Heterotrophic Omega-3 production in a novel microalga

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DECLARATION

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma, and to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due references is made in the text of this thesis or in the notes.

TARANDEEP KAUR

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ABBREVIATIONS

µL, mL	microliter, milliliter
µM, mM	micromolar, millimolar
kJ/g	kilojoule per gram
g/L	gram per liter
mg/g	milligram per gram
mg/L	milligram per Liter
%	percent
w/v	weight by volume
˚C	degree Celsius
rpm	revolution per minute
h	hours
std. dev	standard deviation
ASW	artificial seawater
HPLC	high performance liquid chromatography
MSG	monosodium glutamate
$MgCl_2$.6H2O	magnesium chloride hexahydrate
$CaCl_2.2H_2O$	calcium chloride dihydrate

$MgSO_4.7H2O$	magnesium sulfate
KCl	potassium chloride
Na_2SO_4	sodium sulfate
$(NH_4)_2SO_4$	ammonium sulfate
KH_2PO_4	monopotassium phosphate
Na_2EDTA	ethylenediaminetetraacetic acid
$FeSO_4$	iron(II) sulfate
$MnCl_2.4H_2O$	manganese(II) chloride tetrahydrate
$CoCl_2.6H_2O$	cobalt(II) chloride hexahydrate
DCW	dry cell weight
TAGs	triglycerides
DAGs	diglycerides
MAGs	monoglycerides
FFAs	free fatty acids
PUFAs	polyunsaturated fatty acids
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
TFA	total fatty acids

ABSTRACT

Omega-3 polyunsaturated fatty acids (Omega-3 PUFAs) are considered as essential compounds for human health and nutrition; thus, their demand is escalating. Traditionally, high-value PUFAs (DHA and EPA) are obtained from marine sources fish such as mackerel, salmon, and sardines. However, due to the metal accumulation in the fish oil and overfishing concerns therefore it is important to identify viable alternatives. Microalgae are highly recognized as a suitable platform for the production of omega-3 fatty acids. Moreover, Plants are also known to produce PUFAs but the quantities of PUFAs are not enough to meet market demand. Thus, the main aim of the study was to produce omega-3 fatty acids rich in DHA upon using an in-house heterotrophic microalgal strain S2 which is a thraustochytrid. Studies were conducted to improve omega-3 fatty acids proportions by optimizing various fermentation conditions. Various up-stream processing parameters such as alcohols, sodium salts, salts in the medium, glycerol concentrations, intermittent vs fixed carbon feeding, aeration, use of chemical modulators etc. were studied to achieve highest PUFAs yields. Maximum DHA content (44.23% of TFA) was obtained when carbon was fed at the concentration of 35 g/L at 72 h of fermentation, whereas the biomass recorded was 18.6 g/L, respectively. Strain S2 (thraustochytrid) was used for the first time for the production of omega-3 fatty acids. Thus, the study demonstrated heterotrophic production of omega-3 fatty acids thus achieving its objectives.

CHAPTER-1 (GENERAL INTRODUCTION AND LITERATURE REVIEW)

1.1. Introduction

Omega-3 or ω-3 fatty acids are the polyunsaturated fatty acids (PUFAs) where n-3 denotes the position of first double bond located at the third carbon from the methyl end of the fatty acid chain (Fig.1.) (Patel et al., 2020).

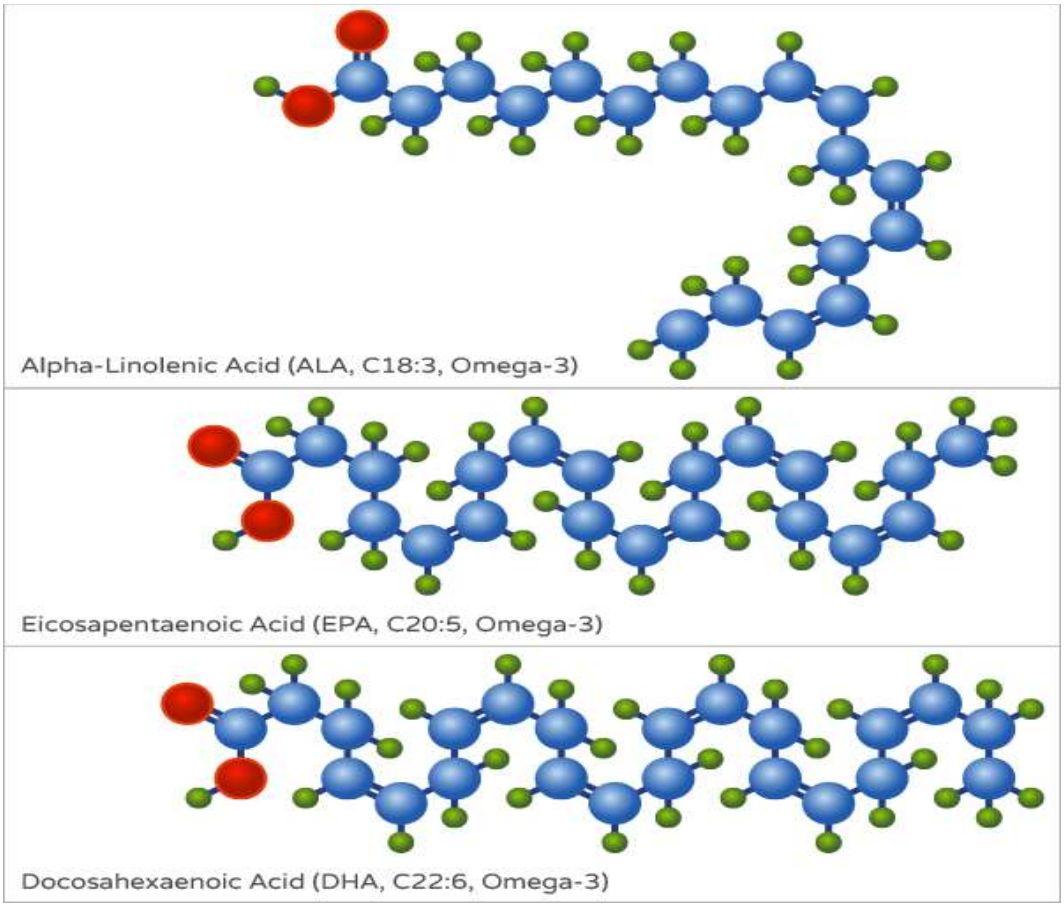


Fig.1. Chemical structure of omega-3 fatty acids

These are the essential fatty acids, a class of nutrients which are regulate the body to function, membrane fluidity and permeability that helps in relaxation and contraction of muscles, blood clotting, fertility, cell division as well as it constitutes the major fatty acid in brain's gray matter

and retina (Gupta et al., 2012, Patel et al., 2020). Nutritionally, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are the most important fatty acids which helps in providing various health benefits to the human body especially in improving cardiac problems such as blood pressure, and stroke (Deckelbaum and Torrejon, 2012). In addition, they are beneficial in dealing with depression, asthma, brain disorders etc. EPA and DHA are traditionally being obtained from fishes such as salmon, mullet (Oliver et al., 2020). In the year 2019, the global market size of omega-3 production was estimated to be $19.7 billion whereas by the year 2030, it is estimated to achieve the market value of $49.7 billion (Arif et al., 2020). However, to fulfil such demand and distortion of aquatic ecosystem, fishes are no longer a sustainable source of omega-3 PUFAs (Ryckebosch et al., 2012). Moreover, there are some of the health concerns related to the use of fish oil, for example, the presence of mercury contaminants in fish oil and this has driven the research into developing an alternate source of omega-3 oils, such as microalgal oils. Additionally, this oil can be consumed by vegetarian population. There are various sources of producing EPA and DHA namely bacteria, fungi, plants, and microalgae (Řezanka et al., 2021). Fungi has long growth periods; plants need large areas of land and water to grow upon and are unable to produce very long chain polyunsaturated fatty acids due to lack of enzymes . Microalgae are the essential producers of EPA and DHA in aquatic environment having magnificent properties of growing fast naturally under different autotrophic, heterotrophic, and mixotrophic culture conditions with the ability of producing high EPA and DHA (Russo et al., 2021). It can be grown anywhere, has the ability to fix atmospheric carbon, and has shorter harvesting times (Katiyar and Arora, 2020). Thus, microalgae can be used as a potential candidate for the industrial production of sustainable omega- 3 oils (Finco et al., 2017). Fig.2. illustrates the comparison of plants, fish and microalgal oil in terms of EPA and DHA production (Finco et al., 2017). Microalgal oil holds the promising fact of producing

sustainable, reliable, ecofriendly oil but the energy demands for the same are quite questionable and demands substantial improvement. Currently, research has been focused on the development of approaches by combining biological and engineering processes. There are some of the major challenges of producing large-scale microalgal culture such as reduced contamination, developing cheap and efficient methods and techniques, etc. for the betterment of nutritional supplementation (Griffiths et al., 2011). In this study, the review focuses on upstream fermentation which can result into better yields of biomass, lipids as well as DHA by optimizing various culture conditions using an in-house strain S2 (Thraustochytrids).

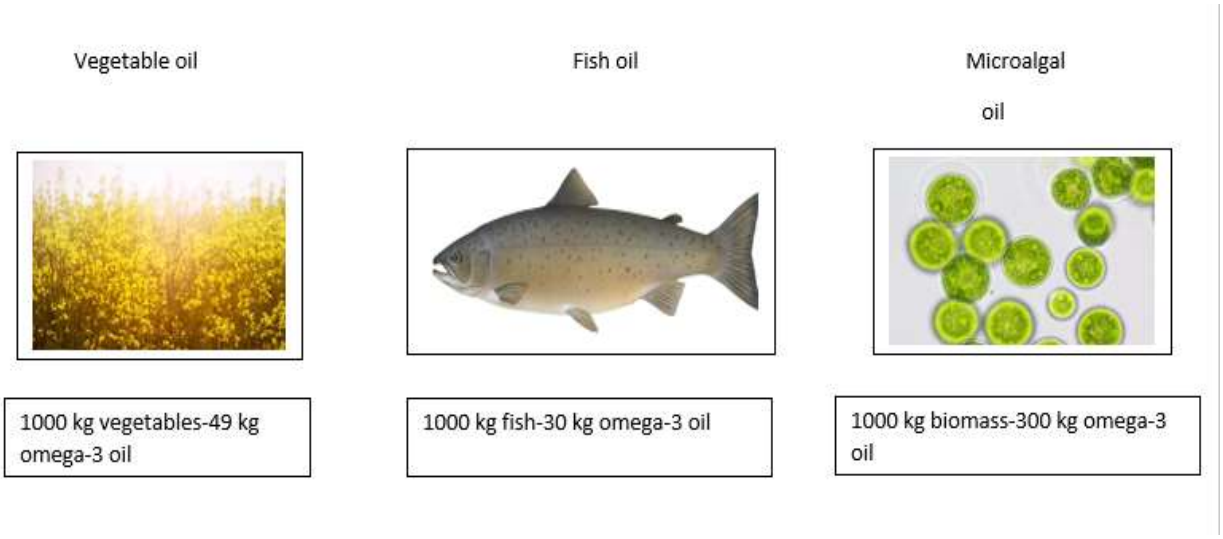


Fig.2. Production of omega-3 oil by different sources and their respective yield comparison.

1.1.1 Microalgae: Omega-3 Producers

Microalgae are singe-cell plant like organisms without leaves or roots. It comprises of four major groups of compounds such as proteins, carbohydrates, nucleic acids, and lipids in varying proportions based on algae class. The most energy-rich compound is lipid (37.6 kJ/g), followed by proteins (16.7 kJ/g), and carbohydrates (15.7 kJ/g) approximately (Sajjadi et al., 2018). The lipids contain polar and non-polar lipids, proportion of which varies according to different growth phases

of algae. Polar lipids are the structural lipids such as glycolipids and phospholipids whereas non-polar lipids are the storage lipids constituting triglycerides (TAGs), diglycerides (DAGs), monoglycerides (MAGs) and free fatty acids (FFAs). Polyunsaturated fatty acids (PUFAs) comprise the structural lipid fraction , while the other fatty acids comprises the storage lipid fraction (Sajjadi et al., 2018). Microalgae has proved to be one of the potential feedstocks available for omega-3 production because of its advantages like high photosynthesis capacity, can be grown in waste water and saline water, have the capacity to mitigate CO_2 rate, and high lipid and omega-3 contents (Bharadwaj et al., 2020). Under the nutrients starving conditions, the storage compounds such as lipids and carbohydrates accumulate and survive under stressful environment. They contain upto 10-50% of lipids based on their dry weight. Various strains from genera *Phaeodactylum, Nannochloropsis, Thraustochytrium* and *Schizochytrium* have the potential of producing high amount of EPA and DHA (30-40% TFA). High amount of DHA and EPA can be obtained by controlling various parameters such as pH, temperature and growth conditions (Adarme-Vega et al., 2012).

1.1.2 Accumulation of omega- 3 due to manipulation of fermentation conditions

Lipids accumulation can be enhanced by manipulating the growth conditions (Wu et al., 2005). For example, EPA content in *Pavlova lutheri* increased from 20% to 30% under low temperature of 15ºC. Similar trend has been observed in case of *Phaeodactylum tricornutum* under low temperature of 10ºC. Total DHA concentration increased upto 60% in *Crypthecodinium cohnii ATCC 30556* when grown under saline conditions of 9 g/L of NaCl. Among the various microalgae, the study has used *Schizochytrium sp.* which has recently gained a attention. It belongs to a group of marine microorganisms- Thraustochytrids (microalgae or microalgae like species) consisting high lipid content, upto 55-70% of dry biomass (Singh et al., 2014). The accumulated

lipids have significant levels of saturated fatty acids and n-6 docosapentaenoic acid, and a very high level of DHA (upto 49% of TFA). Overall, *Schizochytrium sp.* have a great potential to serve as an alternative source of polyunsaturated fatty acids (PUFAs) (Savchenko et al., 2021). More details about heterotrophic microalgae and fermentation optimization have been provided in sections 2.3 and 2.4.

1.2. Metabolic engineering (manipulation) of microalgae for higher omega-3 contents

Metabolomics, a field which gives a deep insight into intracellular metabolites and discover potential biomarkers, have been widely used to investigate into the biochemical reaction network to environmental, genetic or development signals. For instance, a combination of metabolomics and multivariate analysis disclosed the mechanism lying behind the positive effect of gibberellin on lipid and DHA biosynthesis in *Aurantiochytrium* sp. (Geng et al., 2019). For the enhancement of fatty acids production in microalgae, metabolic engineering is a promising technique other than external stress. Basically, *de novo* synthesis of fatty acids occurs in chloroplasts which involves the carboxylation and condensation of acetyl-CoA to malonyl-CoA, which further forms long chain fatty acids with the help of elongation reactions using malonyl ACP as a substrate. Afterwards, those are transferred to glycerol-3-phosphate to from triacylglycerol (TAG) via an intermediate phosphatidic acid in endoplasmic reticulum (De Bhowmick et al., 2015). In the fatty acid synthesis (FAS), polyunsaturated fatty acids(PUFAs) are synthesized by the starting point (C18:3), which is converted into C18:4 by Δ6 desaturase and by elongase into C20:4. This is then converted into C20:5 by Δ6 desaturase, followed by the formation of C22:5 by elongation and then finally converted into DHA (22:6) by Δ6 desaturase. Hence, PUFAs can only be synthesized with an adequate supply of C18:3 as a substrate (Huidan et al., 2021). Fig.3. demonstrates the

synthesis of omega-3's via elongation and desaturation of long chain fatty acids (Zhang et al., 2017).

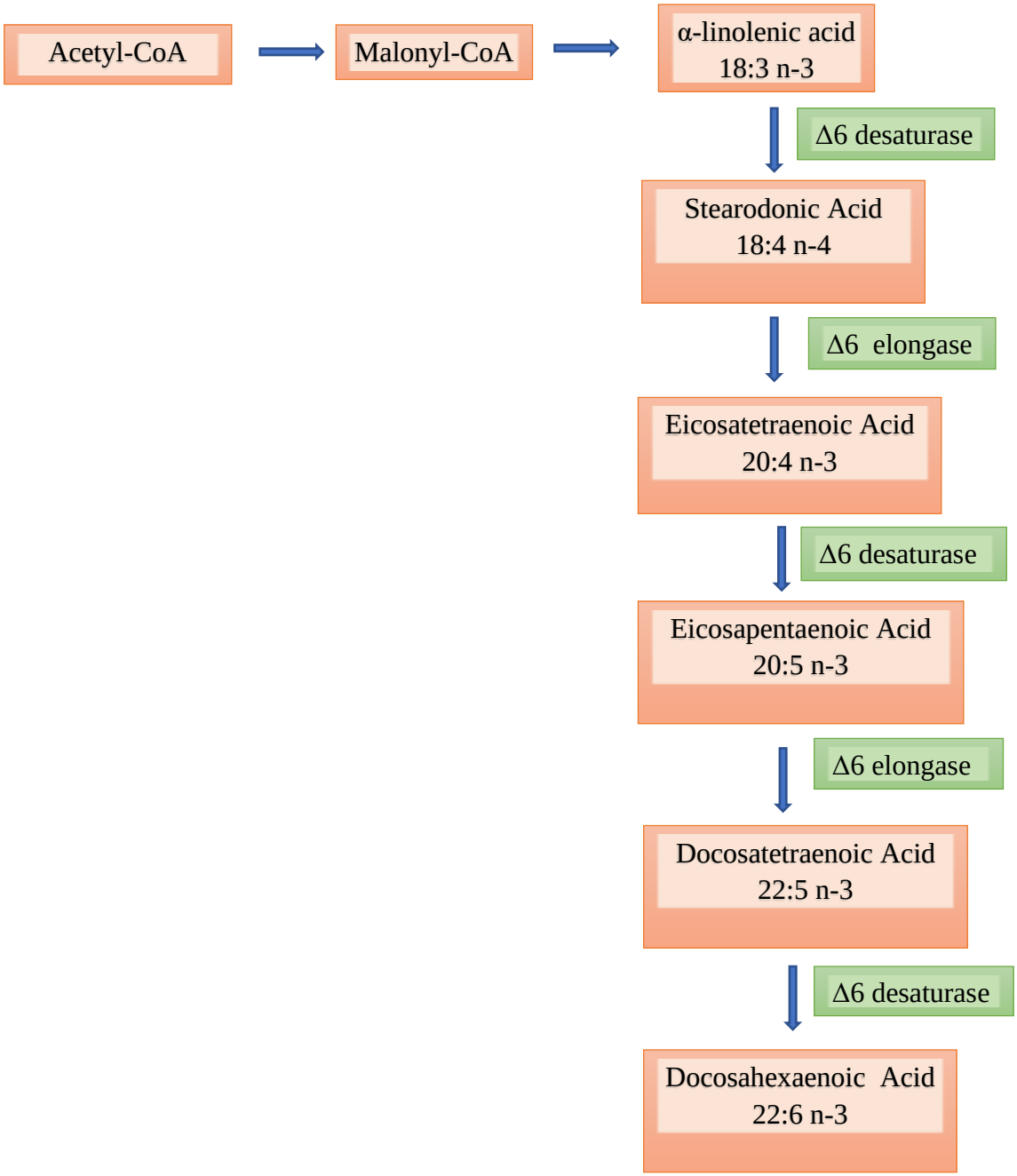


Fig.3. Conventional Fatty Acid synthesis pathway in microbes/ microalgae for biosynthesis of polyunsaturated fatty acids (EPA and DHA)

1.2.1 Heterotrophic microalgae

Most of microalgae grow photo autotrophically with the use of CO_2 but some have the ability to grow heterotrophically by using organic substances as sole carbon and energy sources. A heterotroph is a microorganism which needs organic carbon for its growth. These heterotrophs can be further divided into photoheterotrophs (requires light) and chemoheterotrophs (requires chemical). In other words, they cannot synthesize their own food, and basically depend on complex organic substances for their nutrition (Fernández et al., 2021).

Various photosynthetic microalgae, which are of high importance being able to produce wide range of high value products having applications in industries, such as chemicals, pharmaceuticals, nutraceuticals (PUFAs). A number of heterotrophic microalgae (*Chlorella*, *Thraustochytrium*), (Verma et al., 2020) consume organic molecules as a primary source of nutrition, do not need sunlight for energy. In comparison to photoautotrophs, they do not require light, have the characteristics of maximum growth rates, resulting into high cell biomass and lipid productivities (Hossain and Mahlia, 2019, Liu et al., 2014).

1.3. Various parameters for the cultivation of heterotrophic microalgae that support omega-3 FA production

Some important parameters that need to be considered for microalgal cultivation are discussed below:-

1.3.1 Temperature

It plays a vital role in the biological process of a microalgae for its efficient growth. Like light, different microalgae species needs different temperature for their growth. Temperature lower or higher than the optimum temperature can prevent or disturb the growth of microalgae which

ultimately affects the overall biomass production. High temperature can cause the inactivation of proteins whereas low temperature can impact on the enzyme kinetics. It has been reported that optimum temperature for most of the microalgae ranges between 20-30ºC, showing maximum growth depending upon their geographic areas (Brindhadevi et al., 2021). However, thermophilic algae can grow at a temperature of 80ºC (Khoo et al., 2020).

1.3.2 pH

Similar to other factors, pH also plays a key role in the growth. Most microalgal species showed high growth from pH 6 to 8.76 (Andrade et al., 2021) . Maximum growth has been observed at the pH of 9-10 in *Chlorella vulgaris* with adaptation of pH from 4-10. In the conditions of acidity at pH 3-4, fungal contamination can be prevented (Khoo et al., 2020). Various strains of thraustochytrids can be grown on pH ranging from 4 to 8. For Instance, when species *Aurantiochytrium limanicum* was grown at a pH ranging from 6 to 7, the highest biomass (13.69 g/L) and lipid content (52.34%) was recorded (Pawar et al., 2021). Thus, this demonstrates that pH plays a vital role while cultivating microalgal cells.

1.3.3 Mixing

It is another factor which is quite important for cultivating microalgae because it is highly necessary to mix all the nutrients, temperature, pH, homogenously in a culture as it helps in the prevention of sedimentation, clumps, also provides light everywhere needed (Khoo et al., 2020). Also, excessive concentrations of Dissolved oxygen can inhibit the growth of microalgal biomass (Kazbar et al., 2019).

1.3.4 Nutrients

Microalgae needs different nutrients at specific ratios for their growth. For example, sodium bicarbonate and CO_2 are needed for autotrophs as their only carbon source. Starch, glycerol, glucose, sucrose, and nitrogen sources are required for the heterotrophs whereas combined carbon sources are used in case of mixotrophs. Hence, it is very important to provide all the nutrients at optimum pH and temperature (Khoo et al., 2020).

1.3.4.1 Effect of Nitrogen

The most important nutrient helps increasing the biomass is nitrogen (Khoo et al., 2020). Nitrogen is an important component of cell structure and functional process of microalgae since it is a key component of proteins, amino acids, nucleic acids, enzymes, and photosynthetic pigments. Microalgal species needs nitrogen in different forms in the concentration range of 1-10%. Like other factors, inadequate supply of nitrogen may hinder the growth and overall yield. The most common used nitrogen sources are nitrate and urea. It has been reported that potassium nitrate gave the highest growth of *Chlorella vulgaris* at 0.81/day with biomass production of 0.57 g/L/day with highest lipid productivity of 47.1 g/L/day (Khoo et al., 2020). Algae have the capacity to utilize nitrogen in the form of nitrate, nitrite, ammonia, and urea (Sajjadi et al., 2018).

1.3.4.2 Effect of Carbon

Generally, 50% of microalgal biomass is comprised of carbon on dry weight basis. For instance, *Chlorella* cells when grown heterotrophically, with 10g/L of glucose as carbon source accumulated 28% and 45% more lipids and carbohydrates, respectively when compared to its autotrophic cultivation. A previous study mentioned that all microalgae except *Nannochloropsis oculata* can grow heterotrophically with 5g/L of glucose and maximum growth was observed in

Crypthecodinium cohnii either using glucose or acetate as carbon source (Vazhappilly and Chen, 1998). There are many microalgal species that can grow heterotrophically on organic substances such as monosaccharides, volatile fatty acids, glycerol and urea (Zuccaro et al., 2020). The main ways of organic carbon uptake into the cells are diffusion, active transportation, and phosphorylation. A semi-optimized growth condition was used in *Schizochytrium limacinum* KH105, in a baffled flask, medium consisting glucose (30 g/L) as a carbon source, yeast extract (5 g/L) and polypetone (15 g/L), that yielded 11.5 g/ L of DCW, 5.3 g of TFA/ L, 1.2 g DHA/L after 2 days of fermentation (Shene et al., 2010). Crude glycerol was also used as a sole carbon source for the production of omega-3 oils for *S. mangrovei sp.* Sk-02, resulting DCW upto 28 g/L, with DHA accounted for 6 g/L (Shene et al., 2010).

Thus, it is necessary to maintain the conditions because the unfavorable conditions may result in accumulating the neutral lipids. Marine thraustochytrids have the ability to synthesize DHA, distributed in cell membrane lipids and neutral lipids (Shene et al., 2020) whereas the DHA present in neutral lipids can be transferred to polar lipids by degrading saturated fatty acids (Chang et al., 2021). So, a balance is important to enhance the lipids growth under stressed conditions. Thraustochytrid species such as *Schizochytrium sp.* DT7 and *Schizochytrium* sp. N-1 can produce DHA upto 28.6% and 31.6% of total fatty acids. The omega-3 fatty acid content (mg/g oil) of the total lipid extract from various microalgae species is presented in Table.1 (Ryckebosch et al., 2014, Kamlangdee and Fan, 2014) .

Table.1. Various microalgae producing polyunsaturated fatty acids.

Species/ FAs	***Nannochloropsis gaditana* (mg/g)**	***Nannochloropsis oculata* (mg/g)**	***Pavlova lutheri* (mg/g)**	***Schizochytrium sp.* DT7 (%)**	***Schizochytrium* sp. N-1 (%)**	***Schizochytrium sp.* N-9 (%)**
ALA (C18:3n-3)	0.3 ± 0.03	0.7 ± 0.1	10.0 ± 0.3	ND	0.4	0.1
SDA (C18:4n-3)	0.3 ±0.1	-	17.0 ± 0.5	ND	ND	ND
EPA (C20:5n-3)	175 ± 12	193 ± 24	92 ± 2	ND	1.1	7.4
DPA (C22:5n-3)	-	-	-	NA	NA	NA
DHA (C22:6n-3)	-	-	40.9 ± 0.9	28.6	31.6	31.4

ND- Not detected, NA- Not available, FAs- fatty acids, ALA- α-Linoleic Acid, SDA- Stearidonic Acid, EPA- Eicosapentaenoic Acid, DPA- Docosapentaenoic Acid, DHA- Docosahexaenoic Acid

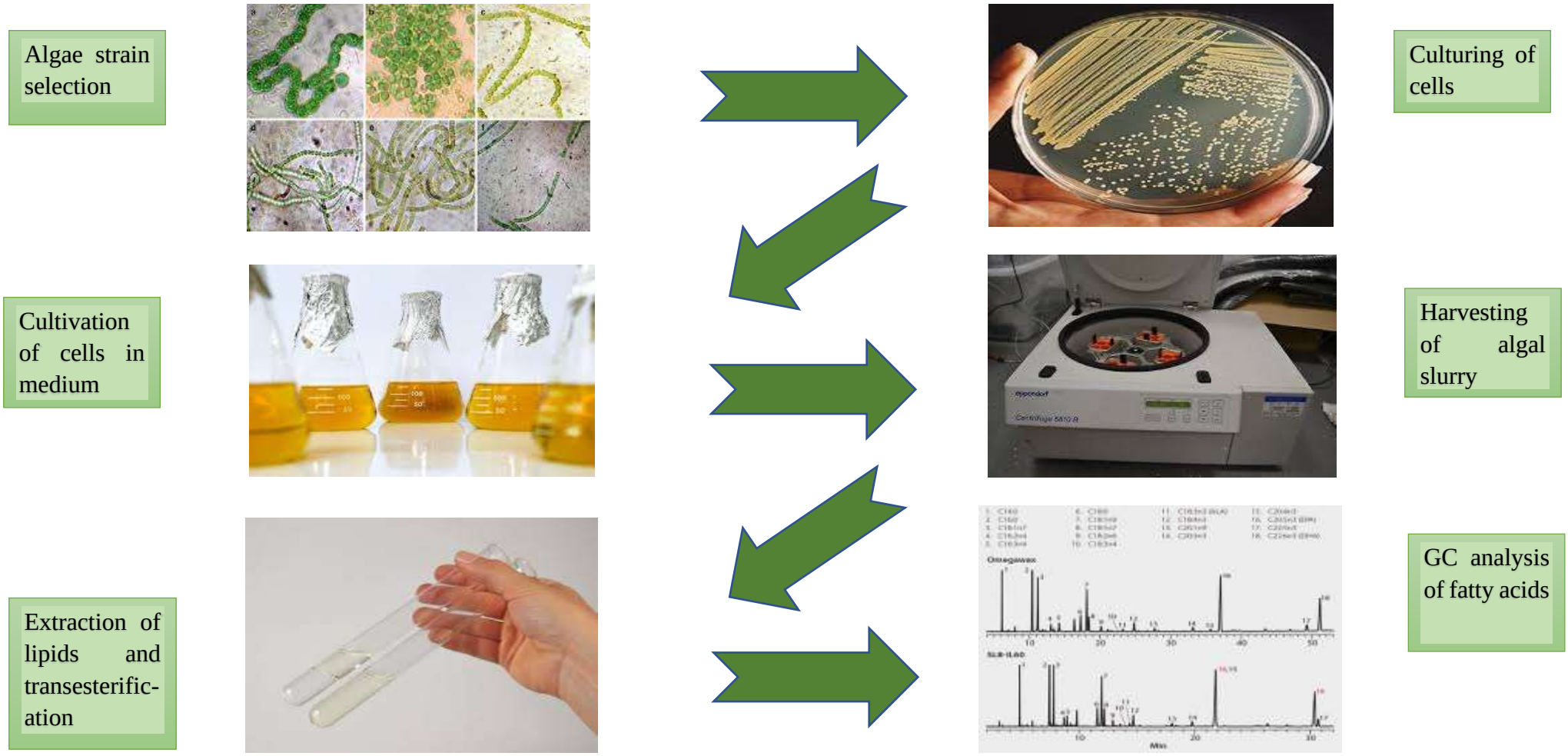


Fig.4. Schematic outlay of the process for the production of microbial lipid

The major steps involved in the lipid production are presented in Fig.4. First step involves the strain selection and cultivation followed by harvesting and extraction of lipids (Delrue et al., 2013). Harvesting techniques, used for biomass separation are explained briefly in section 2.6. Lipids are extracted from the harvested dried biomass using various approaches as described in section 2.7.

Microalgal cells constitutes lipids in their cell bodies, out of which 95% are triglycerides, and rest are mono and diglycerides with some ratios of free fatty acids such as stearic acid, palmitic acid, and oleic acid (Milledge et al., 2014). Different species of microalgae exhibit varied lipid content varied from (2.40-62.0% dry wt. basis). *Chlorella vulgaris*, *Scenedesmus sp*. with sodium bicarbonate, *Haematococcus* produced the lipid content of 11.0-43.0%, 20%, 14.0-18.0% of dry biomass (Deshmukh et al., 2019). Dry cell weight as well as DHA production in *Schizochytrium mangrovei* G13, upon using sodium sulfate (20 g/L) as a substitution with ASW, was 14 g/L and 2.17 g/L at 107 h (Shene et al., 2010).

1.4. Microalgae cultivation

Bioreactors have been used for growing microalgae under different conditions for algal biomass production. The different types of bioreactors are presented in Table.2. Depending upon the type of microalgae (phototrophic or heterotrophic), specific species and its conditions, bioreactor is selected. (Hossain and Mahlia, 2019) .

Table.2. Various bioreactor types and microalgae strain used for omega-3 fatty acid production.

Type of bioreactor	Description	Microalgae candidates with productivity ($g.L^{-1}.d^{-1}$)	Advantages	Limitations
Flat- plate	Rectangular boxes consisting of translucent glass or plastic (PVC)	*Nannochloropsis sp.* (0.85), *S. platensis* (2.15)	High temperature capture efficiency, easy to scale up, high biomass productivity	Large surface area needed, scale up requires high cost, wall growth
Vertical column	Either draft tubes or split cylinders (Diameters up to 19m)	*P. cruentum (0.5)*	Easy to sterilize and scale up, excellent mixing rate moderate control of temperature and growth.	Expensive and sophisticated construction materials needed.
Horizontal or inclined tubular	A number of clear transparent horizontal tubes (diameter ≤10 cm) consisting of glass or plastic material.	*Chlorella sorokiniana, P. cruentum (0.36)*	Good high utilization, moderate biomass productivity, cost-effective.	Large land space needed, fouling, pH gradient, possibility of wall growth.
Internally illuminated	Internally illuminated with fluorescent lamps, impellers for agitation, and spargers for aeration	*Chlorella pyrenoidosa*	Easily heat-sterilized under pressure, low possibility of contamination.	Extra technical efforts are needed for outdoor cultivation.
Airlift	Simple vertical cylinder with an air inlet at bottom constructed with glass or plastic	*Phaeodactylum tricornutum, Dunaliella tertiolecta*	Excellent mass transfer rate, high volume, excellent aeration.	High energy consumption, foaming lot of bubble formation.

1.4.1 Use of Activators and, growth enhancers to improve omega-3 FA production

In microalgal strain, innovative fermentation techniques such as subjecting the cells to alcohols, sodium salts, chemical modulators, feeding strategies have been used in order to enhance the lipid and DHA production. Previous studies and researchers have also mainly focused on the high DHA production because there are many success stories of clinical trials associated with a number of nutrient-based interventions which have resulted in improving cognitive health (Charles et al., 2019). There are some strains that can accumulate upto 50 to 70% lipids comprising 30 to 70% of DHA (Kumar et al., 2019).

Short chain alcohols such as ethanol methanol and butanol are being used in the fermentation medium using Thraustochytrids which have resulted in upregulating the lipid content as well as Astaxanthin (Zhang et al., 2017, Du et al., 2019). Moreover, volatile fatty acids (VFAs) six or fewer carbon fatty acid chains are also being used, that can be easily distilled at atmospheric pressure (Patel et al., 2020). Several reports indicated that only few species of marine microalgae could utilize VFAs and reported inhibitory effect (Fei et al., 2015). Furthermore, various chemical modulators such as BHA (Butylated hydroxyanisole), 2,4-D (Dichlorophenoxyacetic acid) and ETA (Ethanolamine) were also explored in the fermentation study to evaluate the ability of strain S2 to utilize those modulators for growth and lipid production. In previous study, the accumulation of lipids increased up to 8-20% after treating the *C. cohnii* cells with BHA (Butylated hydroxyanisole), or combination of chemical modulators composed of BHA, ETA (Ethanolamine) and BNOA (napthoxyacetic acid) (Wang et al., 2018).

Moreover, glycerol when used at 16 g/L, which is a low-cost carbon source used in previous studies exhibited upto 40% lipid content (Sengmee et al., 2017, Saenge et al., 2011). When 4% (w/v) glycerol was used as a carbon source, DHA upto 36% of total fatty acids was reported (Gupta et

al., 2013). Thus, the study aimed to manipulate the fermentation strategies which might lead to enhanced DHA production.

1.5. Harvesting of biomass

It is the process where algal biomass gets separated from the media after completion of fermentation. Cells having a small size of 2-20 µm makes the process costly and insufficient due to its colloidal stability. There are several types of harvesting techniques, such as physical, chemical, enzymatic, etc. that are discussed below. In some researches the combination of two approaches have been used (Yin et al., 2020).

1.5.1 Chemical methods

Flocculation is used to harvest the algal biomass in which various types of flocculants and coagulants are used to form the huge, scattered units which then settles down. There are three chemical flocculants such as metal salts, $FeCl_3$, $AlCl_3$, etc., inorganic polymers like polyelectrolyte etc. and organic polymers like cellulose, chitosan etc. (Yin et al., 2020).

1.5.2 Physical methods

Various physical methods have been used for the harvesting of microalgal biomass. Some examples of this technique are: Centrifugation, Filtration, Gravity sedimentation, Flotation, and Electrical methods (Yin et al., 2020).

1.5.3 Biological methods

The bioflocculation is a simple process and has easy operating system. In this technique, flocculation occurs due to the secretion of biopolymers, extracellular polymeric substances

secretion (EPS). It can be further divided into four sections:- bacterial flocculation, actinomycetes flocculation, fungal flocculation and plant based bioflocculation (Yin et al., 2020).

1.5.4 Nanomaterial assisted methods

It is one of the reliable techniques for microalgal harvesting as it is does not consumes much time, simple to operate and is reusable. The principle behind the approach is the attachment of cells with the magnetic molecules (Yin et al., 2020).

Table.3. Advantages and disadvantages of various harvesting techniques:-

Harvesting techniques	**Harvesting methods**	**Harvesting conditions**	**Recovery**	**Advantages**	**Disadvantages**
Chemical methods	Inorganic flocculants	pH, charge density, electronegativity	High	Mature technology, high efficiency, biodegradable	Contamination
	Inorganic polymers	Molecular weight	High	Simple, safe, fast	Recycling of media is limited
	Organic polymers	-	High	-	pH dependent
Physical methods	Centrifugation	Particle size	Low-high	Reliable, high recovery	Energy intensive, high capital investment
Biological methods	Bioflocculation	pH of culture	High	No contamination, low cost	Slow growth, time investment
Magnetic methods	Nanoparticles	-	High	No pollution, can be reused	Costly

1.6. Drying biomass

After harvesting, biomass is generally dried for stability and storage purposes. Up to 15% of the moisture is removed after harvesting but it can spoil the slurry within hours, so it is imperative to make the slurry dry. Drying is important to make the biomass more concentrated at 90-95% solid. There are various drying methods such as solar drying, spray drying, freeze drying etc. discussed by (Khoo et al., 2020) that are mentioned below with their advantages and disadvantages, respectively. Methods such as solar drying, oven drying and spray drying are simple to use but there are chances of getting contamination to the samples, or they may lead to lipids degradation.

Table.4. Advantages and disadvantages of different drying processes

Drying methods	**Advantages**	**Disadvantages**
Solar drying	Simple and most reliable, cost effectiveness	Slow rate of drying, requires large space, may cause contamination
Microwave/oven drying	Low operating cost, simple, fast, high product quality	Lipids may get degraded, requires pretreatment
Spray drying	Effective for high value product	High energy consumption, expensive
Freeze drying	Gentle process	Slow, high energy demand

1.7. Lipids extraction

1.7.1 Extraction of lipids from dry algal biomass

Freeze-drying is the method which has been generally used for drying the biomass using vacuum at low temperature. Several methods have been proposed for extracting the lipids or oils from the biomass. However, some techniques that have been discussed require combination of one or two methods prior to lipid extraction. Because the cell wall of thraustochytrids is easy to disrupt with

just solvents (Chloroform: Methanol), leading the solvents to easily penetrate into cell wall thus the work tends to use solvent extraction technique has been extensively used without any mechanical method. Howlader and French, (2020), Kumar et al., (2020), Mubarak et al., (2015) have discussed some of the methods as mentioned below (Fig.5.) (Howlader and French, 2020), (Kumar et al., 2020), (Mubarak et al., 2015).

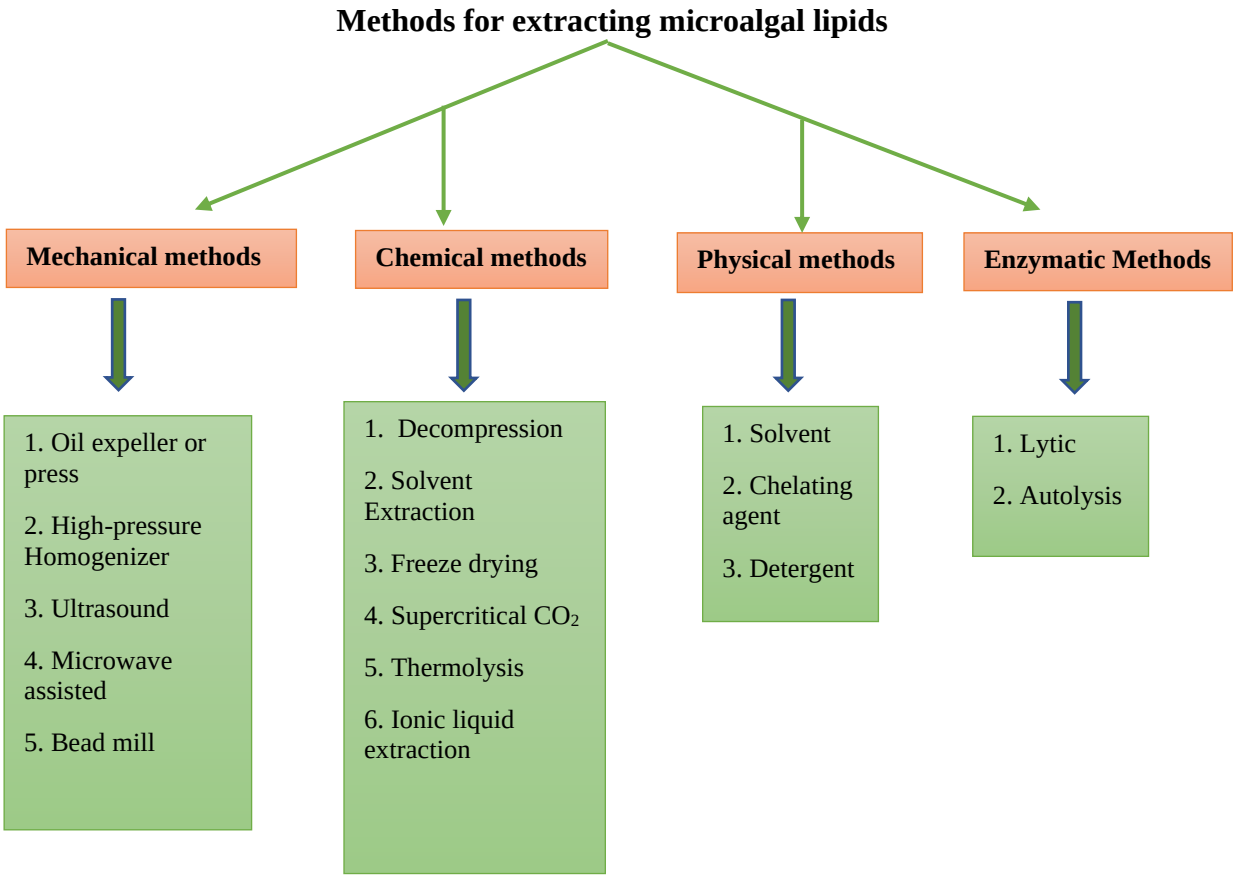


Fig.5. Different methods used for extracting lipids from dried biomass

(Howlader and French, 2020), (Kumar et al., 2020), (Mubarak et al., 2015)

1.7.1.1 High-pressure Homogenization (HPH)

It is the most widely used technique for the extraction of intracellular products. It is commonly used in dairy industry for extracting out and purifying the protein. A high pressure of about 50-1500 bar is favorable with multiple passes to break the cell wall, leading to the release of intracellular products. Then the target compound is extracted using filtration or solvents. It has been observed by that lipid recovery (57.4%) in *Nannochloropsis sp*. was achieved by using solvent (hexane) extraction after the HPH (Yao et al., 2018). It not only leads to the extraction of high lipids but eradicates the high cost of the process as well which is the main drawback of the process (Howlader and French, 2020).

1.7.1.2 Microwave-Assisted Pretreatment

It is another reliable method for the improvement of lipid recovery used widely for laboratory purpose for both wet as well as dry biomass. It works on the principle of the electromagnetic radiation having frequency of 0.3-300 GHz to break the cell with non-contact heat resource and thus extraction can be performed on the product using solvents. de Moura et al., (2018) have reported that in comparison to the ultrasound sonication methods, lipid recovery was 20% more using this method (de Moura et al., 2018).

1.7.1.3 Ultrasound sonication

It is one of the most frequently used methods for the lipid extraction. It causes the high sheer in the cell walls due to the cavitation of microbubbles (de Carvalho et al., 2020). A study demonstrated that using ultrasonication at the power of 20 W with frequency of 20 kHz improved the lipid content in *Schizochytrium sp.* to 890 mg/L in comparison to the control (550 mg/L) (Ren et al., 2019).

1.8. Recent advancements in the extraction techniques

New class of solvents known as "Green solvent" have emerged in the market so that the hazardous solvents which are made up of non-renewable resources can be replaced. These new solvents are eco-friendly, made naturally or are made up of agricultural residues. These are non- toxic, renewable and biodegradable, which is a matter of interest for current research (Karim et al., 2020). These are expensive solvents hence they were not used in this study. These are divided into four groups-supercritical fluids, neoteric, bio-based, and supramolecular solvents (Torres-Valenzuela et al., 2020).

1.8.1 Supercritical fluids

These are the materials having their pressure and temperature above their critical values. They have the characteristics of both gas and liquid as they consist gas like viscosity and liquid like density which makes them perfectly suitable for the extraction due to the easy diffusion of fluids and provides extraction product rapidly (Torres-Valenzuela et al., 2020).

1.8.2 Neoteric solvents

These are the solvents novel in their structures known for their physical and chemical properties and used for various application by bringing the variation in their chemical compounds. Fluorous solvents which consist of highly fluorinated substances, ionic solvents made up of salts like cations and anions and eutectic solvents composed of lewis acids and bases are some of the examples of this type of solvents (Torres-Valenzuela et al., 2020).

1.8.3 Bio-based solvents

These are the solvents obtained from biomass like crops, waste products, wood chips, etc. and are renewable. They are used in biorefinery resulting into the recovery of high value products up to

maximum content. Examples include like alcohol, esters, glycerols etc. These are used for the extraction of bioactive material more efficiently than any other traditional solvent (Torres-Valenzuela et al., 2020).

1.8.4 Supramolecular solvents

These liquids are water immiscible liquids produced spontaneously from amphilies aqueous solutions. They are mostly used for the purification of organic substances like hydrocarbons, pesticides etc. (Torres-Valenzuela et al., 2020).

These solvents are in a huge demand which led to increased research from past few years as they are originated from agri-waste which helps both in cost reduction as well as making environment clean. In general, it can be concluded that with the proper selection, these solvents have the ability to afford high extraction yields in different agri-wastes (Cvjetko Bubalo et al., 2015). Some of these green solvents are being used for the lipids extraction from microalgae (Kumar et al., 2017). The development of more sustainable and cost-effective extraction process is a critical step towards the recovery of low-cost bioactive products (Cvjetko Bubalo et al., 2015).

1.9. Hypothesis and experimental aims

Due to the rising awareness among consumers about healthy and balanced diet, the market for omega-3 fatty acids especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has escalated.

- Can existing in house strain (thraustochytrid) upon using organic compounds in the fermentation medium influence metabolic shift to produce higher DHA yields?
- This project is based on the hypothesis of achieving enhanced omega-3 fatty acids from microalgae by manipulating the medium conditions in upstream processing.

Objectives:

- The first objective of the study was to improve omega-3 fatty acids proportions by optimizing different culture conditions.
- The second objective of research was aimed at the use of cost-effective organic compounds such as glycerol as a carbon sources to enhance the production of omega-3 fatty acids.

CHAPTER-2 (MATERIALS AND METHODS)

2.1. Chemicals

All the chemicals used in this research were of analytical and HPLC grade. Media components such as glycerol, yeast extract, glucose, mycological peptone, sodium acetate, sodium propionate, sodium chloride, Monosodium Glutamate, methanol, Chemical modulators such as- Butylated Hydroxy anisole (BHA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Ethanolamine (ETA), (Sigma Aldrich pty. Ltd., Australia) and sea salt (Instant Ocean, USA) were used for biomass production whereas, solvents such as hexane, ethanol, ethyl acetate, chloroform, toluene, sodium chloride, potassium bicarbonate, anhydrous sodium sulphate, were all of analytical grade (Merck) which were used for lipid extraction.

2.2. Microalgal Strain and cultivation

In this study, an inhouse strain S2 (thraustochytrid), collected from pristine SA (South Australia) waters were provided for optimizing biomass and lipid production. This strain is unique as it produces lipids that are rich in omega-3 fatty acids was used for the study which was provided by Dr. Adarsha Gupta. The isolate was cultivated on a GYP medium, containing (g/L):- glucose, 5; yeast extract, 2; peptone, 2; and Artificial Seawater, 50%; which was then used for inoculum preparation. The medium was autoclaved at 121ºC for 20 min and then incubated at 150 rpm for 48 h at 25 ºC. Seed medium (0.2% yeast extract, 0.2% mycological peptone, ASW 50%, pH 6.5) and fermentation medium having various carbon, nitrogen, sources as named above were used with different concentrations mentioned in detail below. Seed medium (20 mL in 100 mL) was then inoculated from the colonies grown in the agar plates and was grown for 48 h in shake flasks (250 mL) at 25 ºC at 150 rpm in an incubator shaker. The biomass was then harvested using

centrifuge (10,000 x g, 10 min) (Eppendorf 5810R) followed by freeze drying of the biomass for further use.

2.3. Fermentation Medium

Several fermentation conditions were tested in this study to improve the DHA production. All fermentations were carried in Erlenmeyer flask (100 mL) containing 20 mL of a fermentation medium. The composition of the fermentation medium varied as per below mentioned parameters.

2.3.1 *Effects of Alcohols as additional carbon source on lipids and omega-3 production*

Different alcohols- ethanol, methanol was used as additional carbon source with glucose to examine their effects on lipids and omega- 3 content. The concentration of these alcohols (2%, 4%, 6%, and 8%, v/v) was used in the fermentation medium (20 mL). A flask containing only glucose was kept as control at similar concentration. The concentration of other medium components were: Yeast Extract, 0.4%; Peptone, 0.4%; $MgCl_2.6H_2O$, 0.64%; $CaCl_2.2H_2O$, 0.1%; $MgSO_4.7H_2O$, 0.5%; KCl, 0.2% (Du et al., 2019).

2.3.2 *Effects of sodium salts as sole carbon source on lipids and omega-3 production*

Two different types of sodium salts- sodium acetate and sodium propionate were used as sole carbon source. The fermentation medium contained: Sodium Acetate (2% and 4%); Sodium Propionate (2% and 4%); Yeast Extract, 0.2%; Peptone, 0.2%; Artificial Seawater, 50%; and a flask (control) containing only.

2.3.3 *Effects of different salts water vs artificial sea water on lipids and omega-3 production*

The fermentation medium was composed of Glucose, 5% and 8%; yeast extract, 0.04% with the difference of ASW. The ASW was made from the following salts Na_2SO_4, 1.0%; $(NH_4)_2SO_4$,

0.08%; KH_2PO_4, 0.4%; KCl, 0.02%; $MgSO_4$, 0.2%; Monosodium glutamate, 2%; $CaCl_2$, 0.01% and the trance elements: Na_2EDTA, 0.6%; $FeSO_4$, 0.029%; $MnCl_2.4H_2O$, 0.086%; $ZnSO_4$, 0.08%; $CoCl_2.6H_2O$, 0.001%; $Na_2MoO_4.2H_2O$, 0.001%; $NiSO_4.6H_2O$, 0.006%; and $CuSO_4.5H_2O$, 0.06% vs the ASW used in the lab with salts already in it.

2.3.4 *Effect of chemical modulators in fermentation medium on lipids and omega-3 production*

Chemical modulators Butylated Hydroxy anisole (BHA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Ethanolamine (ETA), investigated in this study were added to the fermentation medium. The stock concentrations of each were: 400 µM; 100 mg/mL; and 5M; respectively. BHA and 2,4-D were dissolved in ethanol whereas ETA was dissolved in ddH2O followed by filter sterilization using 0.22 µm syringe filters. Concentration of each used were: BHA, 0.1 µM, 0.054 µM; 2,4-D, 10 mg/L, 20mg/L; ETA, 2mM, 5mM. In addition, the combination of these three modulators (BHA, 2,4-D, ETA) in concentrations of (0.1 µM, 10 mg/L, 2mM) and (0.054 µM, 20 mg/L, 5mM) were used. Other components used were: Glucose, 5%; Peptone, 0.04%; Yeast extract, 0.04%; and Artificial Seawater 50%.

2.3.5 *Effect of monosodium glutamate in medium as a sole nitrogen source on lipids and omega-3 production*

Monosodium Glutamate or MSG is a source of nitrogen replacing peptone in the medium (Shene et al., 2020) The fermentation medium was composed of: glucose, 2%; Yeast extract, 0.6%; MSG, 0.03%, 0.06% and 0.09%; Vitamin solution, 360 µL as well as trace element solution, 2.5 mL in 150 mL. Flasks containing peptone (0.03%, 0.06% and 0.09%) were kept as a control.

2.3.6 *Effect of glycerol as sole carbon source on lipids and omega-3 production*

It has been frequently mentioned in literature to replace glucose as carbon source with crude or pure forms (Huang et al., 2012). In this study, glycerol (pure form), was used as a sole carbon

source (2% and 5%, w/v) in a medium containing yeast extract, 2% and peptone, 2% . In addition, the effect of higher concentration of glycerol (10%), was investigated in a medium containing yeast extract, 4% and peptone, 4%.

2.3.7 *Effect of aeration on lipids and omega-3 production*

This experiment was carried out to investigate the effect of aeration by using Erlenmeyer and baffled flask (250 mL) during fermentation. The medium consisted of: glucose, 12%; Na_2SO_4, 1.2%; Yeast extract, 0.5%; Peptone, 0.5%; $MgSO_4$, 0.2%; $(NH_4)_2SO_4$, 0.1%; KH_2PO_4, 0.1%; K_2SO_4, 0.065%; KCl, 0.05%; $CaCl_2.2H_2O$, 0.017%; Trace element solution: 1ml/L, Vitamin Solution- 2ml/L. Trace element solution was consisted of (mg/L): $FeSO_4.7H_2O$, 10; $CaSO_4$, 3.2; $MnCl_2.4H_2O$, 3; $ZnSO_4.7H_2O$, 3; $NiSO_4.6H_2O$, 2; $CuSO_4.5H_2O$, 2; $CoCl_2.6H_2O$, 0.04; $Na_2MoO_4.2H_2O$, 0.004 whereas vitamin solution consisted (mg/L)- Thiamine, 9.5; cyanocobalamin, 0.15 (Ling et al., 2015).

2.3.8 *Effect of salts on lipids and omega-3 production*

Na_2SO_4 and NaCl were used to investigate if they can be used as a replacement with ASW (Chen et al., 2016). The concentration of both the salts in the medium were 1% and 2% each; Glucose, 8%; $NaNO_3$, 0.3%; Yeast Extract, 1.5%; KH_2PO_4, 0.15% and $MgSO_4.7H20$, 0.05% which were compared to control treatment (50 % ASW).

2.3.9 *Effect of fixed vs intermittent feeding on lipids and omega-3 production*

Carbon and nitrogen source were intermittently fed after every 24, 48 and 72 h of fermentation. Nitrogen feeding was in the concentration of 0.04% and 0.08% for all the three treatments whereas carbon feeding was at 1.5% and 3.5% after 48 and 72 h, respectively. Other medium components were similar as used in the section 2.3.7.

2.4. Cell dry weight determination

To estimate the cell dry weight, the fermentation medium was harvested at the end of 5 days and centrifuged at 10,000 rpm for 10 min to get the pellet separated from the supernatant. The pellet was then washed twice with ddH_2O (double distilled water) in order to get rid of any traces of media. The pellet was then freeze-dried for upto 48 h and stored at -20ºC prior to lipid extraction. The cell dry weight (CDW) was then determined after freeze-drying the cells. All the experiments were performed in duplicates.

2.5. Lipid extraction, esterification, and GC analysis

Lipid extraction was performed with little modifications to the method described by Dr. Adarsha Gupta (Gupta et al., 2016). 10 mg of dried cells were weighed in microcentrifuge tubes for lipid extraction. A solvent mixture of Chloroform: Methanol (2:1) was used for fatty acid extraction followed by 3x vortex and centrifugation at 10,000 g for 10 minutes. Lipids were dried at 50ºC. Lipid content (% dry wt. basis) was calculated gravimetrically. Furthermore, Fatty acid methyl esters (FAMEs) were prepared using 500 µL toluene in a tube along with addition of 200 µL internal standard, methyl nonadecanoate (C19:0) and 200 µL of butylated hydroxytoluene (BHT). Acidic methanol (500 µL) was also added to the glass vial for the overnight incubation at 50ºC. The methanol reagent was prepared by adding 1 mL acetyl chloride dropwise to 10 mL methanol on ice. It was stirred for at least 1 hour. FAMEs were extracted into hexane. The hexane layer was removed and dried by using sodium sulphate. The samples were analyzed by a Gas Chromatography system (GC-BID, Shimadzu GC 2090N), equipped with flame ionization detector (FID) and connected to a BID 2030 unit (Shimadzu) for identification of fatty acid methyl esters (FAMEs) (split injection, 1/150). The GC was equipped with a capillary column (FAMEWAX C18 column, 30 m x 0.32 mm ID (inner diameter)). Helium was used as the carrier

gas at a flow rate of 1.5 mL/min. The oven program is held at 150º C (5-min hold), ramped to 250º C at a rate of 10º C per min and held at 250º C for 1 min. The injector was maintained at 250 °C and a sample volume of 1 µL was injected. Fatty acids were identified and quantified by comparing the retention time data with the peak areas of external standards (Sigma Aldrich CRM47885) using chromatography software (Shimadzu, Australia), and then converted into percentage. Results are presented as mean ± SD of duplicates.

CHAPTER-3 (RESULTS)

Various fermentation studies were investigated to enhance DHA levels in thraustochytrid strain S2.

3.1 *Effect of alcohols as an additional carbon source for biomass and lipids production with increase in glucose concentration*

Ethanol and methanol when used at 2% concentration in addition to glucose in the medium showed 1.9 and 1.6 g/L of DCW whereas control accounted for 4.2 g/L of DCW. On increasing the concentration of alcohols from 2% to 4%, 2.7 and 3.1 g/L of biomass were recorded when grown under ethanol and methanol treatments. The control flask with 4% glucose showed 13.6 g/L of DCW. On further increasing the concentration of alcohols from 6% to 8%, no biomass growth was recorded for ethanol whereas methanol showed 2.5 g/L of DCW, almost similar to previous concentrations of 4%. On the other hand, control flask showed 17.2 g/L DCW when grown at a higher glucose concentration (8%) (Fig 6A).

When ethanol concentration was increased from 2% to 4%, the lipid content increased significantly from 10% to 20%, respectively (Fig 6B). Similarly, increased lipid content upto 40% was observed with increasing concentrations of methanol from 2 to 6%. Notably, highest lipid proportion of 60% was detected with control treatments under high concentrations of glucose (8%).

Ethanol addition also significantly altered the fatty acid profiles of strain S2 (Fig 6C). At low concentration (2%), DHA was 18.22% of TFA, and it was the main polyunsaturated fatty acid recorded, whereas the amount of DHA decreased to 11.80% of TFA when the concentration of ethanol was increased to 4%. No data was recorded for DHA under high concentrations of ethanol. When treated with methanol, approx. similar DHA contents were recorded (20.6%, 20.7%, 19.3%

of TFA) under the concentrations of 2, 4 and 6%, respectively. However, the control flask exhibited highest DHA proportion (23.1% of TFA) at low concentration of glucose (2%). The proportion of Palmitic acid (C16:0) was 37% of TFA.

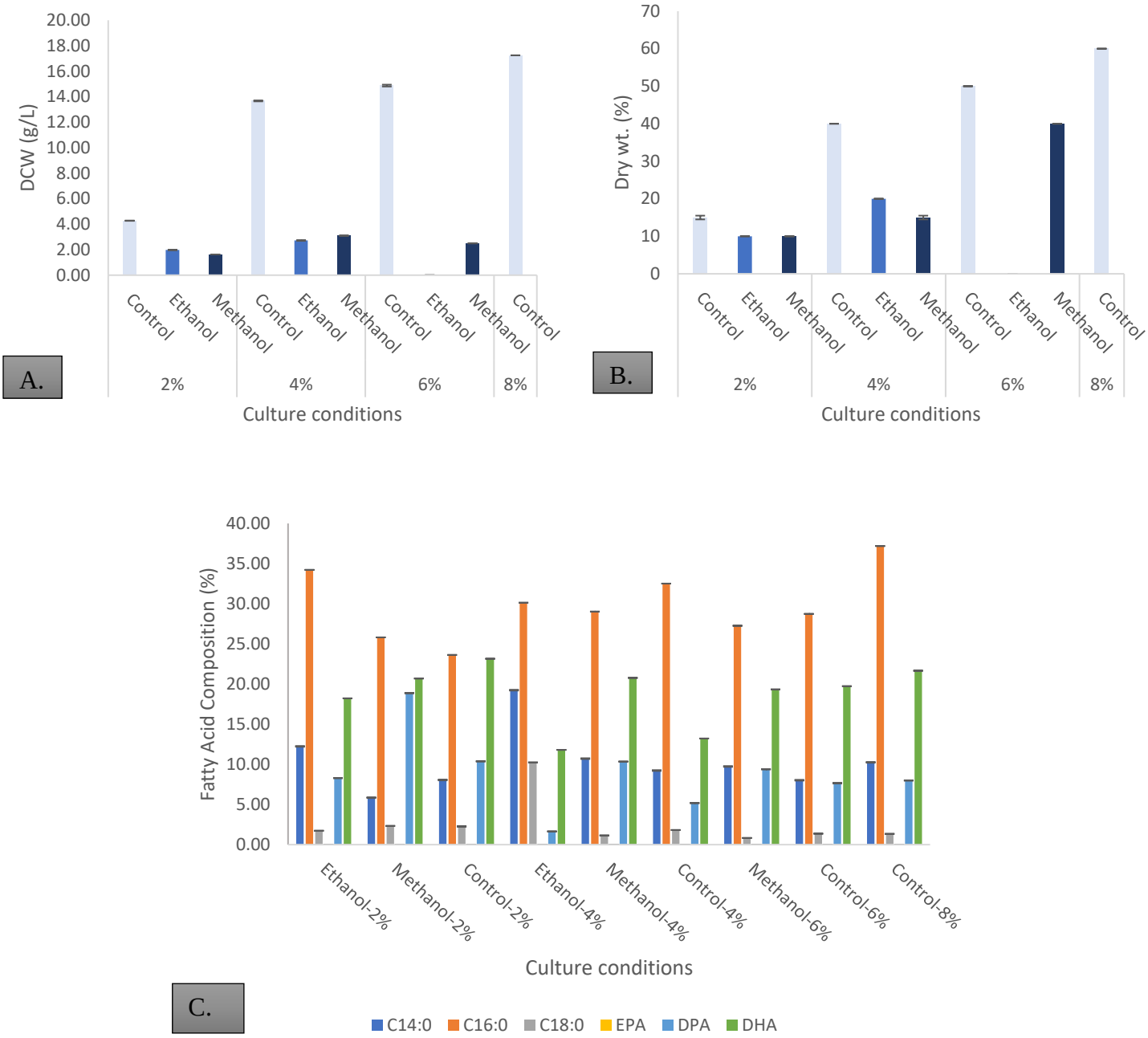


Fig.6. Effect of ethanol and methanol with respect to glucose on dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates. (Control- Glucose with no alcohols, Treatment- alcohols+glucose)

3.2 *Use of sodium salts as sole carbon source for lipids and omega-3 production*

When strain S2 was cultivated at 2%, glucose (control) and sodium acetate, similar amount of biomass produced i.e 6.6 and 6.7 g/L (Fig 7A), respectively. Strain was cultivated on 2% sodium acetate and sodium propionate in order to investigate their effect on low concentration. However, similar growth was observed at 4% concentration which signifies that increasing concentration does not change the growth pattern in control (glucose) as well as sodium acetate. Whereas biomass growth declined (5.3 g/L and 1.3 g/L), respectively when sodium propionate was used at similar concentrations in the fermentation medium.

Lipid accumulation was recorded 45% upon using sodium acetate (2-4%) in the fermentation medium, whereas sodium propionate exhibited only 10-15% of lipids irrespective of the concentrations used (Fig 7B). However, lipids content was 40% and 35% in control at 2% and 4%, respectively.

When strain S2 was cultivated on 2% and 4% on control, sodium acetate and sodium propionate, DPA and DHA were the long chain polyunsaturated fatty acids (PUFAs) recorded (Fig 7C). sodium acetate significantly altered the DHA proportions of strain S2. Sodium acetate under 2 and 4% concentration, recorded highest DHA content of all the other treatment (24% and 25.3% of TFA), whereas because of the low biomass and low lipid content, sodium propionate accounted for DHA at 15.78% and 9.1% of TFA. Highest proportion of Palmitic acid (33.5% of TFA) was recorded in sodium propionate at 4%. However, control recorded 23.1% and 22.4% of DHA of TFA under concentrations of 2 and 4%, respectively.

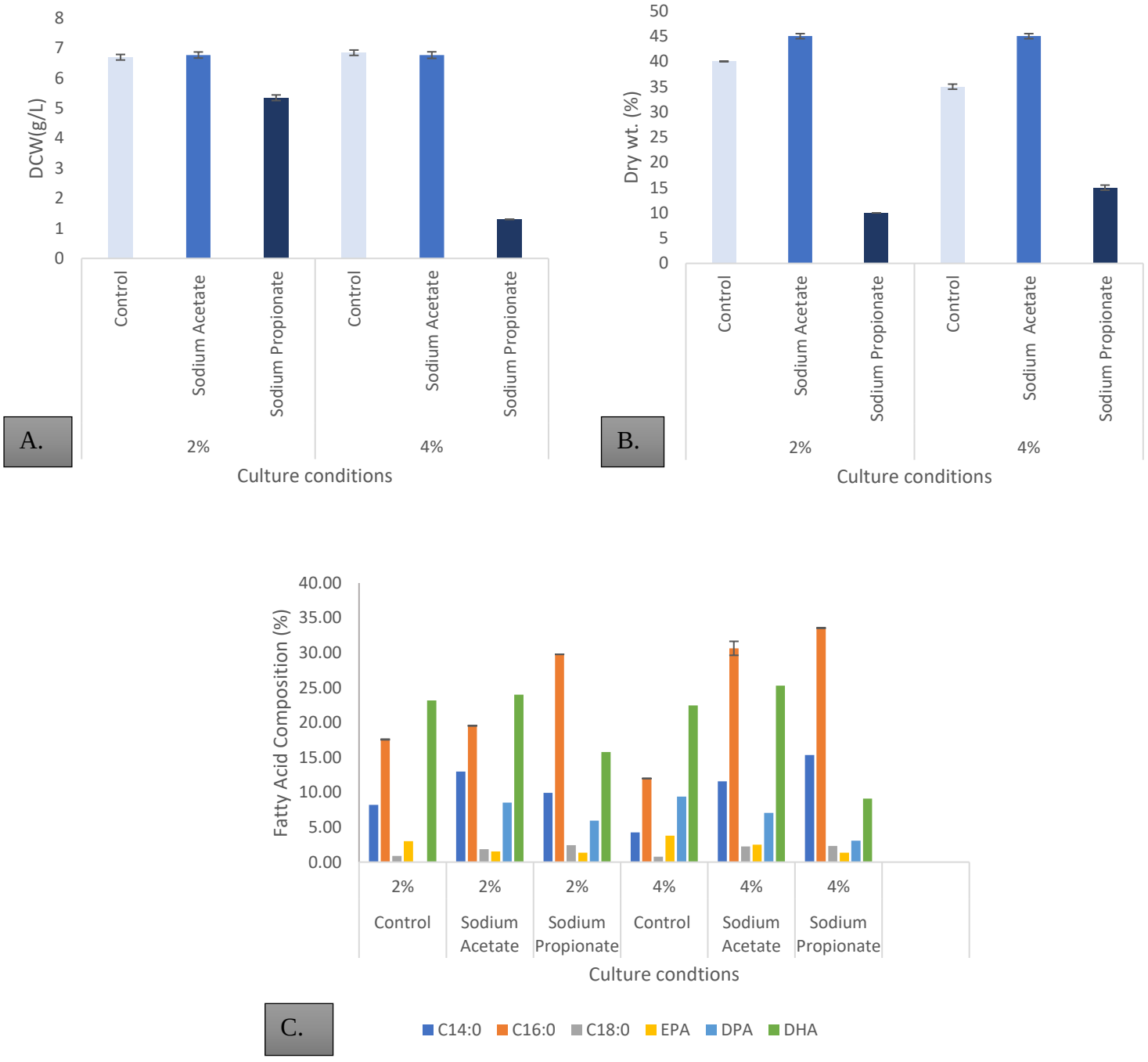


Fig.7. Effect of sodium salts on dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates. (Control- Glucose, Treatment- sodium acetate and sodium propionate-2% and 4% with no glucose)

3.3 *Assessment of different salts water vs artificial sea water (ASW) on lipids and omega-3 production*

Strain S2 was tested for the ability to produce biomass and lipid on both different salts water (50%) and Artificial Seawater (50%) (ASW) in shake flasks. Although both media supported cell growth as well as lipid accumulation (Fig 8A and 8B). 50% ASW exhibited highest biomass from 11.7g/L to 13 g/L at high concentrations of glucose (5% and 8%), respectively, whereas 8.6 g/L and 9.1 g/L of biomass was observed under same concentrations of control (Fig 7A).

When medium was supplemented with 50 % ASW, lipids content was 45% and 55% under glucose concentration of 5% and 8%, respectively (Fig 8B). However, lipid content was 30 % with different salts water under both glucose concentrations.

Fatty Acid Compositions were recorded when the cells were exposed to two different kinds of salts water (Fig 8C). DPA and DHA were the main two polyunsaturated fatty acids (PUFAs) recorded. Since biomass and lipid content were high, DHA content accounted were 21.1% and 31.2% of TFA which were the highest, for 50% ASW under 5 and 8% of glucose, respectively. Highest proportion of C16:0 (Palmitic acid, 37.8% of TFA) was recorded in 50% salts water at 8% glucose. However, 19.5% and 21.4% of DHA proportion of TFA were recorded with 50% different salts water at the concentrations of 5% and 8%, respectively.

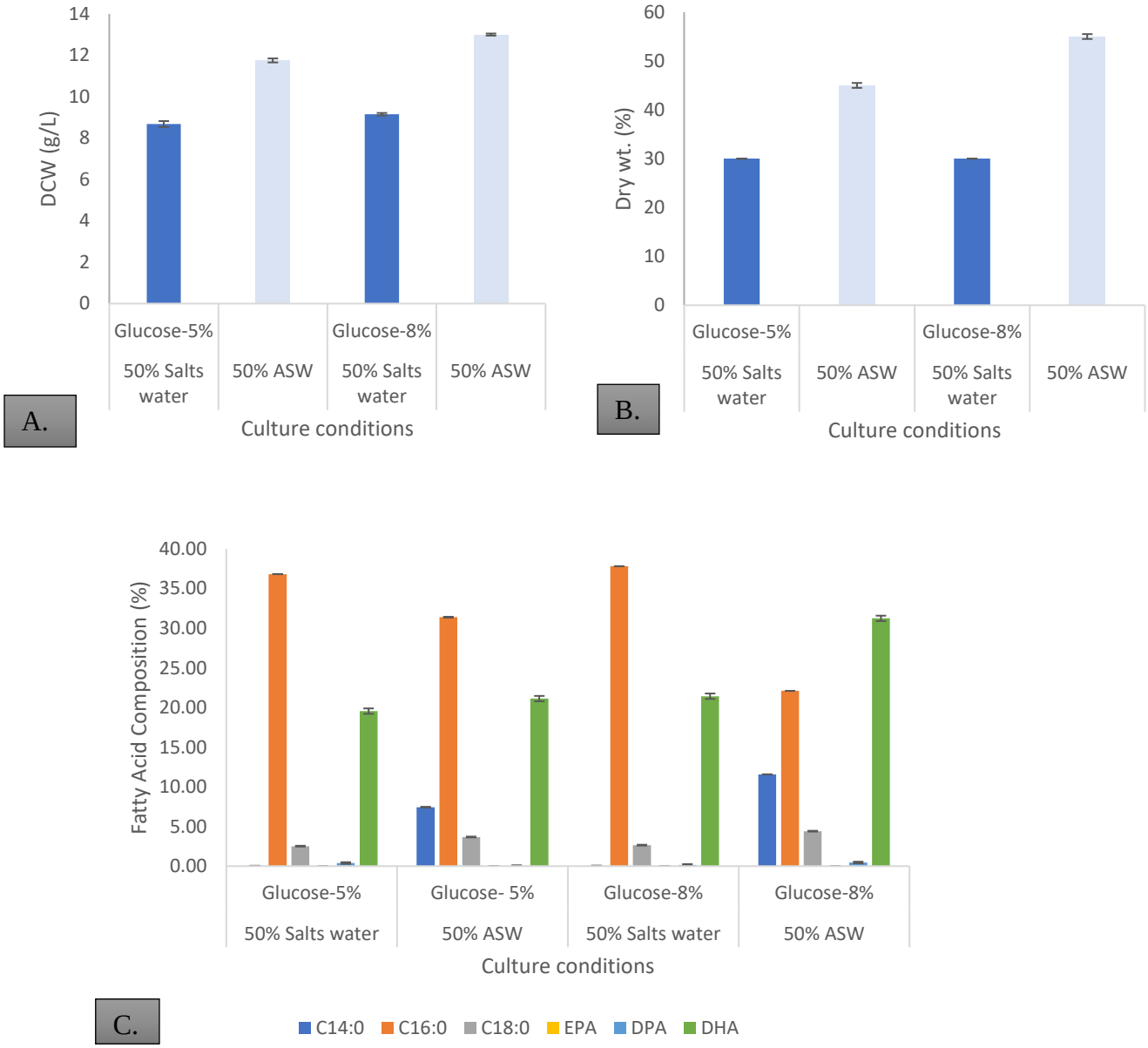


Fig.8. Effect of different salts water vs ASW on dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates. (control- 50% ASW with 5% and 8% glucose, Treatment- different salts water with same concentrations of glucose)

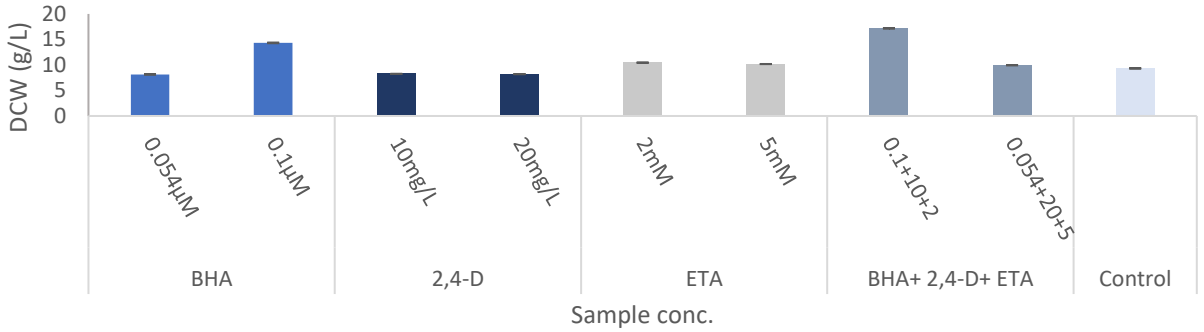
3.4 *Role of Chemical modulators in fermentation medium on biomass, lipids, and omega-3 production*

In this study, three chemical modulators BHA, 2,4-D and ETA were selected to investigate their effects on DCW as well as lipids accumulation. Different concentrations of these chemical modulators were taken because these were the best concentrations chosen from previous studies. Under the tested condition, BHA alone accounted for 14.32 g/L of DCW when used under the concentration of 0.1 µM (Fig 9A). However, 2, 4-D under the concentration of 10 mg/L and 20 mg/L resulted into 8.2 g/L and 8.2 g/L of biomass. A slight increase in biomass was recorded with ETA (10.45 g/L, 10.175 g/L) in comparison to 2, 4-D, under the concentrations of 2 mM and 5 mM, respectively. Combined concentrations of these modulators were also tested to examine their effects on biomass as well as lipids accumulation. As expected, BHA+ 2,4-D+ ETA (0.1 µM +10 mg/L +2 mM) exhibited highest biomass (17.1 g/L) of all the treatments whereas it was 9.9 g/L biomass was recovered under the combined concentrations of BHA+ 2,4-D+ ETA (0.054 µM +20 mg/L +5 mM). However, control accounted for 9.3 g/L of biomass.

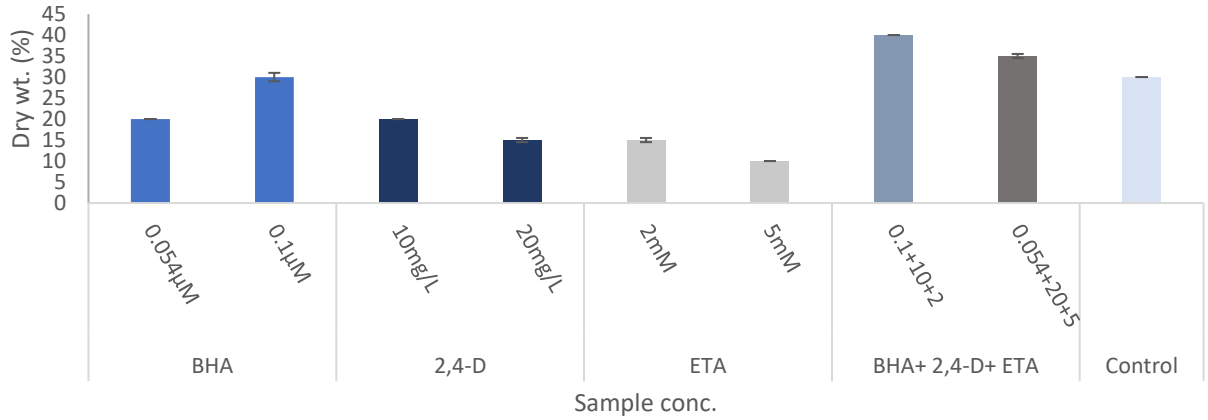
On the other hand, lipid contents were 30%, 20% and 15% for BHA, 2, 4-D and ETA when used under the concentrations of 0.1 µM, 10 mg/L and 2 mM (Fig 9B) which were the highest values recorded in comparison to other concentrations used. However, when those concentrations were used in combination to stimulate lipid accumulation, the lipids contents were 40% as compared to control (30%).

The fatty acid profiles were evaluated and BHA (0.1 µM), 2, 4-D (10 mg/L), ETA (2 mM) recorded for 31.22%, 25.54% and 23.99% of DHA of TFA, respectively, whereas it was 24.2%, 24.7% and 23.6% of TFA when the modulators were used in the concentrations of BHA (0.054 µM), 2, 4-D (20 mg/L), ETA (5 mM), respectively (Fig 9C). Furthermore, BHA+ 2,4-D+ ETA (

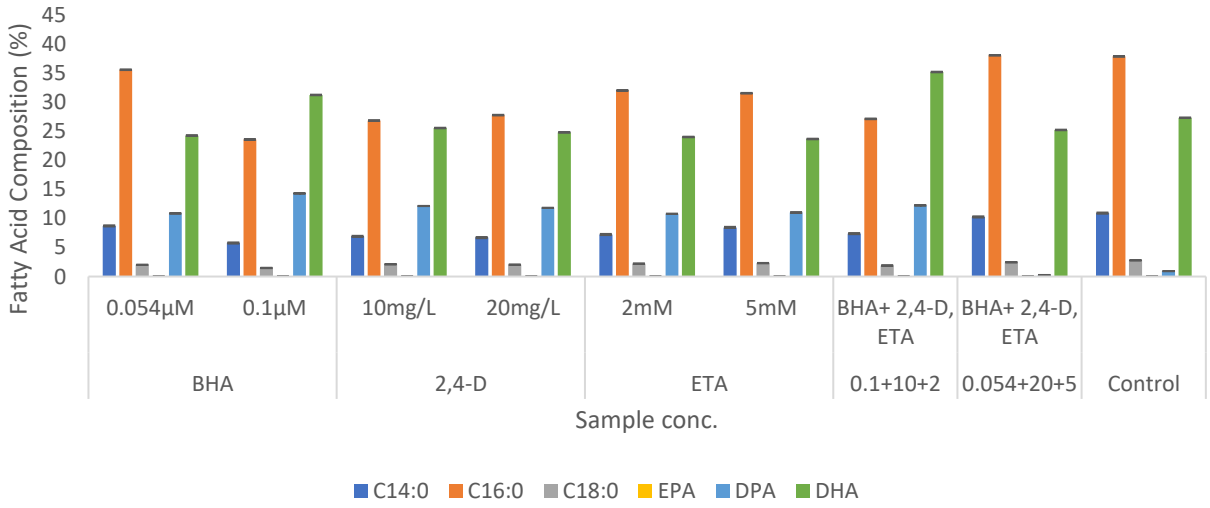
0.1 µM +10 mg/L +2 mM) exhibited highest DHA proportion of all treatments (35.1% of TFA). High proportion of C16:0 (Palmitic acid, 38% of TFA) was recorded in the combined concentrations BHA+ 2,4-D+ ETA (0.054 µM +20 mg/L +5 mM). of chemical modulators. The control accounted for 27.3% of DHA content of TFA.



A.



B.



C.

Fig.9. Effect of different chemical modulators on dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates. (Control- Glucose medium without chemical modulators, Treatment- BHA, 2,4-D, ETA)

3.5 *Use of monosodium Glutamate in media as sole nitrogen source for biomass and lipids production*

Monosodium Glutamate was selected to examine its effect on biomass growth, lipid content, DHA yield, and as a replacement to peptone (control) as a nitrogen source in the culture medium. Biomass growth (7.05 g/L, 6.8 g/L and 6.8 g/L) declined when MSG concentration (0.03%, 0.06% and 0.09%), were used as nitrogen source in the fermentation medium. However, it first showed an increasing trend and then remained same with control, which were 8 g/L, 8.9 g/L and 8.8 g/L, respectively under similar concentrations but with peptone (Fig 10A).

The fatty acid profiles with MSG were recorded as 10%, 25% and 20%, respectively, which exhibited an increasing and then decreasing trend, whereas peptone showed highest lipid content of 15%, 35% and 40%, at the concentrations of 0.03%, 0.06% and 0.09%, respectively (Fig 10B). Overall, lipid accumulated showed an increasing trend when used peptone.

DHA proportion was found to be decreasing with increasing MSG concentrations (0.03%, 0.06% and 0.09%), respectively (Fig 10C). On the other hand, with control (0.03%), maximum DHA was detected as 35.3% of TFA, whereas it decreased (22.6% of TFA) at higher peptone concentration (0.09%). The proportion of C16:0 (Palmitic acid) was 34.1% of TFA in control at 0.09% of peptone.

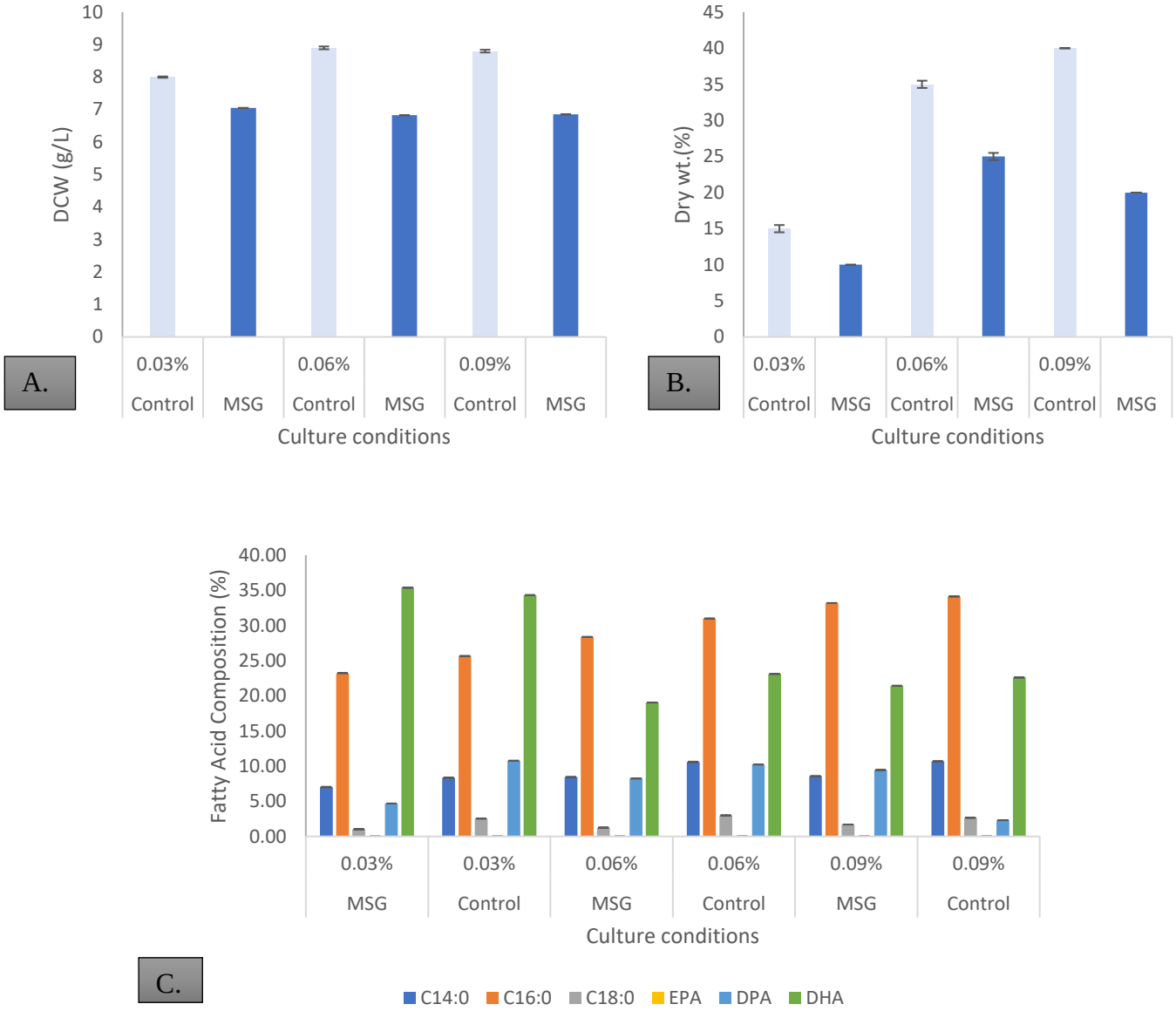


Fig.10. Effect of monosodium glutamate (MSG) on dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates. (Control-Peptone, Treatment- MSG)

3.6 *Use of glycerol as sole carbon source for biomass and lipids production*

It was observed that low concentration of glycerol (treatment) (2%, v/v), showed 5.7 g/L of biomass whereas control (glucose) showed 4.2 g/L (Fig 11A). On further increasing the glycerol concentration from (5-10%), biomass increased (13.8 g/L to 25.9 g/L), which was recorded higher than control (13 g/L to 22.7 g/L) from 5 to 10%, respectively.

Lipid content was 15% when glycerol was used at 2%,whereas it was 10% when Glucose was used (Fig 11B). However, when the concentration of glycerol increased from 5% to 10%, the lipid content observed was 45% and 60% in comparison to control which was 40% and 55%, respectively.

When initial glycerol and glucose concentration was 2%, DHA was accounted as 23.1% and 22.9% of TFA. Moreover, when the concentration of glycerol increased from 5% to 10%, DHA concentration increased from 23.7% to 35.4% of TFA. However, it was 20.2% and 25.6% of TFA, when glucose was used from 5% to 10%. Overall, glycerol showed high DHA content than control with increase in concentrations from low to high (Fig 11C). The proportion of C16:0 (Palmitic acid) was 31.5% of TFA in control in 5% concentration of glucose.

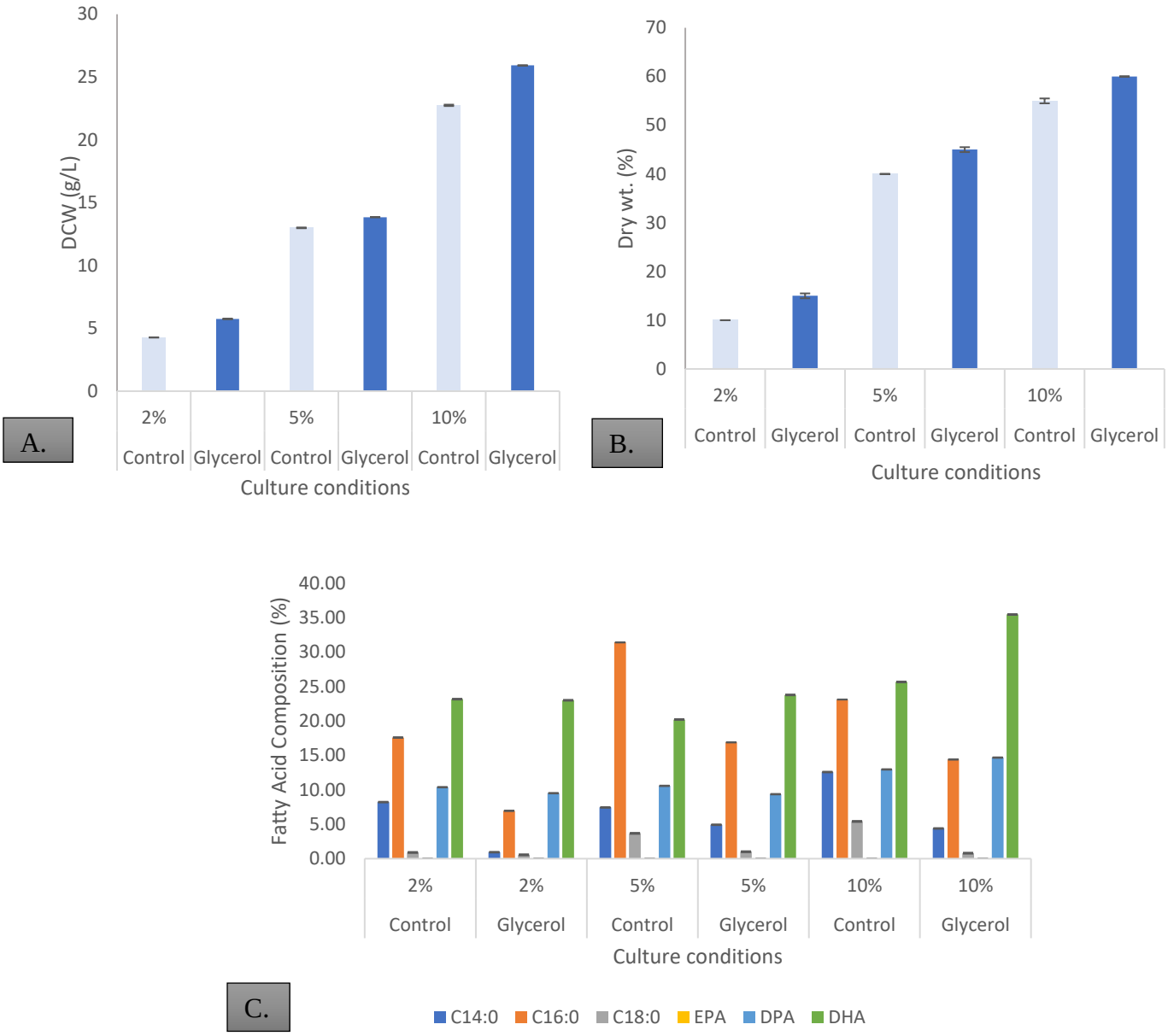


Fig.11. Glycerol influences dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates. (control- glucose, Treatment- glycerol)

3.7 *Aeration improves biomass productivity*

Baffled (improves mixing) and unbaffled Erlenmeyer flasks (referred as "normal" here), as shown in Fig 12(i) and 12(ii), were used for cultivating strain S2 under identical shaking speed. As shown in Fig 13A and 13B, cells grown in Baffled flasks achieved higher biomass than unbaffled flasks. The glucose and nitrogen consumption of cells were high, resulting in higher biomass (13.5 g/L) than unbaffled flasks (10.2 g/L). On the contrary, lipid proportion in unbaffled flasks were higher (40%) in comparison to baffled ones(20%) (Fig 13B).

DHA production was higher in normal flasks (41.8% of TFA) than baffled flasks (33.5% of TFA) (Fig 13C), which was higher DHA observed in the experiments performed above. Moreover, high proportion of C16:0 (48.2% of TFA) (Palmitic acid) was recorded in the normal flask.

Fig.12. (i) and (ii) represents the difference between baffled and unbaffled flask.

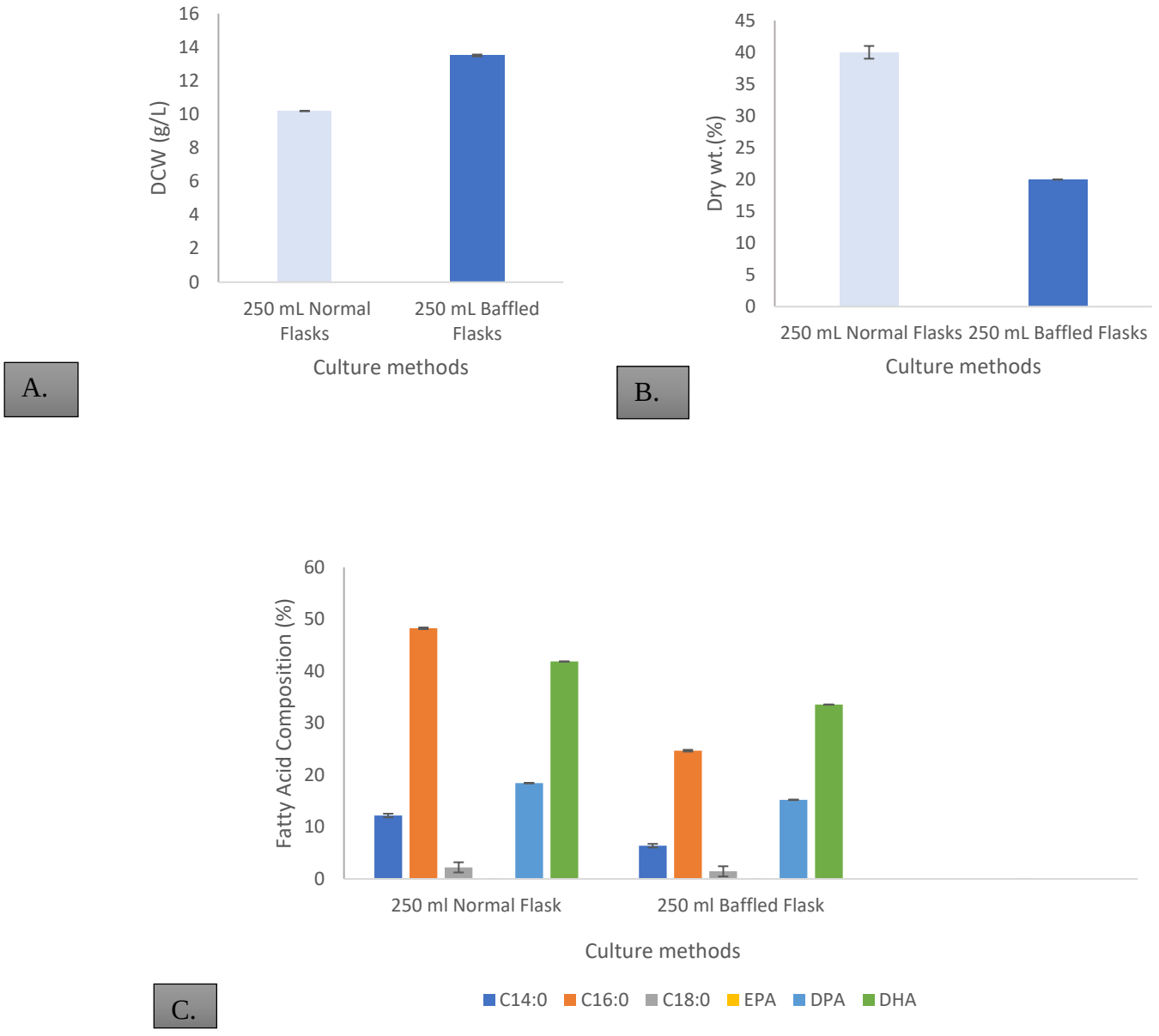


Fig.13. Effect of aeration on dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates.

3.8 *Effect of Na_2SO_4 and NaCl on biomass and lipids production*

Non-chloride sodium salts were tested and compared to investigate if it could be used as a substitute of sea salt. Results demonstrated that 2% NaCl can be replaced by 1% Na_2SO_4 in order to get high biomass and lipid content (Fig 14A). The biomass recovered from 1% Na_2SO_4 was 3.4 g/L which was higher than NaCl (2.8 g/L and 3.4 g/L) used in the concentration of 1% and 2%, respectively. Thus, it was observed that ASW leads to highest of the lipids accumulation (55%) followed by non-chloride sodium salt (Fig 14B). Furthermore, when fatty acids profile was analyzed Fig (14C), it was observed that there was high DHA content at 1% Na_2SO_4 (41.9% of TFA). This was the maximum DHA proportion recorded when compared to all treatments in sections above.

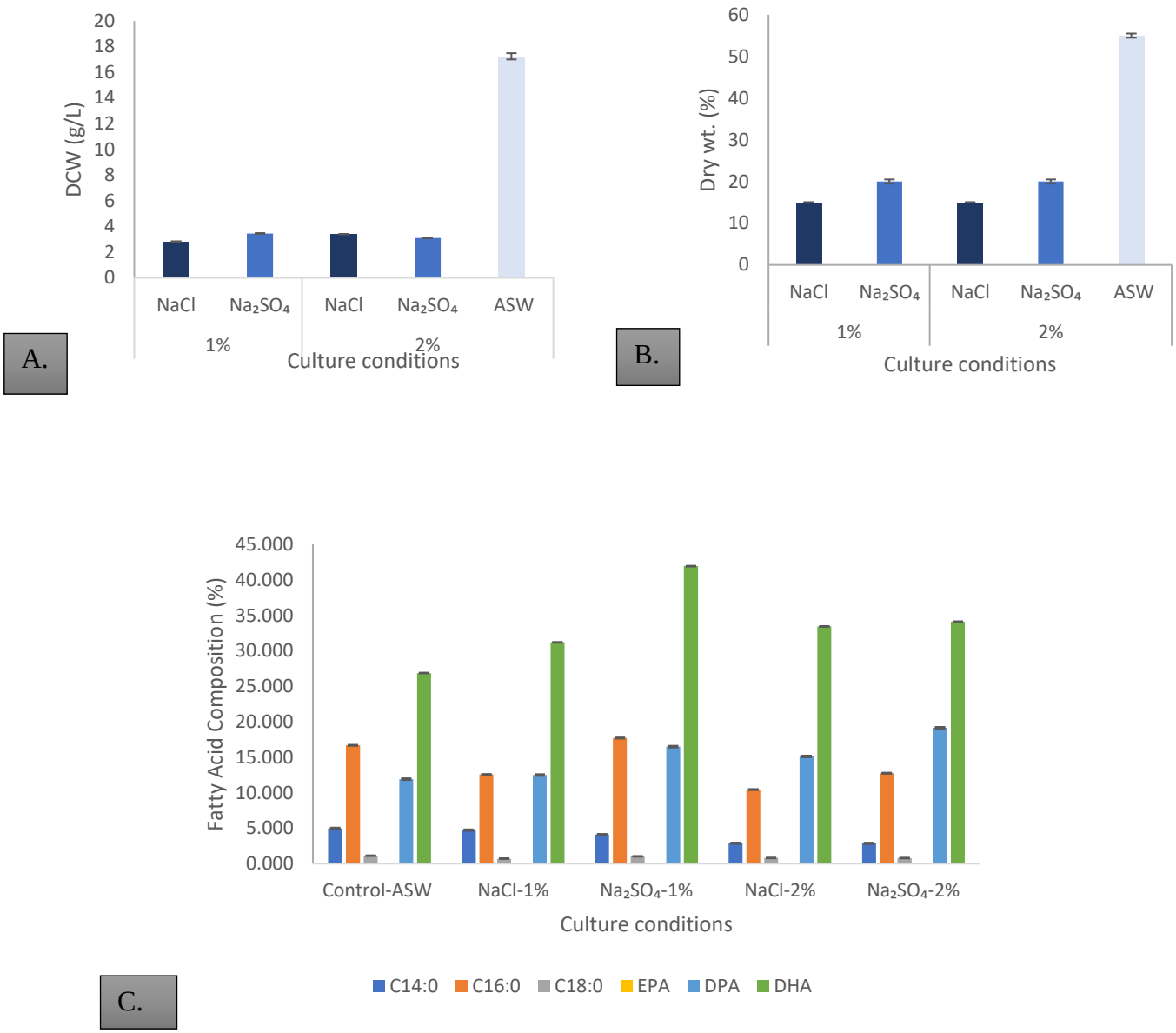


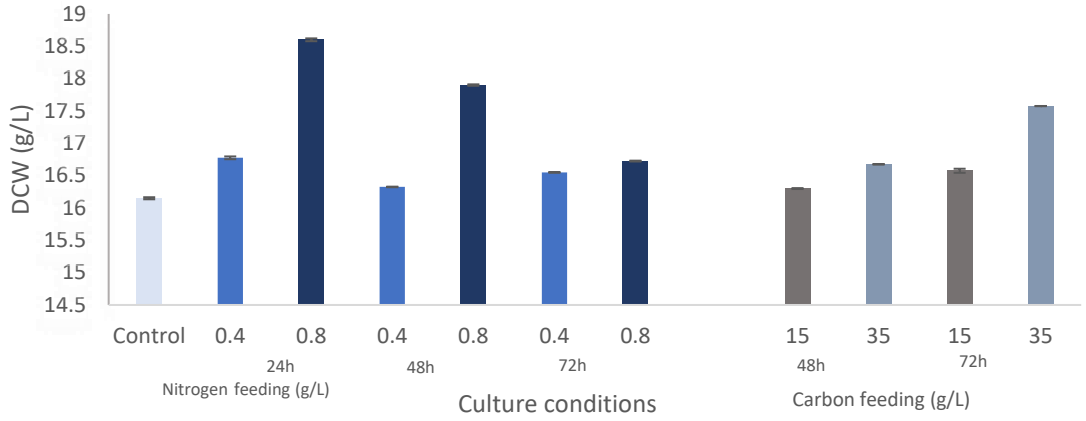
Fig.14. Effect of Na_2SO_4 and NaCl on dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates. (Control- ASW, Treatment- NaCl and Na_2SO_4)

3.9 *Carbon feeding influences biomass and lipids production during fermentation*

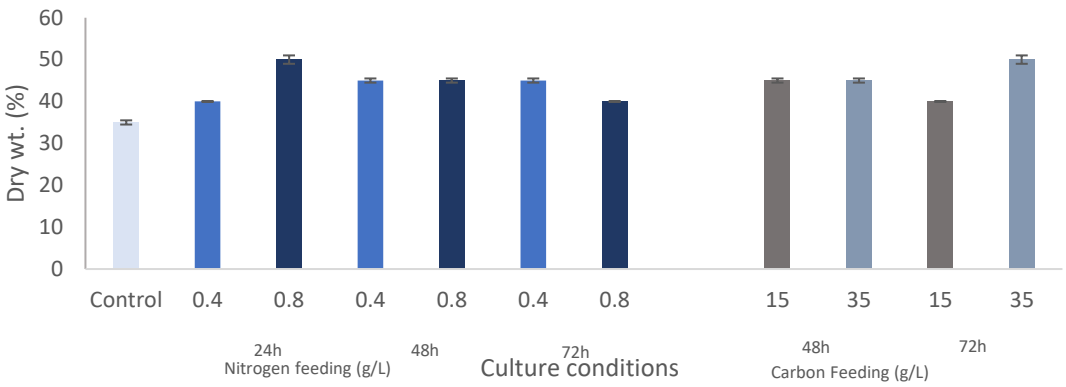
Carbon and nitrogen sources were fed intermittently (C- 15 g/L and 35 g/L, N- 0.4 g/L and 0.8 g/L) at various intervals of time (24h, 48h and 72h of fermentation) whereas there was no feeding in control flask. Highest DCW (18.6 g/L) was observed when 0.8 g/L nitrogen was fed intermittently at 24h of fermentation (Fig 15A). Second highest biomass (17.5 g/L) was observed when 35 g/L of carbon was fed at 72h of fermentation.

Similar trends were observed for lipid accumulation (Fig 15B). When nitrogen (0.8 g/L fed at 24 h) and carbon (35 g/L fed at 72 h), fed during the course of fermentation, more lipids (50%) were accumulated than control (35%). Highest DHA percentage was observed (44.2% of TFA,- maximum achieved so far) when carbon (35 g/L) was fed at 72 h. Carbon (15 g/L) at 72 h accounted only lipid accumulation 28.5% of TFA. However, in control it was 25.8% of TFA (Fig 15C). Furthermore, high proportion of C16:0 (45% of TFA) (Palmitic acid) was recorded at 72 h of carbon feeding (35 g/L) .

A.



B.



C.

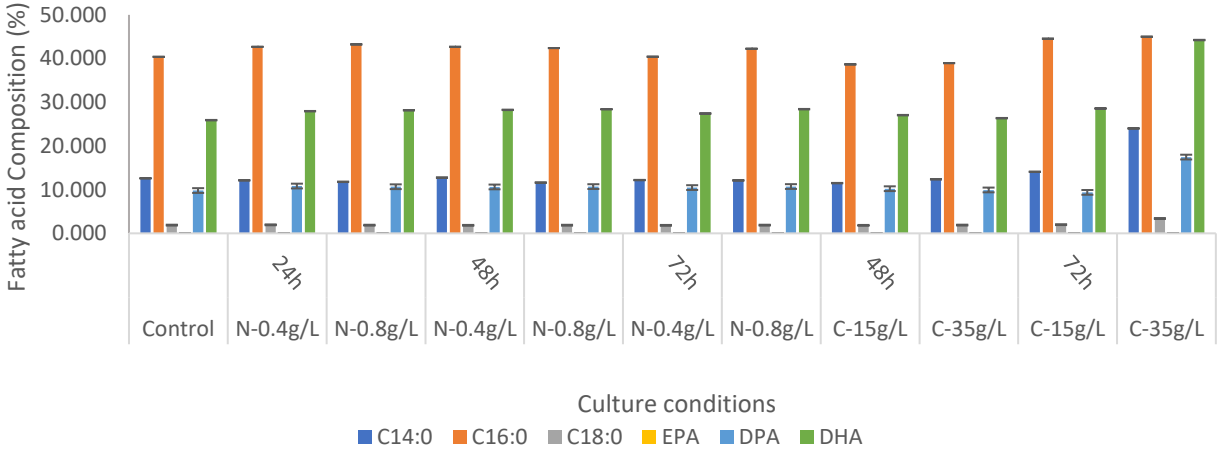


Fig.15. Effect of Intermittent vs Fixed feeding on dry cell weight (DCW) (A), Lipid Content (B), and Fatty Acid Composition (C) of strain S2. Data are presented as mean ± SD of duplicates. (Control- without intermittent feeding, Treatment- with intermittent feeding of carbon and nitrogen)

CHAPTER-4 (DISCUSSION)

There is considerable market interest in developing sustainable platforms/technologies which may contribute to enhancing the production of lipids such as PUFAs, proteins and other valuable products (Napier et al., 2021). Thraustochytrids are considered as natural omega-3 producers as they can accumulate upto 70% of DHA of TFAs (Patel et al., 2020). Thus, the dry cell weight (DCW), lipid accumulation and fatty acid composition of thraustochytrid strain S2 were investigated and revealed that it is strongly dependent on carbon-nitrogen sources as well as other culture conditions. It is hypothesized that there are two existing pathways for PUFA synthesis in Thraustochytrids: the oxygen-dependent aerobic fatty acid synthase pathway (FAS), also called as elongase-desaturase pathway, and the second one is oxygen-independent anerobic polyketide synthase pathway (PKS)- also known as PUFA synthase pathway (Morabito et al., 2019, Patel et al., 2020). When strain S2 was cultured in the media containing alcohols as an additional carbon source such as ethanol and methanol (2%, 4%), only small amount of alcohols were utilized as most of the alcohol remained in the medium, leading to lesser DHA concentration when compared control treatment (23.15% of TFA). A series of enzymes such as 3-ketoacyl synthase (KS), 3-ketoacyl-ACP reductase (KR), enoyl reductase (ER), dehydrase/isomerase are necessary during full fatty acid synthesis pathway. The alcohols showed an inhibitory effect on the S2 cells, probably due to downregulation of various genes such as *G6PD* (*glucose-6-phosphate dehydrogenase*) and *6PGD* (*6-Phosphogluconate dehydrogenase*), which are the key enzymes responsible for generating NADPH through pentose phosphate pathway (PPP). The inhibitory effects of methanol and ethanol on biomass and lipids have also been reported on other microalgal strains as well (Miazek et al., 2017). For example, Methanol at 3.96 g/L, inhibited the growth of *Chlorella vulgaris* and *Selenastrum capricornutum* resulting in 4.68 g/L and 6.33 g/L of biomass.

Additionally, ethanol at concentration of 0.39g/L, exhibited inhibition of biomass of *Isochrysis galbana* and *Heterosigma akashiwo* (15 g/L, 2.5 g/L), respectively. (Miazek et al., 2017). Thus, unabundant supply of NADPH probably leads to low DHA production in an alcoholic medium. The cells were exposed in the medium containing volatile fatty acids (VFAs) such as acetic acid, butyric acid and valeric acid (Fei et al., 2015), but no growth was observed in the fermentation medium (data not shown). Hence, available sodium salts (sodium acetate and sodium propionate) of volatile fatty acids as sole carbon sources, were used to check their effect on the growth of strain S2. It was clear from the data as low DHA content was observed (Fig 7 A, B, C). Low DHA content may be because of the insufficient amounts of desaturases and elongases. High DHA yield (24%) was observed at 4% sodium acetate that might be due to a possible cause of direct production of acetyl-CoA via another route, which is key intermediate for fatty acid synthesis (Patel et al., 2020). When strain S2 was grown in the medium containing 4% sodium acetate, there was a direct conversion of acetyl CoA in the cytosol by acetyl-CoA synthase in one step whereas on the contrary, when the cells grew in a medium of glucose as a sole carbon source, the cells entered into glycolysis, then formed pyruvate, went to mitochondria and incorporated into TCA cycle which is a multi-step reaction (Patel et al., 2020). Sodium acetate was reported to increase biomass (1.4 g/L) production in *N. Oleoabundans* (Silva et al., 2016)

Artificial seawater supports the lipid accumulation and DHA content which might be because of ionic and balanced environment to the cells that helped in enhancing the PUFA synthesis when used with 8% glucose whereas the artificial seawater prepared in the lab from various salts led to low DHA production (Fig 7C). Although, various chemicals have been reported for enhancing the growth of microalgal cells but BHA, 2,4-D and ETA as chemical modulators did not support the plant cells growth in the literature (Wang et al., 2018), but these modulators supported to provide

high biomass and lipid yield for strain S2. High biomass and lipid yields were obtained when BHA, 2,4-D and ETA were used in the concentrations of 0.1 µM, 10 mg/L and 2 mM, respectively. BHA was the modulator led to high biomass and lipid content in the medium probable due to the fact of upregulating the effect of genes involved in PUFAs biosynthetic pathway. In addition, the combination effects were studied that showed high biomass, lipid and DHA production (17.175 g/L, 40%, 35.172% of TFAs), respectively. These modulators did not show any toxic effect on the S2 cells and resulted in enhanced DHA production. *H. pluvialis* cells were subjected to BHA treatment and the resultant lipid content was 42.84%, which was 1.22 times higher than treatment without BHA (35.19%) (Ding et al., 2019).

High biomass, lipid and DHA content was recorded in control than in the medium containing Peptone as nitrogen source, which may be attributed to the fact that microalgae cells could absorb peptone faster than MSG (Anwar et al., 2018). When peptone was provided at concentration of 0.09%, microalgae started the conversion of this nitrogen source into proteins, nucleic acid, etc. Hence, faster the microalgal cells absorb the nitrogen source, the better growth will be observed (Anwar et al., 2018). For example, when *Chlorella. saccharophila* was cultivated in a medium, containing 0.05% peptone, 36.3% of lipid content (% w/w) was recorded (Isleten-Hosoglu et al., 2012). Furthermore, increased concentration of glycerol favoured the lipid, biomass and DHA yield (Morais et al., 2021).

Moreover, it was noted that agitation had a key impact on the culture conditions. Baffled flask tends to provide high biomass because of better mixing that provided more dissolved oxygen (DO) compared to unbaffled Erlenmeyer flasks (Ling et al., 2015). Agitation is crucial for the cultivation of a microorganism, it helps to maintain homogenous cultivation environment to avoid substrate concentration gradient and facilitates mass transfer and oxygen transfer in the fermentation

medium to enhance availability of nutrients and oxygen to cells (Chen and Yang, 2018). It was reported in studies conducted by Ling et., al (2015) that the baffled flasks for strain *Schizochytrium* sp. increased the biomass, lipid production as well as DHA concentration (Ochsenreither et al., 2016). On the contrary, it was observed that using baffled flasks for *M. isabelline*, lipid production was not affected whereas it led to increased biomass production probably due to the fact that more agitation produces more foam and foam act as oxygen barriers which might resulted in less oil and DHA production in the cells in baffled flasks than normal flasks (Chatzifragkou et al., 2011).

Moreover, when the medium was supplemented with 1% Na_2SO_4 to testify the substitution of chloride ions, the DHA content increased than 2% NaCl which simplifies sodium sulfate was found to be superior to sodium chloride, causing an isotonic environment in the medium. Formation of chloride ions lead to the formation of corrosion and salinity in the mediums (Altun and Sen, 2004).

The intermittent feeding led to highest DHA production (44.23% of TFA). The resulting high DHA and biomass with nitrogen feeding at 72h than other feeding, was probably due to the activation of more acetyl CoA carboxylase for lipids accumulation (Hsieh and Wu, 2009). Further, more formation of acetyl Co-A led to the synthesis of PUFAs at high concentration of carbon feeding (35 g/L) at 72h resulting in highest DHA production (Qu et al., 2013). When 25-30 g/L of carbon was fed during pilot scale fermentation (500L), with 10% (v/v) seed culture cultivated for two generations (24 h for each generation). DHA content reached upto 55.02% (Guo et al., 2020). Further, when thraustochytrid strain- *Thraustochytriidae* PKU#Mn16 was subjected to fermentation using mixed substrate feeding strategy (30 g/L of glucose and glycerol), biomass and DHA yields improved to 544.05 mg/L-h^{-1} and 100.76 mg/L-h^{-1}, respectively, due to the involvement of various genes in DNA replication, carbon metabolism and β-oxidation (Ye et al.,

2020). Thus, these results clearly demonstrated that the feeding of carbon and nitrogen assisted in enhancing the production of lipids and DHA (Ling et al., 2015).

CONCLUSION

Production of long chain fatty acids can be effectively achieved by using aquatic protists. High value bio actives such as omega-3 LC-PUFA can be obtained by cultivating Thraustochytrids using different medium compositions (Russo et al., 2021). Quality, safety, and ethical issues related to this oil has led consumer motivation to pay more attention towards it than it was for fish oil. These health nutrients could be developed in an environmental and sustainable way thus, feeding larger populations (Chang et al., 2015). In this study, numerous up-stream experiments were performed to achieve DHA production. Highest DHA content of 44.2% of TFA was achieved when 35 g/L of carbon was fed intermittently at 72h of fermentation compared to control. This showed that DHA proportion could be enhanced by using fermentation strategies, including different medium manipulation conditions. Further advancements to improve Omega-3 productivity are required to ensure that: (1) aquaculture feeds should be originated from land-based resources to contribute to the recovery of global fish stock; (2) Mutagenic sources are able to produce high proportions of VLC-PUFAs, however need regulatory approvals and find consumer acceptance; (3) the cultivation of microalgae in a cost- efficient manner on cheaper substrates to produce DHA cost-effectively thus, fulfilling the global demand. The future may witness the combination of the above sources, that may lead to the development of more sustainable production of omega-3 fatty acids (Adarme-Vega et al., 2014). Thus, microbial production of ω-3 oils is a promising approach for fulfilling the increasing demands of EPA and DHA, considering its various health benefits, therapeutic values, and food supplements (Remize et al., 2021).

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