxanthin (adapted from Brotosudarmo *et al*., 2020)

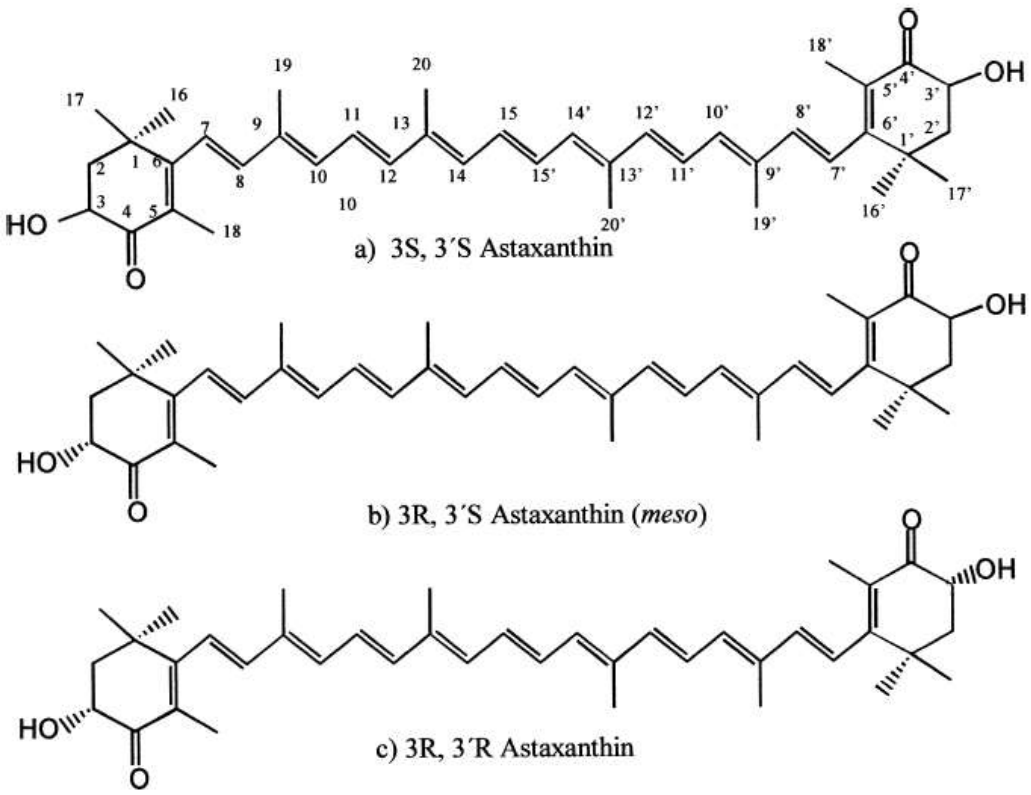

Figure 2.7 Astaxanthin optical isomers: (a) 3S,3'S, (b) 3R,3'S and (c) 3R,3'R (adapted from Brotosudarmo *et al*., 2020)

Astaxanthin can be synthesized in nature or chemically. Animals are unable to synthesize carotenoids *de novo*, so they obtain astaxanthin through dietary intake and ingestion of microalgae and phytoplankton, which are natural astaxanthin producers. Astaxanthin content in shrimp/crab shells is <0.025% dry weight and these crustacean by-products serve as natural supplies of the pigment. Natural astaxanthin is also obtained from synthesizing organisms and microorganisms such as microalgae, bacteria, yeasts, protists and plants. Organisms that synthesize astaxanthin include: microalgae (*H. pluvialis*, *Chlorella zofingiensis*, *Chlamydomonas nivalis*, *Chlorococcum sp*, *Scenedesmus acutus*, *Neochloris wimmeri*); bacteria (*Agrohacterium aurantiacum*, *Paracoccus carotinifaciens*, *Brevundimonas scallop*); yeasts (*X. dendrorhous*); protista (*Aurantiochytrium sp*, *Thraustochytrium sp*) and plants (*Adonis aestivalis*, *Adonis annua*). Studies on yeast, plant and microalgae production capacities showed that wild-type production yields of astaxanthin are significantly variant between different hosts as metabolic fluxes differ between families, species and even within a multicellular organism. Microalga *C. zofingiensis* produced about 0.1% of astaxanthin by dry weight, Nicotiana tabacum produced around 0.24%, *X. dendrorhous* produced between 0.4 and 2.5%, and microalgae *H. pluvialis* produced the most among wild-type organisms at around 3% of dry weight. (Novovesk *et al*., 2019).

Astaxanthin is synthetically produced since 1990 and it is the most abundant method of commercial production found in the world market (95% of the total) because of the lower production cost (approximately 1000 dollars/kg) compared to natural astaxanthin (Perez-Lopez *et al*., 2014). However, this is disadvantaged by the use of petrochemical resources as raw materials and multistep of chemical reactions in synthetic astaxanthin production. There is also controversy of using synthetic

astaxanthin as the origin source might cause harmful effects. Artificial synthetic antioxidants are not favoured for human health because of their negative effects like carcinogenicity and toxicity (Moure *et al*., 2001), This has led to the growing demand for natural products and has increased the search for natural astaxanthin sources in recent years (Khoo *et al*., 2019). Natural astaxanthin production is highly important not only for humans and farmed animals, safeguarding their health but also society and environment, promoting sustainable development. With increase in the demand for natural ingredients, the microbial fermentation technology is implemented to produce astaxanthin. However, the production of natural astaxanthin from natural sources is limited nowadays and substantial efforts have been invested to reduce the production cost of natural astaxanthin. Conditions have been studied and applied to improve the production of astaxanthin in the industry and the interest to find innovative approaches to increase its production persists. Significant technological advancement and cost-efficient methodologies increase the production of astaxanthin. For instance, micro-modules and photo-bioreactors are used to produce astaxanthin, with the aim of minimizing water usage and saving energy. On the other hand, manufacturing technologies including sealed microalgae cultivation reduces the risks of foreign object and agricultural residue entrapment, microbial contamination, while improving yield and reducing waste.

2.3 Applications of astaxanthin

The application of astaxanthin in various industries such as the chemical, pharmaceutical, food, animal feed, poultry and cosmetic has been receiving growing attention in recent years. Astaxanthin is the most important and the most costly carotenoid pigment applied in aquaculture. It is one of the main pigments included in farmed fish feeds and gives the flesh of salmonids, shrimps, lobsters and crayfish the desirable pinkish-red hue as they are unable to synthesize astaxanthin and do not have access to natural sources of carotenoids. Studies showed that feed supplemented with astaxanthin had successfully increase the fish skin pigmentation (Putra *et al*., 2020). Pigmentation is one of the most significant quality criteria of ornamental fish that dictates their market value. Besides pigmentation purpose to increase consumer appeal and acceptance, astaxanthin is a key nutrient for adequate growing, reproducing and sustaining of commercially valuable species in aquaculture (Paripatananont *et al.*, 1999). Studies performed in crustaceans 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 (Betancor *et al*., 2012). It has many other important functions in fish mainly to their reproduction: acceleration of sexual maturity, increasing fertilization and egg survival and a better embryo development (Scabini *et al*., 2011). *X. dendrohrous* has been widely studied in salmonid pigmentation testing with diets containing *X. dendrohrous*, showing similar efficiency to that achieved using synthetic astaxanthin (Whyte *et al*., 2001). For the poultry industry, studies showed that astaxanthin improved the yellow pigmentation of feet, skin and beaks in chickens. Similar effects of astaxanthin as natural pigment on yolk colour in layers has also been reported, besides improving egg quality during storage. Increased fertility, faster weight gain, higher breast muscle weights and higher feed

utilization efficiency were reported for broilers on astaxanthin meal diet (Yang *et al*., 2006).

In addition to its effect on pigmentation, the ability of the astaxanthin to interact with chemically reactive oxygen species such as free radicals and singlet oxygen is one of its most significant properties. Studies showed that astaxanthin has higher antioxidant activity in comparison to carotenoids like α-carotene, β-carotene, lutein, and lycopene, or α-tocopherol and coenzyme Q10; moreover, it induces paroxidanase which is an antioxidant enzyme (Mori *et al*., 2013; Dose *et al*., 2016; Mezzomo & Ferreira, 2016). It is biologically more active than other antioxidants as it can integrate from inside the cell membrane to the outside (Pashkow *et al*., 2008). The polyene chain only captures radicals inside the cell membrane whereas the astaxanthin end ring does so on the surface as well as inside the cell membrane (Goto *et al*., 2001). Astaxanthin dissipates energy through interaction with the solvent after the quenching of singlet oxygen, the carotenoid structure then returns to its initial form (Dose *et al*., 2016; Visioli & Artaria, 2017). Due to its potent antioxidant activity, astaxanthin has been reported to prevent lipid peroxidation and low-density lipoprotein oxidation apart from enhancing the serum lipid profile (Yoshida *et al*., 2010). It was also demonstrated to strengthen the cytotoxic activity of natural killer cells (Park *et al*., 2010a), improve liver function, and significantly influence the biodefense mechanisms (Amar *et al*., 2001). Besides antioxidation activity, it was also reported as a potent biomolecule with anti-inflammation, anti-diabetic, anti-cancer effects besides exhibiting protective effects against cardiovascular, skin, gastro, neuro and immune diseases. It was also shown as a possible antibacterial agent in inhibiting Gram positive and Gram negative bacteria (Ushakumari & Ramanujan, 2013).

2.4 Market and safety requirements of astaxanthin

Due to its potential health and ornamental benefits for human and animal, the commercial attractiveness and market for astaxanthin as a dietary supplement in human and animal diets has expanded significantly in recent years. Besides, pigmentation is one of the most significant quality criteria of ornamental value in products that is dictating their market price and astaxanthin has been used to enhance the colouration of farmed fish and egg yolk. In the feed industry, there is a strong sentiment towards using natural ingredients that are free of chemicals, antibiotics and synthetic colours. This is largely due to consumer demand for natural products or organic farming in agriculture, aquaculture and poultry, along with legislation reducing chemical additives and requiring the inclusion of natural rather than synthetic astaxanthin on a continuous basis (Batghar *et al*., 2018; Tran *et al*., 2019). *X. dendrohrous* is currently available as a fine powder that can be used in animal feed as a natural source of astaxanthin, protein and other nutrients. Astaxanthin is present in fish feed formulations in the aquaculture industry, representing a significant cost of the feed. Thus, accurate astaxanthin determination in biological matrices is needed to determine how much astaxanthin should have been given to the fishes in order to minimise as much as possible the cost of the diets.

Astaxanthin commands high demand in the market and it is ranked as the second most important carotenoid after capsanthin in the international market. It is a high-value specialty product and costs approximately US$ 5000–6000 per kg. Its global market size and value exceeded US$ 288.7 million in 2017 and is predicted to reach US$ 426.9 million by 2022, dominating the carotenoid industry, with a forecasted 8.1% compound annual growth rate until 2022 (Mcwilliams, 2018). With increase in the demand for natural ingredients, the microbial fermentation technology was

implemented to produce astaxanthin and research has been focused in the production of natural astaxanthin through bioprocessing design and development, aiming to overcome problems associated with chemical synthesis. However, the basic production cost using algae *H. pluvialis* was at least 3.5 times higher than chemical synthesis, making the yeast *X. dendrorhous* fermentation a possible alternative source for natural astaxanthin with a lower total basic cost (Dang Nguyen, 2013).

Astaxanthin is highly affected by the environment once exposed to light, oxygen and temperature. Different types of carriers, storage techniques and condition have been applied to improve the stability of astaxanthin (Lin *et al*., 2016). Astaxanthin products are developed in the dosage forms as capsules, tablets, oils, syrups, soft gels, creams, biomass and granulated powders for commercial uses (Ambati *et al*., 2014). The Food and Drug Administration of United States (FDA, USA) has approved the use of astaxanthin as food colouring agent for specific uses in fish and animal feeds (Pashkow *et al*., 2008). According to European Commission, the safety consumption of astaxanthin for marine animals is 100 mg astaxanthin/kg (Bampidis *et al*., 2019). Studies showed that no adverse effect was reported in rats given the oral dosage as high as 950-1000 mg astaxanthin/kg per day for 90 days (Katsumata *et al*., 2014; Lin *et al*., 2017). In human, the consumption dosage was extended from 4 to 100 mg per day for maximum up to a year (Fassett & Coombes, 2009). The recommended safety dosage for adult human from the food supplement was 8 mg astaxanthin/kg per day (Turck *et al*., 2020). Natural astaxanthin is generally recognized as safe (GRAS) and has been approved as a food colourant and dietary supplement by the US Food and Drug Administration for specific uses in food and feed. It can be sold as a dietary supplement and has been approved as a food dye within the E number system (E161j)

by the European Commission for use in the beverage and food industries (Solymosi *et al*., 2015)

2.5 Breast Cancer

Cancer has an important psychosocial effect on patients and their families other than imposing a huge financial burden on patients, families and the society. It is the fourth leading cause of death in Malaysia, contributing 12.6% of all deaths in general hospitals and 26.7% in private hospitals in 2016. The trend has shown increment to 12.6% in 2016 from 11.3% in 2007 (Health Informatics Centre, 2016). The cancer incidence in Malaysia, in males was 86.9 and in females was 99.3 per 100,000 populations for the period from 2007-2011 (Azizah *et al*., 2016). For breast cancer, incidence rate in Malaysia was 47.5 per 100,000 women relative to 90.3 in Ireland and 84.9 in the US in 2018. Nonetheless, breast cancer prevalence has been rapidly rising in most traditionally low-risk countries, presumably as the results of Westernized lifestyle, and breast cancer is currently the most regularly detected female cancer in South-East Asia (26.4%) and in Malaysia (33%)(Bray *et al*., 2018).

Breast cancer has the highest incidence rate among the female population and it is 100 times more probably to occur in a woman than in a man (Wu *et al*., 2004). Women face a higher risk of breast cancer because they have much more breast tissue than men do and estrogen encourages breast cancer development. Many risk factors for breast cancer have been pinpointed, including genetic, lifestyle and environmental factors. The established risk factors for breast cancer include female gender, age, race, hereditary factors, benign breast disease, breast cancer history, early menarche, late menopause, postmenopausal obesity, first full-term pregnancy at late age, low physical activity and high-dose exposure to ionizing radiation. Other risk factors that have been

postulated for breast cancer are never having been pregnant or having only one rather than multiple pregnancy, postmenopausal hormone replacement therapy, consumption of oral contraceptives, specific dietary practices, tobacco smoking and alcohol consumption.

Breast cancer is cancer that forms in the cells of the breasts and the symptoms of breast cancer may include changes in size, shape and the appearance of the breast, inverted nipple, a lump, redness or changing of the skin around the breast. Determination the stages of breast cancer was based on the tumor size and the metastatic condition of the cancer. The stages of cancer are divided into 4 stages in TNM (tumor, node and the metastasis) staging system of the disease and is critical for a better grasp of the disease and the future treatment plan. The staging of cancer started with a small tumor in a particular organ and is not invasive. Stage 2 represented with a larger size of tumor compared to stage 1 and showed a possible shifting into the other body parts. For stage 3, the tumor had grown relatively large and start to grow to the other body parts and moving on to stage 4 the tumor had spread to the other parts of the body and there is possible uprise of second cancer. Stage 2 to 4 breast cancer treatment usually includes radiation therapy and surgery, often with chemotherapy before (neoadjuvant) or after (adjuvant) surgery. According to NCI (2018), the average 5-year relative survival for breast cancer in Malaysia was 66.8% for those diagnosed between 2007 and 2011 and followed up to 2016. The 5-year relative survival in Malaysia was higher than India and Thailand but lower than Singapore, China, Korea, Japan, Australia, New Zealand, USA and most of the European countries (Allemani *et al*., 2019). The relative survival at stage I and stage II was the highest, exceeding 80% up to 10 years for stage I and up to 5 years for stage II. Survivals declined at a faster

rate for stage III and stage IV. Hazard ratio was 1.41 at stage II, 2.71 at stage III and was significantly high at stage IV, which was 7.52 compared to stage I.

In addition to pathological grade and stage, upgraded breast cancer staging system now includes progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) status in order to guide the choice of the appropriate and targeted therapy. Different etiologic pathways are thought to be associated with different breast cancer subtypes classified by hormone receptor (HR) status (Gaudet *et al*., 2018). With that, four subtypes were identified as basal like, luminal, claudin-low and triple negative cell lines. The most common cell lines represented in the anticancer research including MCF-7 and MDA-MB-231 as they emerged a total difference in the cell characteristics. MCF-7, characterized by ER+, PR- and HER2-, is the most commonly used Luminal A subtype breast cancer cell line in researches because it has very high hormone sensitivity due to its high ER expression. Meanwhile, MDA-MB-231 is one of the claudin-low (triple negative, ER-, PR-, and HER2-) breast cancer subtype (Perou & Borresen-Dale, 2011).

2.6 Current breast cancer treatment and its limitations

Breast cancer treatment has evolved over time, both surgically and medically. Breast cancer care in the modern era is becoming more personalised and reliant on multimodal approaches. Commonly practice cancer therapy nowadays includes surgery, chemotherapy, radiotherapy, hormone therapy and immunotherapy. The different surgical approaches include mastectomy alone or with reconstruction, and lumpectomy or breast conserving therapy (BCT) that removes only the part of the breast that has cancer. Chemotherapy is usually prescribed to systemic disease as opposed to localised lesions that can be treated with surgery or radiation.

Antineoplastic agents are used to try to undermine tumor cells by interfering with cellular functions such as replication (Singh *et al*., 2018). The downside of the chemotherapy usually came along with extreme fatigue, nausea, loss of appetite, hair loss and liver damage, other than concerns for hypercalcemia, neutropenia and preservation of fertility (Yip *et al*., 2014). Radiotherapy is one of the common treatment provided by targeting cancer cells at the specific region with high energy X-ray. The adverse effects include fatigue, breast swelling, redness and/or discolouration of the skin, or pain or burning of the skin, sometimes with blistering or peeling (Tao *et al*., 2015). Hormone therapy is a treatment for cancer that slows or stops the growth of cancers that use hormones to grow and is one of systemic therapies involving medication (Tao *et al*., 2015). It is effective for majority tumors tested positive for either progesterone or estrogen receptors (PR positive or ER positive). Hormonal therapy may be administered pre-surgery to shrink a tumor and stop the metastasis process of the cancer thus make surgery easier to carry out. Tamoxifen is a drug that obstructs estrogen from attaching to breast cancer cells especially for those women who have been through menopause. Yet, the general side effects of tamoxifen include hot flashes and vaginal dryness, discharge or bleeding. Immunotherapy is a newer form of treatment that uses body immune system to fight the cancer. There are many ways in performing this therapy including the immune checkpoint inhibitors and monoclonal antibodies designed specifically to target the cancer cells. Atezolizumab and Pembrolizumab are examples of drugs sanctioned by the FDA for the immunotherapy of breast cancer. Immunotherapy will cause a milder side effects compared to the other types of treatment and patients who received this treatment were likely to experience fever, diarrhea, vomiting and headache. Tumor necrosis factor (TNF), interferon-γ (IFN-γ), and forkhead box P3 (FOXP3) are immunological