Astaxanthin production from *Schizochytrium* sp and its stabilisation through encapsulation

A thesis submitted to Flinders University for the degree of

Master of Biotechnology

College of Medicine and Public Health

Ratnakinnera Kode

Master of Agriculture

Student ID:2252206

9th May 2023

TABLE OF CONTENTS

Chapter Two

Chapter Three

ABSTRACT

Astaxanthin is a red coloured carotenoid that occurs naturally. It is widely used as a nutraceutical because it has several health benefits including antioxidant activity, anti-inflammatory effects, improved eye health, enhanced skin health, and better cardiovascular health. The green algae *Haematoccus pluvalis* is the commercially viable natural source, however, it accounts for only 1% of total astaxanthin production. Recently, the marine protist *Schizochytrium* sp. has been documented as a source of polyunsaturated, squalene and carotenoid production. In this study, a newly isolated *Schizochytrium* strain (DT3) was used for astaxanthin production. *Schizochytrium* strain was treated with different ethanol, and methanol concentrations to investigate the effects on astaxanthin accumulation. Using an ultrasonication process, astaxanthin was extracted from a freeze dried *Schizochytrium* biomass. It was observed that 3% ethanol concentration promoted astaxanthin accumulation in 168 h. Further, biological activity analysis displayed that extracted astaxanthin exhibited the highest radical scavenging activity (ABTS) when compared to Trolox as a positive control. Astaxanthin obtained through *Schizochytrium* fermentation was encapsulated with high pressure homogeniser technique employing various polymers/wall materials. The egg albumin, gum arabica and sodium alginate combination showed 68% entrapment efficiency of astaxanthin enriched biomass. The study showed that the encapsulation efficiency of astaxanthin improved as the concentrations of enriched biomass or astaxanthin oil increased. The results showed that the highest encapsulation efficiency (74%) was observed when combination of pea protein, sodium alginate and gum arabica investigated as wall materials. The study demonstrated an increased encapsulation efficiency of astaxanthin, making the process suitable for food and pharmaceutical applications.

DECLARATION

Declaration

I certify that, aside from any references made in the text or the notes, this thesis does not contain any information that has been used to confer a degree or diploma and that, to the best of my knowledge and belief, it does not contain any information that has been previously published or written by someone else.

Ratnakinnera Kode

09-05-2023

ACKNOWLEDGEMENTS

First, I would like to express my gratitude to Associate Professor Munish Puri, Dr. Adarsh Gupta (Post-doctoral Associate), and Dr. Mariam AI Hattab (Post-doctoral Associate), for supervising me. I also express my thanks to Shweta Sahni, and Vidana Arachchige Appuhami for providing assistance while pursuing my research work in the laboratory. They have always helped me out when I've had issues or questions concerning my research or thesis writing. Throughout my Master's project, they gave me excellent guidance. I am really grateful for the supervisor's kindness and advice while I was preparing my thesis.

I am grateful for the help, advice, and pleasant experiences I had in the lab with the faculty and research assistants from the Department of Medical Biotechnology.

Finally, I want to express my gratitude to my family for their unending help and support throughout my education and my thesis research. This accomplishment would not have been possible without them.

LIST OF FIGURES

LIST OF TABLES

Abbreviations

CAGR	Compound annual growth rate
µl, ml	microliter, millilitre
µM, mM	micromolar, millimolar
%	percent
Approx.	approximately
Hr	hour
CaCl2	calcium chloride
Conc	concentration
HCL	Hydrochloric acid
PEF	Pulse electric field

Chapter 1

1.Introduction and Literature review

1.1. Importance and current market scenario of Astaxanthin

Microalgae are considered a promising platform for sustainable production of many bioactive compounds (Hussian, 2018). Carotenoids are phytochemicals responsible for the various colours of food and also play a key role in dietary supplement to maintain the health and immunity of human, and animals. Around 700 naturally occurring carotenoids are known, and they are responsible for colour of plant leaves, flowers, fruits, birds, fish, and insects (Sankari et al.,2016). Carotenoids are classed as xanthophylls or carotenes based on the degree of oxygenation. Carotenes include β-carotene, xanthophylls, α- carotene or lycopene While xanthophylls includes astaxanthin, lutein, zeaxanthin, and canthaxanthin. Astaxanthin is a natural carotenoid with a red colour that is soluble in fat. (Bhosale et al.,2005). It is found in a variety of organisms, including algae, yeast, salmon, trout, krill, shrimp, crayfish, and seaweed. Its unique chemical structure gives it a range of potential health benefits (Fig 1), including antioxidant and anti-inflammatory properties. (Aneesh et al., 2022)

Figure removed due to copyright restriction

Figure 1: Biological properties of Astaxanthin (This figure is modified from Martins et al., 2021., and Davinelli et al., 2018)

Due to astaxanthin's substantial anti-oxidative capabilities, numerous studies have been conducted to examine its potential medical applications in the prevention and treatment of diseases caused by reactive oxygen species, including cancer and neurological disorders. (Kavitha et al., 2013).

The astaxanthin market is anticipated to develop at a 17.2% CAGR from 2023 to 2030, reaching a value of USD 6.90 billion. For instance, Health Canada granted NextFern Technologies regulatory certification for its Astafern in July 2021. (ltd, Astaxanthin market size, Share & Trends analysis report, 2023 - 2030). By 2026, the Indian pharmaceutical industry is expected to generate $55 billion. (Market, 2022). Astaxanthin producer US-based Algalif got non-GMO certification (Astalif, 5%, and Oleoresin, 10%). Their products are of high quality. (Algalif's astaxanthin awarded non-GMO status, 2022). The primary drivers of market expansion are the rising demand for nutraceuticals and the expanding applications of carotenoids in aquaculture and the animal feed industry. (Fig 2).

Figure removed due to copyright restriction

Figure 2: Market value of astaxanthin based on its application in various sectors. (This picture has been reproduced from dataintelo.com/ astaxanthin market, 2022.)

1.2 Chemical structure and sources of Astaxanthin

Astaxanthin (3,3' -dihydroxy-β-β carotene 4,4'-dione), a carotenoid of economic significance, is a fat-soluble carotenoid that dissolves in almost all organic solvents including chloroform, ether, acetone, and ethanol. It is insoluble in water and has a melting point of 224°C. The molecular weight of astaxanthin is 596.86 g/mol. It has greater antioxidant activity than other carotenoids due to its peculiar chemical structure with polar end groups that quench free radicals. It potentially removes the high energy electrons because of its double bond in its central segment (Fig 3). (Davinelli et al., 2018). It's worth noting that astaxanthin occurs naturally in the three stereoisomeric forms transform (3S, 3'S), but synthetic astaxanthin is made up of two optical isomers: meso form (3R, 3'R), (3R, 3"

S), and (3S, 30'S) dominates the world astaxanthin market. However, astaxanthin from microbial sources are mainly trans isomer 3S, 3'S which enjoys great consumer preference owing to its better pigmentation capabilities (Barbosa et al., 1999).

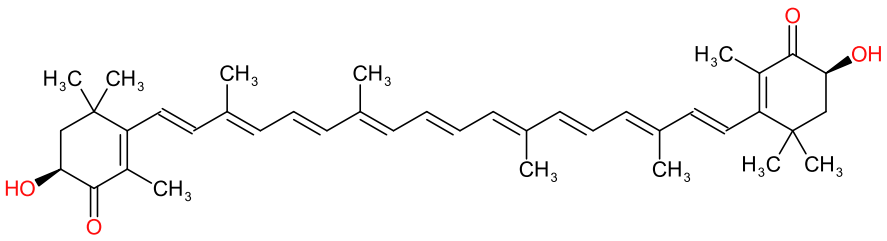


Figure 3: Chemical structure of Astaxanthin

A freshwater alga is the primary natural source of astaxanthin used for commercial purposes. When under stress such as nutrient deprivation, salt stress, high temperatures, or strong sun intensities thick-walled globular cysts form that contain astaxanthin. This natural astaxanthin is more biologically active than synthetic astaxanthin (Table1). They are suitable for the industrial-scale mass production of astaxanthin due to these characteristics. Moreover, Carotenoid derived from microalgae offers wide range of benefits (Aneesh et al., 2022).

Table1: Difference between synthetic and natural astaxanthin

Characteristics	Synthetic astaxanthin	Natural astaxanthin	Reference
Isomers	1:2:1	1:2:22	(Zhang et al., 2021)
Structure	Non esterified structure	95% of molecules are esterified	(Li et al., 2020)
Industrial application	Aquaculture feed	Application in food, cosmetics, nutraceuticals, aquaculture feed	(Li et al., 2020)

1.3 Microbial production of Astaxanthin

Microalgal sources of astaxanthin include *Chlorella zofingiensis* (Bar et al. 1995), *Botryococcus braunii* (Grung et al. 1994), *Chlamydomonas nivalis* (Bidigare et al. 1993), *Thraustochytrium sp*. S7 (Singh et al., 2015). The most frequent natural source of astaxanthin on the market is *H. pluvialis*. However, growing microalgae in open systems poses several challenges. For example, there is a risk of undesirable microbes contaminating ponds. Because of this risk, expensive high capacity photobioreactors are required. Furthermore, *Haematococcus pluvialis* develops slowly and produces little biomass when grown in standard media. (Ranjbar et al., 2008). The marine microalgae belonging to family Thraustochytritaceae synthesise carotenoids most probably to escape oxidative stress and to prevent storage fats from oxidation (Burja et al., 2006). *Aurantiochytrium, Thraustochytrium, Schizochytrium, Aplanochytrium, Labyrinthuloides*, and other strains of thraustochytrids grow heterotrophically and synthesise astaxanthin. When compared to other astaxanthin-producing strains, *Schizochytrium* microalgae have a low astaxanthin concentration. However, the amount of carotenoid synthesis depends entirely on the careful strain selection, downstream processing optimisation, and process integration are necessary for cost-effective microbial astaxanthin production. Optimal growing conditions that promote cell growth and raise the yield of biomass and astaxanthin is significant. (Table 3).

Impact of different carbon sources on the productivity of astaxanthin

1.3.1. C-source promotes biomass growth and astaxanthin production

For many microorganisms, glucose is a crucial supply of carbon, and it's commonly used as a substrate for the synthesis of astaxanthin (Table 2). For some cases, adding glucose to the culture medium can boost the biomass and astaxanthin concentration of the microorganisms. A study (Xiao et al., 2019) mentioned the investigation of glucose. The findings showed that astaxanthin was achieved at a concentration of 1.5 g/L at 40 g/L and reached a maximum astaxanthin concentration of 0.5g/g dry cell mass at 100 g/L. Also, they noticed that raising yeast and peptone (16g/L) levels resulted in the highest astaxanthin concertation. In a study, high concentrations of astaxanthin and canthaxanthin were produced in the *Aurantiochytrium* sp PH-7A and RH-7A-7 strains via chemical mutagenesis. Research on metabolism has shown that oxidative stress affects the accumulation of carotenoids. When ferrous ion (Fe^{2+}), an agent for oxidative stress, was introduced to the culture media, the mutant's production of astaxanthin increased significantly (Watanabe et al., 2018).

Table 2: Effect of various carbon sources on production of astaxanthin

Species	Carbon and N-sources	Astaxanthin (Yield)	References
Aurantiochytrium sp.	Methanol, 1-Propanol 1-Butanol	0.327±0.029mg/L 1.592±0.168mg/L 0.952±0.072mg/L	(Sakamoto, T. *et al.*, 2023)
Chlorella zofingiensis	Ammonia	1.22±0.07mg/g	(Liu et al., 2013)
Chlorella zofingiensis	Urea	1.17±0.04mg/g	(Liu et al., 2013)
Paracoccus carotinifaciens	Biotin Sucrose Corn steep liquor	864mg/L	(Hirasawa, K. *et al.*, 2017)
Paracoccus carotinifaciens *Bacillus megatarium*	Corn cob hydrolysate	10.2mg/L	(Khomlaem et al., 2021)
Schizochytrium limanicum	Butanol	107.74±9.24µg/g	(Zhang et al., 2017)
Thraustochytrium sp	Biotin, ascorbic acid, And Glycerol	168±7µg/g (Total carotenoid) 7.1±0.4%	(Leyton et al., 2022)

Thraustochytrium sp	Glycerol	131.56µg/g	(Singh et al.,2015).
Thraustochytrium sp, *Aurantiochytrium* sp.	Glucose and nitrogen	21.1±1.2µg/g 52.0±1.3µg/g	(Furlan et al., 2019)
Xanthophyllomyces sp.	Ethanol	1.05mg/L	(Kikukawa et al., 2022)

1.3.2. Ethanol promotes astaxanthin accumulation

Many microorganisms have been proven to grow and function more efficiently when exposed to ethanol, and in some instances, it has also been demonstrated to boost astaxanthin production (Fig 4). The effect of ethanol on astaxanthin production was observed (Sakamoto et al., 2023). The astaxanthin productivity of *Aurantiochytrium* sp. (2.231mg/L) was increased by 2% ethanol. Moreover, in 2% ethanol concentration, the mutant strain OM3-3 produced 5.075 mg/L of astaxanthin. A study looked into how ethanol affected the build-up of astaxanthin in *Haematococcus pluvalis*. The outcomes showed that even in low-light situations, ethanol addition caused astaxanthin accumulation. At 3% (v/v) ethanol, the astaxanthin productivity was capable of reaching 11.26 mg/L, which was 2.03 times higher than that of the control (Wen et al., 2015). In a study, Zhu et al., (2022) examined the effect of different ethanol concentrations on the synthesis of astaxanthin *S. limacinum B4D1*. The findings revealed that high amounts of ethanol (3% or above) increased the production of astaxanthin and altered the fatty acid ratio. Furthermore, after four days of treatment, it was revealed that the addition of ethanol (0.4%) considerably raised the cellular astaxanthin concentration to 3.85%. (Liu et al., 2019).

1.3.3. Methanol promotes astaxanthin accumulation

Methanol is known to enhance the growth and metabolic activities of a number of microorganisms and in certain cases, it has been shown to increase astaxanthin production. Lee et al. (2017) looked into the impact of methanol on *Haematococcus pluvialis* microalga astaxanthin synthesis. They discovered that adding methanol to the culture medium significantly increased the biomass and astaxanthin concentration of the algae. Their hypothesis states that methanol functions as a carbon source to encourage the production of astaxanthin. In a different study, the effects of the methanol treatment were examined (Du et al., 2019). The results showed that astaxanthin production increased

along with the increase in methanol concentration, peaking at 5.6%, which was 2,000 times more than the control. However, 5.6% methanol, the biomass and lipid content decreased.

1.3.4. Other Carbon sources promotes carotenoid accumulation

Other carbon sources that have been shown to increase carotenoid accumulation in microbes include glycerol, acetate, and lactate. In a study it is proven that, adding oxaloacetate to microalgae growing media increases astaxanthin concentration in the cells. This is because, oxaloacetate can act as a precursor for the production of astaxanthin, and its addition can boost the availability of this precursor for the cells. Yu et al. (2022) examined the effect of oxaloacetate on *Haematococcus pluvialis* microalga astaxanthin synthesis. The results showed that, under nitrogen deprivation on day 7, the addition of 10 mM- oxaloacetate increased the cellular astaxanthin content approximately 7.18-fold.

The effectiveness of using several carbon sources (CO (2), glucose, glycerol, starch, urea, and glycine) for *C. acidophila's* efficient growth and carotenoid synthesis was examined in a study (Cuaresma et al., 2010). Results indicate that different carbon sources produced varying algal biomass productivities, with urea being similarly effective as CO (2) when used as the only carbon source (20 g dry biomass m (-2) day (-1), according to the research. Mixotrophic growth on glucose was also effective in producing biomass (14 g dry biomass m (-2) day (-1)).

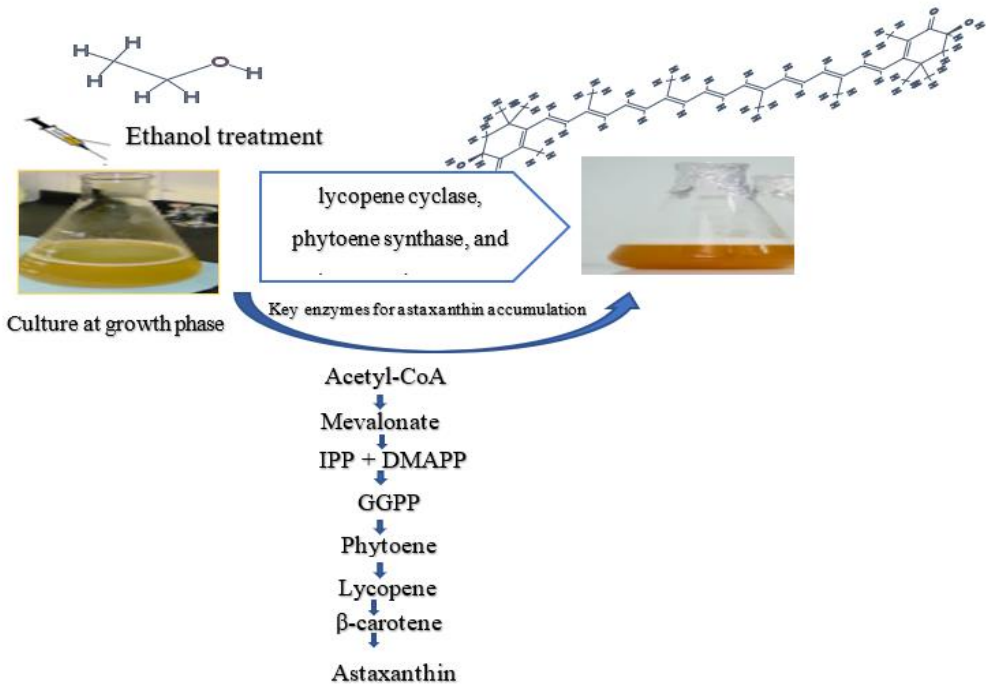


Figure 4: The biochemical pathway for astaxanthin accumulation in a *Schizochytrium* sp.

Table 3: Microbial production of Astaxanthin

Microalgae	Technology used	Cell dry weight	Lipids (% TFA)	Astaxanthin (ug/g)	Extraction method	Reference
Chromochloris zofingiensis	Fermentation High illumination Light and Dark cycle Low illumination	3.65±0.21g/L 7.30±0.02g/L 6.75±0.35 g/L	-	7.11mg/g	Solvent extraction	(Chen et al., 2017)
Schizochytrium limacinum B4D1	Fermentation	25.69g/L	23.97%(DHA)	107.74±9.24	Sonication	(Zhang et al., 2017)
Schizochytrium sp. SHG 104	Fermentation	10.8	45.8% 32.1%(DHA)	4.6mg/L	Solvent extraction	(Park et al., 2018)
TISTR 9500 strain (Chlorococcaceae family)	Fermentation KCl stress N starvation	-	12.79±0.03 28.07±0.79 (Total FA %)	3.85±0.11 1.18±0.01 µg/mg DW	Ultrasonication	(Janchot et al., 2019)
Thraustochytrium	Batch and Repeated batch culture	4.2±1.1g/L 3.3±0.1g/L	EPA (16.5%) DHA 42.67%	168±7	Sonication	(Leyton et al., 2022)

Table :4 Microbial sources and factors effecting the yield of astaxanthin

Species	Class/Mode of Cultivation	Culture condition	Extraction Method	Astaxanthin Yield	Reference
Alphaproteobacteri a	Coculture *Paracoccus haeundaensis,*	Incubated for 72h at 25°C	Treated with 6 (chloroform):4 (Methanol)	821.09±30.98 µg/g Concentration /dried cell weight	(Choi et al., 2021)
Chlorella zofingieniss	Chlorophyceae / (Mixotrophic mode of cultivation)	12h(Light): 12h (dark) cycle under illumination above 80µmol photons $m^{-2}s^{-1}$ under illumination above 80µmol photons $m^{-2}s^{-1}$	Bed beating	14.82±2.68 µg/g	(Chen, Liu and Wei, 2017)
Chlorophyceae/ Autotrophic mode of cultivation	*Chlorococcum sp*	Illumination 22µmol photons $m^{-2}s^{-1}$, H_2O_2 0.1mM.	Freeze drying Using methanol /dichloro methane (3:1, v/v) .	6.5±0.3 µg/g	(Yin-Nin Ma and Chen, 2000)

Chlorophyceae/ Autotrophic	*Coelastrum sp.*	Illumination white light 35±5 µmol photons $m^{-2}s^{-1}$ and increased to 165±5 µmol photons $m^{-2}s^{-1}$ after stationary phase, Black light at 365nm/1hr per day/15 days	Acid method acetone	0.999 g/ml	(Kaha et al., 2021).
Haematococcus pluvialis	Chlorophyceae/ (Mixotrophic mode of cultivation)	12h(Light):12h(dark) cycle under illumination at 104µmol photons $m^{-2}s^{-1}$	Ultrasonication with ethyl acetate added	24.55±2.83 µg/g	(Pan-utai et al., 2021)
Haematococcus pluvialis	Chlorophyceae/ (Mixotrophic mode of cultivation)	16h(Light):8h(dark) cycle illumination at 75µmol photons $m^{-2}s^{-1}$	Ultrasonication with liquid nitrogen, hexane, and acetone (1:1v/v) added.	123.76±15.26 µg/g	(Coutinho Rodrigues et al., 2021)

Labyrinthulea/ (Heterotrophic)	*Aurantiochytrium Sk 4* Wild type	In dark condition at 25°C at 150rpm,	Solvent extraction	28.2±2.9 µg/g	(Ye et al., 2019)
Labyrinthulea/ (Heterotrophic)	*Thraustochytrium* sp. s7	Incubated 10-15 days at 17°C	Ultrasonication	156.07±4.15 µg g^{-1}	(Singh et al., 2015)
Tremellomycetes/ Heterotrophic	*Xanthophyllomyces dendrorhous*	Incubated at 20 ◦C for five days/600rpm	Enzymatic extraction using Accellerase, Glucanex	3.6 mg/L	(Harith et al., 2020)

1.4. Extraction of astaxanthin

In order to extract astaxanthin, cells may be disrupted using a variety of traditional and advanced methods, such as milling, homogenization, ultrasonic pulsed electric field (PEF), multi-enzyme, hydrochloric acid, high pressure micro-fluidization, and ionic liquids (Table 4). Tan et al. (2021) compared the impacts of various techniques on the astaxanthin composition. The results showed that the yield, purity, and stability of astaxanthin varied depending on factors such as the type of microorganisms used, cell densities, and processing conditions. These findings highlight the importance of selecting the most effective cell disruption method to increase astaxanthin production and purity.

1.4.1. Chemical assisted extraction

Solvent-assisted extraction is a method for extracting compounds from a sample while using a solvent. A nonpolar solvent, like hexane, acetone, or ethyl acetate, is frequently utilized in the case of astaxanthin, a lipophilic carotenoid. The astaxanthin is dissolved by the solvent and is then separated from the other components of the sample. In one study (Dong et al., 2014), astaxanthin was extracted from *Haematococcus pluvialis* using four different methods: methanol extraction followed by acetone extraction (MET-ACE, 2-step extraction), hydrochloric acid pre-treatment followed by acetone extraction (HCl-ACE), hexane/isopropanol (6:4: v/v) mixture solvents extraction (HEX-IPA), and soy oil extraction. Results showed that the HCl-ACE approach would give the highest oil and astaxanthin content.

1.4.2. Bead milling assisted extraction

The process involves grinding the microalgae biomass with small glass or ceramic beads in a high-speed bead mill. The beads and the biomass are agitated in a chamber, creating a high-shear environment that disrupts the cell walls and releases the intracellular components. A study was conducted (Liu et al., 2021) on bead milling method for mechanical disruption of yeast cells. It was filled with 65% v/v of 0.4 mm zirconia beads, system with a constant 2039 rpm agitation and temperature was kept under control at 20 °C. For measures of product release and cell disintegration, samples were obtained every minute. The results showed that a recover 75.0±2.2% of the lipids and 70.6±3.0% of the carotenoids with a selectivity of 99.9±0.0% and 98.1±0.1%, respectively. Furthermore, 14.9±0.6% of the proteins were retrieved. However, this technique is strain specific. Based on the studies it is more effective in certain studies and less effective in others. The drawback to this technique is that it produces heat.

1.4.3. Ultrasound assisted sonication

Ultrasound-assisted sonication extraction is a technique that uses high-frequency sound waves to break down the cell walls of a sample, allowing for the extraction of compounds such as astaxanthin. A study conducted by Pan-utai et al., (2021) compared mechanical and chemical extraction methods of astaxanthin. The result indicated that the ultrasonic technique with solvents improved the astaxanthin yield (see Fig 5). The highest astaxanthin production was observed with ethyl acetate after 25 min of ultrasonication. Singh et al. (2015). conducted a study on the four mechanical methods for cell rupture, including three-minute maceration using a mortar and pestle after quick freezing of biomass with liquid nitrogen. 50 cycles of ultrasonication at 20 kHz (kilohertz) for 30 minutes. Homogenization at 10,500 rpm for 30 minutes. According to the findings, the astaxanthin yield increased with the use of ultrasonication approach compared to direct extraction (from 26.77±1.23 μg/g to 156.07±4.15 μg/g with ultrasonication), accounting for a 6-fold rise in extraction efficiency.

Figure removed due to copyright restriction

Figure 5: Principle of ultrasonication (This figure is reproduced from Shojaeiarani et al., 2020)

1.5. Encapsulation of astaxanthin

Encapsulation refers to immobilizing a bioactive compound, such as astaxanthin, within a protective matrix/material, known as the wall material, to improve its stability, solubility, and bioavailability. Encapsulation of bioactive can provide several advantages over free form. First, encapsulation can protect astaxanthin from oxidation and degradation, which can lessen its efficiency and potency. The astaxanthin may be shielded from outside elements including light, oxygen, and moisture through encapsulation, which can prevent deterioration. Second, astaxanthin's bioavailability may be enhanced via encapsulating. Because astaxanthin is a fat-soluble substance, it is best absorbed when

there are fats or oils present. Astaxanthin can be administered via encapsulation in a more bioavailable manner, such as in a lipid-based capsule or emulsion (Khalid & Barrow, 2018). Astaxanthin can be protected from harmful elements and have its shelf life increased through encapsulation. The most crucial elements for the encapsulation process are encapsulating chemicals, temperature, and drying techniques (Hussein et al., 2006). Most astaxanthin delivery systems through encapsulation, including improving organoleptic characteristics by covering up unpleasant flavours or odours, enhancing cell membrane transport that increases bioavailability, and controlled release rate (Martinez-Alvarez et al., 2020).

There are various techniques such as coacervation, extrusion, spray drying, solvent evaporation, emulsification, fluidized bed, phase separation, interfacial polymerization, and plasma polymer-based encapsulation are studied for bioactive encapsulation (Table 5,6). Because of space limitation only some of the methods are described below

1.5.1 Emulsification

Emulsions are frequently used to stabilize astaxanthin. Proteins, in particularly, are excellent emulsifiers and are therefore used in wide range of food emulsions. Anarjan et al (2011) used sodium caseinate to stabilize AXT nano dispersion using the emulsification-evaporation method followed. Khalid et al. (2017) used straight through microchannel emulsification to create astaxanthin loaded emulsions with excellent storage stability, lasting more than 15 days without a significant increase in droplet size. (Khalid et al., 2017). In another study, astaxanthin-containing microcapsules were produced using spray drying and emulsion solvent evaporation. The impact of polylactic acid concentration on astaxanthin dispersion and microcapsules was studied and the results showed excellent stability and biocompatibility of microcapsules, with an encapsulation efficiency of 70.82 % (Fang et al., 2022).

Figure removed due to copyright restriction

Figure 6: High energy emulsification (This figure is reproduced from Nikolic et al., 2020)

1.5.2 High pressure homogenization

For the encapsulation of bioactive substances, such as astaxanthin, in a variety of matrices, the high-pressure homogenization (HPH) is a widely utilised approach (Fig 6). The process of high-pressure homogenizer involves three crucial parameters that influence the outcome, namely temperature, pressure, and the number of cycles (Espitia et al., 2018). W/O/W double filled hydrogel was studied to examine its stability. As a surfactant for W1/O, 4% PGPR was specifically combined with soybean oil, and as a gelling agent, gelatine was first dissolved in water (W1). After 7 minutes of stirring at 15000 rpm, a W1/O emulsion was created. The W1/O emulsion was then homogenised at 13000 rpm for 5 min with a sodium caseinate solution (W2) to create the W/O/W double emulsion. The Gellan gum solution was combined with the W1/O/W2 emulsions using a homogenizer at 13000 rpm for 5 minutes to create steady W1/O/W2 emulsions. The double emulsions were stable for 30 days while being stored, with the maximum encapsulation efficiency of phycocyanin and astaxanthin being 94.1% and 90.82%, respectively (Yu et al., 2022). Astaxanthin was encapsulated in solid lipid nanoparticles utilising stearic acid, glycerine monostearate, and glycerol distearates in soybean oil as the dispersed phase and deionized water with Tween 20 as the continuous phase. Using high pressure homogenization, the various phases were blended together. The particle size and thermal stability of astaxanthin in nanoparticles were enhanced 1% (Li et al., 2015).

Table 5: Types of encapsulation technologies employed Astaxanthin stabilisation

Core material	Wall material	Technology used	Encapsulation efficiency	Reference
Asta-C22:6	Whey protein isolate and hydroxypropyl-β-cyclodextrin	Freeze drying Spray drying	72.5%	(Gao et al., 2022)
Astaxanthin rich oil extract	Whey protein and Persian gum	Lyophilization	42.95±0.86 46.05±0.48 49.85±0.37	(Bassijeh et al., 2020)
Astaxanthin & Phycocyanin	Polyglycerol polyricinoleate and gelatin soyabean oil sodium caseinate	W/O/W emulsion	94.1%	(Yu et al., 2022)
Astaxanthin	Soy phosphatidylcholine cholesterol	Liposomes	97.68± 0.34%	(Pan et al., 2018)
Astaxanthin oleoresin	Sodium caseinate and k-carrageenan	Freeze drying	85%	(Huang et al., 2022)
Astaxanthin	PLGA with chitosan nano particles	PLGA nanoparticles	85%	(Liu et al., 2019)
Astaxanthin	Carboxymethyl cellulose sodium and	Freeze drying	58.76% 52.88%	(Feng et al., 2018)

	microcrystalline cellulose			
β-Carotene	High denatured peanut protein and sesbania gum	Cold plasma	97%	(Yu et al., 2021)
Camelina oil extract	Chicken egg albumin, Gum Arabica Tannic acid	-	70%	(Xie et al., 2020)

1.5.3 Types of wall material used for encapsulations

Wall material (made of natural and synthetic polymers) play a key role in encapsulation of bioactive. There are two types of natural polymers: protein-based (such as albumin and gelatine) and polysaccharide-based (such as agarose, alginate, dextran, and chitosan, etc) that are used in the literature for achieving bioactive entrapment (Fig 7). These organic polymers are naturally biodegradable and biocompatible. Synthetic polymers such as poly lactic acid, poly (lactic-co-glycolic acid), and polycaprolactone etc. have been studied for astaxanthin encapsulation. The physical and chemical characteristics of the resulting micro-/nano capsules are determined by the selection of a suitable wall material (Vijeth et al., 2019).

Figure removed due to copyright restriction

Figure7: Wall materials used in encapsulation of bioactive (This figure was reproduced from Jayanudin et al., 2016)

1.6 Strengths of encapsulation

There are several potential uses for encapsulation in the food, agricultural, and pharmaceutical industries since it creates a physical barrier between the core molecule and the surrounding media. (Martinez-Alvarez et al., 2020). Finer particle size, high nutrient adsorption content, and better stability, including resistance to heat and salt, were all associated with the formation of double-layer structures on the oil-water interface. (Yu et al., 2021.) Enhancing the bioavailability and defending the vulnerable molecule from oxidation and degradation is possible with astaxanthin encapsulation. This method of delivery can aid in delivering the entire range of astaxanthin's health advantages, including its anti-inflammatory and antioxidant capabilities. It is a risk-free and efficient way to take advantage of astaxanthin's potential benefits for general wellbeing and good heal. The advantages and disadvantages of various methods used for astaxanthin encapsulation are summarised in Table 6.

1.7 Research gap

Choosing the right encapsulating materials is essential to preventing astaxanthin's deterioration and enhancing its absorption. Encapsulation is one method for preserving astaxanthin from deterioration, however it is unclear how this will affect how well the body will absorb and use it. Although several encapsulating methods have been studied, they still need to be optimised in order to boost the stability and efficiency of astaxanthin encapsulation. By choosing the right encapsulating material, astaxanthin's stability and bioavailability can be significantly affected. In some studies, synthetic emulsifiers, such as Tween 20 and Tween 80, were used (Anarjan et al., 2010). Some of these studies might not be suitable for mass production, as well. Additionally, more research is needed to compare

the bioavailability of free astaxanthin to that of astaxanthin that has been encapsulated. While some research has been done on astaxanthin encapsulation's potential applications, including those in the food and cosmetics industries, there is still a need to investigate the entire range of these uses.

1.8 Opportunities and threats of astaxanthin encapsulation

Encapsulation aids to keep the astaxanthin from oxidation degradation, thus assists in improving its shelf life (Table 6). This enables astaxanthin to be transported and stored without losing any of its effectiveness over time (Vakarelova et al., 2017). In a study, astaxanthin encapsulated with zein and oligochitosan was used to increase the antioxidant activity of liquor, vinegar. (Jiang & Zhu, 2019). Moreover, it provides for controlled release of the nutrient, enabling targeted distribution of the substance to particular locations inside the body. The bioavailability and efficacy of astaxanthin supplementation may be improved as a result. Furthermore, it can improve astaxanthin absorption by shielding it from the unpleasant stomach digesting environment and increasing astaxanthin uptake by the body's cells. This may increase the astaxanthin supplement's general efficacy.

The process of encapsulating astaxanthin can be costly, which may raise the cost of astaxanthin supplements. Astaxanthin encapsulation necessitates strict quality control to ensure that the capsules contain the correct amount of astaxanthin and are free of contaminants. Although astaxanthin is known to have a variety of health benefits, more research is needed to determine the optimal astaxanthin dosage, formulation, and delivery strategy.

Table 6: Various types of encapsulation techniques based on advantages and disadvantages

Methods	Wall material examples	Advantage	Disadvantage	Application	Reference
Coacervation	Gelatin**,** cashew gum, Gum Arabic	Good dispersion, colouring functionality, can encapsulate heat sensitive bioactive	Exabit low mechanical strength because of weak binding between bonds	Functional foods	(Wang et al., 2018)
Extrusion	Sodium alginate low methoxyl pectin's	Thermal stability	Electrostatic extrusion cannot be operated on commercial level	Functional foods, Pharmaceuticals	(Lin et al., 2016); (Bamidele et al., 2020)
Emulsification / High pressure homogenization/ high internal phase emulsions/ Microfluidizer/Ultra sonication	Chitosan, Glyceryl behenate, Oleic acid, Lecithin, steric acid, precirol ATO 5., Starch-Soya	Thick interfacial layer avoids oxidation of droplets, Stability improvement at various temperatures, increase the physical and	Overprocessing may occur. High temperature can lead to reduce the emulsifying properties of the surface-active agents.	Beverages, non-alcoholic beers	(Tamjidi et al., 2017)

	protein	electrical stability with the Starch-Soya protein wall material.			
Freeze drying	Gelatine, cashew gum	Suitable for sensitive bioactive compounds	Expensive, High-energy consumption 24-48h drying process	Functional food	(Rezvankhah et al., 2019)
Spray drying	Whey protein isolates, Gum Arabic, Chitosan	Increase of Bio accessibility, good Dispersion, High Thermal stability	High viscosity leads to nozzle obstruction which influence the droplet and encapsulation efficiency	Functional foods	(Würth, et al., 2017); (Chen et al., 2016).

1.9 Objective of the study

- Astaxanthin is a valuable compound with numerous health benefits, including antioxidant, anti-inflammatory, and anticancer properties. Studying the growth pattern of microorganisms that produce astaxanthin is important for optimizing the production process, and developing sustainable production strategies, for astaxanthin production. Furthermore, delivering this microbially produced astaxanthin via encapsulation would improve its stability, controlled release, and bioavailability, Therefore, the purpose of this study was to investigate the effect of carbon sources on astaxanthin accumulation and growth pattern in microorganisms in order to identify the most effective carbon source, optimize the production process, and encapsulate astaxanthin-enriched biomass and oil using a natural biopolymer-based emulsion method.

The specific aims of the study

- Microbial production of astaxanthin from *Schizochytrium*. sp on a cost-effective carbon-source
- Developing a method for astaxanthin encapsulation and stabilization.

Hypothesis

The carotenoid (astaxanthin) yields can be increased by employing the bioprocessing optimization technique. Furthermore, it is anticipated that encapsulation of astaxanthin using various polymers would result in higher efficiency and stability.

Chapter 2

2.Materials and methods

2.1: Chemicals

All chemicals used in the study were of analytical grade. Glucose (Sigma-Aldrich, USA), yeast extract (Thermofisher, France), peptone (OXOID, UK), magnesium sulfate, agar (Sigma-Aldrich, USA). (Merck, Germany), and sea salt (Instant Ocean, France) were used for biomass production. Acetone (Sigma-Aldrich, USA), ethyl acetate (RCI Labscan Limited), chloroform (from Merck), and methanol (from Sigma-Aldrich, USA) were used for astaxanthin and lipid extraction. Gelatin (Sigma-Aldrich, USA), maltodextrin (Melbourne Food Depot, Australia), sodium alginate (Sigma-Aldrich, USA), gum arabica (Marrickville, Australia), egg albumin (Sigma-Aldrich, USA), astaxanthin enriched oil (Sports research Australia) were used for encapsulation of astaxanthin.

2.2. Cultivation of *Schizochytrium sp*

In this investigation, an in-house strain of *Schizochytrium* DT3 preserved at the Bioprocessing Lab, Medical Biotechnology, College of Medicine and Public Health (CMPH), Flinders University, was used. This strain was isolated in Victorian waters by Dr. Adarsha Gupta, a post-doctoral researcher working with Associate Professor Munish Puri (Gupta et al., 2016). The strain was maintained at 25°C on an agar medium comprising 10 g/L of glucose, 1 g/L of yeast extract, 1 g/L of peptone, 10 g/L of agar, 500ml of artificial saltwater, and 500 ml of MilliQ water (Supplementary Information S1; see Appendix). The inoculum was made in 500 ml of GYP medium, which contained 25 g/L of glucose, 2 g/L of yeast extract, 2 g/L of peptone, and 500ml of artificial saltwater. It was then incubated for 48 hours at 25 °C with 150 rpm. (Gupta et al., 2013b). Thereafter, 5% inoculum (v/v) was transferred to fermentation medium containing glucose 80 g/L, yeast extract 4 g/L, peptone 0.4 g/L, monosodium glutamate 20 g/L, magnesium sulphate 10 g/L and incubated at 25°C and 150 rpm for 7 days (Fig 8).

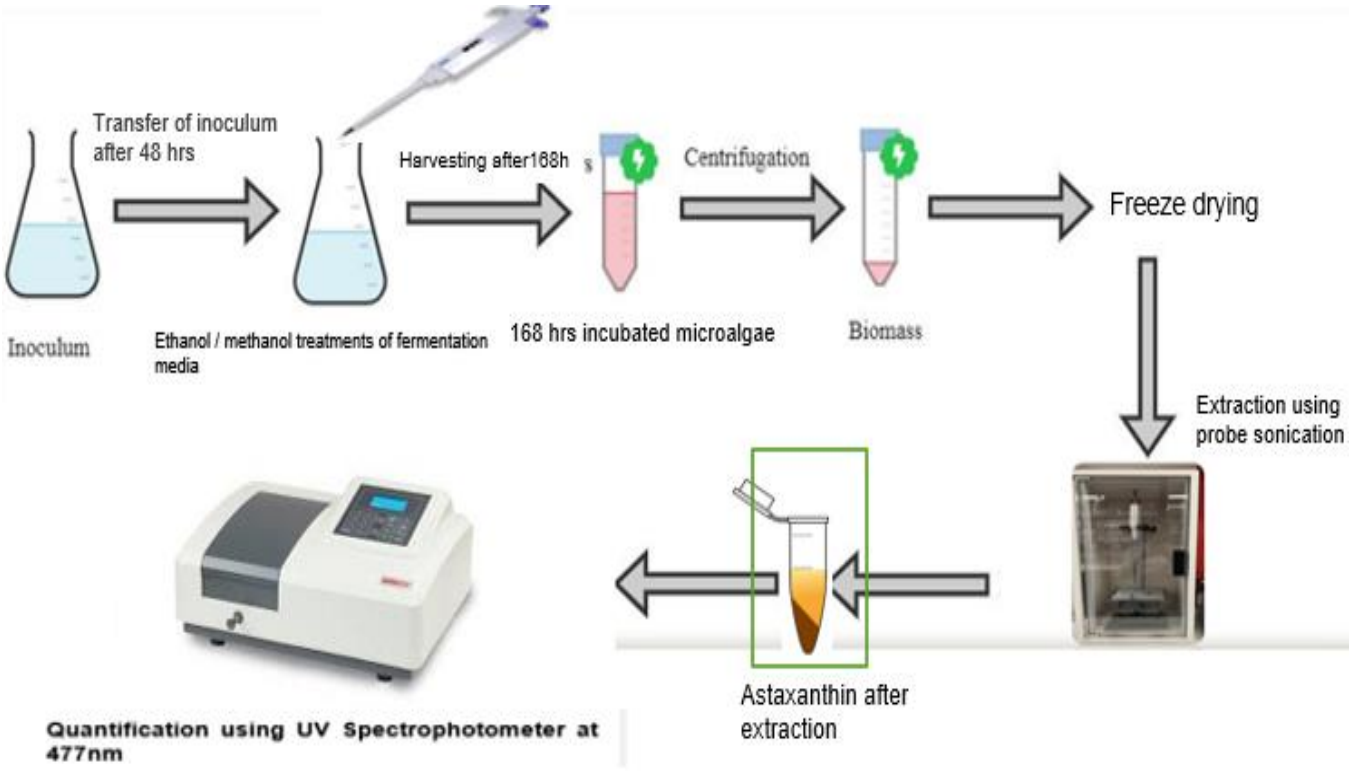


Figure 8: Illustration showing steps followed for astaxanthin production from *Schizochytrium sp.*

2.2.1. Effect of glucose concentration on astaxanthin production

Astaxanthin synthesis is dependent on glucose, which is a critical carbon source for microalgae growth. It has been discovered that as the glucose concentration rises, microalgae acquire more astaxanthin (Zhang et al., 2019). In this study, the effect of different glucose concentrations (5% and 8%) on the biomass and astaxanthin production was investigated.

2.2.2. Effect of ethanol and methanol treatments on astaxanthin accumulation

It has previously been observed that ethanol and methanol treatments can promote astaxanthin accumulation in some microorganisms. (Du et al., 2019). To test the effect of solvents on astaxanthin accumulation, several concentrations of ethanol (2% to 4% v/v) and methanol

(1%,1.25%,1.5%,1.75%, or 2%, v/v) treatments were used in the fermentation medium after autoclave on the zeroth day and kept for incubation for 7 days.

2.2.3. Effect of temperature and incubation period

In terms of astaxanthin accumulation, different strains respond differently to temperature and incubation time. To optimise astaxanthin accumulation, the optimal temperature and incubation duration for each strain must be determined. To address this, the *Schizochytrium* DT3 culture media were harvested at various incubation period (5, 6, and 7 days) to examine biomass and astaxanthin accumulation. In addition, the effect of temperature on biomass and astaxanthin production was investigated at 25°C and 30°C.

2.2.4. Biomass yield determination

The fermentation process was carried out in a 100 ml flask with a 20 ml fermentation medium for various incubation period. Biomass samples were harvested at intervals of 5, 6, and 7 days by centrifugation (3500 rpm for 10 min). The supernatant was removed, and the pellet was freeze dried (Christ, Beta 2-8 LS Cbasic) before being weighed. For later use, freeze-dried biomass was kept in storage at -20°C. Biomass content (in g/L) was determined as described before (Gupta et al., 2016) as follows:

Dry cell weight (DCW) = (W1-W2)/20×1000

Where W1 is the empty weight of the tube and W2 is the freeze-dried biomass with tube. 20 is the culture volume (ml)

2.2.5. Astaxanthin extraction

Freeze-dried cells (25 mg) were taken in a 2 ml microcentrifuge tube, to which 1 ml of acetone was added. This sample was sonicated for 30 min (Sonics vibra-cell) (20% amplitude), followed by centrifugation at 4500 rpm for 10 min. The supernatant was collected in a 2 ml microcentrifuge tube for quantification after the extraction was carried out twice. (Singh et al., 2015).

Astaxanthin concentration was measured at 477 nm using UV spectrophotometer against an acetone blank. The astaxanthin content was determined using an astaxanthin calibration curve at 477 nm (Supplementary Information S3; see Appendix). Astaxanthin content was calculated as follows:

Astaxanthin (µg) = [(OD477 -0.0155)/0.1405]× V

Extraction Yield (µg)/g = (Astaxanthin× V)/DB

Where, astaxanthin is astaxanthin concentration in µg/ml, V is the volume of solvent in ml and DB is the dried biomass in g.

2.2.6. HPLC analysis of astaxanthin

Astaxanthin was analysed by high performance liquid chromatography (HPLC) as described before (Singh et al., 2015). Reverse phase HPLC (Shimadzu prominence ultra-Fast liquid chromatography, Column**:** Phenomenex PhenoSphere Next 5u C18 150 x 4.6 mm (PN 00F-4308-E0)) was used for carotenoid separation with detection at 477 nm wavelength with a bandwidth of 4 nm. Samples were filtered before injecting into the system. The LC system was equilibrated using the initial conditions for the separation (100% A, 0.75 mL/min) for 15 minutes (Table 7). After equilibration the sample was injected, and the initial conditions were held for 10 minutes before the flow rate was ramped to 1.5 mL/min and the mobile phase composition changed to 100% B over 20 minutes. The final LC conditions were held for a further 10 minutes. UV-V is absorbance was measured using a PDA detector with a wavelength range of 250-520 nm

Table 7: Parameters followed for HPLC analysis

Time	%A	%B	Flowrate (ml/min)
-15 mins (equilibration)	100	0	0.75
10 mins	100	0	0.75

30 mins	0	100	1.5
40 mins	0	100	1.5

2.2.7. Lipid extraction

To extract the lipids, freeze-dried biomass (10 mg) had been subjected to solvent extraction using a chloroform: methanol mixture (2:1, v/v) and vortexed for two minutes. The supernatant was then obtained by centrifugation (10,000 rpm, 10 min). Afterwards, all the extracts were mixed, put through a 0.22 m filter, and collected in a glass vial. This process was repeated three times. Using a Dry Block Heater (Ratek Instruments) set to 50°C to evaporate the solvent, the lipid weight was calculated gravimetrically as previously described (Gupta et al., 2016).

$$\text{Total lipid content (\%)} = \frac{\text{Weight of the dried lipids}}{\text{Weight of the dried biomass}} \times 100$$

2.2.8. Fatty acid analysis of *Schizochytrium* sp. grown on ethanol and methanol

Lipids were transesterified and fatty acid methyl esters (FAMEs) were analysed using a previously described method (Gupta et al., 2016). In a test tube, 1 mL of toluene was used to dissolve 10 mg of oil, and 200 µL of an antioxidant (100 mg of BHT in 100 mL of toluene) and an internal standard (50 mg of methyl nonadecanoate in 10 ml of toluene) were added. After adding 500 mL of methanol reagent, which contained 1 mL of acetyl chloride and 10 mL of methanol, the mixture was left for overnight at 50°C in a glass vial with a cap for transesterification. Then, 1 mL of sodium chloride solution (5% w/v in MQ water) was added after bringing the solution to room temperature followed by the addition of hexane (1 mL). The hexane layer was collected and washed with 1 mL of 2% w/v potassium bicarbonate solution in MQ water. It is necessary to remove the hexane layer and dry it over salt sulphate. The samples were stored in GC vials before GC analysis.

Using a FAMEWAX column (30 m x 0.32 mm ID (inner diameter)) and a Shimadzu Gas Chromatography (GC, 2090N) equipped with a flame ionisation detector (FID) and connected to a BID 2030-unit, FAMEs analysis was carried out. With split injection (1/150) and a constant column flow rate of 5 mL/min, the inlet temperature was kept at 25°C. The oven programme was executed at 150°C for five minutes, then ramped up to 250°C at a rate of 10°C per minute, and then held at 250 °C for one minute. The peak regions of authentic FAMEs standards (Sigma Aldrich CRM47885) were compared to determine the fatty acid esters.

2.3. Antioxidant assay of astaxanthin enriched biomass

The inclusion of keto and hydroxyl moieties on each ionone ring in astaxanthin creates a unique chemical structure that is responsible for the substance's potent antioxidant properties (Brotosudarmo et al., 2020). In an ABTS (2,2'-azino-bis (3-ethylobenzothiazoline-6-sulfonicacid) radical scavenging activity assay, antioxidant activity was examined. When strong antioxidants react with ABTS radicals, the colour of the radicals turns almost translucent (Fig 9) Antioxidant assay was performed with slight modifications (Samuel et al., 2022). Previously, 7.5 mM of ABTS and 140 mM of Potassium persulphate solution were prepared. In a flask, 5 ml of the ABTS and 88 µl of potassium persulphate was incubated at room temperature for 16 h in the dark. Trolox and ABTS were used as standard and positive control (concentrations from 0.006 to 0.048 mM) Methanol was used as a negative control. 30 µl of the working ABTS solution were mixed with 970 µL of the different concentrations of the extract (astaxanthin in methanol 1.2–2 µg/mL). The absorbance was measured at 734 nm after each concentration of astaxanthin was added to an ABTS radical solution that had been reacting at room temperature for 15 minutes to compare it to the control group without the addition of sample.

$$\text{Radical Scavenging (\%)} = \frac{(\text{Control} - \text{ABTS Sample}) * 100}{\text{Control}}$$

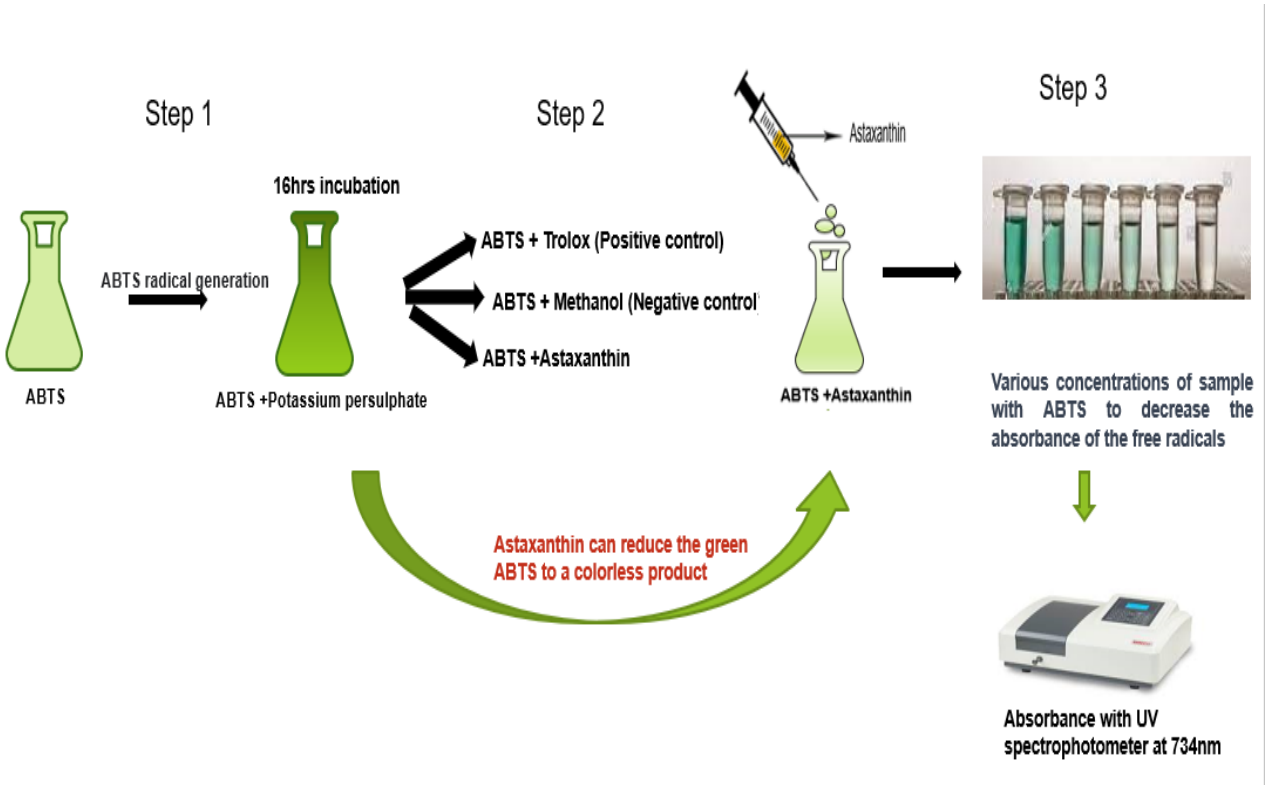


Figure 9: Illustration showing process for conducting an antioxidant assay.

2.4. Encapsulation of astaxanthin

Using a variety of polymers, astaxanthin-enriched biomass was encapsulated. In water, sodium alginate (1% w/v), maltodextrin (2.5% w/v), and gelatine (2.5% w/v) were dissolved. In order to create the emulsion, this solution was homogenised for 10 min (8000 rpm Ultra-Turrax T25), Further, hemp oil and biomass were added and homogenised for 10 min (13500rpm Ultra-Turrax T25) (Fig 10).

2.4.1 Effect of egg-albumin as a wall material on astaxanthin encapsulation

Similarly, astaxanthin-enriched biomass was encapsulated using egg albumin (0.5% w/v), gum arabica (0.5% w/v), and sodium alginate (1% w/v) in water. This emulsion was extruded dropwise in a calcium chloride solution (0.2 M), and the efficiency of the emulsion as well as generated beads were noted based on a previous protocol (Xie et al., 2020). Calcium chloride is commonly used as a crosslinking agent for the hardening of microcapsules during

encapsulation. The process of encapsulation involves enclosing a core material within a shell or membrane to protect it from the external environment or to control its release. (Niizawa et al., 2019).

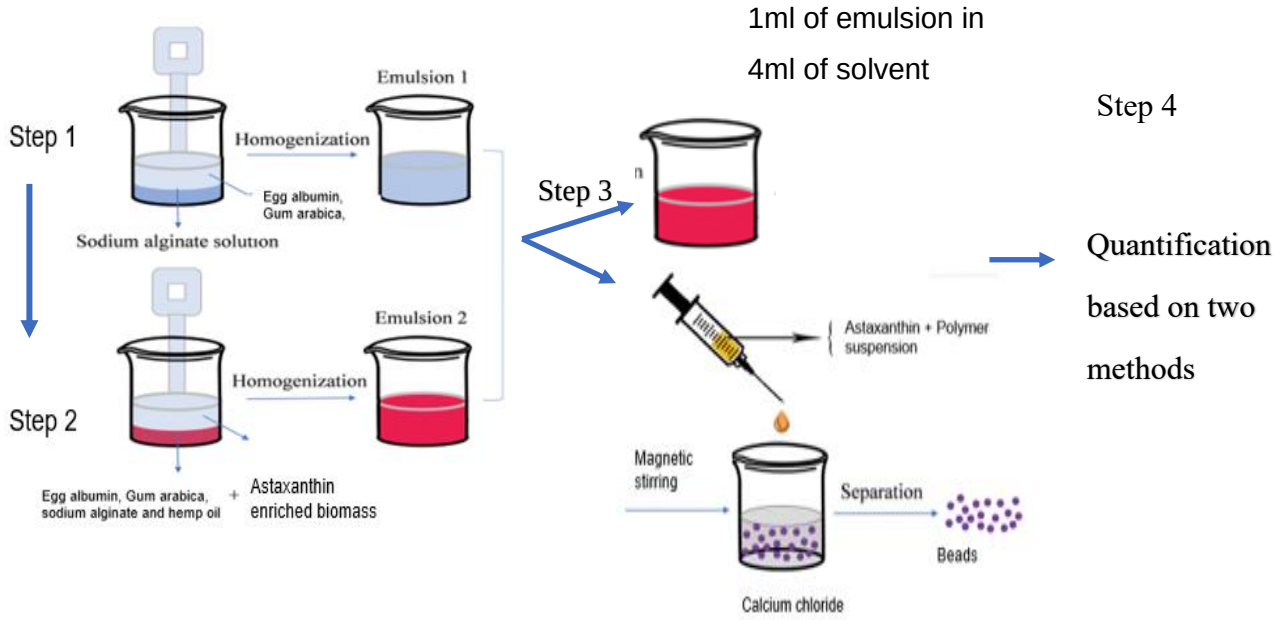


Figure 10: Illustration showing emulsification and encapsulation of Astaxanthin-enriched biomass

2.4.2. Effect of pea protein as a wall material on astaxanthin encapsulation

Similarly, pea protein (that replaced egg albumin) was used with above procedure to examine the effect of other protein wall material on encapsulation efficiency as well as stability of emulsion. Pea protein (0.5% w/v), gum arabica (0.5% w/v), and sodium alginate (0.5% w/v) were used for encapsulation.

2.4.3 Encapsulation of astaxanthin enriched oil using pea protein

Using commercially available astaxanthin enriched oil (in coconut oil) and a variety of polymers (pea protein, gum arabica and sodium alginate) encapsulation was performed. The

encapsulation efficiency of astaxanthin enriched astaxanthin enriched oil with astaxanthin enriched biomass was compared. A preliminary oil-in water emulsion was prepared by homogenizing astaxanthin enriched oil with wall material stock solution.

2.4.4. Effect of bioactive load

Various bioactive concentrations (astaxanthin biomass) were used to optimise the process. The effect of increasing bioactive load on astaxanthin-encapsulation efficiency was investigated by using astaxanthin enriched biomass (1.5%, 2% and 2.5%, w/v), and astaxanthin oil (30 µl,40 µl, and 50 µl; v/v) for encapsulation.

2.4.5. Effect of wall material concentration

The concentration of the wall material used in encapsulation can have a significant impact on the encapsulation efficiency and stability of the encapsulated compound (Ruengdech & Siripatrawan, 2022). Egg albumin and gum arabica concentrations (0.5, and 0.25 w/v) were steadily decreased to optimise the process wall material concentrations.

2.4.6. Encapsulation efficiency

Encapsulation efficiency was determined with slight modifications (Niizawa et al., 2019). The emulsion was resuspended in 4 ml ethyl acetate and ethanol (1:1, v/v) and disintegrated with homogeniser for 5 minutes at 20,000 rpm. The supernatant obtained after centrifugation at 3500 rpm for 15 min was used to determine the actual amount of astaxanthin. The astaxanthin content was determined using an astaxanthin calibration curve at 477 nm (Supplementary Information S4; see Appendix). The astaxanthin release rate was calculated from the following equation.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Actual amount of astaxanthin}}{\text{Theoretical amount of astaxanthin}} \times 100$$

2.4.7. Storage stability

The quality and effectiveness of astaxanthin encapsulation were significantly impacted by its stability as it holds up during storage. The prepared emulsion was transferred into a glass test

tube and tightly sealed. A portion of the sample was regularly withdrawn (every 24 hours) for observational test analysis as previously described (Sun et al., 2022) while the sealed tubes were kept in low light environments at 23°C and 4°C for 2 days. The amount of astaxanthin in the sample was determined using the encapsulation efficiency. Visual examination (taking pictures) was used to record the changes in the emulsion's appearance during the period of storage.

2.5 Statistical analysis

The results were averages and standard deviation derived from two replicated measurements

Chapter 3

3. Results and discussion

3.1 Effect of carbon source on production of biomass and astaxanthin

Schizochytrium sp**.** has been recognised as a promising source for the production of astaxanthin. However, the production of biomass and astaxanthin in microorganisms can be significantly influenced by the carbon source. It is possible to improve the yields and augment the effectiveness of microbial bioprocessing by optimising the carbon concentration. To address this, the effects of glucose on astaxanthin accumulation were investigated at various concentrations (5% and 8%). The results showed a drastic increase of astaxanthin as well as biomass yield with increasing glucose concentration. At 5% glucose concentration, the biomass yield was 10 g/L, which increased to 13 g/L at 8% glucose. Moreover, astaxanthin yield improved from 33 µg/g (at glucose 5%) to 123 µg/g (at glucose 8%) (Fig 11). The results obtained in this study are in agreement with trends noted in literature. A previous study investigated the effect of glucose on the cell structure as well as regulation of astaxanthin biosynthesis (Zhang et al. 2019). They discovered that glucose might increase the expression of the PSY (15-cis-phytoene/all trans-phytoene synthase), LCYb (Lycopene β-cyclase), CHYb (β-carotene 3-hydroxylase), and BKT (β carotene ketolase) genes, causing an increase in astaxanthin synthesis. These results suggested that glucose may control astaxanthin synthesis by influencing cell structure. Ip et al., (2004) investigated the growth and production of carotenoid from green microalga *Chlorella zofingiensis* grown in mixotrophic culture at various glucose concentrations (5 to 40 g/L) and nitrate. The results revealed that addition of glucose at 5 g/L showed 2.5±0.3 mg/L of astaxanthin and at 30 g/L showed 12.5±0.8 g/L of astaxanthin yield. Based on these findings, it can be concluded that increasing glucose concentration increased the growth and carotenoid accumulation.

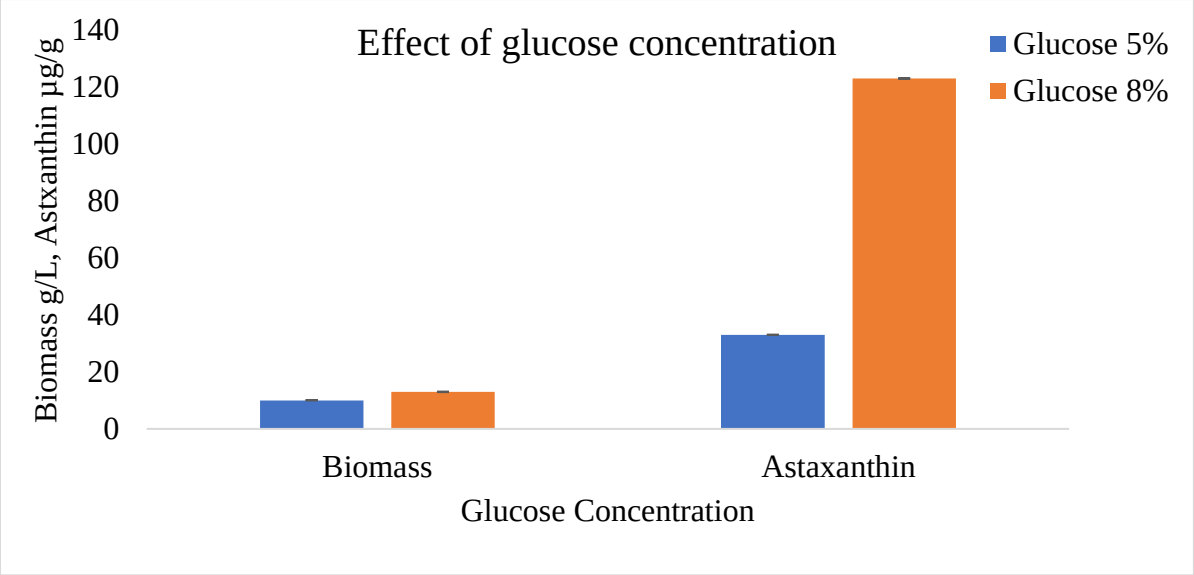


Figure 11: Effect of glucose concentration on biomass(g/L), and astaxanthin(µg/g) yield in control. The data is presented as the mean ±SD of two replicates.

3.1.1. Effect of ethanol on biomass, lipid and astaxanthin production

In this study, ethanol at various concentrations (2%, 2.5%, 3%, 3.5%, and 4%) was added to the fermentation medium. The results showed that ethanol supplementation influenced the astaxanthin, lipid, and dry cell weight (DCW) yields. The lipid content and DCW decreased as the ethanol concentration increased from 2% to 4%. When the ethanol concentration reaches 4%, the lipid content decreased significantly. It was found that the cells treated with ethanol formed less lipid structures than cells in the control group (Fig 16).

When the *Schizochytrium* DT3 strain was exposed to ethanol at a range of concentrations, the accumulation of astaxanthin drastically changed. The biomass began to change colour under treatments with lower concentrations of ethanol (2%) whereas the biomass colour changed to reddish during treatments with higher concentrations of ethanol (3%) due to increasing astaxanthin accumulation (Supplementary Information S2; see Appendix). As the ethanol concentration increased from 2% to 3%, overall astaxanthin content significantly increased (Fig 12). This might be attributed to the stress response due to ethanol, which might have resulted in the activation of astaxanthin synthesis-related genes (Zhu et al., 2022). At 4% ethanol concentration, astaxanthin yield decreased. Furthermore, ethanol at high concentration

can be toxic to cells, which could have an adverse effect on astaxanthin synthesis (Fig 13). Moreover, with increasing glucose concentration, astaxanthin yield increased from 132 µg/g (at glucose 5%) to 387 µg/g (at glucose 8%) in the biomass samples treated with ethanol 3% concentration (Fig 12 and 13). The results obtained in this study are in agreement with previous study (Wen et al. 2015). They reported that even in low-light situations, ethanol addition improved astaxanthin accumulation in *Schizochytrium*. At 3% (v/v) ethanol, astaxanthin productivity reached 11.26 mg/L/d, which was 2.03 times higher than that of the control. Liu et al., (2019) examined the impact of ethanol on astaxanthin production and studied the molecular processes behind the astaxanthin biosynthetic pathway and regulation in *H. pluvialis*. On the fourth day of treatment, they found that the addition of ethanol (0.4%) considerably increased the cellular astaxanthin concentration to 3.85%. In total, 95% of the astaxanthin that had accumulated had been esterified and was particularly rich in monoesters. Ethanol changed the physiology and structure of cell walls, observed during ultrastructural research investigations.

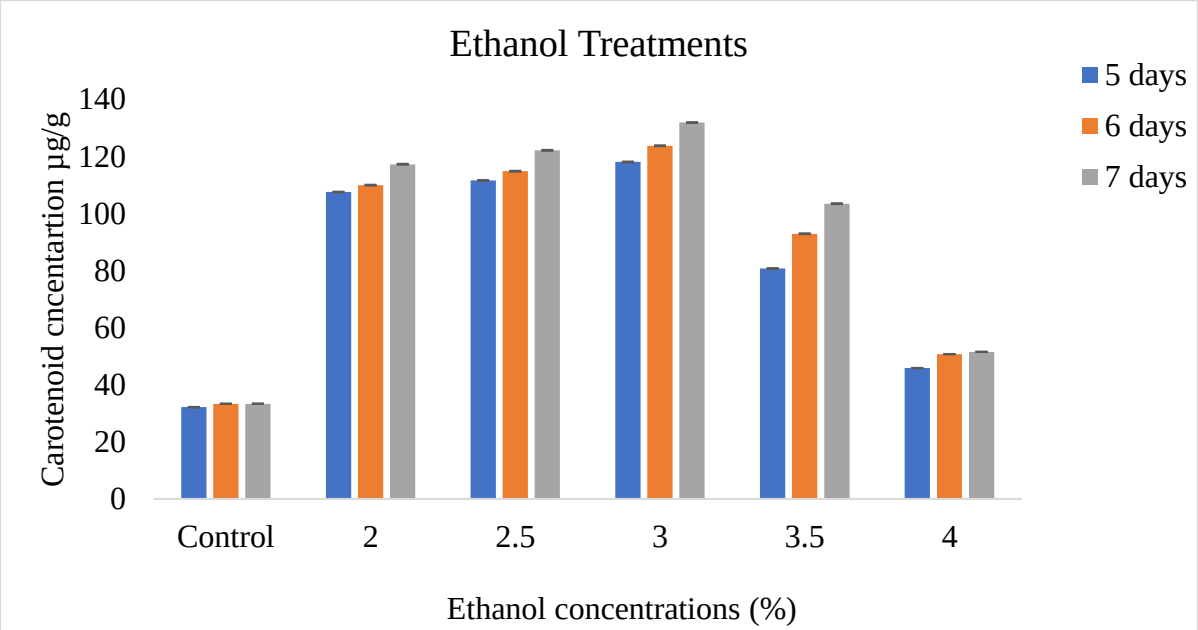


Figure 12: Effect of increasing ethanol concentration (2%,2.5%,3%,3.5% and 4%), glucose 5% with increasing incubation time on *Schizochytrium* astaxanthin yield (µg/g). The data is presented as the mean ±SD of two replicates.

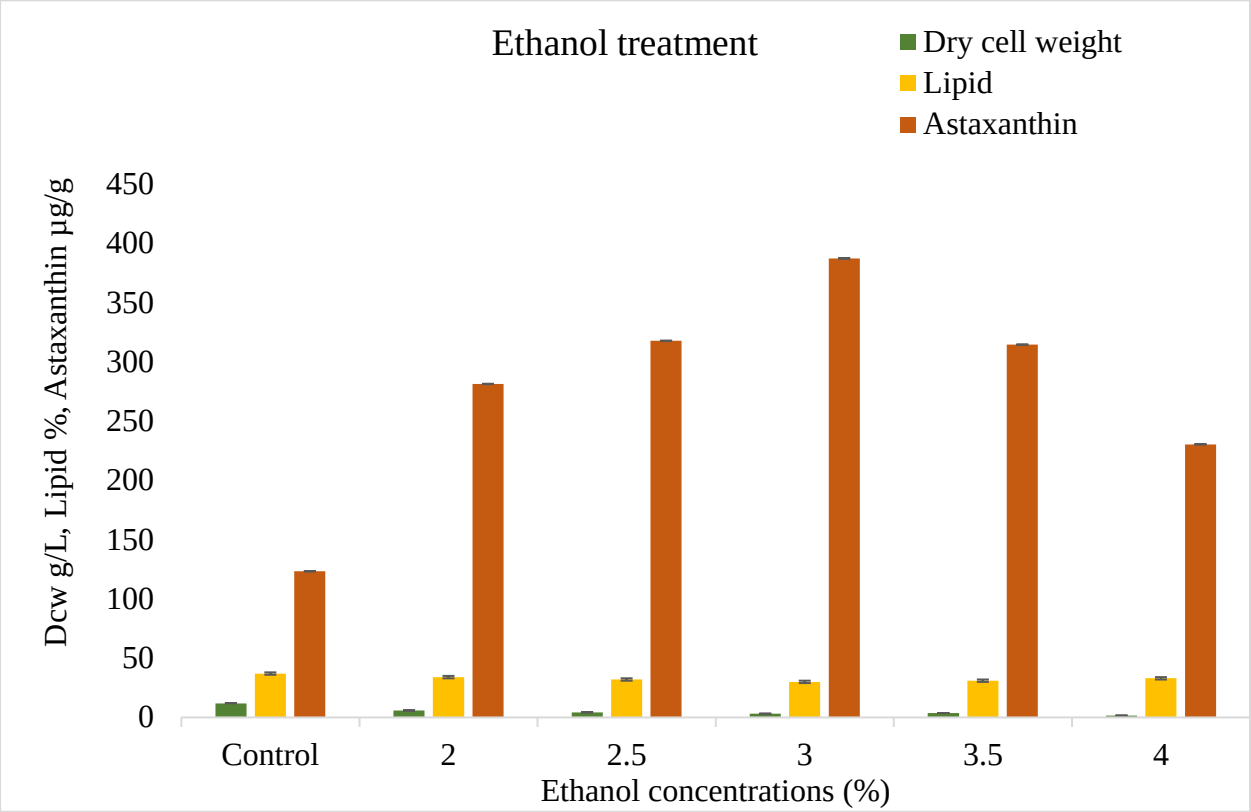


Figure 13: *Schizochytrium* in response to increasing glucose concentration (8%) with samples treated with ethanol. The data is presented as the mean ±SD of two replicates.

3.1.2: Effect of methanol on biomass, lipid and astaxanthin production

In this study, various methanol concentrations (1%, 1.25%,1.5%, 1.75% and 2%) were added to the fermentation medium using *Schizochytrium* DT3 strain. Results revealed that DCW, lipid, and astaxanthin initially decreased with 1% of methanol treatment compared to the control, but gradually increased with increasing methanol concentration (1% to 2%) over various time intervals (5, 6 and 7 days) (Fig 14, 15 and 16). Moreover, with increasing glucose concentration, astaxanthin yield increased from 54 µg/g (at glucose 5%) to 183 µg/g (at glucose 8%) in the biomass samples treated with methanol 2% concentration (Fig 14,15). The results obtained in this study are in agreement with trends noted in literature. In previous study, when methanol concentration of 3.2% was used, astaxanthin started to build up, and peaked at 5.6%, a 2,000-fold increase compared to control (Du et al., 2019). But higher methanol concentration

(5.6%) reduced the biomass, lipid and astaxanthin. Thus, it was concluded that in microorganisms, astaxanthin content can be affected by methanol concentration in both favourable and unfavourable ways. On the one hand, methanol can cause stress in microorganisms, resulting in improved astaxanthin. In addition, high methanol concentrations can also impede their development and metabolism, which lowers biomass production. So, it is significant to optimising the methanol treatment conditions.

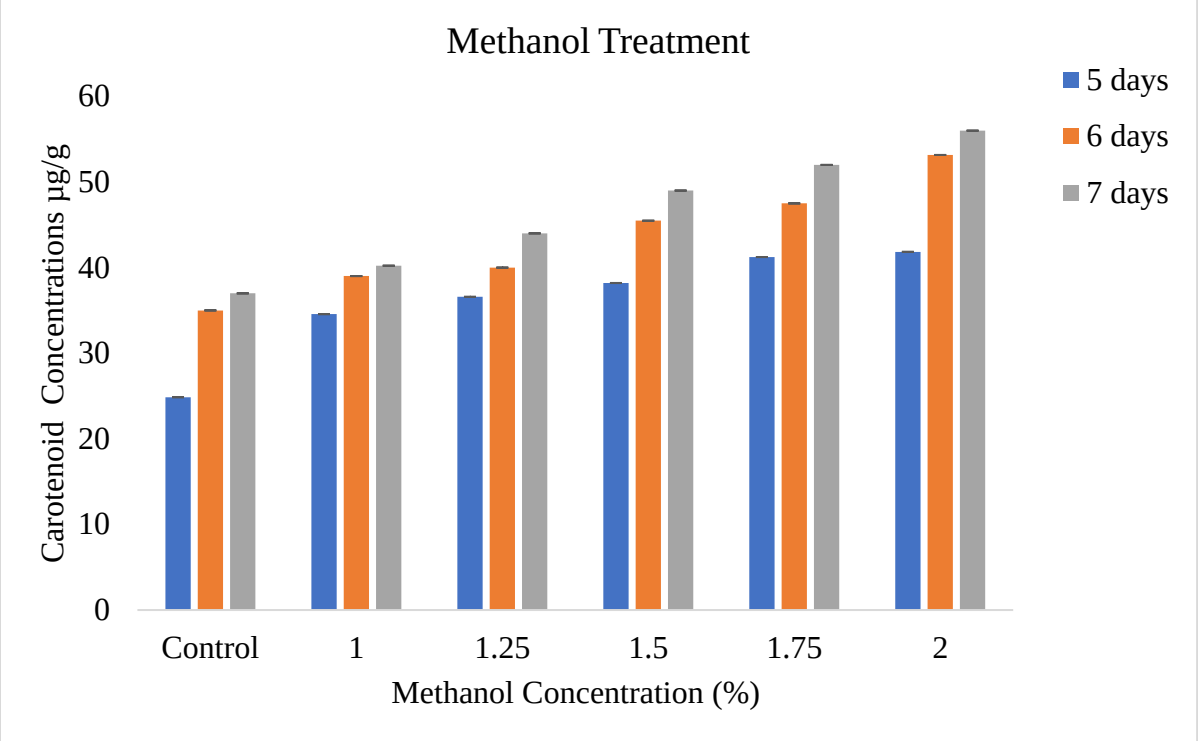


Figure 14: Effect of increasing methanol concentration (1%, 1.25%, 1.5%, 1.75% and 2%), glucose 5% with increasing incubation period on *Schizochytrium* astaxanthin yield (µg/g). The data is presented as the mean ±SD of two replicates.

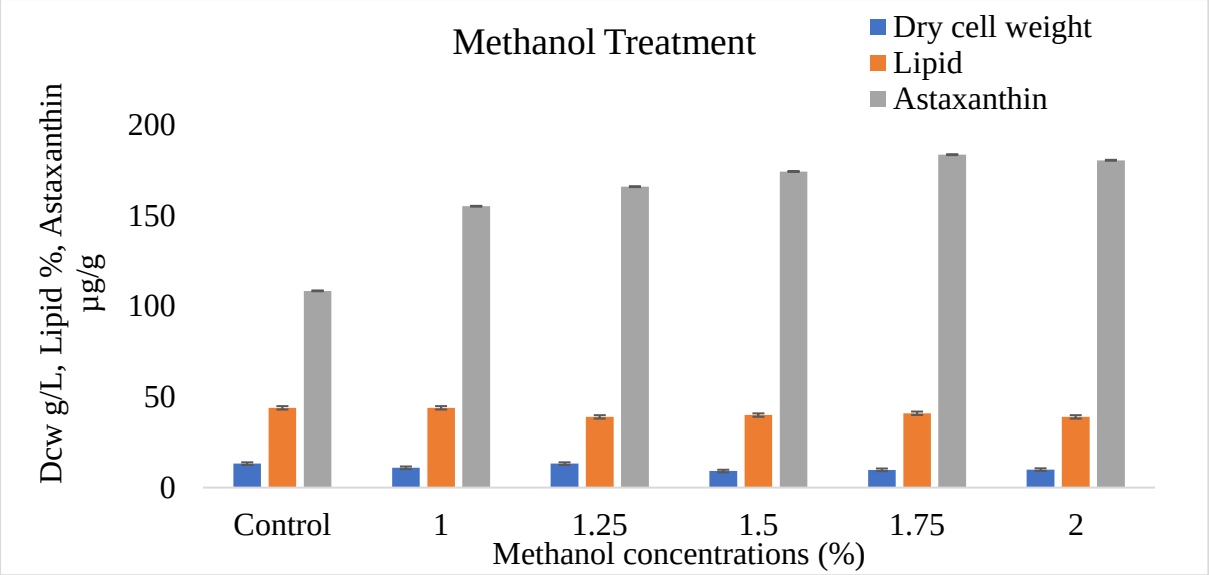


Figure 15: Effect of different methanol concentration (1%, 1.25%, 1.5%, 1.75% and 2%) on *Schizochytrium* biomass (g/L), lipid (%) and astaxanthin yield (µg/g) compared to control. Glucose concentration of 8% was used in the fermentation. The data is presented as the mean ±SD of two replicates.

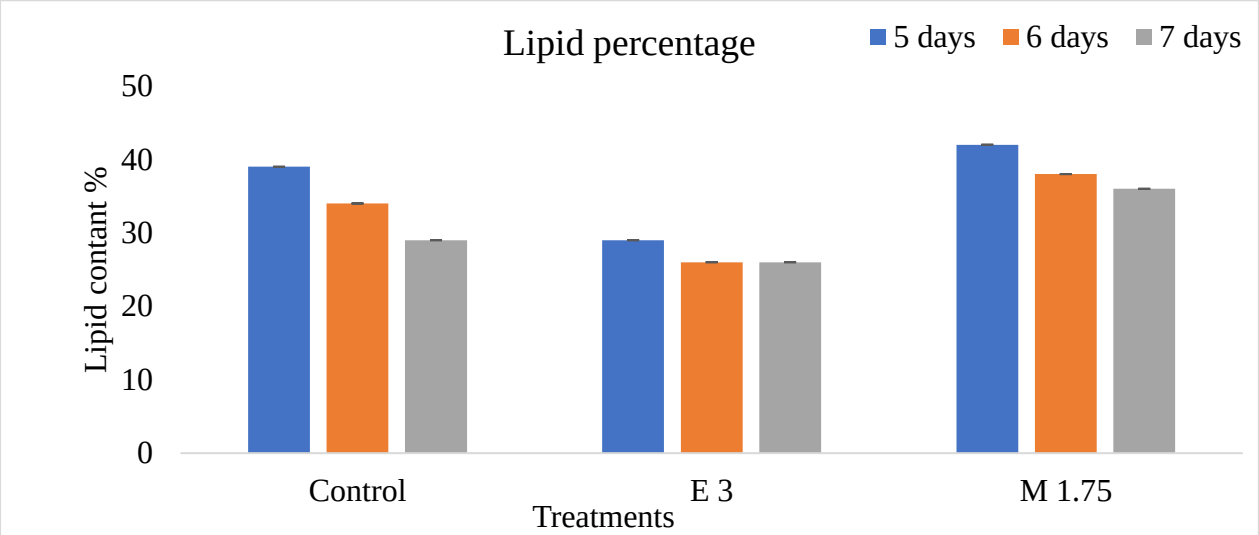


Figure 16: Effect of optimised ethanol(E) and methanol(M) concentration on lipid yield in control. The data is presented as the mean ±SD of two replicates.

3.1.3 Effect of temperature and incubation period on accumulation of biomass, lipid and astaxanthin

In terms of astaxanthin accumulation, some microalgae may thrive between 15°C and 25°C, while others might exhibit better accumulation between 25°C and 30°C (Wan et al., 2014). The *Schizochytrium* DT3 strain was cultivated for 5, 6, and 7 days at 25°C and 30°C to determine the optimum temperature conditions. Results showed that longer incubation times (7 days) increased astaxanthin accumulation in both ethanol 118 µg/g (5 days incubation) to 132 µg/g (7 days incubation) and methanol treated biomass 42 µg/g (5 days incubation) to 54 µg/g (7 days incubation) (Fig 17 and 18). However, biomass decreased from 13 g/L to 10 g/L in control with increasing incubation period (Fig 17). According to the findings, longer the incubation period, the more time the microalgae have during the stationary phase to synthesise the secondary metabolite astaxanthin. There is a chance that this increase in astaxanthin production will result in less biomass and lipid production. (Xing et al., 2022)

In this study, astaxanthin content improved at 25°C (132µg/g) when compared to 30°C (118µg/g) (Fig 17). The development and metabolism of microorganisms can be impacted by temperature changes in the pace of enzymatic reactions, fluidity of cell membranes, and nutrient uptake (Wan et al., 2014). In general, increasing temperature accelerates cellular processes and enzymatic activities, resulting in accelerated cell development and metabolism. However, if the temperature is too high, it can denaturize proteins and enzymes, which will slow down metabolism and growth. Giannelli et al. (2015) studied the impact of temperature on biomass and astaxanthin content. The findings suggested that increasing temperature, up to 28 °C, could assist astaxanthin accumulation at the highest cell concentration at 30.5°C was only 35% of that at 20°C, and the rate of cell growth dropped with temperature increases over the ideal range.

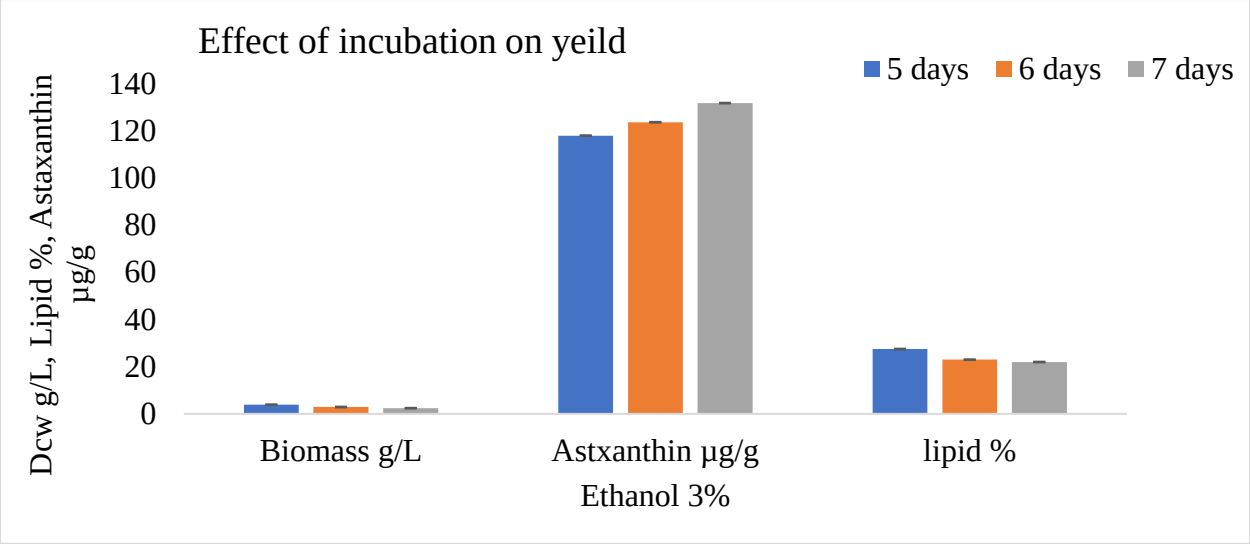


Figure 17: Effect of incubation on biomass (g/L), astaxanthin (µg/g), and lipid yield (%) of samples treated with ethanol 3%. The data is presented as the mean ±SD of two replicates.

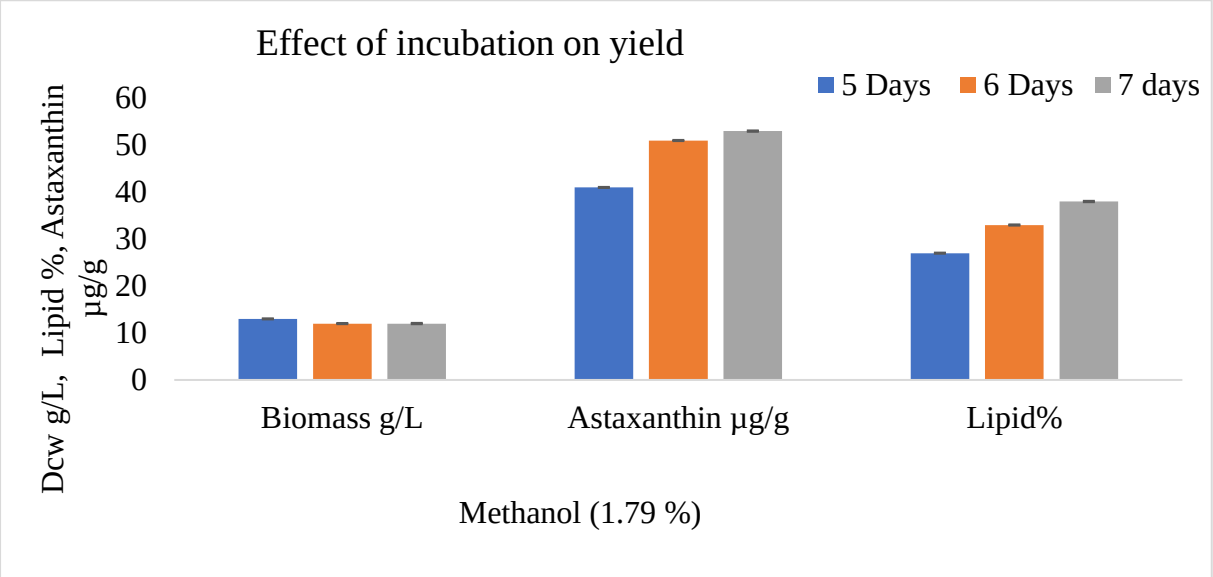


Figure 18: Effect of incubation on biomass(g/L), astaxanthin(µg/g) and lipid yield (%) of samples treated with methanol 1.79%. The data is presented as the mean ±SD of two replicates.

3.2 HPLC analysis of astaxanthin enriched biomass

High-performance liquid chromatography (HPLC) analytical technique was used for the detection and quantification of astaxanthin. An HPLC analysis of astaxanthin extracted from *Schizochytrium* sp. showed a retention time approx. 3.5 mins (Supplementary Information S7; see Appendix) This allowed the quantification of astaxanthin in all biomass samples. The peak height is then compared to the calibration curve to determine the concentration of astaxanthin in the sample. The results revealed that astaxanthin extracted from biomass showed a linear calibration curve with a correlation coefficient of 0.999 with relative standard deviation of 2.2144 (Supplementary Information S5 S6; see Appendix). The calibration curve was used to determine the concentration of astaxanthin in the sample, which was found to be 0.034231 (control) to 0.3841 µg/ml (3% ethanol treated sample). Based on the results, it was found that there was a 11-fold increase of astaxanthin in 3% ethanol treated samples compared to the control. The results obtained in this study are in agreement with trends noted in literature. According to a study on the HPLC analysis of astaxanthin, the correlation coefficients for the regression lines for astaxanthin obtained were 0.9981, demonstrating the strong association between peak area and concentration (Tzanova et al., 2016). In addition, Koopmann et al. (2022) studied on detection and quantification of astaxanthin. For the purpose of determining the calibration curve, the obtained peak regions were integrated. The peak of all-E astaxanthin had a retention duration of 7.50 minutes. The correlation f(x) = 1693.4x - 990.57 with a coefficient of determination of 0.9985 was produced through linear regression.

3.3.1 Fatty acid analysis of *Schizochytrium* sp. grown on ethanol

Fatty acid analysis of *Schizochytrium* DT3 biomass showed a variety of fatty acids, with DHA (C22:6, docosahexaenoic acid) and C16:0 (palmitic acid) representing essential polyunsaturated and saturated fatty acids, respectively (Table 8). According to the data, the amount of C16:0 did not alter as a result of the ethanol treatments, but it had a detrimental effect on DHA (Table 8). Palmitic acid levels were found to be high in all samples when compared to DHA, ARA (arachidonic acid), and EPA (Eicosapentaenoic acid). Saturated fatty acids were found in high concentrations in ethanol-treated samples. However, treatment

with ethanol (3%) reduced total oil content and PUFA percentage. The results obtained in this study are in agreement with trends noted in literature. In one study, (Wang et al., 2012) examined the effect of several carbon sources on the fatty acid composition of *Chlorella sp.* They found that using ethanol as a carbon source increased the percentage of saturated fatty acids, such as palmitic acid (C16:0). (Liu et al., 2019) reported similar results about the impact of ethanol carbon sources on the fatty acid profile of *Haematococcus pluvialis*. It was also found that using ethanol as a carbon source caused a drop in the proportion of unsaturated fatty acids, such as linoleic acid and an increase in saturated fatty acids, such as palmitic acid (C16:0), and stearic acid (C18:0) (Fang et al., 2004).

3.3.2 Fatty acid analysis of *Schizochytrium* sp. grown on methanol

Addition of methanol significantly changed the fatty acid composition of *Schizochytrium* DT3 strain. Gas chromatography analysis showed that main fatty acid composition was palmitic acid (C16:0), DHA (C22:6) and myristic acid (C14:0) (Table 8). The total oil content also increased with the methanol 1.75% compared to the control (Table 8). It has been reported that methanol addition in a fermentation medium of *Chlorella sp.* resulted in a significant rise in both the total lipid content and the proportion of unsaturated fatty acids, in the cells (Choi et al., 2011). In another study, Du et al., (2019) reported that the fatty acid profile of *Schizochytrium limacinum* B4D1 grown in a methanol supplemented medium resulted in increase of DHA (C22:6, docosahexaenoic acid) and EPA (C20:5 Eicosapentaenoic acid). With increasing methanol concentration EPA got increased. In another study methanol effect on *Chlorella sp.* was examined. The results displayed that under lower methanol concentrations, the morphology of the cells changed marginally (3.2%) but significantly (4.8%) under higher methanol concentrations. *Chlorella sp.* growth and lipid synthesis were improved by methanol at 7.9 g/L (1 v/v%) in the presence of light and 5% CO2. As CO2 was present, methanol assimilation was enhanced, and methanol addition increased the concentration of palmitic (C16:0) and oleic (C18:1) acids. (Choi et al., 2011).

Table 8: Effects of ethanol and methanol concentration on the fatty acid composition (%) of *Schizochytrium* DT3 strain.

Fatty acids	Control (1)	Control (2)	Ethanol treated sample 1	Ethanol treated sample 2	Methanol treated sample 1	Methanol treated sample 2
FAMEs	1	2	3	4	5	6
C14:0	12.3	12.5	4.3	4.3	14.5	14.5
C15:0	8.2	8.5	25.0	24.2	9.5	9.7
C16:0	36.5	35.4	34.6	35.9	35.6	36.2
C17:0	2.6	2.7	8.8	8.4	2.7	2.7
C18:0	2.3	2.4	2.9	2.5	2.3	2.3
C20:4	1.4	1.3	1.9	2.0	1.0	1.1
C20:5	4.2	4.5	5.8	5.6	3.6	3.7
C22:5	8.7	9.3	6.0	4.3	8.2	7.5
C22:6	21.1	21.1	10.8	12.9	19.2	18.7
SFA%	61.8	61.6	75.5	75.2	64.9	65.6
MUFA%	2.2	1.9	0.0	0.0	2.7	2.9
PUFA%	36.0	36.6	24.5	24.8	32.4	31.5
Oil (%)	27	35	12	11	42	37

*(Fatty acids such as myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), (C20:5 Eicosapentaenoic acid), DPA (C22:5, Docosapentaenoic acid), and DHA (C22:6, docosahexaenoic acid) as observed in a fatty acid profile of the *Schizochytrium sp* . The given values are presented as % of total fatty acids TFA). The data is presented as the mean ±SD of replicates. [This data was thankfully analysed and provided by Ms Vidana Arachchige Appuhami]

3.4 Antioxidant activity of astaxanthin enriched Biomass

The ABTS + scavenging activity of astaxanthin and Trolox in this investigation showed a similar pattern with an increase in astaxanthin concentrations. Both astaxanthin and Trolox had a linear increase in their capacity to scavenge ABTS + radicals (Supplementary Information S8,9; see Appendix) Astaxanthin combination treatments resulted in an increase in the scavenging activity of ABTS radicals in a concentration dependent fashion with an IC50 0.78 µg/mL with activity percentage of highest 83% (Fig 19). Due to its great stability, astaxanthin isolated from *Schizochytrium* sp. can sustain its antioxidant action even in environments with heat, light, and oxidative stress. This can be attributed to its distinct molecular makeup and lipophilic properties, which prevent it from degrading and losing its biological function (Liu et al., 2019). Using the ABTS assay, Tran *et al.* (2023) examined the antioxidant activity of astaxanthin isolated from *Rhodosporidium torulodies G17*. With an IC50 value of 0.97±0.01µg/g, they discovered that astaxanthin demonstrated significant antioxidant activity. In another study astaxanthin from Shrimp shell displayed a concentration dependent ABTS scavenging activity, with an EC50 value of 7.7± 0.6 µg/mL. This outcome supported the notion that astaxanthin had a discernible impact on scavenging ABTS radicals. (Chintong et al., 2019).

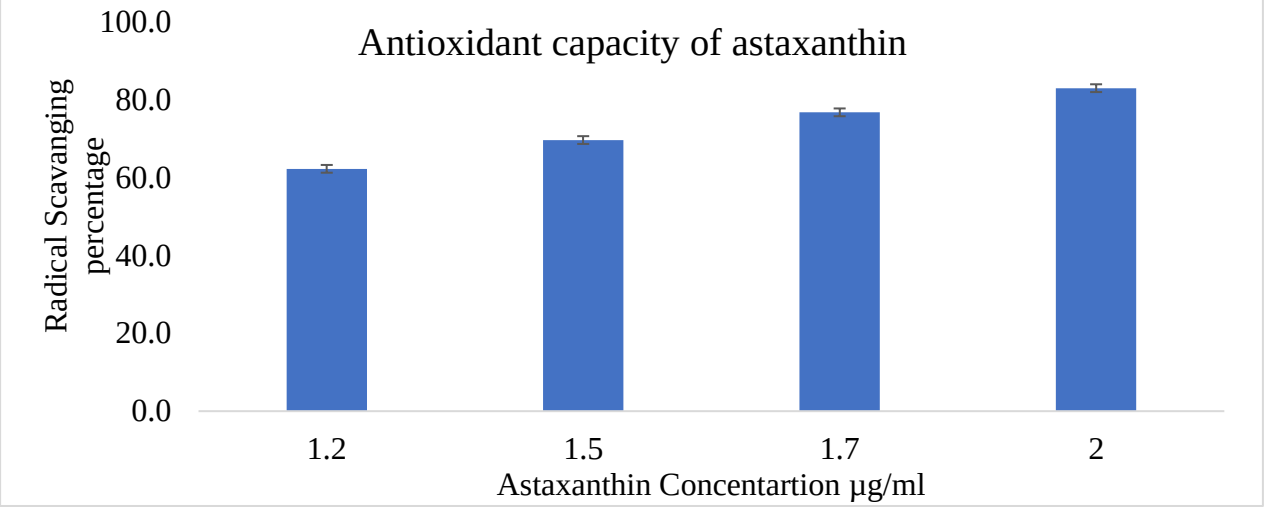


Figure 19: Antioxidant capacity of astaxanthin enriched biomass of *Schizochytrium* DT3. The data is presented as the mean ±SD of two replicates.

3.5 Encapsulation of Astaxanthin from *Schizochytrium* sp.

Encapsulation is a technique that is used for entrapping a biologically active molecule that enhances its stability, solubility, and bioavailability while also offering regulated release of this vital nutrient (Eun et al., 2019). In the literature, various proteins and polysaccharides have been used for achieving encapsulation (Robert et al., 2003; and Deng et al., 2014).

3.5.1 Encapsulation astaxanthin using Gelatin, maltodextrin and sodium alginate

A mixture of wall material such as, maltodextrin, gelatine, and sodium alginate (0.5% wall material concentration) were utilised in this investigation to encapsulate the astaxanthin enriched *Schizochytrium* sp dried biomass (2%). When mixture was extruded dropwise into calcium chloride solution, spherical beads (230 beads) were observed. An encapsulation efficiency (23 %) was achieved. which indicated that maltodextrin and gelatine. are ineffective as wall materials. However, the encapsulation efficiency of astaxanthin was improved (43.6%), when high-speed homogenisation was conducted. Astaxanthin is extremely hydrophobic and have a propensity to aggregate in aquatic environments. Maltodextrin and gelatine-based coatings are hydrophilic, which indicates they have a significant affinity for water thus, facilitating astaxanthin leakage. As a result, they were unable to efficiently encapsulate hydrophobic compounds such as astaxanthin. (Chaudhary, et al., 2021).

3.5.2 Encapsulation astaxanthin using egg albumin, gum Arabica and sodium alginate

A mixture of wall material (egg albumin, gum arabica, and sodium alginate at 0.5% respective concentration) were utilised in this investigation to encapsulate the astaxanthin enriched *Schizochytrium* sp dried biomass (2%, w/v). When emulsion mixture was extruded dropwise into calcium chloride solution, spherical beads (250 beads) were observed. An encapsulation efficiency (44 %) was achieved. The encapsulation efficiency of astaxanthin was improved (66%), when high-speed homogenisation was conducted. (Table 9). With increase in biomass concentration (2.5%, w/v), further improvement in encapsulation efficiency (68.7%) was observed. These observations were in agreement with literature findings where egg albumin

and gum arabica were found to be suitable for encapsulating hydrophobic compounds (Gu et al., 2018; and Gu et al., 2017). These wall materials do provide a stable microenvironment around the pigments by preventing the carotenoid from aggregation and increasing their solubility in water-based systems. GA (gum arabica) is a powerful emulsifier at the oil-water interface due to the hydrophobic protein portion's strong surface activity that is covalently bound to hydrophilic polysaccharide structures (Ozturk et al., 2015). To combat the low water solubility and oxidative stability, encapsulation of camelina oil was examined (Xie et al., 2020). With egg albumin and gum arabica, the maximum efficiency (74%) was reported at pH 7.

The egg albumin (>98% purity; sourced from Sigma at $102/g) is a costly matrix; thus, it was decided to explore a cost-effective protein source that meets large scale encapsulation requirements. The pea protein (alternative protein source for enhancing human nutrition available at $55/Kg; Bulk Nutrients, New Zealand) was used to encapsulate astaxanthin enriched biomass by high-speed homogenisation process. An encapsulation efficiency (63.7%) was observed when pea protein, maltodextrin and sodium alginate was used (Table 9). The encapsulation efficiency was moderately less (~4%) when compared to egg albumin, however, considering cost-comparison pea protein was used for future experiments.
Additionally, pea protein is regarded as a suitable material for encapsulation due to its high solubility, low allergenicity, and good emulsifying properties (Guo et al., 2022).

Table 9: Encapsulation efficiency of astaxanthin enriched biomass

Wall material	Encapsulation Efficiency (%)	*Encapsulation Efficiency (%) at 4°C (24 h)	*Encapsulation Efficiency (%) at room temperature (24 h)
Maltodextrin+ Gelatin+ Sodium alginate (Beads)	22.9		

Maltodextrin+ Gelatin +Sodium alginate (Emulsion)	43.6	37.4	33.1
Egg albumin+ Gum Arabica +Sodium alginate (Beads)	44		
Egg albumin+ Gum Arabica +Sodium alginate (Emulsion with 2% Biomass)	66	60.1	61.2
Egg albumin+ Gum Arabica +Sodium alginate (Emulsion with 2.5% Biomass)	68.7	64.8	62.3
Pea protein +Gum Arabica +Sodium alginate (emulsion) with 2.5% Biomass	63.7	59	54.7

*The data presented in this table on encapsulation efficiency as a function of stability is discussed under stability section (3.6). The data is presented as the mean ±SD of two replicates.

3.5.3 Encapsulation efficiency of astaxanthin enriched oil using pea protein

For translation purposes, the evolved encapsulation methodology of *Schizochytrium* biomass, pea protein was also used to encapsulate the astaxanthin enriched oil. The freshly prepared emulsion showed red appearance and an orange colour due to the presence of astaxanthin. An encapsulation efficiency (68.5 %) of astaxanthin in the initial emulsion with pea protein and gum arabica was observed (Table 10). Pea protein has strong emulsifying abilities, which means it can aid in forming stable astaxanthin emulsions and dispersions in aqueous solutions. This is crucial for improving astaxanthin's bioavailability and solubility in food and beverage applications. With the increasing of astaxanthin oil concentration (30µl to 50µl), encapsulation efficiency got increased from 68.5% to 74.4% (Table 10). This was an encouraging observation as a similar study when conducted on encapsulation of curcumin using pea protein reported an encapsulation efficiency of 85%. The results demonstrated that increased loading capability

and encapsulation efficiency with PPI-HMP (provide full form) complex (Guo et al., 2020). In a study surfactant type of effects on the encapsulation efficiency and loading capacity of complexes containing resveratrol-loaded complexes were examined. Encapsulation efficiency values of the resveratrol-loaded high methoxy pectin-rhamnolipid-pea protein isolate (96.98±0.40%), high methoxyl pectin-tea saponin-pea protein isolate (93.52±0.52%), and high methoxyl pectin-Ethyl lauroyl arginate-pea protein isolate (86.19±0.69%) (Guo et al., 2020).

Table 10: Encapsulation efficiency of astaxanthin enriched oil

Wall material	Encapsulation Efficiency (%)	*Encapsulation Efficiency (%) at 4°C (24 h)	*Encapsulation Efficiency (%) at room temperature (24 h)
Pea protein + Gum Arabica +Sodium alginate (emulsion) with 30µl astaxanthin oil	68.5	63	49
Pea protein +Gum Arabica +Sodium alginate (emulsion) with 40µl astaxanthin oil	71.9	60	45
Pea protein+ Gum Arabica +Sodium alginate (emulsion) with 50µl astaxanthin oil	74.4	60	42

*The data presented in this table on encapsulation efficiency as a function of stability is discussed under stability section (3.6). The data is presented as the mean ±SD of two replicates.

3.6 Stability of encapsulated astaxanthin enriched biomass

When compared to egg albumin and gum arabica, wall materials such as gelatine and maltodextrin showed poorer stability and displayed phase separation, and aggregation. Moreover, the encapsulation efficiency reduced to 37.4-33.1% from 43.6% (Table 9). Phase separation, temperature, or pH caused aggregation in the emulsion. The texture and appearance of the product was observed to change as a result of emulsion aggregation (Fig 22). Due to its hygroscopic nature, maltodextrin has a propensity to absorb moisture from its surroundings. This might shorten the shelf life of the encapsulated astaxanthin and cause it to degrade over time. In one of the investigations, when wheat gluten was mixed with maltodextrin (ratio 1:2) the surface hydrophobicity decreased noticeably. This decrease in surface hydrophobicity was linked to protein aggregation mediated by hydrophobic interactions. (Wang et al., 2019)

Astaxanthin-enriched biomass with wall components such egg albumin, gum arabica, and sodium alginate, presented better stability. At room temperature (24 h storage), an encapsulation efficiency (60%) was observed. It demonstrated steady emulsion and effective astaxanthin encapsulation due to the egg albumins and gum arabica's high emulsifying characteristics. Furthermore, gum arabica, an amphiphilic polysaccharide, and egg albumin, which is water soluble and widely used as a foam and emulsion stabilising agent. An amphiphilic wall material can form a stable layer surrounding the pigment by interacting with the aqueous environment and the hydrophobic sections with the astaxanthin. This improves astaxanthin stability and dispersion in aquatic environments. In one of the investigations (Xie et al., 2020), an emulsion made with gum arabica and egg albumin and containing astaxanthin oil provided protection against heat stress.

3.6.1. Stability of encapsulated astaxanthin enriched oil

Stability of pea protein and gum arabica, on the other hand, showed a phase separation with extended storage duration which led to decreased relative encapsulation efficiency (Fig 20, and 23). However, no aggregation or flocculation was observed. The encapsulation efficiency decreased from 68.5 % to 55.6-45.8% while using 30µl (Fig 20) and then from 74.4 to 63.7

when using 50µl of astaxanthin oil (Fig 21). The results were in agreement with literature findings where β -carotene retention and chemical stability were observed to decrease with pea protein - high methoxyl pectin colloidal particles (Yi et al., 2021). The emulsion stabilised by the HMP-Rha-PPI-Cur complex displayed improved physical stability. Another study found that after UV exposure and heat treatment, the retention rates for β-carotene in the high methoxyl pectin-Rhamnolipid-Pea protein isolate- curcumin complex-stabilized emulsion were 17.75 0.02 and 33.64 0.02%, respectively (Guo et al., 2022).

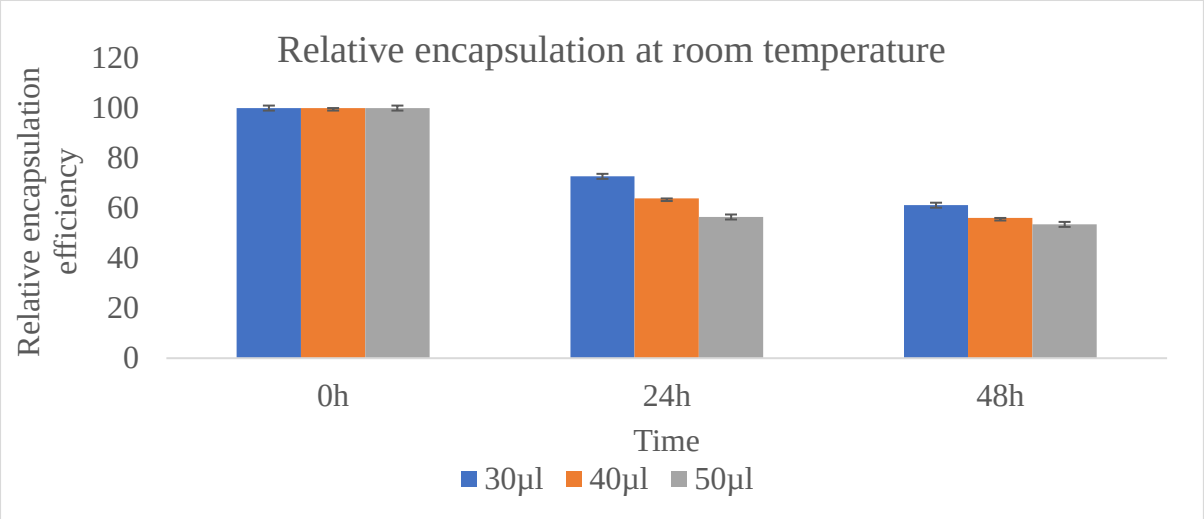


Figure 20: Relative encapsulation efficiency with increasing bioactive concentration (30,40 and 50µl) at room temperature. The data is presented as the mean ±SD of two replicates.

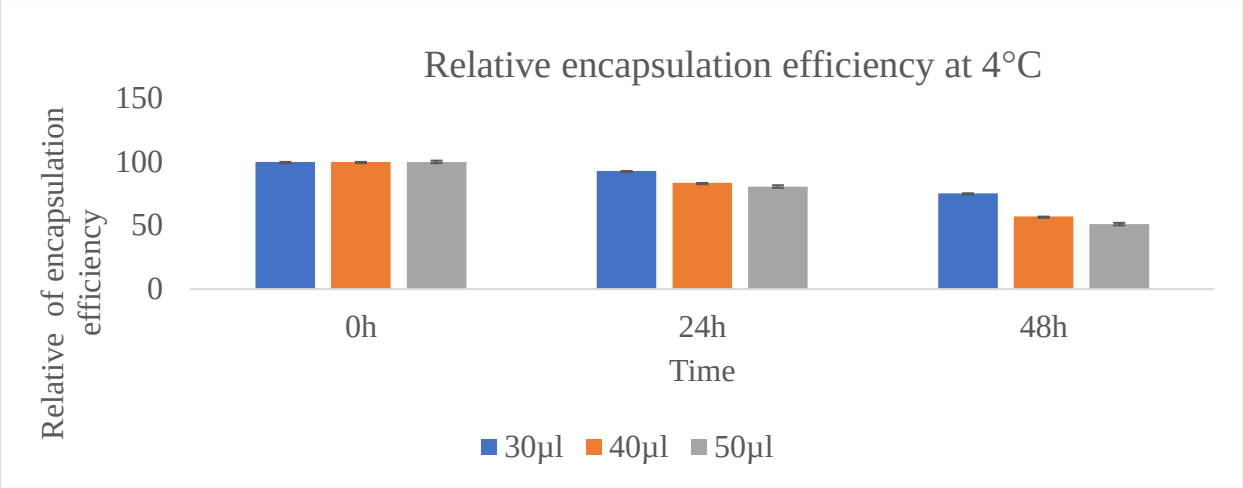


Figure 21: Relative encapsulation efficiency with increasing bioactive concentration (30,40 and 50 µl) at 4°C. The data is presented as the mean ±SD of two replicates.

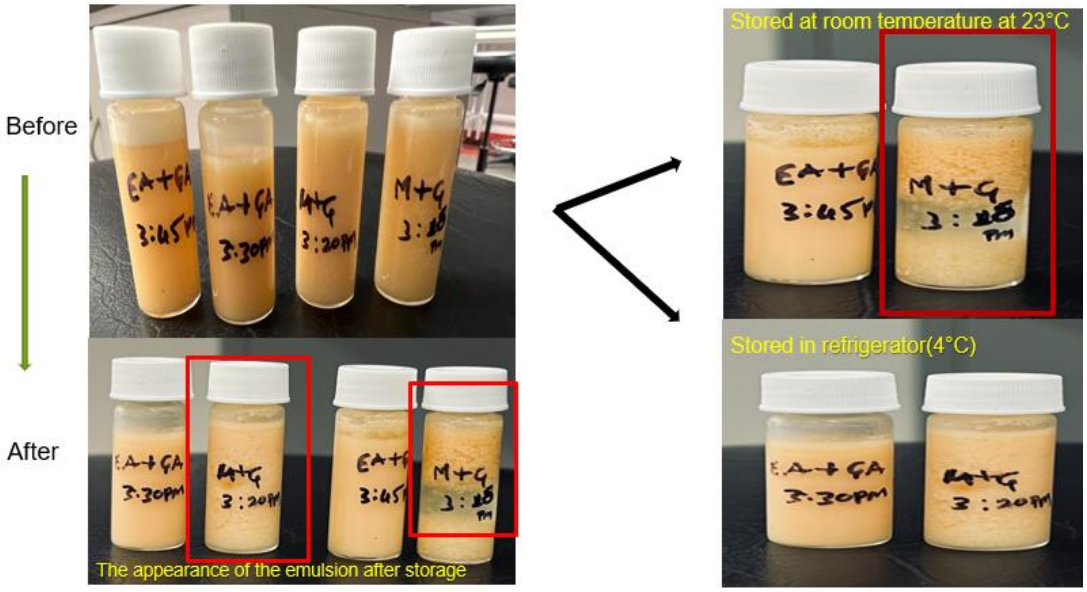


Figure 22: The stability of the astaxanthin enriched *Schizochytrium* biomass when observed with different polymers (the emulsions were kept overnight at RT and 4°C).

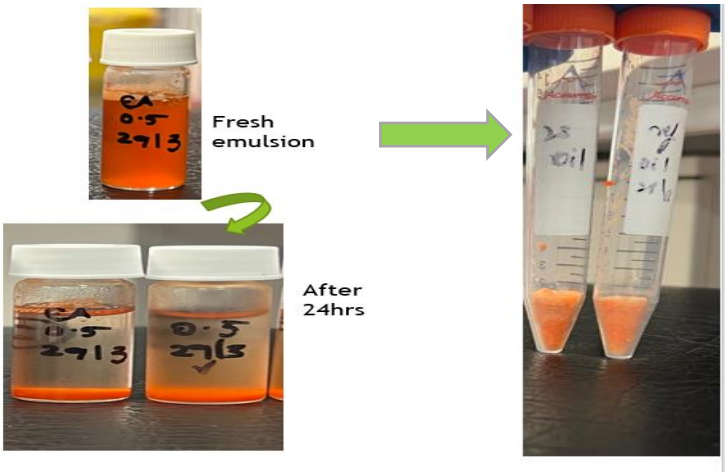


Figure 23: The stability of the astaxanthin enriched oil-*based* emulsion

Pea protein in combination with gum arabica and sodium alginate led to better encapsulation of astaxanthin enriched biomass/oil, however, further studies such as phase separation kinetics and role of pH are warranted as a follow up investigation. The use of pea protein for the encapsulation of astaxanthin (carotenoids) may be advantageous for the food and nutraceutical industries. To ascertain its feasibility and cost-effectiveness, follow-up research may be conducted to scale up the encapsulation process.

.

Conclusion:

In this study, an alternate stress approach was applied to accumulate astaxanthin in *Schizochytrium sp*. Treatment with ethanol (387µg/g) was shown to be more effective than methanol (183µg/g) in increasing astaxanthin accumulation. Astaxanthin produced by *Schizochytrium* fermentation was encapsulated in a variety of polymers/ wall materials using a high-pressure homogenisation. The EA:GA:SA (egg albumin, gum arabica, and sodium alginate) and PA:GA:SA (pea protein, gum arabica, and sodium alginate) emulsion methods were used successfully to encapsulate astaxanthin. When astaxanthin-enriched biomass was encapsulated using egg albumin and gum arabica, it demonstrated encapsulation efficiency (68%) with a better stability. The encapsulated astaxanthin oil demonstrated high encapsulation efficiency (74.4%), thus enhancing its potential for food and therapeutic applications.

References

Anarjan, N., Mirhosseini, H., Baharin, B. S., & Tan, C. P. (2010). Effect of processing conditions on physicochemical properties of astaxanthin nanodispersions. *Food Chemistry*, *123*(2), 477–483.

Anarjan, N., Tan, C. P., Ling, T. C., Lye, K. L., Malmiri, H. J., Nehdi, I. A., Baharin, B. S. (2011). Effect of organic-phase solvents on physicochemical properties and cellular uptake of astaxanthin nanodispersions. *Journal of Agricultural and Food Chemistry*, *59*(16), 8733–8741.

Aneesh, P. A., Ajeeshkumar, K. K., Lekshmi, R. G. K., Anandan, R., Ravishankar, C. N., & Mathew, S. (2022). Bioactivities of Astaxanthin from natural sources, augmenting its biomedical potential: A Review. *Trends in Food Science & Technology*, *125*, 81–90.

Bar, E., Rise, M., Vishkautsan, M., & Arad, S. (Malis). (1995). Pigment and structural changes in *chlorella zofingiensis* upon light and Nitrogen Stress. *Journal of Plant Physiology*, *146*(4), 527–534.

Bamidele, O. P., & Emmambux, M. N. (2020). Encapsulation of bioactive compounds by "extrusion" technologies: A Review. *Critical Reviews in Food Science and Nutrition*, *61*(18), 3100–3118.

Barbosa, M. J., Morais, R., & Choubert, G. (1999). Effect of carotenoid source and dietary lipid content on blood astaxanthin concentration in rainbow trout (Oncorhynchus mykiss). *Aquaculture*, *176*(3–4), 331–341.

Bassijeh, A., Ansari, S., & Hosseini, S. M. (2020). Astaxanthin encapsulation in multilayer emulsions stabilized by complex coacervates of whey protein isolate and Persian gum and its use as a natural colorant in a model beverage. *Food Research International*, *137*, 109689.

Bhosale, P. and Bernstein, P., (2005). “Microbial xanthophylls”. *Applied Microbiology and Biotechnology*, 68(4), pp.445-455.

Bidigare, Robert. R., Ondrusek, M. E., Kennicutt, M. C., Iturriaga, R., Harvey, H. R., Hoham, R. W., & Macko, Stephen. A. (1993). Evidence a photoprotective for secondary carotenoids of snow algae1. *Journal of Phycology*, *29*(4), 427–434.

Brotosudarmo, T. H., Limantara, L., Setiyono, E., & Heriyanto. (2020). Structures of astaxanthin and their consequences for therapeutic application. *International Journal of Food Science*, *2020*, 1–16.

Burja, A. M., Radianingtyas, H., Windust, A., & Barrow, C. J. (2006). Isolation and characterization of polyunsaturated fatty acid producing *Thraustochytrium* species: Screening of strains and optimization of omega-3 production. *Applied Microbiology and Biotechnology*, *72*(6), 1161–1169.

Chaudhary, V., Thakur, N., Kajla, P., Thakur, S., & Punia, S. (2021). Application of encapsulation technology in edible films: Carrier of bioactive compounds. *Frontiers in Sustainable Food Systems*, *5*.

Chen, J., Liu, L., & Wei, D. (2017). Enhanced production of astaxanthin by chromochloris zofingiensis in a microplate-based culture system under high light irradiation. *Bioresource Technology*, *245*, 518–529.

Chintong, S., Phatvej, W., Rerk-Am, U., Waiprib, Y., & Klaypradit, W. (2019). In vitro antioxidant, antityrosinase, and cytotoxic activities of astaxanthin from shrimp waste. *Antioxidants*, *8*(5), 128.

Choi, W.-Y., Oh, S.-H., Seo, Y.-C., Kim, G.-B., Kang, D.-H., Lee, S.-Y., Jung, K.-H., Cho, J.-S., Ahn, J.-H., Choi, G.-P., & Lee, H.-Y. (2011). Effects of methanol on cell growth and lipid production from mixotrophic cultivation of chlorella sp. *Biotechnology and Bioprocess Engineering*, *16*(5), 946–955.

Choi, S. S., Seo, Y. B., Nam, S.-W., & Kim, G.-D. (2021). Enhanced production of astaxanthin by co-culture of *paracoccus haeundaensis* and lactic acid bacteria. *Frontiers in Marine Science*, *7*.

Coutinho Rodrigues, O. H., Itokazu, A. G., Rörig, L., Maraschin, M., Corrêa, R. G., Pimentel-Almeida, W., & Moresco, R. (2021). Evaluation of astaxanthin biosynthesis by haematococcus pluvialis grown in culture medium added of cassava wastewater. *International Biodeterioration & Biodegradation*, *163*, 105269.

Cuaresma, M., Casal, C., Forján, E., & Vílchez, C. (2010). Productivity and selective accumulation of carotenoids of the novel extremophile microalga *Chlamydomonas acidophila* grown with different carbon sources in batch systems. *Journal of Industrial Microbiology & Biotechnology*, *38*(1), 167–177.

Dataintelo.com (2022). Market report/global-synthetic astaxanthin-market. (retrieved from [online] <https:// dataintelo.com /report/global-syntheticastaxanthin-market/> [Accessed 29 April 2022].

Davinelli, S., Nielsen, M., & Scapagnini, G. (2018). Astaxanthin in skin health, repair, and disease: A comprehensive review. *Nutrients*, *10*(4), 522.

Deng, X.-X., Chen, Z., Huang, Q., Fu, X., & Tang, C.-H. (2014). Spray-drying microencapsulation of β-carotene by soy protein isolate and/or Osa-modified starch. *Journal of Applied Polymer Science*, *131*(12).

Dong, S., Huang, Y., Zhang, R., Wang, S., & Liu, Y. (2014). Four different methods comparison for extraction of astaxanthin from green alga*haematococcus pluvialis*. *The Scientific World Journal*, *2014*, 1–7.

Du, H., Liao, X., Gao, Z., Li, Y., Lei, Y., Chen, W., Chen, L., Fan, X., Zhang, K., Chen, S., Ma, Y., Meng, C., & Li, D. (2019). Effects of methanol on carotenoids as well as biomass

and fatty acid biosynthesis in *schizochytrium limacinum* b4d1. *Applied and Environmental Microbiology*, *85*(19).

Espitia, P. J., Fuenmayor, C. A., & Otoni, C. G. (2018). Nanoemulsions: Synthesis, characterization, and application in bio-based active food packaging. *Comprehensive Reviews in Food Science and Food Safety*, *18*(1), 264–285.

Eun, J.-B., Maruf, A., Das, P. R., & Nam, S.-H. (2019). A review of encapsulation of carotenoids using spray drying and freeze drying. *Critical Reviews in Food Science and Nutrition*, *60*(21), 3547–3572.

Fang, X., Wei, C., Zhao-Ling, C., & Fan, O. (2004). Effects of organic carbon sources on cell growth and eicosapentaenoic acid content of nannochloropsis sp. *Journal of Applied Phycology*, *16*(6), 499–503.

Fang, R., Liang, H., Li, J., Chen, Y., Luo, X., Li, Y., Li, B., & Liu, S. (2022). Microencapsulation of Astaxanthin based on emulsion solvent evaporation and subsequent spray drying. *Journal of Food Science*, *87*(3), 998–1008.

Feng, Z.-Z., Li, M.-Y., Wang, Y.-T., & Zhu, M.-J. (2018). Astaxanthin from *Phaffia Rhodozyma*: Microencapsulation with carboxymethyl cellulose sodium and microcrystalline cellulose and effects of microencapsulated astaxanthin on yogurt properties. *LWT*, *96*, 152–160.

Finance.yahoo.com. (2022). Yahoo is part of the Yahoo family of brands. (retrieved from [online]:<https://finance.yahoo.com/news/worldwide-astaxanthin-industry-expected-reach153300947.html> [Accessed 22 April 2022]

Furlan, V. J., Batista, I., Bandarra, N., Mendes, R., & Cardoso, C. (2019). Conditions for the production of carotenoids by *Thraustochytrium* SP. ATCC 26185 and *Aurantiochytrium* SP. ATCC PRA-276. *Journal of Aquatic Food Product Technology*, *28*(5), 465–477.

Gao, Q., Qiao, X., Yang, L., Cao, Y., Li, Z., Xu, J., & Xue, C. (2022). Effects of microencapsulation in dairy matrix on the quality characteristics and bioavailability of docosahexaenoic acid astaxanthin. *Journal of the Science of Food and Agriculture*, *102*(13), 5711–5719.

Giannelli, L., Yamada, H., Katsuda, T., & Yamaji, H. (2015). Effects of temperature on the astaxanthin productivity and light harvesting characteristics of the green alga *Haematococcus pluvialis. Journal of Bioscience and Bioengineering*, *119*(3), 345–350.

Grung, M., Metzger, P., Berkaloff, C., & Liaaen-Jensen, S. (1994). Studies on the formation and localization of primary and secondary carotenoids in the green alga *Botryococcus braunii*, including the regreening process. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *107*(2), 265–272.

Gupta, A., Wilkens, S., Adcock, J. L., Puri, M., & Barrow, C. J. (2013). Pollen baiting facilitates the isolation of marine thraustochytrids with potential in omega-3 and biodiesel production. *Journal of Industrial Microbiology and Biotechnology*, *40*(11), 1231–1240.

Gupta, A., Singh, D., Byreddy, A. R., Thyagarajan, T., Sonkar, S. P., Mathur, A. S., Tuli, D. K., Barrow, C. J., & Puri, M. (2015). Exploring omega-3 fatty acids, enzymes and biodiesel producing thraustochytrids from Australian and Indian Marine Biodiversity. *Biotechnology Journal*, *11*(3), 345–355.

Gu, L., Su, Y., Zhang, M., Chang, C., Li, J., McClements, D. J., & Yang, Y. (2017). Protection of β-carotene from chemical degradation in emulsion-based delivery systems using antioxidant interfacial complexes: Catechin-egg white protein conjugates. *Food Research International*, *96*, 84–93.

Gu, L., Pan, C., Su, Y., Zhang, R., Xiao, H., McClements, D. J., & Yang, Y. (2018). *in vitro* bioavailability, cellular antioxidant activity, and cytotoxicity of β-carotene-loaded

emulsions stabilized by catechin–egg white protein conjugates. *Journal of Agricultural and Food Chemistry*, *66*(7), 1649–1657.

Guo, Q., Su, J., Shu, X., Yuan, F., Mao, L., Liu, J., & Gao, Y. (2020). Production and characterization of pea protein isolate-pectin complexes for delivery of curcumin: Effect of esterified degree of pectin. *Food Hydrocolloids*, *105*, 105777.

Guo, Q., Su, J., Shu, X., Yuan, F., Mao, L., & Gao, Y. (2020). Development of high methoxyl pectin-surfactant-pea protein isolate ternary complexes: Fabrication, characterization and delivery of resveratrol. *Food Chemistry*, *321*, 126706.

Guo, Q., Bayram, I., Shu, X., Su, J., Liao, W., Wang, Y., & Gao, Y. (2022). Improvement of stability and bioaccessibility of β-carotene by curcumin in pea protein isolate-based complexes-stabilized emulsions: Effect of protein complexation by pectin and small molecular surfactants. *Food Chemistry*, *367*, 130726.

Harith, Z. T., de Andrade Lima, M., Charalampopoulos, D., & Chatzifragkou, A. (2020). Optimised production and extraction of astaxanthin from the yeast *xanthophyllomyces dendrorhous*. *Microorganisms*, *8*(3), 430.

Hirasawa, K. *et al*. (2017). "Method for producing astaxanthin by fermentation". Patent number US 9,605,323 B2.

Huang, J., Feng, X., Zhang, S., Wang, L., Yue, J., & Chu, L. (2022). Preparation and characterization of astaxanthin-loaded microcapsules and its application in effervescent tablets. *Journal of the Science of Food and Agriculture*, *103*(3), 1421–1431.

Hussein, G., Goto, H., Oda, S., Sankawa, U., Matsumoto, K., & Watanabe, H. (2006). Antihypertensive potential and mechanism of action of astaxanthin: III. antioxidant and histopathological effects in spontaneously hypertensive rats. *Biological and Pharmaceutical Bulletin*, *29*(4), 684–688.

Hussian, A. E. (2018). The role of microalgae in renewable energy production: Challenges and opportunities. *Marine Ecology - Biotic and Abiotic Interactions*.

Ip, P.-F., Wong, K.-H., & Chen, F. (2004). Enhanced production of Astaxanthin by the Green Microalga *Chlorella zofingiensis* in mixotrophic culture. *Process Biochemistry*, *39*(11), 1761–1766.

Irshad, M., Myint, A. A., Hong, M. E., Kim, J., & Sim, S. J. (2019). One-pot, simultaneous cell wall disruption and complete extraction of astaxanthin from *Haematococcus pluvialis* at room temperature. *ACS Sustainable Chemistry & Engineering*, *7*(16), 13898–13910.

Janchot, K., Rauytanapanit, M., Honda, M., Hibino, T., Sirisattha, S., Praneenararat, T., Kageyama, H., & Waditee-Sirisattha, R. (2019). Effects of potassium chloride-induced stress on the carotenoids canthaxanthin, astaxanthin, and lipid accumulations in the green chlorococcal microalga straintistr9500. *Journal of Eukaryotic Microbiology*, *66*(5), 778–787.

Jayanudin, J., Rochmadi, R., Fahrurrozi, Moh., & Wirawan, S. (2016). Microencapsulation technology of Ginger Oleoresin with Chitosan as wall material: A Review. *Journal of Applied Pharmaceutical Science*, 209–223.

Jiang, G.-L., & Zhu, M.-J. (2019). Preparation of astaxanthin-encapsulated complex with Zein and Oligochitosan and its application in food processing. *LWT*, *106*, 179–185.

Kaha, M., Iwamoto, K., Yahya, N. A., Suhaimi, N., Sugiura, N., Hara, H., Othman, N., Zakaria, Z., & Suzuki, K. (2021). Enhancement of astaxanthin accumulation using black light in *coelastrum* and monoraphidium isolated from Malaysia. *Scientific Reports*, *11*(1).

Kavitha, K., Kowshik, J., Kishore, T. K., Baba, A. B., & Nagini, S. (2013). Astaxanthin inhibits NF-KB and Wnt/β-catenin signalling pathways via inactivation of Erk/MAPK and PI3K/AKT to induce intrinsic apoptosis in a hamster model of oral cancer. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1830*(10), 4433–4444.

Khalid, N., Shu, G., Holland, B. J., Kobayashi, I., Nakajima, M., & Barrow, C. J. (2017). Formulation and characterization of O/w nano emulsions encapsulating high concentration of astaxanthin. *Food Research International*, *102*, 364–371.

Khalid, N., Shu, G., Kobayashi, I., Nakajima, M., & Barrow, C. J. (2017). Formulation and characterization of monodisperse O/W emulsions encapsulating astaxanthin extracts using microchannel emulsification: Insights of formulation and stability evaluation. *Colloids and Surfaces B: Biointerfaces*, *157*, 355–365.

Khalid, N. and Barrow, C.J. (2018). "Critical Review of Encapsulation Methods for stabilization and delivery of astaxanthin," *Journal of Food Bioactives*,

Khomlaem, C., Aloui, H., Oh, W.-G., & Kim, B. S. (2021). High cell density culture of *Paracoccus* sp. LL1 in membrane bioreactor for enhanced co-production of polyhydroxyalkanoates and astaxanthin. *International Journal of Biological Macromolecules*, *192*, 289–297.

Kikukawa, H., Shimizu, C., Hirono-Hara, Y., & Hara, K. Y. (2022). Effect of ethanol on Astaxanthin and fatty acid production in the red yeast *xanthophyllomyces dendrorhous. Journal of Applied Microbiology*, *132*(3), 2034–2041.

Koopmann, I. K., Kramer, A., & Labes, A. (2022). Development and validation of reliable astaxanthin quantification from natural sources. *PLOS ONE*, *17*(12).

Leyton, A., Shene, C., Chisti, Y., & Asenjo, J. A. (2022). Production of carotenoids and phospholipids by *Thraustochytrium* sp. in batch and repeated-batch culture. *Marine Drugs*, *20*(7), 416.

Lin, S.-F., Chen, Y.-C., Chen, R.-N., Chen, L.-C., Ho, H.-O., Tsung, Y.-H., Sheu, M.-T., & Liu, D.-Z. (2016). Improving the stability of astaxanthin by microencapsulation in calcium alginate beads. *PLOS ONE*, *11*(4).

Liu, J., Sun, Z., Zhong, Y., Gerken, H., Huang, J., & Chen, F. (2013). Utilization of cane molasses towards cost-saving astaxanthin production by a *chlorella zofingiensis* mutant. *Journal of Applied Phycology*, *25*(5), 1447–1456.

Li, M., Zahi, M. R., Yuan, Q., Tian, F., & Liang, H. (2015). Preparation and stability of astaxanthin solid lipid nanoparticles based on stearic acid. *European Journal of Lipid Science and Technology*, *118*(4), 592–602.

Li, K., Cheng, J., Ye, Q., He, Y., Zhou, J., & Cen, K. (2017). In vivo kinetics of lipids and astaxanthin evolution in *Haematococcus pluvialis* mutant under 15% CO2 using Raman microspectroscopy. *Bioresource Technology*, *244*, 1439–1444.

Li, X., Wang, X., Duan, C., Yi, S., Gao, Z., Xiao, C., Agathos, S. N., Wang, G., & Li, J. (2020). Biotechnological production of astaxanthin from the Microalga *Haematococcus pluvialis. Biotechnology Advances*, *43*, 107602.

Liu, C., Zhang, S., McClements, D. J., Wang, D., & Xu, Y. (2019). Design of astaxanthin-loaded core–shell nanoparticles consisting of chitosan oligosaccharides and poly (lactic-*co*-glycolic acid): Enhancement of water solubility, stability, and bioavailability. *Journal of Agricultural and Food Chemistry*, *67*(18), 5113–5121.

Liu, Y.-H., Alimujiang, A., Wang, X., Luo, S.-W., Balamurugan, S., Yang, W.-D., Liu, J.-S., Zhang, L., & Li, H.-Y. (2019). Ethanol induced jasmonate pathway promotes astaxanthin hyperaccumulation in *Haematococcus pluvialis*. *Bioresource Technology*, *289*, 121720.

Liu, Z., van den Berg, C., Weusthuis, R. A., Dragone, G., & Mussatto, S. I. (2021). Strategies for an improved extraction and separation of lipids and carotenoids from oleaginous yeast. *Separation and Purification Technology*, *257*, 117946.

ltd, R. M. (2023). "Astaxanthin market size, Share & Trends analysis report by product (dried algae meal or biomass, oil, Softgel, liquid), by source (natural, synthetic), by application,

by region, and segment forecasts, 2023 – 2030", *Research and Markets - Market Research Reports*

retrieved from (https://www.researchandmarkets.com/reports/5450246/astaxanthin-market-size-share-and-trends-analysis (Accessed: 02 March 2023).

Martins, C. B., Assunçao, M. F. G., Ferreira, J. D., & Santos, L. M. A. (2021). Astaxanthin in Microalgae Eustigmatophyceae. *Global Perspectives on Astaxanthin*, 61–70.

Martínez-Álvarez, Ó., Calvo, M. M., & Gómez-Estaca, J. (2020). Recent advances in Astaxanthin Micro/Nanoencapsulation to improve its stability and functionality as a food ingredient. *Marine Drugs*, *18*(8), 406.

Market, A. (2022). Astaxanthin *Market Growth, Size, and Forecast to 2026.* |MarketsandMarkets. [online] Marketsandmarkets.com. [Accessed 29 April 2022]

Niizawa, I., Espinaco, B. Y., Zorrilla, S. E., & Sihufe, G. A. (2019). Natural astaxanthin encapsulation: Use of response surface methodology for the design of alginate beads. *International Journal of Biological Macromolecules*, *121*, 601–608.

Nikolić, I., Gledovic, A., Tamburic, S., Major, T., & Savić, S. (2020). Nanoemulsions as carriers for natural antioxidants: Formulation Development and Optimisation. *Emulsion-based Encapsulation of Antioxidants*, 149–195.

Ozturk, B., Argin, S., Ozilgen, M., & McClements, D. J. (2015). Formation and stabilization of nanoemulsion-based vitamin E delivery systems using natural biopolymers: Whey protein isolate and Gum Arabic. *Food Chemistry*, *188*, 256–263.

Pan, L., Zhang, S., Gu, K., & Zhang, N. (2018). Preparation of astaxanthin-loaded liposomes: Characterization, storage stability and antioxidant activity. *CyTA - Journal of Food*, *16*(1), 607–618.

Pan-utai, W., Boonpok, S., & Pornpukdeewattana, S. (2021). Combination of mechanical and chemical extraction of astaxanthin from *Haematococcus pluvialis* and its properties of microencapsulation. *Biocatalysis and Agricultural Biotechnology*, *33*, 101979.

Park, H., Kwak, M., Seo, J., Ju, J., Heo, S., Park, S., & Hong, W. (2018). Enhanced production of carotenoids using a thraustochytrid microalgal strain containing high levels of docosahexaenoic acid-rich oil. *Bioprocess and Biosystems Engineering*, *41*(9), 1355–1370.

Ranjbar, R., Inoue, R., Katsuda, T., Yamaji, H., & Katoh, S. (2008). High efficiency production of astaxanthin in an airlift photobioreactor. *Journal of Bioscience and Bioengineering*, *106*(2), 204–207.

Rezvankhah, A., Emam-Djomeh, Z., & Askari, G. (2019). Encapsulation and delivery of bioactive compounds using spray and freeze-drying techniques: A Review. *Drying Technology*, *38*(1–2), 235–258.

Robert, P., Carlsson, R. M., Romero, N., & Masson, L. (2003). Stability of spray-dried encapsulated carotenoid pigments from Rosa Mosqueta (*rosa rubiginosa*) oleoresin. *Journal of the American Oil Chemists' Society*, *80*(11), 1115–1120.

Ruengdech, A., & Siripatrawan, U. (2022). Improving encapsulating efficiency, stability, and antioxidant activity of catechin nanoemulsion using foam mat freeze-drying: The effect of wall material types and concentrations. *LWT*, *162*, 113478.

Saha, S. K., McHugh, E., Hayes, J., Moane, S., Walsh, D., & Murray, P. (2013). Effect of various stress-regulatory factors on biomass and lipid production in microalga *Haematococcus pluvialis*. *Bioresource Technology*, *128*, 118–124.

Sakamoto, T., Ikeda, Y., Masuda, N., & Sakuradani, E. (2023). Ethanol enhances astaxanthin production by *aurantiochytrium* sp. O5-1-1. *Journal of Oleo Science*, *72*(4), 441–446.

Sankari, M., Hemachandran, H., Anantharaman, A., Babu, S., Madrid, R. R., C, G. P., Fulzele, D. P., & Siva, R. (2016). Identifying a carotenoid cleavage dioxygenase 4A gene and its efficient agrobacterium-mediated genetic transformation in Bixa orellana L. *Applied Biochemistry and Biotechnology*, *179*(5), 697–714.

Samuel, S. Y., Wang, H.-M. D., Huang, M.-Y., Cheng, Y.-S., Chen, J.-R., Li, W.-H., & Chang, J.-J. (2022). Safety assessment of 3S, 3's astaxanthin derived from metabolically engineered K. Marxianus. *Antioxidants*, *11*(11), 2288.

Sciences, L. and Supplements, D., (2022). Astaxanthin Market Size by Source (Synthetic, Natural), By Application (Dietary Supplement, Personal Care, Pharmaceuticals, Food & Beverages, Animal Feed {Aquaculture, Livestock, Pets}) Industry Outlook Report, Regional Analysis, Application Potential, Pr. [online] Marketresearch.com. [Accessed 29 April 2022]

Shen, Q., & Quek, S. Y. (2014). Microencapsulation of Astaxanthin with blends of milk protein and fiber by spray drying. *Journal of Food Engineering*, *123*, 165–171.

Shojaeiarani, J. *et al*. (2020). "Sonication amplitude and processing time influence the cellulose nanocrystals morphology and dispersion," *Nanocomposites*, 6(1), pp. 41–46.

Singh, D., Gupta, A., Wilkens, S. L., Mathur, A. S., Tuli, D. K., Barrow, C. J., & Puri, M. (2015). Understanding response surface optimisation to the modeling of astaxanthin extraction from a novel strain *Thraustochytrium* SP. S7. *Algal Research*, *11*, 113–120.

Stoklosa, R. J., Johnston, D. B., & Nghiem, N. P. (2019). *Phaffia rhodozyma* cultivation on structural and non-structural sugars from sweet sorghum for astaxanthin generation. *Process Biochemistry*, *83*, 9–17.

Sun, M., Chen, H., Geng, F., Zhou, Q., Hao, Q., Zhang, S., Chen, Y., & Deng, Q. (2022). Fabrication and characterization of botanical-based double-layered emulsion: Protection of DHA and astaxanthin based on interface remodelling. *Foods*, *11*(22), 3557.

Tamjidi, F., Shahedi, M., Varshosaz, J., & Nasirpour, A. (2017). Stability of Astaxanthin-loaded nanostructured lipid carriers as affected by ph, ionic strength, heat treatment, simulated gastric juice and freeze–thawing. *Journal of Food Science and Technology*, *54*(10), 3132–314.

Tan, Y., Ye, Z., Wang, M., Manzoor, M. F., Aadil, R. M., Tan, X., & Liu, Z. (2021). Comparison of different methods for extracting the Astaxanthin from *Haematococcus pluvialis*: Chemical Composition and biological activity. *Molecules*, *26*(12), 3569.

Tran, T. N., Tran, N.-T., Tran, T.-A., Pham, D.-C., Su, C.-H., Nguyen, H. C., Barrow, C. J., & Ngo, D.-N. (2023). Highly active astaxanthin production from waste molasses by mutated *rhodosporidium toruloides* G17. *Fermentation*, *9*(2), 148.

Tzanova, M., Argirova, M., & Atanasov, V. (2016). HPLC quantification of astaxanthin and canthaxanthin in Salmonidae Eggs. *Biomedical Chromatography*, *31*(4).

Vakarelova, M., Zanoni, F., Lardo, P., Rossin, G., Mainente, F., Chignola, R., Menin, A., Rizzi, C., & Zoccatelli, G. (2017). Production of stable food-grade microencapsulated astaxanthin by vibrating nozzle technology. *Food Chemistry*, *221*, 289–295.

Vijeth, S., B. Heggannavar, G., & Y. Kariduraganavar, M. (2019). Encapsulating wall materials for micro-/nanocapsules. *Microencapsulation - Processes, Technologies and Industrial Applications*.

Wang, Y., Chen, T., & Qin, S. (2012). Differential fatty acid profiles of *chlorella kessleri* grown with organic materials. *Journal of Chemical Technology & Biotechnology*, *88*(4), 651–657.

Wan, M., Hou, D., Li, Y., Fan, J., Huang, J., Liang, S., Wang, W., Pan, R., Wang, J., & Li, S. (2014). The effective photoinduction of *Haematococcus pluvialis* for accumulating astaxanthin with attached cultivation. *Bioresource Technology*, *163*, 26–32.

Wan, M., Zhang, J., Hou, D., Fan, J., Li, Y., Huang, J., & Wang, J. (2014). The effect of temperature on cell growth and astaxanthin accumulation of *Haematococcus pluvialis* during a light–dark cyclic cultivation. *Bioresource Technology*, *167*, 276–283.

Watanabe, K., Arafiles, K. H., Higashi, R., Okamura, Y., Tajima, T., Matsumura, Y., Nakashimada, Y., Matsuyama, K., & Aki, T. (2018). Isolation of high carotenoid-producing *aurantiochytrium* sp. mutants and improvement of astaxanthin productivity using metabolic information. *Journal of Oleo Science*, *67*(5), 571–578.

Wang, B., Akanbi, T. O., Agyei, D., Holland, B. J., & Barrow, C. J. (2018). Coacervation technique as an encapsulation and delivery tool for hydrophobic biofunctional compounds. *Role of Materials Science in Food Bioengineering*, 235–261.

Wang, Y., Gan, J., Li, Y., Nirasawa, S., & Cheng, Y. (2019). Conformation and emulsifying properties of deamidated wheat gluten-maltodextrin/citrus pectin conjugates and their abilities to stabilize β-carotene emulsions. *Food Hydrocolloids*, *87*, 129–141.

Wen, Z., Liu, Z., Hou, Y., Liu, C., Gao, F., Zheng, Y., & Chen, F. (2015). Ethanol induced astaxanthin accumulation and transcriptional expression of carotenogenic genes in *Haematococcus pluvialis*. *Enzyme and Microbial Technology*, *78*, 10–17.

Würth, R., Foerst, P., & Kulozik, U. (2017). Effects of skim milk concentrate dry matter and spray drying air temperature on formation of capsules with varying particle size and the survival microbial cultures in a microcapsule matrix. *Drying Technology*, *36*(1), 93–99.

Xiao, R., Li, X., Leonard, E., Tharayil, N., & Zheng, Y. (2019). Investigation on the effects of cultivation conditions, fed-batch operation, and enzymatic hydrolysate of corn stover on the astaxanthin production by *Thraustochytrium striatum*. *Algal Research, 39.*

Xie, L., Ciftci, O., & Zhang, Y. (2020). Encapsulation of astaxanthin-enriched Camelina oil extract in ovalbumin/gum arabic stabilized emulsion with/without crosslinking by tannic acid. *ES Food & Agroforestry*.

Xing, H., Zhao, Y., Li, T., Han, B., Zhao, P., & Yu, X. (2022). Enhancing astaxanthin and lipid coproduction in *Haematococcus pluvialis* by the combined induction of plant growth regulators and multiple stresses. *Bioresource Technology*, *344*, 126225.

Ye, J., Liu, M., He, M., Ye, Y., & Huang, J. (2019). Illustrating and enhancing the biosynthesis of astaxanthin and docosahexaenoic acid in *aurantiochytrium* SP. SK4. *Marine Drugs*, *17*(1), 45.

Yi, J., Gan, C., Wen, Z., Fan, Y., & Wu, X. (2021). Development of pea protein and high methoxyl pectin colloidal particles stabilized high internal phase Pickering emulsions for β-carotene protection and delivery. *Food Hydrocolloids*, *113*, 106497.

Yin-Nin Ma, R. and Chen, F., (2000). Induction of astaxanthin formation by reactive oxygen species in mixotrophic culture of *Chlorococcum* sp. Biotechnology Letters, 23(7), pp.519–523.

Yin, F.-W., Zhan, C.-T., Huang, J., Sun, X.-L., Yin, L.-F., Zheng, W.-L., Luo, X., Zhang, Y.-Y., & Fu, Y.-Q. (2022). Efficient co-production of docosahexaenoic acid oil and carotenoids in *aurantiochytrium* SP. using a light intensity gradient strategy. *Applied Biochemistry and Biotechnology*, *195*(1), 623–638.

Yu, J., Zhang, Y., Yan, J., Li, S., & Chen, Y. (2021). A novel glycoprotein emulsion using high-denatured peanut protein and Sesbania gum via cold plasma for encapsulation of β-carotene. *Innovative Food Science & Emerging Technologies*, *74*, 102840.

Yu, H., Wang, H., Su, W., Song, Y., Zaky, A. A., Abd El-Aty, A. M., & Tan, M. (2022). Co-delivery of hydrophobic astaxanthin and hydrophilic phycocyanin by a ph-sensitive water-in-oil-in-water double emulsion-filled Gellan Gum Hydrogel. *Food Hydrocolloids*, *131*, 107810.

Yu, W., Zhang, L., Zhao, J., & Liu, J. (2022). Enhancement of astaxanthin accumulation in *Haematococcus pluvialis* by exogenous oxaloacetate combined with nitrogen deficiency. *Bioresource Technology*, *345*, 126484.

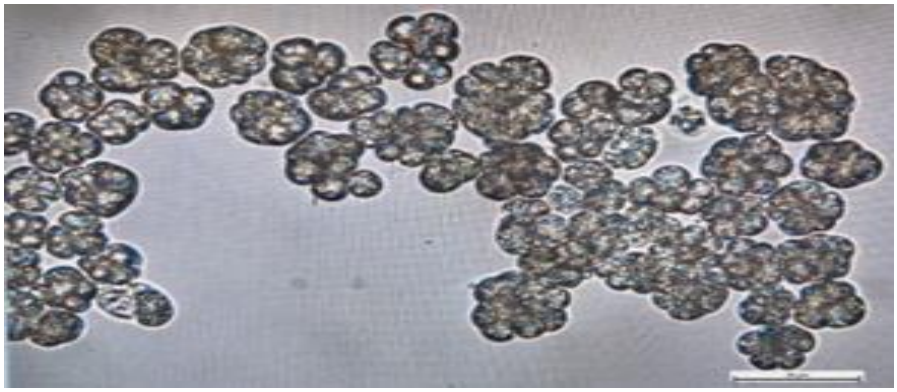
Zhang, C., Jin, Y., Yu, Y., Xiang, J., & Li, F. (2021). Effects of natural astaxanthin from microalgae and chemically synthetic astaxanthin supplementation on two different varieties of the ridgetail white prawn (*exopalaemon carinicauda*). *Algal Research*, *57*.

Zhang, K., Chen, L., Liu, J., Gao, F., He, R., Chen, W., Guo, W., Chen, S., & Li, D. (2017). Effects of butanol on high value product production in *Schizochytrium Limacinum* b4d1. *Enzyme and Microbial Technology*, *102*, 9–15.

Zhang, Z., Sun, D., Zhang, Y., & Chen, F. (2019). Glucose triggers cell structure changes and regulates astaxanthin biosynthesis in *chromochloris zofingiensis*. *Algal Research*, *39*,

Zhu, X., Meng, C., Du, H., Chen, L., Sun, F., Chen, W., Wei, Z., Ren, J., Gao, Z., & Li, D. (2022). Enhancement of astaxanthin production in *Schizochytrium Limacinum* B4D1 under ethanol induction. *Algal Research*, *61*, 102537.

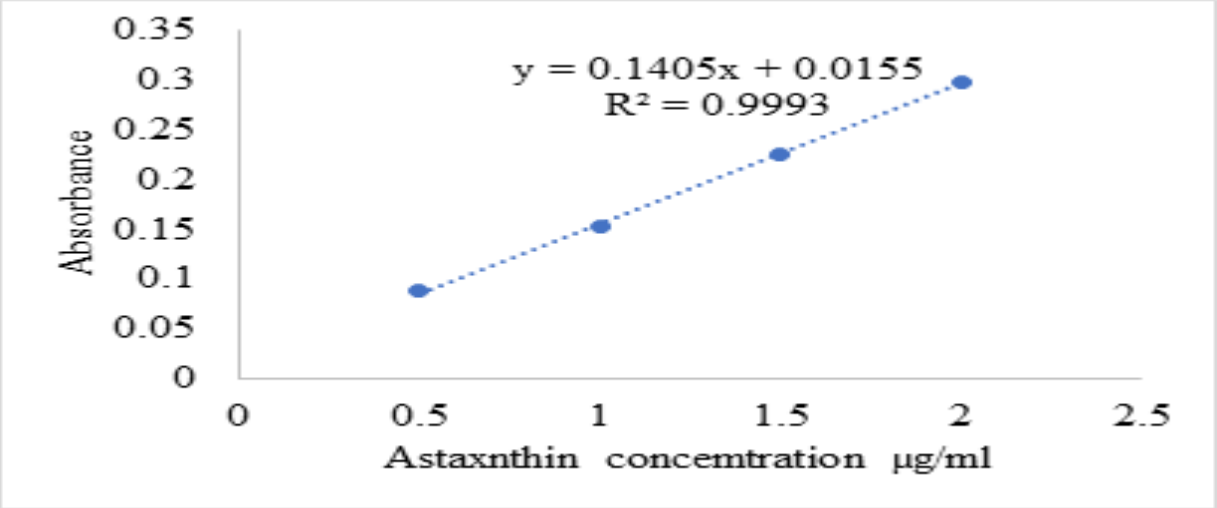
APPENDIX



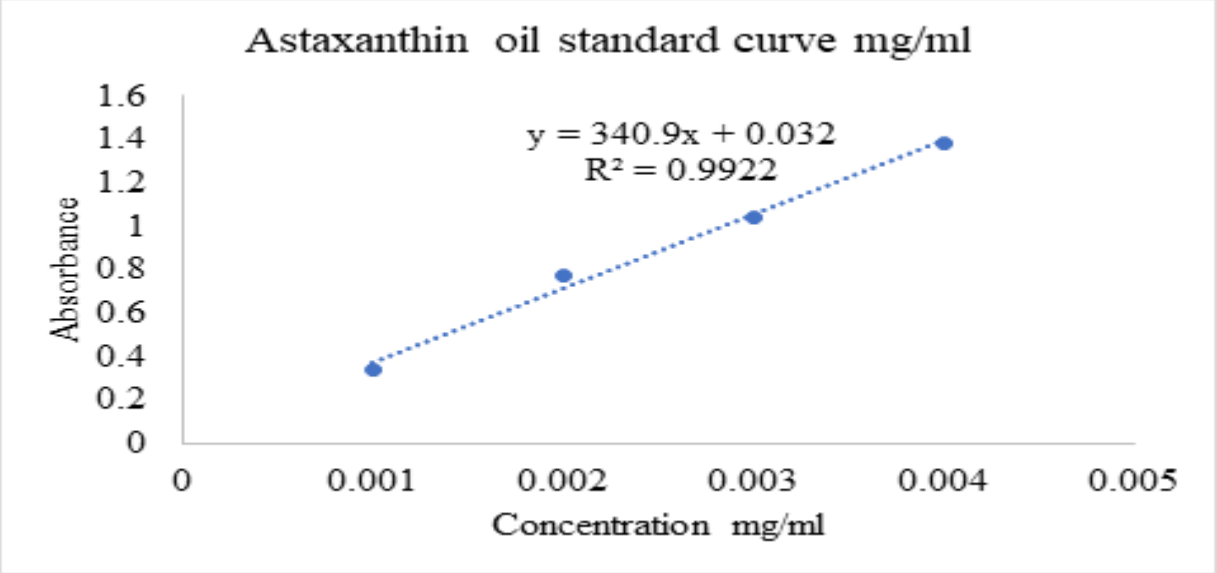
Supplementary 1 (S1): Inoculum of *Schizochytrium* DT3 after 48 h of incubation (The *Schizochytrium* cells as observed under a 40X magnification, Nikon 2S TR)

Control biomass (powder) **Biomass powder (Ethanol 3%**

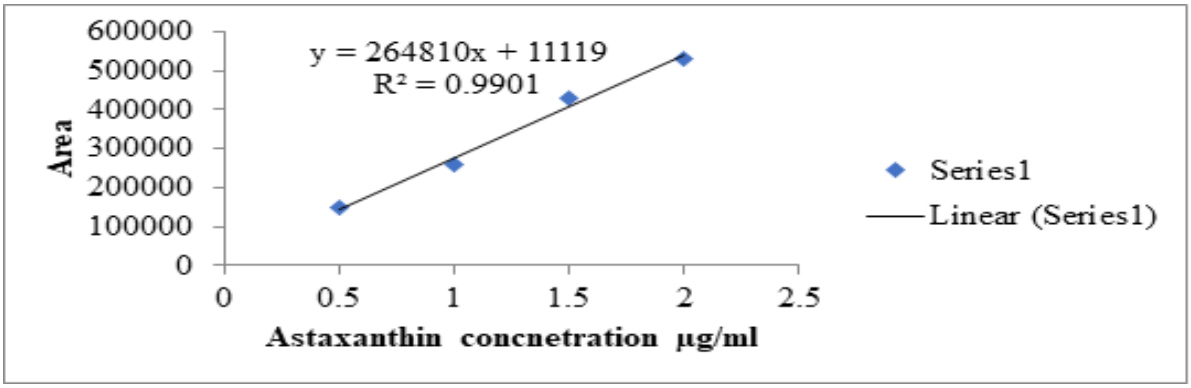
Supplementary 2: *Schizochytrium* DT3 dried biomass (control and treated with ethanol 3%, v/v) had a different power morphology. The treated biomass exhibited intense orange colour.



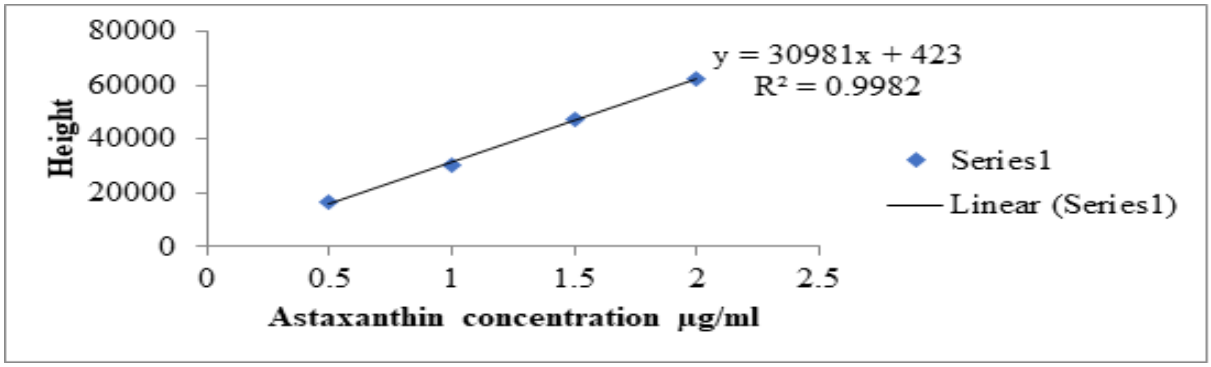
Supplementary 3: Standard curve of astaxanthin



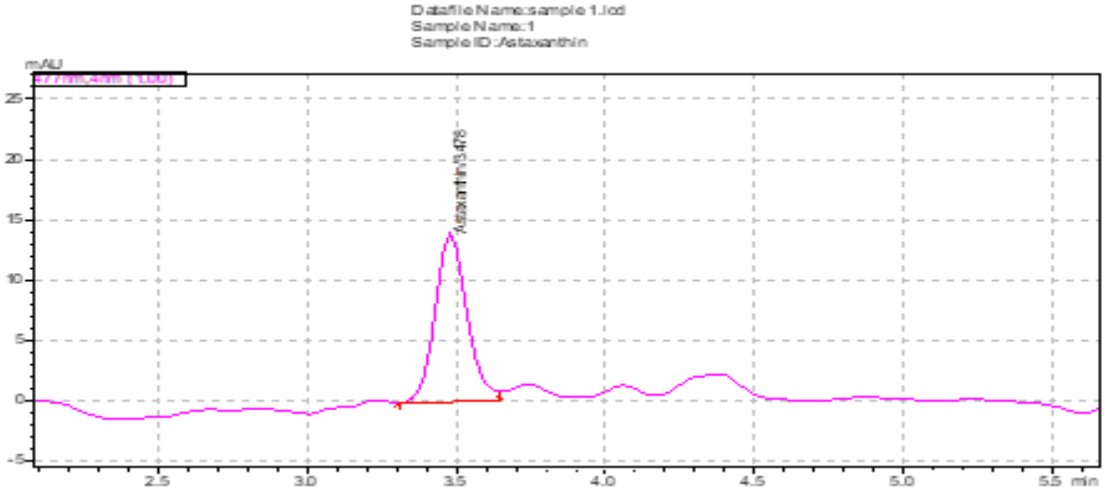
Supplementary 4: Standard curve of astaxanthin oil



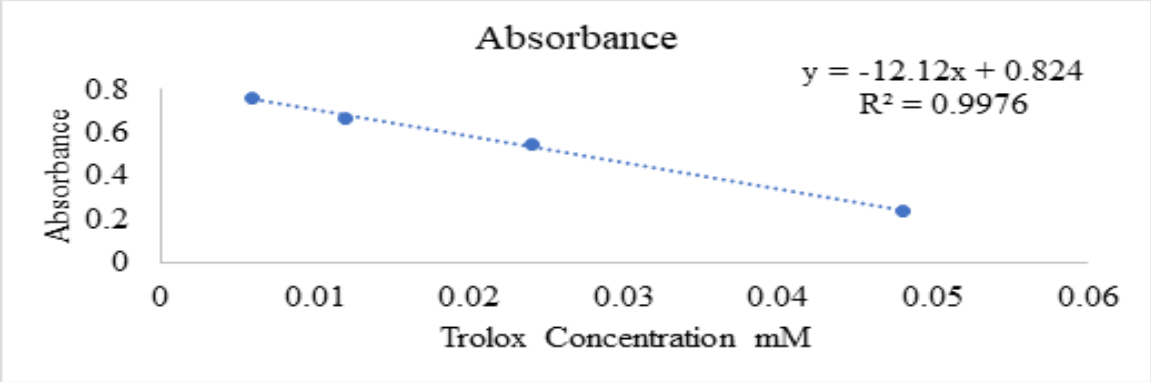
Supplementary 5: Standard curve of astaxanthin using HPLC analysis



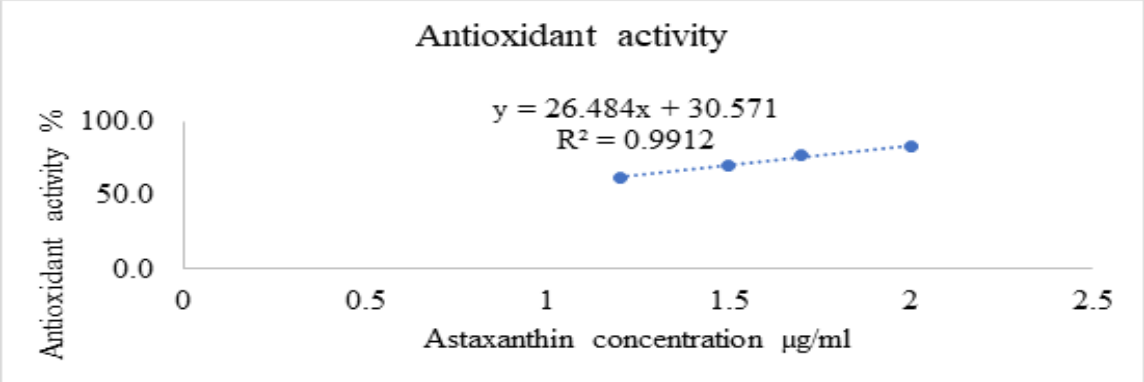
Supplementary 6: Standard curve of astaxanthin using HPLC analysis



Supplementary 7: The astaxanthin concentrations in *Schizochytrium* were determined by HPLC



Supplementary 8: Positive control (Trolox) of antioxidant assay



Supplementary 9: Antioxidant activity of astaxanthin enriched biomass *Schizochytrium* DT3

Turnitin statement

The written submission titled "Astaxanthin synthesis from *Schizochytrium* sp. and its stability through encapsulation" is unique and free of plagiarism. Turnitin used to check the work, and the report shows that [33%]. The references portion makes up 19% of this. Only 14% of them are demonstrated in theory; the majority are scientific terms, formulas, compounds, and species names that cannot be modified. The sources included in the report have been correctly referenced, according to my review of it. Plagiarism has not been found in any of the work. As a result, I certify that this work is entirely own and that it complies with the academic requirements.