SUSTAINABLE PRODUCTION OF ARACHIDONIC ACID (AA) BY A NOVEL THRAUSTOCHYTRID STRAIN

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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 are made in the text of this thesis or in the notes.

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ABBREVIATIONS

I.	mg/L	Milligram per Liter
II.	mg, g	Milligram, gram
III.	µL, mL	Microliter, Milliliter
IV.	%	Percent
V.	w/v	Weight by volume
VI.	^{0}C	Degree Celsius
VII.	d	Day
VIII.	h	Hours
IX.	rpm	Revolution per minute
X.	AA	Arachidonic acid
XI.	ABA	Abscisic acid
XII.	ASW	Artificial seawater
XIII.	ALA	alpha-linolenic acid
XIV.	BA	6-benzyl adenine
XV.	CBS	Centraalbureau voor Schimmel cultures
XVI.	DCW	Dry cell weight
XVII.	DDA	Docosadienoic acid
XVIII.	DE	Differentially expressed
XIX.	DHA	Docosahexaenoic acid
XX.	DNS	3,5 dinitrosalicylic acid
XXI.	DPA	Docosapentaenoic Acid
XXII.	EPA	Eicosapentaenoic acid

XXIII.	EP	Eicosapolyenoic
XXIV.	FA	Fatty acid
XXV.	GA	Gibberellin
XXVI.	GC	Gas chromatography
XXVII.	GYP	Glucose, Yeast, Peptone
XXVIII.	IAA	Indole-3-acetic acid
XXIX.	IBA	Indole-3-butyric acid
XXX.	KT	Furfuryl adenine
XXXI.	LA	Linoleic acid
XXXII.	NaCl	Sodium chloride
XXXIII.	OD	Optical density
XXXIV.	PA	Palmitic acid
XXXV.	Plac.Burma	Plackett-Burman Design
XXXVI.	PUFA	Polyunsaturated fatty acids
XXXVII.	RSM	Response Surface Methodology
XXXVIII.	SCOs	Single cell oils
XXXIX.	std. dev	Standard deviation
XL.	TAG	Triacylglycerol
XLI.	TFA	Total Fatty acids
XLII.	VLCPUFA	Very-long-chain polyunsaturated fatty acids
XLIII.	ω	Omega

ABSTRACT

For a healthy life, it is vital to obtain a higher proportion of polyunsaturated fatty acids (PUFAs) as daily supplements. Various clinical studies have advocated the role of PUFAs, especially arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) in supporting nutrition in infants. However, only limited sources such as fish oil are known for ensuring PUFAs availability in the human diet. Therefore, more sustainable resources are needed to produce PUFAs. AA is found in high proportion in eggs, meat and poultry, and marine fish oil but it cannot be produced by mammalian cells. Therefore, it should be obtained externally through food supplements, which are meeting the increasing demand of the vegetarian population. The microbial source can be an alternative for producing fatty acid, as these are non-polluting, regarded sustainable sources to produce PUFAs, and acceptable by the vegetarians. Thraustochytrids have been applied as a commercial resource for producing PUFAs. These marine microbes have an attractive PUFA profile that comprises of omega-3 and omega-6 fatty acids. The current study investigated the heterotrophic production of omega-6 fatty acids. The objective of this study was to produce AA through thraustochytrid and increase its yield through fermentation approach which is environment friendly. The present study examined the effects of various medium components (carbon, salt, and yeast concentration) and fermentation parameters (pH, temperature, agitation rate) on the production of AA from an in-house thraustochytrid S2 strain. Different chemical modulators (BA, IBA, KT, and IAA) were added to the fermentation media to enhance AA production. Addition of 6-benzyl adenine (BA) at 15 mg/ L resulted in the highest AA accumulation. Under the optimal conditions of pH 6.5, temperature 22 ^{0}C, agitation 150 rpm, salt and yeast concentration (20 g/L), the maximum biomass (22.1 g/L), and AA (3%) production were observed. In addition to AA production, other prominent fatty acids such as palmitic acid

(C16:0), docosahexaenoic acid (C22:6), and palmitoleic acid (C16:1n7) were also identified as part of total fatty acids. Feeding of a precursor rich in unsaturated fatty acids further enhanced AA, DCW, and lipid yields using thraustochytrid S2 strain, thus, achieving the objectives of the study.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Polyunsaturated fatty acids (PUFA) are known as precursors to numerous active molecules in the body. PUFA controls growth, anti-antioxidant and inflammatory activities as well as communicates in and between different organisms, such as fungi, bacteria, plants and animals (Dye et al. 2020; Shanab et al. 2018). Arachidonic acid (AA, 20:4n-6), belongs to omega-6 group of PUFAs and plays an important role in metabolic processes as a precursor to prostaglandins, leukotrienes and eicosanoids and are known as essential PUFAs thus essential for nutrition. Docosahexaenoic acid, (DHA, 22:6n-3), belongs to the omega-3 group and have a beneficial role in infant nutrition and wide range of applications in medicine (Bouwstra et al. 2003; Shanab et al. 2018).

AA is available through natural sources such as egg yolk, adrenal glands, animal liver, however, the quantity of AA is low that these sources cannot fulfill the growing requirements of this fatty acid. The limited availability of AA from natural resources has necessitated the development of microbiological AA production. Currently, various strains of fungi such as *Mortierella* have been developed in the US, Europe, and China for its industrial production (Mamani et al. 2019; Mironov et al. 2018; Zhu, M et al. 2004). These processes have high production lipid cost.

Thraustochytrids, a heterotrophic and unicellular protists, are considered as a sustainable marine resource for producing PUFAs as same are contained as storage and membrane lipids (> half of the total fatty acids) in a cell (Meesapyodsuk et al. 2016; Raghukumar 2008). AA is recognized as one of the polyunsaturated fatty acids (PUFAs) in various thraustochytrids strains (Raghukumar 2008). The growth and PUFA profiles that prominently comprise of AA, DHA and EPA (eicosapentaenoic acid, 20:5 ω3) levels are depended on culture conditions (Kavitha et al. 2016).

1.1 Importance of Fatty acids

Fatty acids are the main component of fat. They exist in the cell membrane as phospholipids. Moreover, they are found in food sources in the form of triglycerides and known as energy substrates in the human body, providing 30% of total energy consumption. Fatty acids are categorized as saturated and unsaturated carboxylic acids according to the variation of carbon chains between the carbon atoms (2 and 36 of carbon atoms) (Fig .1) (Tvrzicka et al. 2011; Vannice et al. 2014).

1.1.1 Very-long-chain polyunsaturated fatty acids (VLCPUFAs)

Very long chain polyunsaturated fatty acids (VLCPUFAs) are characterized under two families according to their configuration of pentadiene of double bonds, such as n-3 (or Omega-3/ω-3) and n-6 (or Omega-6/ ω-6). In the n-3 group, the last double bond is located at the third carbon and it is beyond the methyl end. In the n-6 family, the last double bond is located at the sixth and that is far from the methyl end. Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) are two examples for n-3 family and Arachidonic acid (AA) is an example for n-6 family (Fig.1) (Qiu et al. 2020; Shanab et al. 2018; Tvrzicka et al. 2011). The structures of AA, EPA, and DHA are presented below (Fig 2A-C). DHA and AA are very important for the nutrient requirement of human (Abbadi et al. 2004). According to the recommendation of WHO, the ratio of n-6: n-3 should be less than 10 and it must be obtained from our daily meal (Shanab et al. 2018).

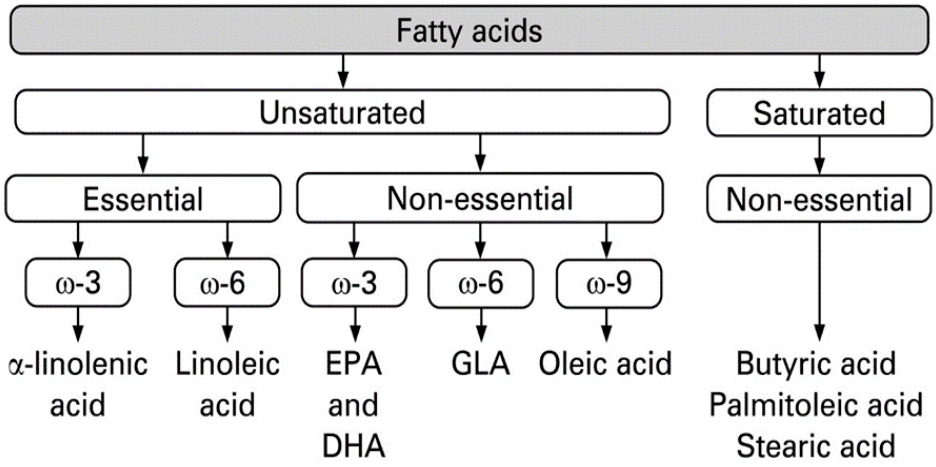


Fig 1 : Categorization of fatty acids according to human metabolism (This figure is reprinted with permission from (Yashodhara et al. 2009))

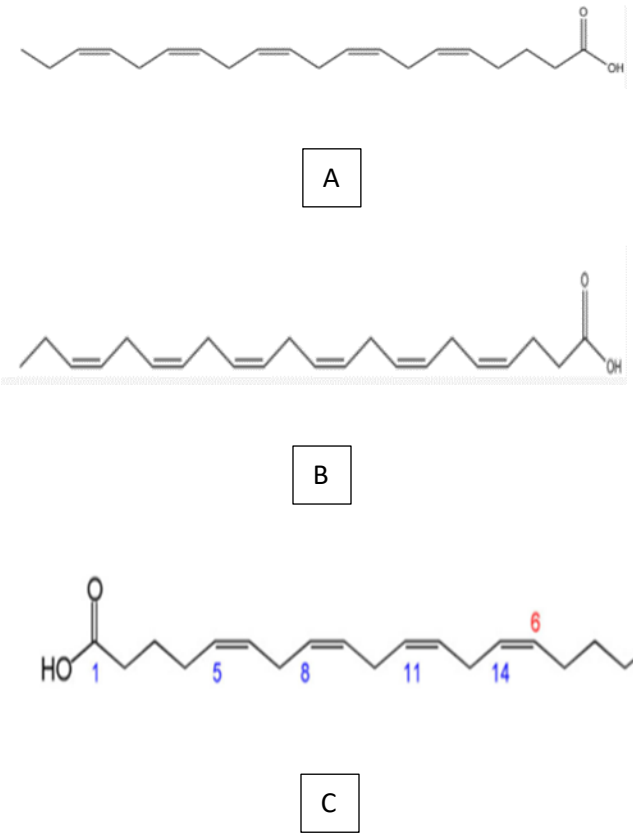


Fig 2: Chemical structure of various polyunsaturated fatty acids of A. EPA, B. DHA and C. AA (This figures are reprinted with permission from (Barbosa et al. 2014; Shanab et al. 2018))

Chemical structure of A. EPA, B. DHA (Adapted from (Barbosa et al. 2014)), C. AA (Adapted from (Shanab et al. 2018))

1.1.2 Distribution of VLPUFAs in nature

VLPUFAs are not synthesized by the human body thus are sourced from external sources such as fish oils. Whereas, Linolenic and linoleic acid are found in the vegetables (green) as well as oils extracted from plants (Abbadi et al. 2004). Fish oil mainly contains n-3 VLPUFAs, while vegetable oil is the major provider of n-6 PUFAs. For a healthy life, it is important to obtain a higher proportion of VLPUFAs as a daily supplement (Shanab et al. 2018). However, due to concerns associated with overfishing, only limited sources of PUFAs are available. Therefore, other novel sources for PUFAs production may be investigated (Shanab et al. 2018).

1.1.3 Production of PUFAs by using microorganisms

Various studies have reported the sources of n-3 and n-6 PUFAs as plants, algae, and other microbes (Shanab et al. 2018). Microalgae are considered as a primary resource for producing PUFAs that contain more nutrients than other sources (Khozin-Goldberg et al. 2011; Kugler et al. 2019). For example, under the nitrogen starvation conditions, green microalga accumulated ω-6 long-chain polyunsaturated fatty acids (LC-PUFA), especially AA (Kugler et al. 2019). In microbes, biosynthesis of PUFA is initiated in the endoplasmic reticulum (ER). In addition, microalgae cultivation is considered a green and sustainable method to produce PUFAs for several applications, such as pharmaceutical, health and nutrition industries (Kugler et al. 2019). Researchers have isolated genes from various fatty acid producing marine microorganisms in order to produce LC-PUFAs through transgenic plants (Khozin-Goldberg et al. 2011), however, due to lesser developed molecular biology tools only a few plant crops field trials got successful (Zhu, Mengmeng et al. 2016). However, it has been suggested that microbial source can supply a natural

diet of fatty acid for vegetarians (Suzuki et al. 2019). When compared with other sources of PUFAs, microbial production would be more environment-friendly (Suzuki et al. 2019).

1.2 Role of fatty acids in emerging plant-based meat market

High consumption of red meat has led to adverse consequences such as cardiovascular, colorectal cancer and mortality (Neacsu et al. 2017; Nieto et al. 2012). In addition, the production of animal foods has contributed towards environmental hazards, such as greenhouse emission. More land and higher nitrogen quantity are required to produce animal-based proteins (Curtain et al. 2019). Therefore, it is important to systematically reduce the production of animal products and enhance the production of plant-based products to improve environmental sustainability.

As a result of COVID-19, a significant growth in plant-based meat market from USD 3.6 billion to USD 4.2 billion (2020-2021) has happened. It is growing at a 17.0% of a compound annual growth rate (CAGR). There are some key factors which affected the development of the magnitude of the plant- based meat market size, such as the popularity of plant-based products among the communities in the world, significant growth of vegan population, increasing awareness of consumers about the health benefits of plant-based meat and rising climate consciousness (Markets and Markets Research 2020). The results from present study would assist in proposing marine thraustochytrid for AA production that can be used as a food supplement for the vegan population thus serving nutritional needs.

Thus, the use of microbial oils rich in PUFAs may be considered as an efficient way in meeting the surging popularity of plant-based meat. It has been reported that plant-based proteins can be an alternative source for meat components, however protein availability has become a challenge for the production of meat components (Curtain et al. 2019). When compared to global production,

Australia is the third country that has a rapidly growing vegan market (Curtain et al. 2019) . It is estimated that the market demand for plant-based meat may increase up to $3 billion by 2030. From 2012 to 2016, the number of vegetarians has significantly increased from 1.7 million to 2.1 million which is 11.2% of the total population (Curtain et al. 2019). However, the knowledge about the plant-based meat production is very limited.

1.3 Importance of AA

Eicosanoids are one of the cis-PUFAs in the membrane lipids. They basically consisted of AA and EPA in less percentage (Dye et al. 2020). AA (n-6 or omega-6) is one of the essential polyunsaturated fatty acids (PUFAs). It belongs to the 5,8,11.14-eicosatetraenoic- acid family. According to the carbon chain, the structure of AA is indicated as $C_{20}H_{32}O_2$, $C_{20:4}$ (Fig.2C). The synthesis of AA occurred through two different pathways (n-3 and n-6) (Fig.4) (Qiu et al. 2020; Shanab et al. 2018) .

1.3.1 Resources of AA

EPA and AA are known as major eicosapolyenoic fatty acids (EP) which are not found in higher plants. In higher plants, PUFAs are produced by linoleic (18:2; LA) as α-linolenic acids (18:3; α-LnA). EPA and AA are abundant in plant pathogens as lipids in oomycetes and involved as microbe-associated molecular patterns (MAMPs) (Dye et al. 2020). Red microalgae consist of a considerable amount of polyunsaturated fatty acids, including eicosapentaenoic and arachidonic acid (Dembitsky et al. 1990).

A previous study demonstrated that AA is found in high proportion in eggs, meat and poultry, and marine fish oil (Shanab et al. 2018; Vannice et al. 2014). Microbes such as algae (cyanobacteria

- blue-green algae), microalgae, macroalgae, lichens, plants and other components (marine fish oil and animal tissues) are known as sources of AA (Shanab et al. 2018).

1.3.2 Metabolism of AA

Very long-chain polyunsaturated fatty acids (VLCPUFAs) are precursors for docosanoids and eicosanoids, which are known as cell signaling molecules. AA is metabolized to eicosanoids that has inflammatory responses that can escalate harmful physiologic reactions. Various organisms, such as eukaryotic microbes, higher animals and lower plant bodies have shown the biosynthesis of VLCPUFAs in an aerobic mode. According to this pathway, AA synthesis occurs, when the oleic acid is modified to the final products of VLCPUFA (Fig. 3). When considering the n-3 and n-6 families of VLCPUFAs, there are two subtypes of synthesis pathways can be identified in phase II. The first pathway is known as the conventional pathway (Δ6 – pathway) found in eukaryotic organisms. The second pathway can be found in some microorganisms and protists. That is known as an alternative pathway (Δ8– pathway) (Fig. 4) (Qiu et al. 2020; Shanab et al. 2018).

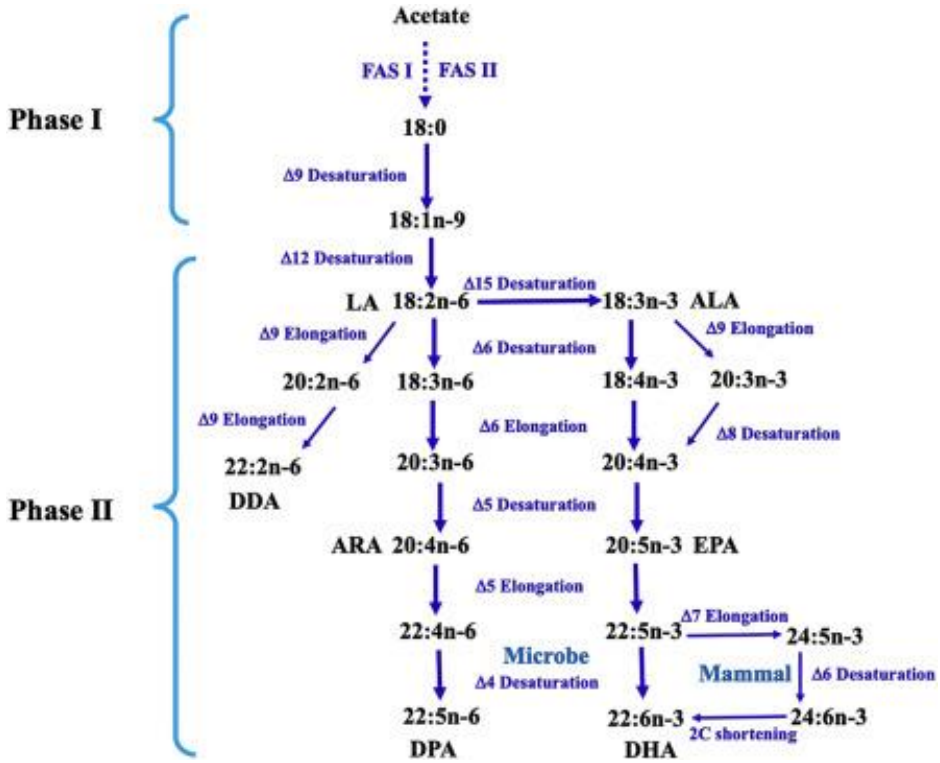


Fig 3: Biosynthesis of VLCPUFA in eukaryotes (aerobic pathway). (LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DDA, docosadienoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid) (This figure is reprinted with permission from (Qiu et al. 2020))

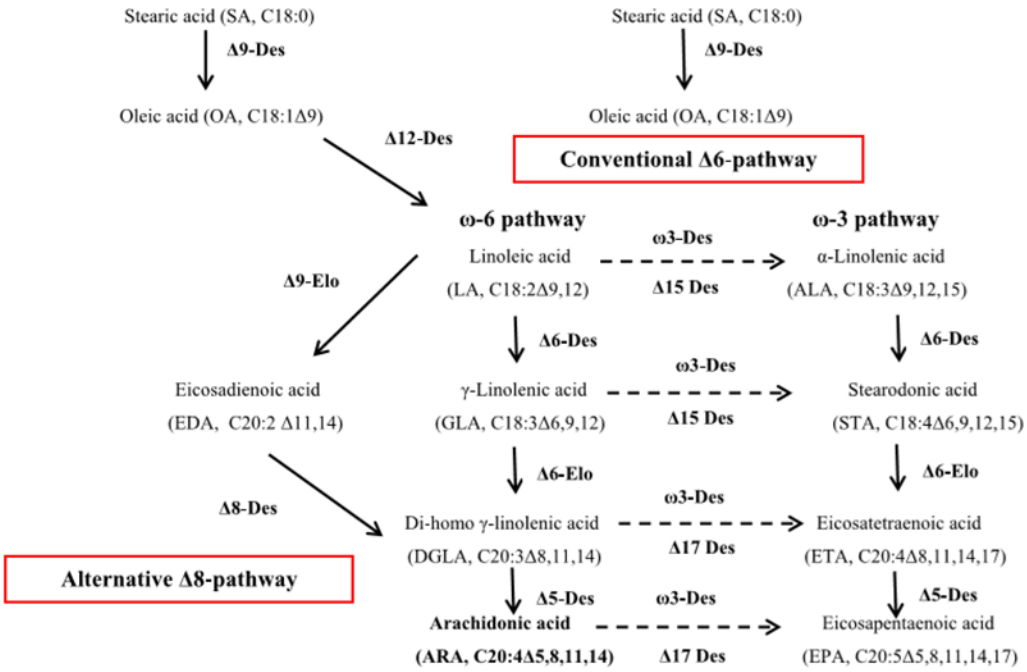


Fig 4: Conventional and alternative pathways for the biosynthesis of AA (This figure is reprinted with permission from (Shanab et al. 2018))

The starting point of AA biosynthesis is linoleic acid. The biochemical pathway of AA is indicated below (Fig. 5) (Brenna 2016).

Linoleic acid → Gamma-linolenic acid → dihomo-gamma-linolenic acid → AA

(18:2→18:3→20:3→20:4)

Fig 5: Flow chart of biosynthesis pathway of AA (This figure is reprinted with permission from (Brenna 2016))

According to the above-mentioned process (Fig. 5), FADS2 which is coding for a Δ6-desaturase that is optimizing the 3 steps are comprised in this scheme. ELOVL protein is involved to initiate the elongation system of 4- protein fatty acid chain and FADS1 that is a Δ5-desaturase enzyme involved in the last step. Here, the FADS2 gene is consists of more than 10 substrates of fatty

acids. During this process, the total catalytic flux of any given substrate and their concentrations are controlled by physiological state and levels of fatty acid content (Brenna 2016).

1.4 Requirements of AA

AA is one of the major nutritionally important VLCPUFAs (Abbadi et al. 2004). It has been estimated that it should be consumed 1.5 g of AA per day by every person (Magnifico 2020). AA is involved in various activities in the body. It is involved to construct the phospholipid structure in the membrane of the brain. AA has demonstrated activities against tumor growth, inflammatory responses, pathogenies activities for acute illnesses, such as coronary heart conditions, cell signaling, parturition and blood clotting (Brenna 2016; Shanab et al. 2018). AA and its metabolites are major elements for the activation of nervous and skeletal muscle systems. It has a specific role in our diet and its inclusion as infant milk substitutes play a significant role in infant development.

The deficiency of AA can cause many problems such as reducing fertility in adults, falling hair and anemia, and degeneration of fatty liver etc. (Shanab et al. 2018). The requirement of AA is higher than DHA in the body. For example, AA is involved as a signaling molecules. Moreover, the concentration of AA is at a higher level than DHA in a number of tissues in the body (Brenna 2016; Shanab et al. 2018). Therefore, it is important to add AA in our daily meal, especially for the infant formula to avoid listed deficiencies.

According to previous research data, AA is highly concentrated in the membrane phospholipid layer of lean meats, especially in poultry meat and pork (Li, D et al. 1998). Australia's National Heart Foundation recommended that the weekly consumption of red meat should be limited to 350 g per week due to the risk associated with the consumption of red meats. But current records have

indicated that Australian weekly red meat consumption is 455 g. It should be decreased to a low level to achieve better health conditions (Curtain et al. 2019).

1.4.1 Development of Infant nutrition

It is investigated that AA and DHA have a special role in the development of the brain and young nervous system in infants (Bouwstra et al. 2003; Schuchardt et al. 2010). When the required quantity of AA is not available in infant milk composition, that may result in repercussions, such as gaining weight (Brenna 2016).

AA and DHA are involved from the early growth periods in human development to late childhood. A considerable amount of AA in membrane cells and high AA is found in neural membranes (Brenna 2016). The European Food Safety Authority has recommended that AA is an alternative nutrient for infants even when DHA is present (Brenna 2016). Moreover, it would be harmful to provide higher DHA concentrations without providing AA for some babies (Brenna 2016). Several studies have shown that the appropriate AA supplementation as well as DHA, is required during the cognitive development in infancy, especially for the structure and functionalities in the brain and retina (Brenna 2016; Hancocks 2020; Nettleton et al. 2019). In addition, AA is involved in blood vessels, blood flow and the formation of bones functions (Hancocks 2020). But the interaction and functions of ω-6 fatty acids are poorly described, therefore it is needed to investigate thoroughly to understand the activation of the AA in the body.

According to the previous studies, only a few infants reported the capability of producing AA initiated by linoleic acid in the components of breast milk according to their requirements. Many infants obtained those from artificial sources. AA is transferred to breast milk. The average number

of daily intakes of breast milk is estimated as 836 mL/day and 41.1 mg fat/mL. Here the 161 mg (0.47% of AA) AA is transferred daily by breast to the child (Brenna 2016).

DHA and AA are transported through the placenta by using active and selective transport types in the developing infant. AA is required for normal body development during the lactation period as well as during early childhood. Due to the influence of certain fatty acid desaturase (FADS) gene polymorphisms in infants, their carrying upper levels precursors of LCPUFAs (especially LA) and lower levels of LC-PUFAs (including AA) can affect their cognitive functions. The requirement of daily intake of both fatty acids in infants is recommended into infant formulae in the instance when it is not possible to obtain breast milk. The reason for the supplementation of AA and DHA in infant formula is to enhance the responses in the immune system and to reduce the risk of being infected with the upper respiratory as well as allergic conditions. Pursuant to previous studies, researchers have recommended that it is necessary to add AA and DHA into the neonates' formula during the first year of their life. Therefore, in many countries, various studies have been conducted to understand the significance of AA and DHA for the baby's nutrient supplementation (Khozin-Goldberg et al. 2011; Nettleton et al. 2019). It has been reported that the requirement of producing a higher AA yield would be a significant factor in AA production in the future. Large-scale production has been established using *Mortierella alpina* (oleaginous filamentous fungus) to fulfill the growing requirement of the infant formula industry (Khozin-Goldberg et al. 2011). As a result, many countries comprised AA-rich oil from *M. alpina* strain for their baby formulae (Khozin-Goldberg et al. 2011). Due to the inadequate AA synthesis in premature babies, World Health Organization (WHO) and Food and Agricultural Organization (FAO) recommended including the AA in the neonates' formula as a non-breastfeeding supplementation. It is emphasized that the

importance to the development of their body systems, such as the retina and central nervous system (Shanab et al. 2018).

DSM, a reputed multinational Dutch company, produces health and nutrition products. It has recently introduced a vegetarian omega powder product (microbial DHA single cell oil B and AA single cell oil powders) that enhances infant nutrition. It is regarded as a vegetarian product that has substantial DHA ω-3 and AA ω-6. It consists of 110 mg (minimum) of AA or DHA. The quality of the product is very high that supplements neonates' formula. It is suggested that this product is safe to use as a supplement of DHA and AA when breastfeeding is not feasible (Hancocks 2020).

1.5 Microbes producing AA

There are several microorganisms that have been reported to produce AA. Some are listed in Table 1. According to the patent (Patent No.: US 7,396,548 B2), AA can be produced by Chlorophytes (*Parietochlods*), Euglenophytes (*Euglena*), Rhodophytes (*Porphyridium*) as well as certain seaweeds (Kyle 2008). Moreover, different fermentation conditions can help increase the AA yields depending on the type of strain. The composition of AA and fatty acid profile of a microbial strain will be changed according to the growth condition. The quantity of the total lipid yield is dependent on medium composition as well as it is influenced by the kind of fatty acid in the microbe (Byreddy et al. 2015). Among all microbial AA producers, non-pathogenic fungi species *Mortierella spp.* are reported as a higher AA producing microbe (about 70% of lipid content). In addition, *Flavobacterium* strain (psychrophilic bacteria) can produce 1.4-2.7% AA. *Phormidium pseudopristleyi* is the only cyanobacterial strain (79S11 and 64S01) that can produce AA at 24% and 32% of their fatty acid contents, respectively (Shanab et al. 2018).

AA has been produced by using different concentrations of 5-Aminolevulinic acid (5-ALA) (growth hormone). It was used as a promoting agent for cell growth in *Porphyridium purpureum* cell cultures (Jiao et al. 2017) (see Table 1). Researchers have shown that *Porphyridium purpureum* is the only microalgae that can produce considerable amount of AA. It has been reported that *Porphyridium purpureum* is able to synthesize 40% of the entire fatty acid content under stress culture conditions, such as pH and temperature, elevated salinity, light intensity (suboptimal) and lack of nutrients (Shanab et al. 2018). A similar microbial culture was used to optimize the aeration rate and light intensity to produce and AA. They have shown that the production of AA can be significantly improved by optimizing these factors. The highest production of AA was achieved with the light intensity of 110 μmol/m2s and aeration rate of 3L/min (Table 1). According to the previous research data, it has been shown that in the large-scale production, the maximum yield (1.37×10^8 cells/L) of *P. purpureum* was under the conditions of 2% CO_2 at 30°C with 0.8 g/L $NaHCO_3$ for the production of AA. It is reported that the application of *Porphyridium purpureum* for commercial value is limited, because of the low production yield. Therefore, it is required to search for more manufacturing procedures that can obtain more effective biomass (Li, S et al. 2020).

Various studies have been reported using *Mortierella alpina* to produce AA using glycerol as a carbon source. It was demonstrated that AA production was dependent on the fungi growth phases, and it was highly sensitive at acidic pH values. The optimum temperature for AA was determined to be 20-22°C (Mironov et al. 2018) (Table 1). A previous study evaluated the production of EPA and AA by *M. alpina* in CBS 528.72 (Centraalbureau voor Schimmelcultures (CBS)), by using solid-state fermentation (Zeinab Asadi et al. 2018). Date waste (carbon source) and soybean meal (nitrogen source) were used as substrates (Table 1). Similarly, potato chips wastes were used as a

carbon source to produce biomass, lipid and AA (Table 1) (Goyzueta-Mamani et al. 2021). In addition, different fed-batch strategies have been applied to reduce the fermentation period from 7 days to 5 days with the yield of AA-rich oil from 0.9 g/(L·d) to 1.3 g/(L·d) when compared to batch cultivation (Table 1) (Ji et al. 2014). Another study evaluated the effects of selected parameters for AA production (Malaiwong et al. 2016). They used Plackett-Burman statistical design technique and found that medium volume (% v/v) and the temperature were main parameters for AA production. Moreover, only soy isolates and glucose affected AA production (Table 1). Recently, phytohormones such as 6-benzyl adenine (BA), indole-3-acetic acid (IAA), furfuryl adenine (KT), indole-3-butyric acid (IBA), gibberellin (GA) and abscisic acid (ABA) were used to improve AA biosynthesis by using *M. alpina* (Zhang et al. 2019) (Table1). Apart from that bacterial strains (*Aureispira maritima*) have been used to produce AA by optimizing the fermentation conditions (Table 1).

Table 1: Summary of various microbes used for AA production

Microbial Strains	Promoting agents in AA biosynthesis	AA Yield	Reference
Aureispira maritima **(Bacteria)**	• Tryptone • Temperature • pH • Agitation rate	1.95 g/L	(Saelao et al. 2011)
Mortierella alpina **(Fungi)**	• Fed-batch strategy	6.8 g/L	(Ji et al. 2014)
Porphyridium purpureum **(Red microalga)**	• Aeration rate • Light intensity	115.47 mg/L	(Ludevese-Pascual et al. 2016)
Mortierella alpina **(fungi)**	• pH value • Temperature • Percentage of glucose and medium volume per flask volume (% v/v) • KNO_3 • K_2HPO_4 • Soy isolate concentrations	6.76 g/L	(Malaiwong et al. 2016)
Porphyridium purpureum **(Red microalga)**	• 5-Aminolevulinic acid	170.32 mg/L	(Jiao et al. 2017)
Mortierella alpina **(fungi)**	• pH	25.2 % (20 ^{0}C)	

	• Temperature		(Mironov et al. 2018)
***Mortierella alpina* (fungi)**	• Substrate particle • Soybean oil % • Linseed oil% • Substrate initial moisture% • pH • heating pretreatment • Seed age • C/N • Temperature • Incubation time • Mass on 4th day	4.66 mg/g (Plac.Burma) (14.37±0.06) (RSM)	(Zeinab Asadi et al. 2018)
***Mortierella alpina* (fungi)**	• Phytohormones (BA, IAA, KT, GA, IBA, ABA)	3.35 (15 mg/L BA)	(Zhang et al. 2019)
***Mortierella alpina* (fungi)**	• Batch fermentation • Wastes (potato chips)	Batch fer- 40% optimized culture medium+ yeast	(Goyzueta-Mamani et al. 2021)

1.5.1 Microbial production of AA based on cost-effective substrates

There are many advantages of using heterotrophic culture compared to phototrophic growth conditions. For instance: it is required to supply lighting in the phototropic growth, compared to heterotrophic cultivation. Also, the cellular densities are produced higher in the heterotrophic mode (Harel et al. 2002). In addition, it is necessary to optimize physical conditions, such as salinity, temperature, light, etc. for the growth of microorganisms. It has been proved that nitrogen sources have s more considerable effect on the production of fatty acids. Lack of nitrogen requirement can inhibit the cellular growth and synthesis of substances. Here, glucose and glycerol can be used as carbon sources (Li, S et al. 2020). Rice straw, wastewater (barley distillation), crude glycerol, soybean cake, liquid residues potatoes, and beer manufacturing industries broth (Singh et al. 2020) can be used as a carbon source for economizing AA production cost.

In the present research work, it was hypothesized "Can AA yields be improved through heterotrophic fermentation to facilitate the adoption of single cell oils for infant/vegan nutrition?"

To prove the given hypothesis, the present study investigated various culture parameters of heterotrophic marine protists to produce AA in an environment-friendly way that may be suitable for vegetarians and infants.

The following objectives of this study were pursued:

i) Production of AA through marine protist thraustochytrid S2 by fermentation and

ii) Enhancement of AA yield through heterotrophic fermentation by medium and process optimization.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Various chemicals such as glucose, glycerol, yeast extract, bacteriological peptone, sodium chloride (NaCl), ethanol, phytohormones such as 6-benzyl adenine (BA), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Furfuryl adenine/ Kinetin (KT), (Sigma Aldrich pty. Ltd., Australia) and sea salt (Instant Ocean, USA) were used for biomass production whereas, solvents such as hexane, methanol, chloroform, acetyl chloride, toluene, sodium chloride, potassium bicarbonate, anhydrous sodium sulphate, were all of analytical grade (Merck) which were used for lipid extraction. All the chemicals used in this research were of analytical and HPLC grade.

2.2 Microorganisms

An in-house microalgal strain (Thraustochytrid sp-S2 strain isolated from SA marine waters and known for its high lipid content) was used throughout this study. This axenic strain was provided by my co-supervisor Dr. Adarsha Gupta. The strain was maintained in 100-mm sterile petri dishes containing solid Agar medium. The agar medium had following composition (g/L): 10 agar, 10 glucose, 1 yeast extract, 1 bacteriological peptone, and 50% artificial seawater (ASW). The plates were kept at 22 ^{0}C.

2.3 Fermentation methods

2.3.1 Inoculum preparation

The inoculum (seed culture) was prepared in a medium consisted of (g/L); 5 glucose, 2 yeast extract, 2 bacteriological peptone, which were dissolved in 50% ASW pH 6.5 in 100 ml flask. The medium was sterilized in an autoclave (121 ^{0}C, 20 min). After sterilization, a loop of axenic thraustochytrid strain from agar plate was inoculated to the flask. The flasks were incubated for 48 hours (25 ^{0}C, 150 rpm). The inoculum was observed under electron microscope (40x) to check for

any contamination. The absorbance was measured at 610 nm. When the absorbance reached Optical density of 1, then the cells were inoculated to the production medium.

$$Weight\ of\ glucose\ = \frac{0.5\ g}{100\ ml} \times 20ml\ = 0.1\ g$$

$$Weight\ of\ yeast = \frac{0.2\ g}{100\ ml}\ \times 20ml\ = 0.04\ g$$

$$Weight\ of\ peptone = \frac{0.2\ g}{100\ ml} \times 20\ ml\ = 0.04\ g$$

ASW was prepared by adding 33.33 g of Instant Ocean Sea Salts in 1L of Milli.Q. water.

2.3.2 GYP Medium preparation

The fermentation was performed in a shake flask containing medium components glucose, yeast, and peptone (GYP) (Singh et al. 2020). To determine the effect of physical and chemical parameters, various physical (pH(Malaiwong et al. 2016; Mironov et al. 2018), temperature, agitation and aeration) and chemical parameters (carbon and nitrogen source) were optimized to maximize the biomass and AA production. The GYP medium contained 5% glucose, 0.4 % yeast, 0.04% peptone, and 50% ASW. Medium was sterilized in an autoclave (121 ^{0}C, 20 min). Glucose was separately filtered sterilized and added to the fermentation medium after autoclave. Then, the flasks were incubated for 5 days (25 ^{0}C, 150 rpm) and checked regularly for contamination.

2.3.3 Algal biomass harvesting and freeze drying

The heterotrophically grown marine protist cells were harvested by centrifugation (10,000 rpm, 10 minutes), supernatant was removed, and the pellet was freeze dried (48 hours) to obtain cell dried biomass. The resulting pellet was washed with water to remove traces of any contaminants. Dry weight of freeze dried cell pellet was measured and stored at −20°C (Singh et al. 2020). These dried cells were used for further fatty acid analyses.

2.3.4 Lipid extraction and estimation

The lipid extraction was carried as per the established method using various solvents (Gupta et al. 2016). The dried cell biomass (10 mg) was extracted in chloroform-methanol solution (chloroform: methanol-; 2:1) according to Bligh and Dyer's method (Jiao et al. 2017; Su et al. 2016). They were vortexed for 2 min. Then, the solution was centrifuged (10 000 g for 10 minutes) (Su et al. 2016). This extraction was repeated 3 times and supernatants (x3) were collected in pre-weighed glass vials (clear, 12 mL) (after filtration with 0.22 µm filter). Then solvent containing lipids (supernatant) dried in heat-block (50 ^{0}C) to obtain oil weights. Lipid content was determined gravimetrically.

2.3.5 Fatty Acid Methyl Esters (FAMEs) and Gas chromatography analysis

The dried lipid samples were trans-esterified to prepare fatty acid methyl esters for GC analysis (Gupta et al. 2016). Here fatty acids were converted to methyl esters by using acid-catalyzed trans-esterification process (Byreddy et al. 2015). First, 500 µl of toluene was added to the dried lipids followed by the addition of 10 µl internal standard (50 mg methyl nonadecanoate in 10 ml toluene) and 200 µL antioxidant (100 mg BHT in 100 ml toluene). Then, 500 µL of acidic methanol (containing acetyl chloride; the methanol reagent was prepared by adding 1 ml acetyl chloride dropwise to 10 ml methanol on ice. The methanol reagent was stirred for at least 1 hour). The solution was kept overnight at 50°C to react in a capped glass vial (clear, 1.5 mL vials, silicon capped).

After cooling the solutions to room temperature, 1 ml sodium chloride solution (5% w/v in Milli.Q. water) (for washing) and 1 ml hexane were added (lipids are miscible in hexane layer). The hexane layer (supernatant) was collected, and it was washed 1 ml potassium bicarbonate solution (2% w/v in Milli.Q. water). Hexane layer was removed and dried it over sodium sulfate. The samples were

kept in GC vials (clear, 1.5 mL, with silicon caps, Agilent) (If necessary, hexane layer was evaporated to concentrate the FAMEs).

Fatty acid analysis was performed on a Shimadzu GC 2090N (FAMEWAX column, 30 m x 0.32 mm ID (inner diameter) equipped with flame ionization detector (FID) and connected to a BID 2030 unit (Shimadzu) for identification of fatty acid methyl esters (FAME) (split injection, 1/100). Helium was used as the carrier gas.

The discharge gas rate was set at 50 mL/min. The oven program was programmed at 170°C and ramped to 200°C (at a rate of 5°C per min) and final increment to 240°C (at a rate of 10°C per min) and held at 240°C for 10 min. The detector temperature was held at 240°C. Total run time was 20 min. Sample volume of 1 μL was injected. Fatty acids are quantified by comparison of peak areas of authentic FAMEs standard (Sigma Aldrich CRM47885).

2.4 Experiment layout followed in running experiments

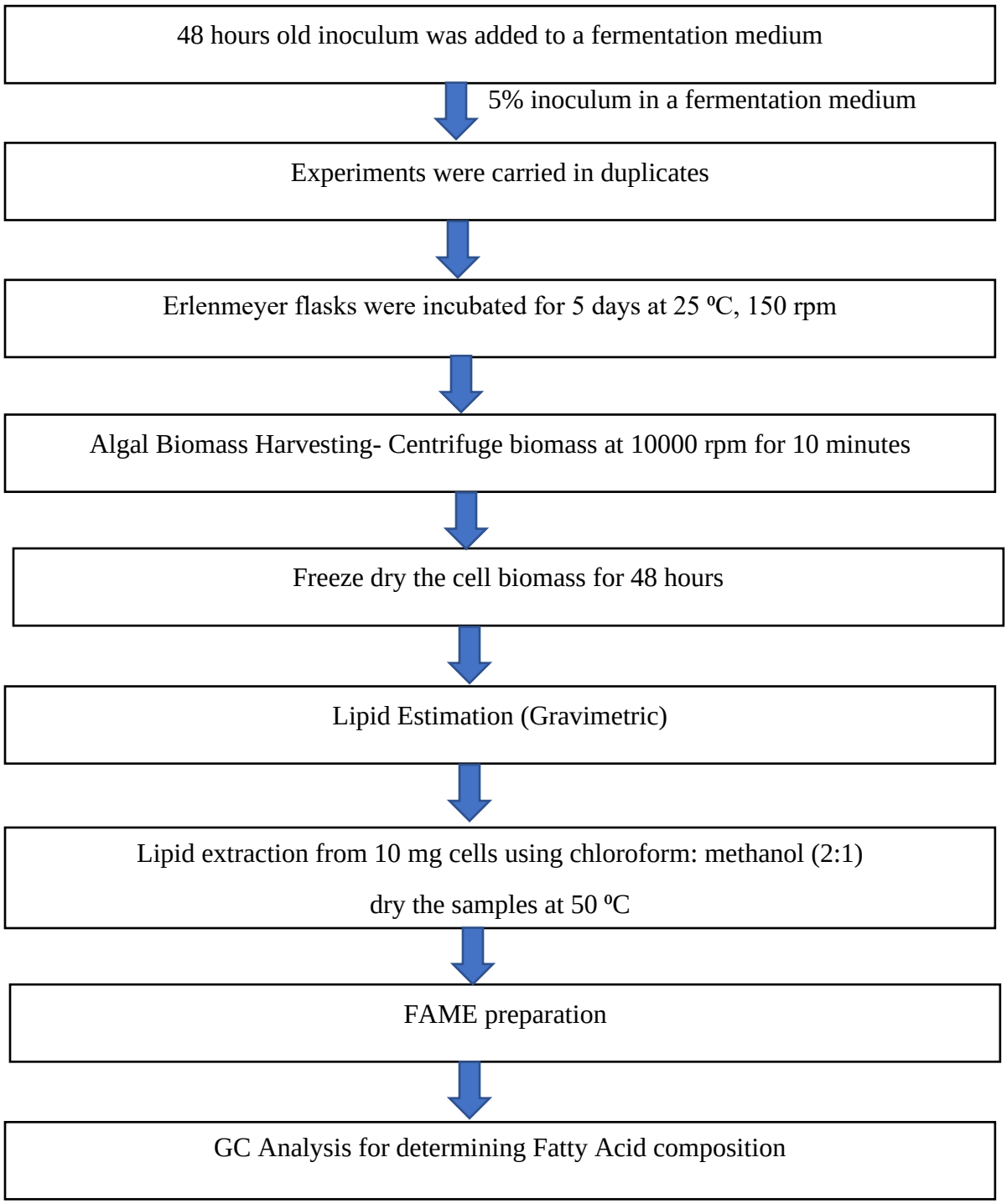


Fig 6: Basic Experimental Design followed throughout the carried research work.

2.5 Estimation of reducing sugars

The glucose concentration in the fermentation broth was estimated by **3,5 dinitrosalicylic acid** (DNS) assay (Miller 1959). The procedure was followed as mentioned below:

A: Preparation of DNS Reagent (100 mL)

Schott bottle was covered with aluminium foil and 1.6g NaOH and 40ml of Milli.Q. water were added. Then the solution was kept stirring on a magnetic stirrer. 1g of DNS was added slowly into the NaOH solution. Then 30 ml of Milli.Q. water was added. After that 3g of Potassium sodium tartrate tetrahydrate (Rochelle salt) were added and 30ml of water was added to make final volume 100 mL with the constant stirring on magnetic stirrer (DNS reagent bottle should be covered with aluminium foil all the time as DNS is light sensitive and may crystallize around the top of the bottle).

B: Preparation of glucose dilutions

Glucose stock solution (1%, w/v) was prepared by dissolving 100 mg glucose in 100 ml Milli.Q. water and it was stored at 4 ^{0}C. The glucose standards (100-800 ug/ml) were prepared in tubes as detailed in table (Appendices A-1).

C: Conducting DNS assay

Glucose concentrations (100-800 ug/ml) were prepared in 10 mL test tubes by making dilutions with Milli.Q water. DNS reagent (1 ml) was added to all test tubes, and they were boiled for 10 minutes, cooled immediately on ice and the absorbance was measured at 540nm using a UV-VIS Spectrophotometer (Pginstruments, T70, UK). The standard curve was prepared by plotting glucose concentration against absorbance at 540nm.

The fermented broth samples were harvested at 24 h interval and analysed for reducing sugar concentration. Before the fermentation, sample was collected from a control flask to quantify initial glucose concentration.

2.6 Production of AA through heterotrophic fermentation

Several fermentation conditions were tested in this study to improve the AA 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.

2.6.1 Effect of pH on biomass, lipid and AA production: The fermentation medium was composed of glucose (5%), yeast extract (0.4%), peptone (0.04%) and ASW.

pH of the fermentation medium was maintained or fixed by using either 0.2 M Hydrochloric acid (HCl) or 0.2M NaOH (Sodium Hydroxide). pH was determined using pH meter (OHAUS, Starter 3100, USA). The pH (6.9) was not changed in the control flask.

When using glycerol as a carbon source in the fermentation medium, 5% of glycerol concentration was used.

Calculations

$$Total\ volume\ of\ flasks = 200\ ml$$

$$Weight\ of\ glycerol = \frac{5g}{100\ ml} \times 200\ ml = 10g$$

After confirming the optimized pH value (6.5), pH of media in rest of the experiments were set at pH 6.5.

2.6.2 Effect of temperature on AA production: The fermentation medium containing glucose was kept at various temperatures (22-28 ^{0}C) to investigate changes in biomass, lipid and AA production.

2.6.3 Effect of carbon source on AA production: The glucose (5%, w/v) and glycerol (5%, v/v) were used in the fermentation medium to investigate changes in biomass, lipid and AA production.

2.6.4 Effect of agitation rate on AA production: The effect of improved aeration on AA production was investigated by carrying fermentation at variable agitation rates (120- 200 rpm).

2.6.5 Effect of salt concentration (NaCl) on AA production: Various concentration of salt (5,10,20,30,40 and 50 g/L) was added to the fermentation medium. The salt was dissolved in a water. The changes in biomass, lipid and AA production were monitored as flasks were kept at 25 ^{0}C, 150 rpm. Here one flask was kept as control (without adding NaCl).

2.6.6 Effect of yeast extract concentration on AA production: Different concentrations (low to high) of yeast were tried in the fermentation medium. The fermentation medium contained 5% glucose, 0.04% peptone, and 50% ASW. The changes in biomass, lipid and AA production were monitored as flasks were kept at 25 ^{0}C, 150 rpm.

2.6.7 Effects of phytohormones on AA production: Various phytohormones, 6-benzyl adenine (BA), indole-3-acetic acid (IAA), furfuryl adenine (KT) and indole-3-butyric acid (IBA) were used to analyze their effect on AA production. The details of phytohormones and their respective concentrations are provided in Table 2. Phytohormones such as IBA and KT were dissolved in Milli.Q. water. However, BA was dissolved in ethanol (100%). A flask containing ethanol (20 mg/ml) in the fermentation medium was kept as control to determine the effect of ethanol alone on AA production.

Table 2: Tested concentrations of various phytohormones and their added volumes

Phytohormones concentration (mg /L)	Volume (μl) of hormone added to fermentation medium (20 mL)
1	2
2.5	5
5	10
10	20
15	30
20	40
Control	0
Ethanol (20 mg/L)	40

2.6.8 Effects of feeding of a precursor rich in unsaturated fatty acids on AA production: Influence of Linseed oil (procured from the supermarket, Coles Wellness Road Organic Flaxseed oil) in improving AA production was investigated. Various concentrations (0.25-2 %, v/v) of linseed oil were added into the fermentation medium and the flasks were kept at 25^0 C and 150 rpm for 120 h.

2.6.9 Effect of carbon feeding (fed-batch) on biomass production: The effect of biomass concentration and glucose consumption on AA production were investigated when heterotrophic fermentation was carried for 120 hours. The samples were withdrawn from the flask at regular intervals of time (24h). The samples were analyzed for microbial growth by measuring Optical Density at 660 nm, and for glucose consumption and lipid production. When analyzing growth

profile, due to high OD values of more than 1 in later days of the growth, all samples were diluted to 1:10. Graph was plotted against incubation time and absorbance. When glucose concentration was depleted during fermentation, fresh glucose (2.5% or 5% w/v) was fed into the medium.

All experiments were carried in duplicates. The results are presented as mean standard deviation (SD) of duplicates repeated twice.

CHAPTER 3

RESULTS AND DISSCUSION

Microbial oils are referred to as Single cell oils (SCOs) since these are produced by various microbes (bacteria, fungi and yeast) for commercial applications. AA and DHA are the main commercial elements of SCOs that are now used extensively as dietary supplements in infant formulas (Ratledge 2004). Thraustochytrids are marine protists that are known to produce signature fatty acid molecules such as DHA, EPA and DPA (Ludevese-Pascual et al. 2016). In this study, various physical and chemical parameters were optimized to increase the biomass, lipid and AA production through a novel thraustochytrid strain S2.

3.1 Identification of thraustochytrids

3.1.1 Thraustochytrids morphology

The cell morphology was observed using an optical microscope (Nikon Eclipse Ts2R Inverted Research Microscope). The cells of different sizes forming clumps (53.44μm; as shown in Fig 7) and some single cells (10.78 μm; lower corner of Fig 7) were observed under a microscope (40x) magnification. Thraustochytrids are single celled organisms that reproduce asexually by the production of zoosporangia (Bongiorni et al. 2005). A defined cell wall was observed in a microscope monitor (based on a capability embedded in the software, Nikon, Japan), indicating no contamination. The thraustochytrids are ubiquitously distributed marine heterotrophic organisms, belonging to *Labyrinthulomycetes*, and found commonly in coastal waters. They comprise nine genera out of which *Thraustochytrium*, *Aurantiochytrium*, *Schizochytrium* and *Ulkenia* are the most investigated (Liu et al. 2017; Marchan et al. 2018).

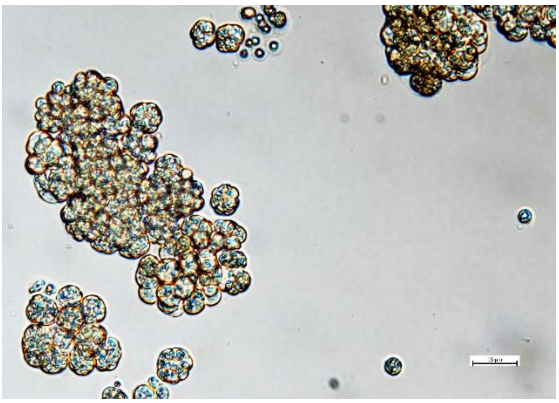


Fig 7: Microscopic image of thraustochytrid strain S2 at 40x magnification (3 days old culture)

The growth of thraustochytrids strains was observed before inoculation to rule out any contamination. If there was any contamination, the inoculation to the fermentation medium was not pursued.

3.1.2 Presence of AA in Thraustochytrid strain

This study was conducted to achieve optimum culture conditions in thraustochytrid fermentation to enhance biomass, lipid and AA production.

3.1.3 Growth profile of in-house heterotrophic microalgal strain S2 (thraustochytrid)

It was observed that the cell growth increased and reached the maximum on the 5^{th} day for AA production. The growth was initially low with the corresponding OD on 1^{st} day which is regarded as a lag phase. The log phase started after 2^{nd} day of fermentation which lasted until 4^{th} day. On 5^{th} day, the biomass growth stabilized indicating the end of the log phase (Fig .8). Previous researchers have investigated that the rate of the cell growth of *Thraustochytrium* sp. started slowly and low OD was observed after this stage (stationary phase) and cells started to decline (death phase) (Arafiles et al. 2011). It was observed during fermentation that glucose concentration reduced after

24 hours. The glucose consumption showed a very high rate during 0-24h (1st day). The glucose was consumed almost completely by the 5th day (Fig .8). Previous researchers have evaluated the rate of the substrate consumption of *Mortierella alpina* where glucose was consumed completely by 6th day (Wu et al. 2017)

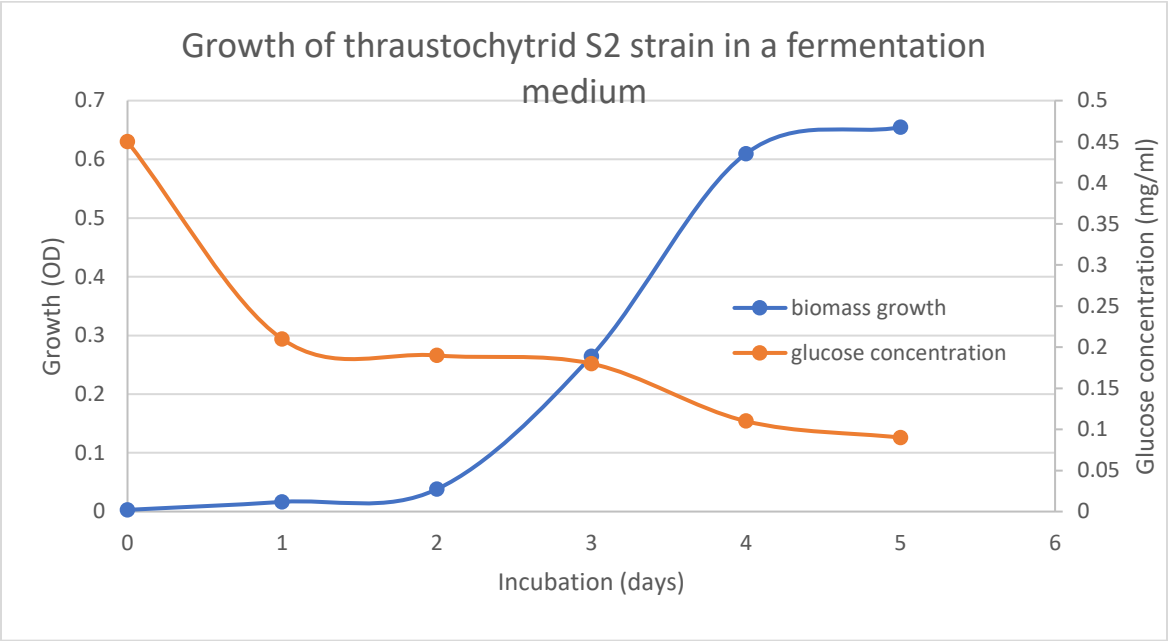


Fig 8: Growth profile of Thraustochytrid strain (S2 strain) when grown in a glucose medium.

3.2 Effect of pH on biomass, lipid and fatty acid composition of Thraustochytrid strain S2

3.2.1 Effect of glucose as a sole carbon source

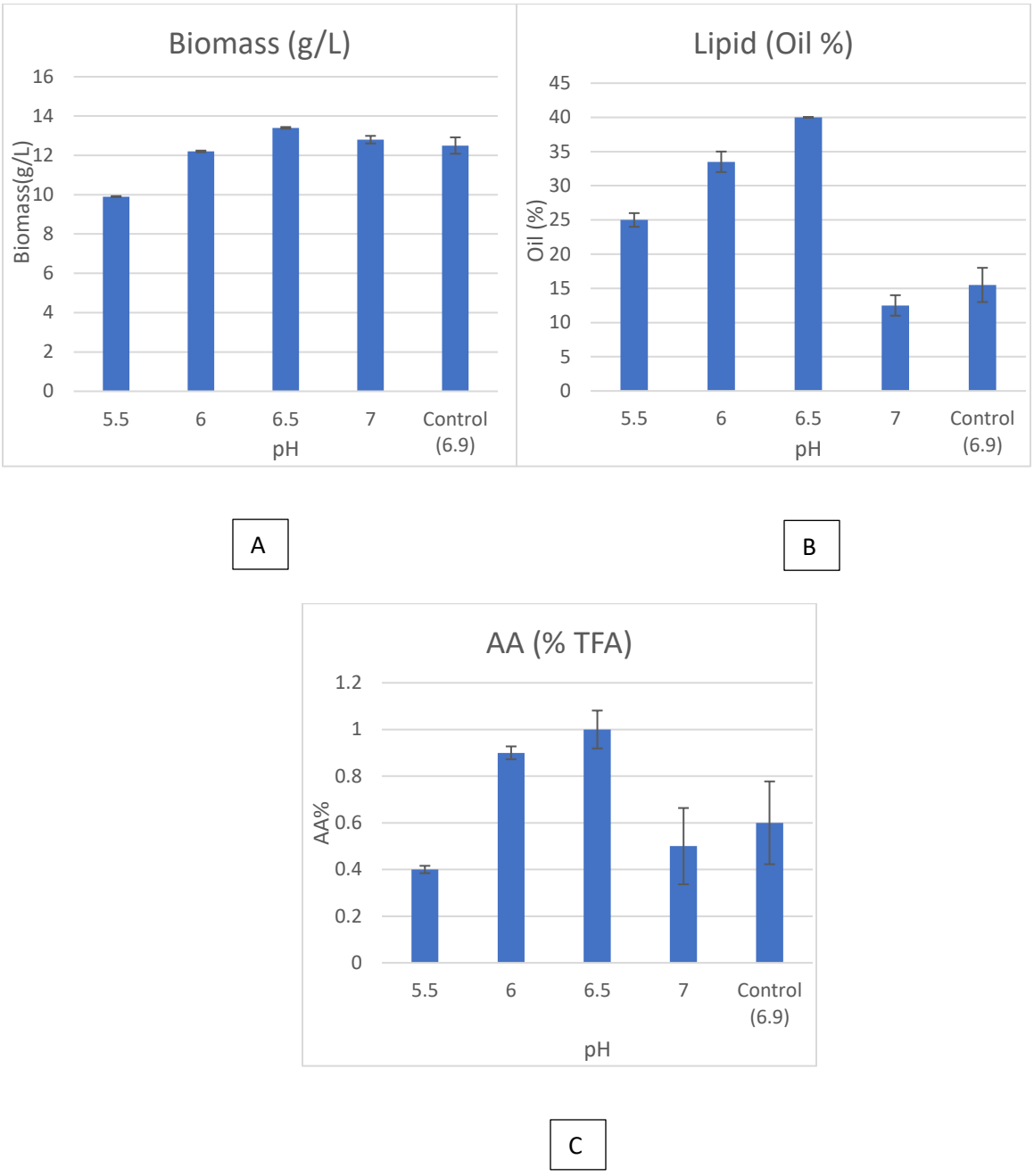


Fig 9: Profile of dry cell weight (DCW) (A), lipid content (B), and AA composition (C) of thraustochytrid strain S2 as a function of pH optimization. Data are presented as mean ± SD of duplicates.

Upon using glucose as a sole carbon source, highest biomass (13.4 g/L), lipid (40 %) and AA (1 %) production was observed. The production of AA increased in parallel with the cell growth and maximum AA yield was achieved at pH 6.5.

Table 3: Fatty acid profile of Thraustochytrid strain S2 under optimized pH conditions (glucose substrate)

	Fatty acid (% of total fatty acids)									
pH values	14:0	15:0	16:0	18:0	18:1	18:2	18:3	20:5	22:5	22:6
5.5	6.8	19.5	15.8	1.3	0.2	-	0.1	1.4	8.6	17.1
6	6.5	5	31.7	1.6	0.3	-	0.2	1.2	8.3	21.4
6.5	9	9.4	24.4	2.2	0.6	-	0.1	0.7	7.9	14.8
7	8.1`	23.1	21.2	1.5	0.5	-	0.05	0.7	5.9	12.4
Control (6.9)	7.8	7.2	30.3	2	0.3	-	0.1	1.6	9.9	23

14:0 (myristic acid), 15:0 (pentadecanoate), 16:0 (palmitic acid), 18:0 (stearic acid), 18:1((oleic Acid), 18:2 (linoleic acid), 18:3 (alpha-linolenic acid, ALA), 20:5 (EPA), 22:5 (docosapentaenoic Acid, DPA), 22:6 (docosahexaenoic acid, DHA).

Highest proportion of Palmitic acid (31.7 % of TFA) was recorded at pH 6 (Table 3). It was interesting to find out that at pH 5.5 and 7, pentadecanoate acid was observed to be highest in proportion (% of TFA) (Table 3).

3.2.2 Effect of glycerol as a sole carbon source

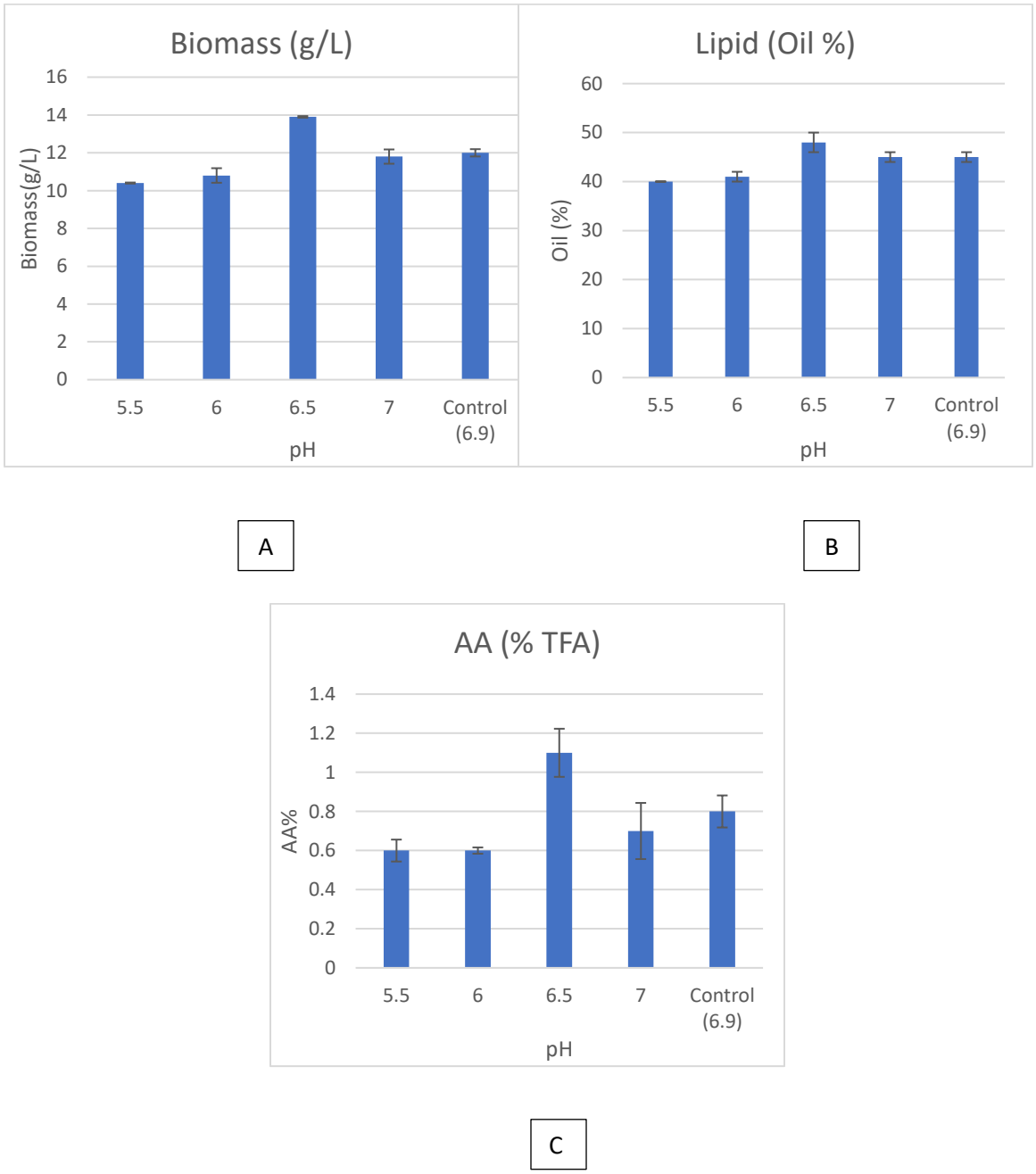


Fig 10: Profile of dry cell weight (DCW) (A), lipid content (B), and AA composition (C) of thraustochytrid strain S2 as a function of pH optimization (glycerol substrate). Data are presented as mean ± SD of duplicates.

When glycerol was used as the sole carbon source, the production of AA increased in parallel with the cell growth. The highest biomass was obtained at 13.9 g/L and lipid and AA were accounted as 48% and 1.1 % respectively at pH 6.5 (Fig .10).

Table 4: Fatty acid profile of Thraustochytrid strain S2 under optimized pH conditions (glycerol substrate)

	Fatty acid (% of total fatty acids)									
pH values	14:0	15:0	16:0	18:0	18:1	18:2	18:3	20:5	22:5	22:6
5.5	7.6	19.1	14.9	1.4	0.3	0.03	0.1	1.6	7.6	15.5
6	8.7	22.2	17	1.7	0.7	0.03	-	1	7.9	12.4
6.5	9.8	12.2	25.2	1.9	0.2	-	-	1.5	6	13
7	6.8	5.9	28.7	1.9	0.2	-	0.03	0.5	8.3	21.9
Control (6.9)	9.2	8.9	33.3	2.3	0.4	0.03	0.1	0.8	7.1	16.2

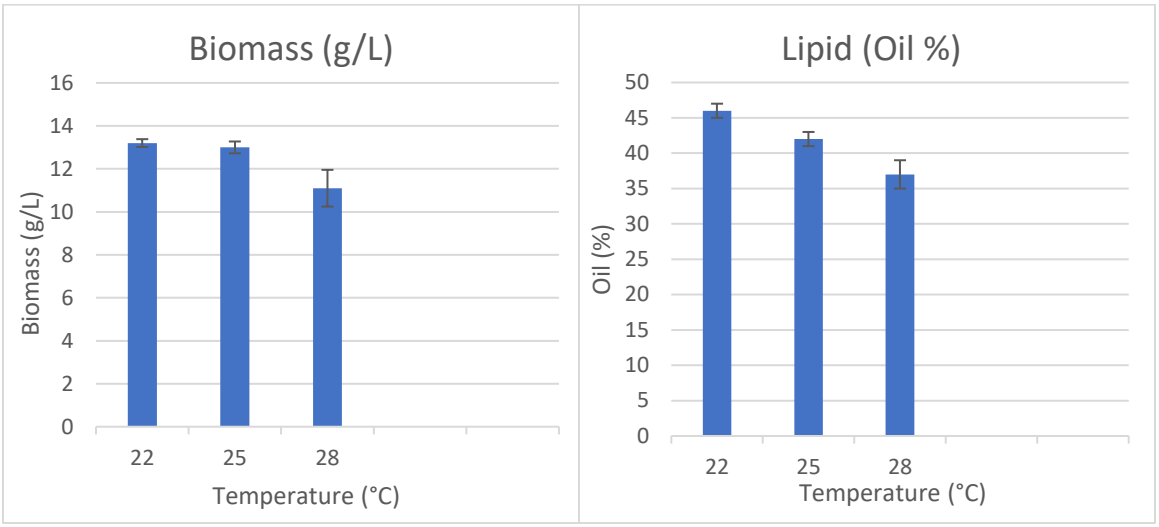
Highest proportion of Palmitic acid (33.3 % of TFA) was recorded at pH 6.9 (Table 4). It was interesting to find out that at pH 5.5 and 6, pentadecanoate acid showed highest proportion (% of TFA) (Table 4).

This present study showed that the dry cell weight (DCW), lipid accumulation and fatty acid composition of the Thraustochytrid strain are dependent on the pH of the fermentation medium. It should be noted that S2 strain showed good growth and accumulated AA under mildly acidic conditions (pH 6 – 6.5). Similar studies have been conducted with *Mortierella* species (fungi) where maximum biomass, lipid and AA production were obtained at different pH values. In an

acidic pH medium, biomass production is suppressed leading to low lipid yields (Mironov et al. 2018). The production of AA was highly sensitive to acidic pH values (pH 3), resulting in the lowest yields. This is in agreement with the present study where low yields of biomass, oil and AA were obtained at low pH value (5.5). Low pH could be inhibitory to the fatty acid enzymes involved in AA synthesis. The activation of desaturases is required to produce unsaturated FA. For example, stearic acid is converted to oleic acid (OA) by delta-D-9-desaturase and OA is converted to linoleic acid by delta-D-12-desaturase and leading to the synthesis of AA after the action of a complex of enzymes (Mironov et al. 2018).

It can be suggested that at low pH values, the activity of delta-D-12 desaturase and the complex of enzymes responsible for the conversion of linoleic acid (C18:2) into AA (C20:4) were inhibited, which resulted in the lower AA synthesis. Our present study found that after pH 6.5; AA and lipid were simultaneously decreased with the decrease in biomass.

3.2.3 Effect of temperature on biomass, lipid and fatty acid production



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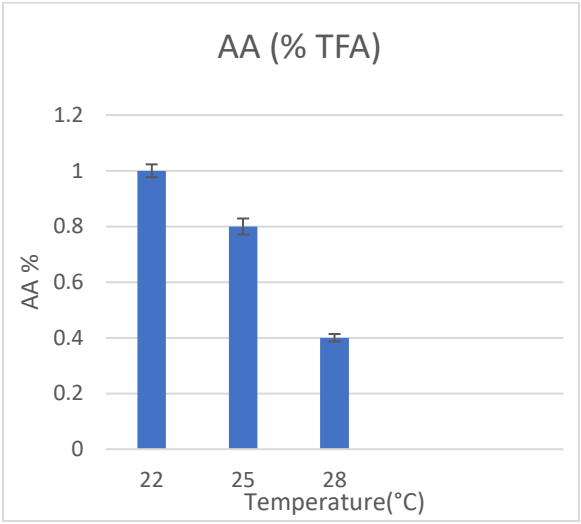


C

Fig 11: Profile of dry cell weight (DCW) (A), lipid content (B), and AA composition (C) of thraustochytrid strain S2 as a function of temperature. Data are presented as mean ± SD of duplicates.

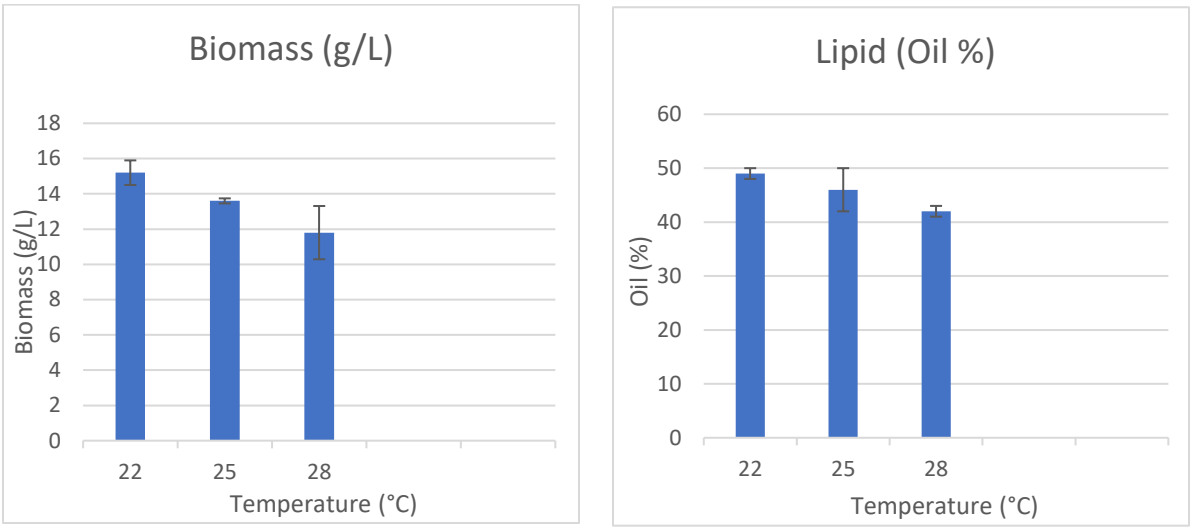
Highest biomass (13.2 g/L) and lipid content (46%) were obtained when the strain was grown in a glucose medium at 22 ^{0}C leading to AA accumulation (1% of TFA) (Fig .11). When the fermentation was carried at 28 ^{0}C, low biomass and AA content (0.4%) was observed than the biomass grown at 22 ^{0}C and 25 ^{0}C as 1% and 0.8% respectively.

Table 5: Fatty acid profile of Thraustochytrid strain S2 under optimized temperature conditions

	Fatty acid (% of total fatty acids)								
Temperature (^{0}C)	14:0	16:0	18:0	18:1	18:2	18:3	20:5	22:5	22.6
22	10.5	22.5	2.1	0.2	0.06	0.04	0.8	6.9	15.4
25	9	24.4	2.2	0.6	-	0.1	0.7	7.7	14.8
28	8.6	27.3	2.5	0.2	-	0.06	0.6	7.5	12.8

Highest proportion of Palmitic acid (27.3 % of TFA) was recorded at 28 ^{0}C (Table 5).

3.2.4 Using glycerol as sole carbon source



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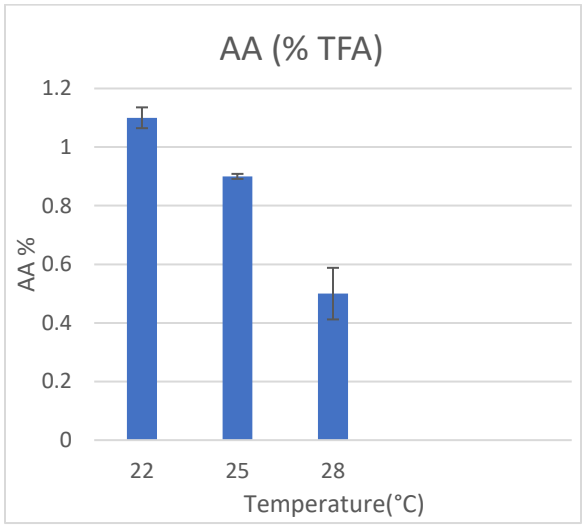


C

Fig 12: Profile of dry cell weight (DCW) (A), lipid content (B), and AA composition (C) of thraustochytrid strain S2 as a function of temperature (glycerol substrate). Data are presented as mean ± SD of duplicates.

Highest AA (1.1% of TFA) was obtained at 22 ^{0}C, with maximum DCW (15.2 g/L) and lipid content (49%) (Fig 12).

Table 6: Fatty acid profile of Thraustochytrid strain S2 under optimized temperature conditions (glycerol substrate)

	Fatty acid (% of total fatty acids)								
Temperature (^{0}C)	14:0	16:0	18:0	18:1	18:2	18:3	20:5	22:5	22.6
22	11.5	20.3	2	0.2	0.04	0.2	1.6	7.2	17
25	9.2	33.4	2.3	0.4	0.03	0.05	0.8	7.1	16.2
28	8.1	37.8	2.9	0.6	0.02	0.04	1.1	7.6	14.3

Highest proportion of Palmitic acid (37.8 % of TFA) was recorded at 28 ^{0}C (Table 6).

Glycerol as a carbon substrate showed no considerable change in the DCW of *M. alpina* (Mironov et al. 2018). However, the increase in temperature resulted in lipid accumulation (highest at 28 ^{0}C and lowest at 20–22 ^{0}C) in the given fungi. An optimum temperature for the growth of fungal strains was observed to be 20 ^{0}C, which was similar to our findings (optimum temperature at 22 ^{0}C). In fungus *Mortierella* , the AA content decreased with an increase in the temperature as observed in this study (Mironov et al. 2018). When glucose was used as a carbon source, the synthesis of PUFAs increased with the decrease in the temperature leading to improved omega-3/6 production (Gupta et al. 2016). Low temperature favors activation of PUFA synthesis enzymes

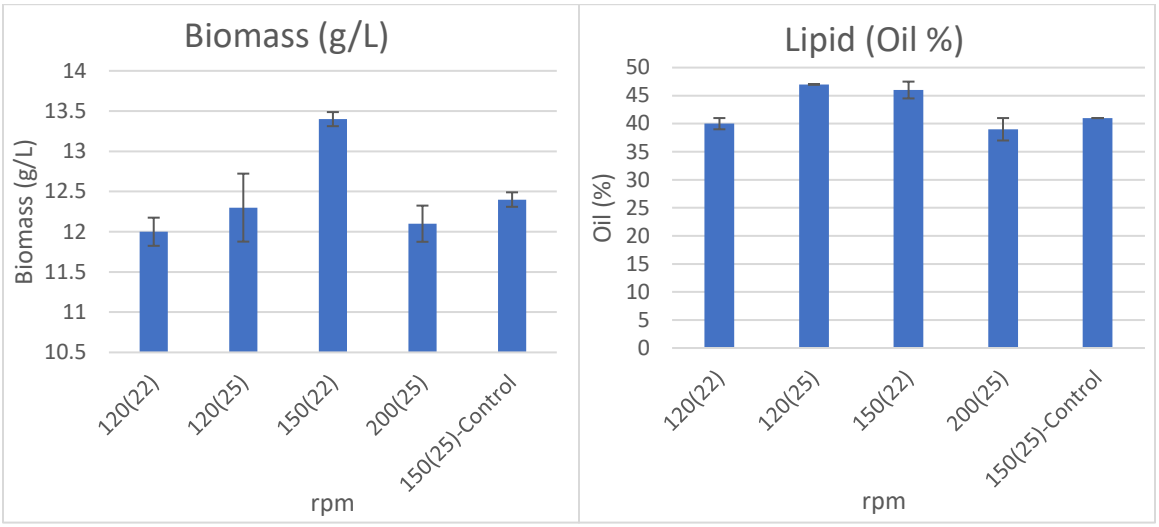
thus supporting the accumulation of EPA and stearic acid in the total lipids, a phenomenon observed in thraustochytrid strains collected from the Antarctic region (Shene et al. 2020).

In this study, we observed that the thraustochytrid's response to lipid accumulation and fatty acid composition of strain S2 was strongly dependent on temperature. This could be due to the effect of temperature on genes expression which is involved in the fatty acids metabolism (Paredes et al. 2020). The number of differentially expressed (DE) genes associated with the metabolism of lipids in thraustochytrid isolates has been investigated and it was found that many DE genes got stimulated by the low temperature (Paredes et al. 2020).

When considering the AA production, linoleic acid (LA) is a precursor of n-6 pathway which is synthetized from palmitic acid (PA, C16:0). It is produced from the fatty acid synthase (FAS) pathway. D9- as well as D12-desaturases are the associated genes that are coding FAS. At the lower temperature, the total lipids associated with DHA and EPA increased, because of the upregulation of the genes coding for a Δ5-desaturase as well as Δ5-elongase (Paredes et al. 2020).

An easily assimilable substrate is considered essential to produce AA through microbial fermentation (Dedyukhina et al. 2012; Mironov et al. 2018). In the pH and temperature experiments, glucose and glycerol were used as carbon sources to produce biomass and AA. Among them, glycerol showed more growth of biomass, oil as well as AA production as 15.2 g/L, 49% and 1.1 % respectively at pH 6.5 and 22 ^{0}C (Fig .12). But when comparing with pH data, glucose substrate resulted in more biomass than glycerol at pH 6, 7, and control (pH 6.9) (Fig .9 and Fig .10). In a previous study, glucose was demonstrated to be a better carbon source for AA production using *Mortierella elongate* species (Yamada et al. 1987). Thus, glucose was used as a sole carbon source for the rest of the experiments.

3.3 Agitation effect on biomass, lipid and fatty acid composition of Thraustochytrid strain S2



A

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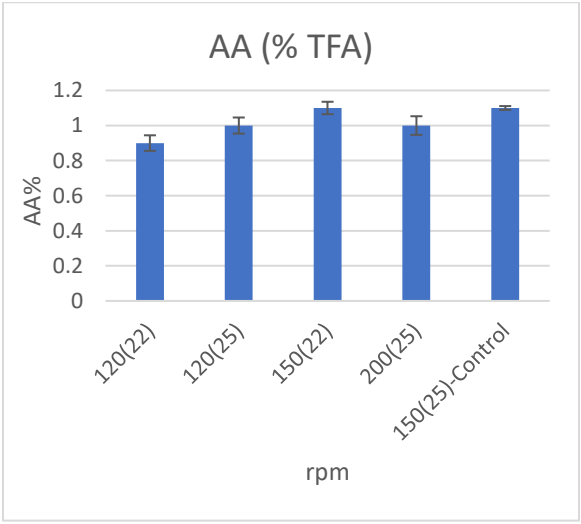


C

Fig 13: Profile of AA production of thraustochytrid strain S2 as a function of agitation. Data are presented as mean ± SD of duplicates.

Highest AA (1.15% of TFA) yield was observed at 150 rpm compared to the cultures grown at 120 rpm and 200 rpm at 25 ^{0}C (AA-1 % of TFA) (Fig 13). At higher agitation rates 200 rpm, biomass and lipid production decreased.

Table 7: Fatty acid profile of Thraustochytrid strain S2 under optimized agitation conditions

Fatty acid (% of total fatty acids)									
rpm	14:0	16:0	18:0	18:1	18:2	18:3	20:5	22:5	22.6
120 (22 ^{0}C)	9.8	23.8	1.8	0.5	-	0.1	1.1	8.1	17.9
120 (25 ^{0}C)	8.9	25.2	1.8	0.4	-	0.2	0.8	7.3	14
150 (22 ^{0}C)	10.5	22.5	2.1	0.2	0.06	0.04	0.8	6.9	15.4
200 (25 ^{0}C)	8.6	28	2.1	0.1	-	0	0.8	7.9	15
Control 150 (25 ^{0}C)	9	24.4	2.2	0.6	-	0.1	0.7	7.7	14.8

Highest proportion of Palmitic acid (28 % of TFA) was recorded at 200 rpm (Table7).

Agitation rate had a significant effect on the production of AA as 170 rpm showed maximum yields of biomass and AA in a previous study (Saelao et al. 2011). Beyond 170 rpm, low biomass was obtained at 200 rpm than 150 rpm, which may be due to the fact that the cells were sensitive to the stress as reported in the literature for a bacteria species (*Aureispira maritima*) (Saelao et al. 2011). Aerobic biological systems are affected by agitation rates. Both cell and metabolites production were affected under less oxygen supply. High agitation can supply more oxygen to the cell growth and intracellular molecular oxygen availability, which is associated with oxygen-

dependent enzymes in the production of PUFAs. When the oxygen levels were too high, the production of AA reduced, because of the cellular adaptation of β -oxidation of the FA (Saelao et al. 2011). Studies related to *Thraustochytrium aureum* showed the highest lipid yields at 200 rpm. When agitation speed increased to 250 rpm, it can result in cell disruption that can change the cell morphology, resulting in decreased PUFAs yields (Hur et al. 2002).

3.4 Role of salt in lipid production

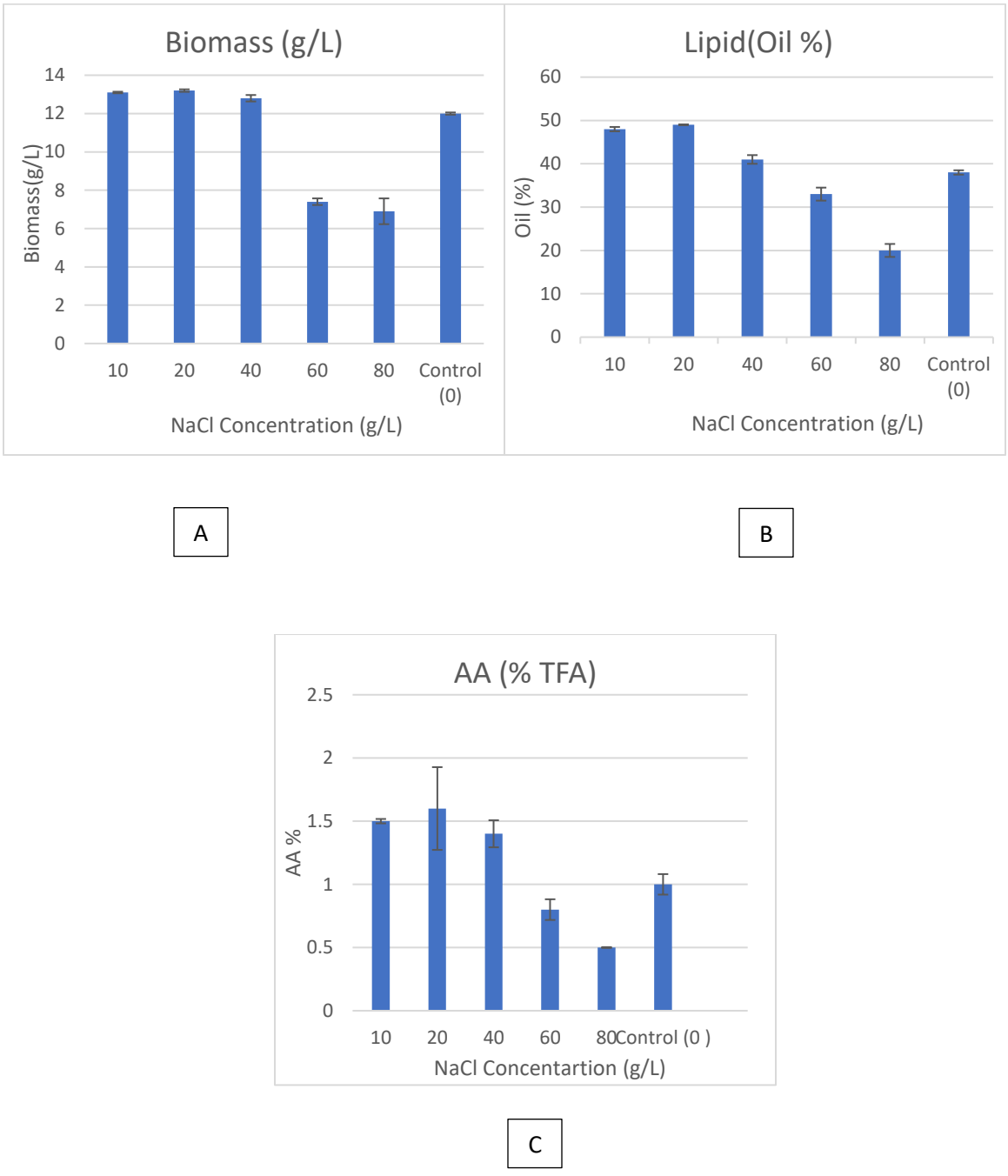


Fig 14: Profile of fatty acid production of thraustochytrid strain S2 as function of salt concentration. Data are presented as mean ± SD of duplicates.

Maximum biomass, lipid and AA production were achieved in the cultures treated with 20 g/L of NaCl (13.2 g/L, 49% and 1.6 % respectively) (Fig .14). The biomass and TFA started to decrease from 40g/L of NaCl up to 100 g/L. The growth of cells was completely repressed at higher salt concentrations (dissolved NaCl in ASW and Milli.Q. water). There was no cell growth when cells were grown in a fermentation medium lacking salt (0 g/L NaCl).

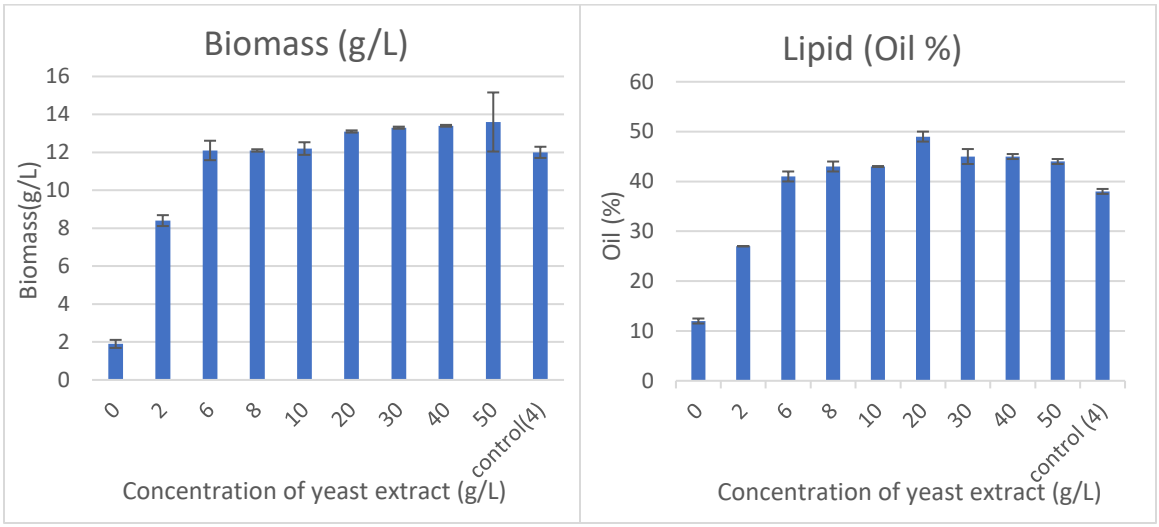
Table 8: Fatty acid profile of Thraustochytrid strain S2 under optimized salt concentrations

	Fatty acid (% of total fatty acids)									
NaCl Concentration (g/L)	14:0	15:0	16:0	18:0	18:1	18:2	18:3	20:5	22:5	22.6
10	4.2	25.9	9.6	1.1	0.4	-	0.1	3.8	9.4	21.1
20	3.8	24	9.7	1	0.5	-	0.1	4.7	10.1	21.6
40	5	20.6	14.7	1.7	2.3	-	-	3.6	7.2	17.3
60	6	20.7	15.4	1.4	1.6	-	0.1	3.1	7.4	21.4
80	5	21.8	16.8	2.2	1.5	0.5	0.2	1.7	5.2	18
Control (0)	9	9.4	24.4	2.2	0.1	-	0.1	0.7	7.9	14.8

During fermentation when percentage of AA was increased, the percentage of linoleic and alpha-linolenic acid were reduced. Highest proportion of Pentadecanoate acid (25.9 % of TFA) was recorded 10 g/L NaCl concentration (Table 8).

Previous studies have reported that the addition of NaCl into fermentation medium had influenced the FA acid concentrations in the microalgal cells (Takagi et al. 2006). Salinity is one of the important parameters that has influenced the growth of Thraustochytrid isolates (Ludevese-Pascual et al. 2016). Similar studies have investigated the effect of NaCl on oil content and PUFA profiles and achieved higher percentages of TAG (Triacylglycerol) and TFA at 2% NaCl concentration. This was in agreement with our study, as the highest lipid yield (49%) was obtained at a similar NaCl concentration. Whereas higher NaCl concentration (4%) showed low yields of biomass and TFA. AA was predominantly accumulated in TAG.;. Diacylglycerol acyltransferase 2 (DGAT2) enzyme is responsible for the addition of acyl-chain to DAG backbone to produce TAG. The gene expression of DGAT2 was higher in the NaCl treated cultures, resulting in increased TFA and TAG (Ho et al. 2008).

3.5 Nitrogen source supports biomass and lipid production in Thraustochytrid strain S2



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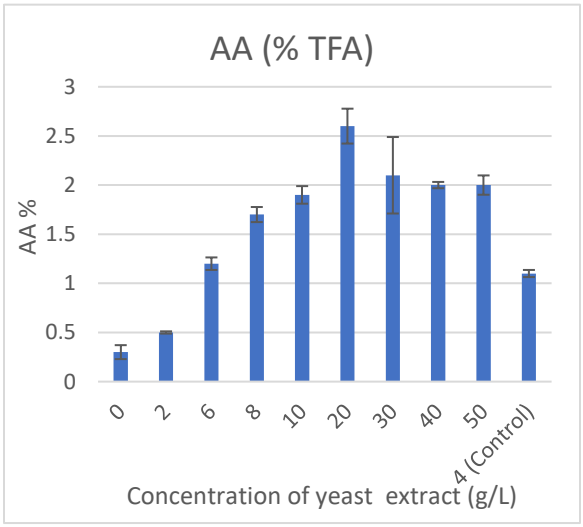


C

Fig 15: Profile of yeast extract concentration on dry cell weight (DCW) (A), lipid content (B), and AA Composition (C) of thraustochytrid strain S2. Data are presented as mean ± SD of duplicates.

It was observed that biomass increased with the increase in yeast extract concentration. Maximum biomass (13.6 g/L) was observed at 50 g/L of yeast extract concentration. However, maximum lipid content (49%) and AA (2.6% of TFA) were observed at 20 g/L of yeast extract (Fig .15).

Table 9: Fatty acid profile of Thraustochytrid strain S2 under optimized yeast extract concentrations

	Fatty acid (% of total fatty acids)								
Yeast extract Concentration (g/L)	14:0	16:0	18:0	18:1	18:2	18:3	20:5	22:5	22.6
0	4.6	27.1	2.4	0.6	-	0.1	1.9	7.8	16.2
2	9.2	25.8	1.9	0.2	-	0.05	0.7	8	16.9
6	8.5	26.2	2.2	0.4	-	0.1	1.4	9.4	19.1
8	8.1	25.8	2.2	0.4	-	0.1	1.7	9.7	19.9
10	7.8	21.2	2.2	1.2	-	-	3.1	11.3	21.6
20	6.4	18.5	2	1. 8	-	-	2.1	10	22
30	6.2	18.4	2.1	1.9	-	-	2	9.9	21.8
40	6.1	17.9	2.1	1.9	-	-	2	9.7	21.1
50	5.4	18	2.2	1.5	-	-	3.5	8.3	19.5
Control (4)	9	24.4	2.2	0.1	-	0.1	0.7	7.9	14.8

Highest proportion of Palmitic acid (27.1 % of TFA) was recorded in a medium that lacked yeast extract. During the fermentation, when percentage of AA was increased, the percentage of linoleic and alpha-linolenic acid were reduced (Table 9).

The increase in yeast extract concentration resulted in increased biomass and AA (Fig 15). In the present study, yeast extract was applied as a nitrogen source. Yeast extract is known as one of the major factors for the culturing of microbes as nitrogen sources can significantly affect the morphology of the cells (Park et al. 1999). Therefore, it can be concluded that a high concentration of yeast extract concentration could change the cellular morphology of S2 stain, resulting in more biomass and AA. Further, the quantity of AA is affected by the nitrogen factor (Park et al. 1999). A similar study reported the effect of tryptone (served as complex nitrogen factor) and it was found that a high concentration of tryptone was necessary for the production of biomass and AA, which was in agreement with our study (Saelao et al. 2011). Therefore, we can conclude that yeast extract served as a nutrient factor for microalgal growth. However, the high price of yeast extract can limit its use in large-scale production of AA (Park et al. 1999). In addition, nitrogen factor can increase the activity of malic enzyme, which is essential for producing NADPH for FA synthesis (Wynn et al. 1999).

3.6 Effect of phytohormones on improving lipid production

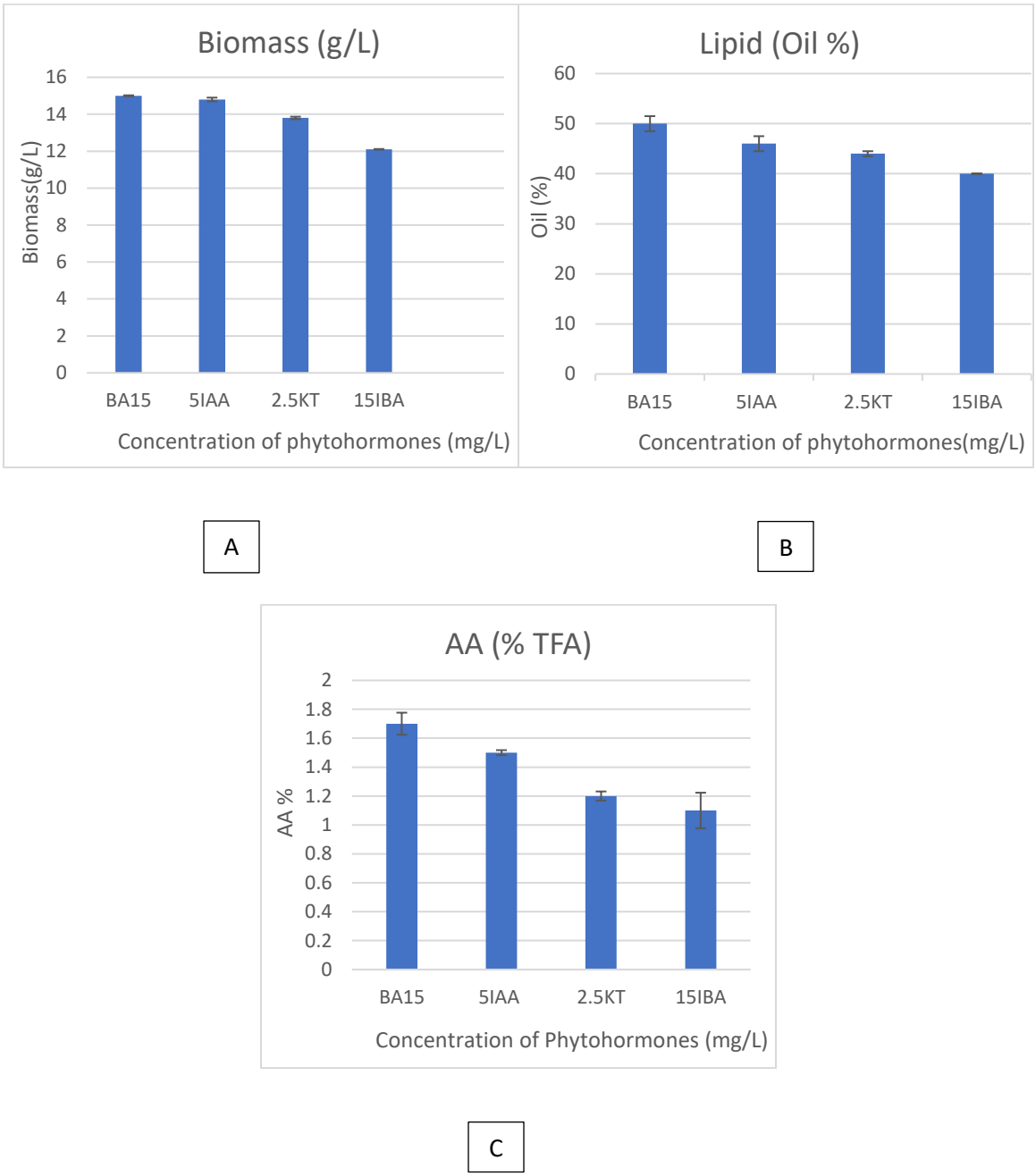


Fig 16: Lipid production as a function of effect of phytohormones on dry cell weight (DCW) (A), Lipid Content (B), and AA composition (C) of strain S2. Data are presented as mean ± SD of duplicates.

This study was carried out to assess the effect of phytohormones on increasing AA production in thraustochytrid strain S2. Here, four chemical modulators such as BA, KT, IAA and IBA were selected to investigate their effects on biomass and lipids production. Different concentrations of these phytohormones (as mentioned in the materials and methods section) were used that have been reported as the best concentrations in previous studies (Zhang et al. 2019). At tested concentrations, the inducing effect of chemical modulators is as follows: BA>IAA> KT>IBA. Highest biomass (15 g/L), lipid (50%) and AA (1.7 % of TFA) were observed in the culture medium supplemented with 15 mg/L of BA (Fig .16). The biomass, lipid and AA in the control were ranged between 11.8-12 g/L, 38-39% and 0.9-1% respectively. However, in comparison with control, IBA did not show a considerable effect on biomass and lipid yields. When the medium was supplemented with ethanol, 12.1 -12.4 g/L of DCW, 42% of lipid and 1-1.1 % of AA were attained. It was noted that ethanol response to enhancing lipid yield was relatively weak when compared with BA.

Chemical modulators can affect the metabolism of microorganism (Jiao et al. 2017). Previous studies reported that auxin promoted cell growth at lower concentrations due to the stimulation of gene expression related to the cellular division and inhibited the cell growth at higher levels as they behaved as herbicides at higher concentrations (González-Garcinuño et al. 2016). It was in agreement with our studies as at higher levels of IAA, the yields remained lower than those of the control. From 5-20 mg/L of IAA, biomass, lipid and AA decreased to 14.8-8.4 g/L, 46- 22%, and 1.5 -0.6 % of TFA, respectively, indicating that IAA inhibited the growth at high concentration (15 and 20 mg/L). According to a recent study, cytokinin (BA and KT) and auxin (IAA) resulted in the increased production of lipid and AA (Zhang et al. 2019). In addition, it was reported that phytohormones could increase the supply of NADPH, aiding the biosynthesis of FA and storage

of FA in fungal strain (*Mortierella* sp.). They found that high BA concentration resulted in high lipid production that was in agreement with our studies. The presence of phytohormones could induce the enzyme activation of ME (in the pyruvate/malate cycle) and G6PD (pentose phosphate pathway) required for the biosynthesis of NADPH. Moreover, they can increase the acetyl-CoA and the storage of triglycerides (Zhang et al. 2019).

3.6.1 Use of Linseed oil in improving lipid and AA

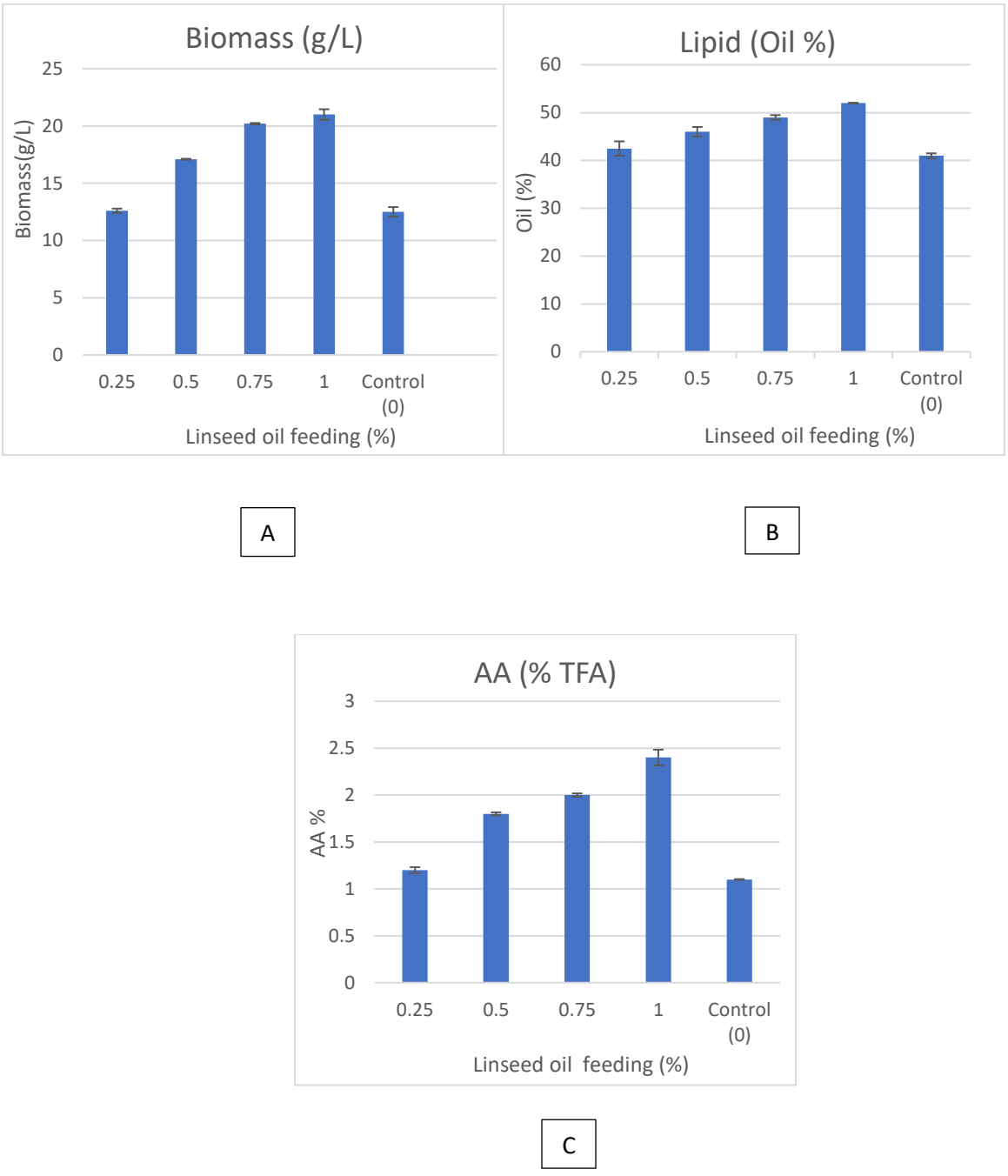


Fig 17: Effect of linseed oil feeding on dry cell weight (DCW) (A), lipid content (B), and AA composition (C) of thraustochytrid strain S2. Data are presented as mean ± SD of duplicates.

At 1% linseed oil feeding during fermentation, highest biomass (21 g/L), lipid (52%) and AA (2.4% of TFA) were observed (Fig .17). The production of AA increased in conjunction with the cell growth and lipid content. On increasing feeding rate from 0.25 % to 1%, improvement in AA from 1.2% and 2.4% of AA (% TFA) was recorded, respectively. Oil-like droplets were observed inside the flask when compared with a control (without feeding), indicating that linseed oil was not completely consumed by the strain.

The improvement in AA yields was encouraging, however, more experiments are warranted to estimate residual AA oil post-fermentation.

Previously, plant oils (olive oil and soybean oil) have been used in the fermentation medium to induce AA synthesis (Shinmen et al. 1989). Cottonseed oil and olive oil were used as carbon substrates, soybean oil as a nitrogen substrate for fungal growth and production of AA. Plant oil, such as soybean contains PUFAs (with linoleic acid and α-linolenic acid) that can induce the EPA and AA production when fed into the fermentation medium (Shinmen et al. 1989; Zeinab Asadi et al. 2018). Literature studies reported that linseed oil showed a significant effect on AA and EPA production than soybean oil. It was observed that the metabolism of linseed oil was more effective for PUFAs production (Zeinab Asadi et al. 2018) . The fungal cells utilized added lipids in the medium to produce PUFAs through FA desaturation and elongation process (Zeinab Asadi et al. 2018). Linseed oil is considered as a rich source of α-linolenic acid (Komprda et al. 2005). From the above findings, we concluded that thraustochytrid strain S2 can partially utilize the linseed oil, including α-linolenic acids to enhance AA production.

3.7 Fed-batch studies-glucose feeding on AA production

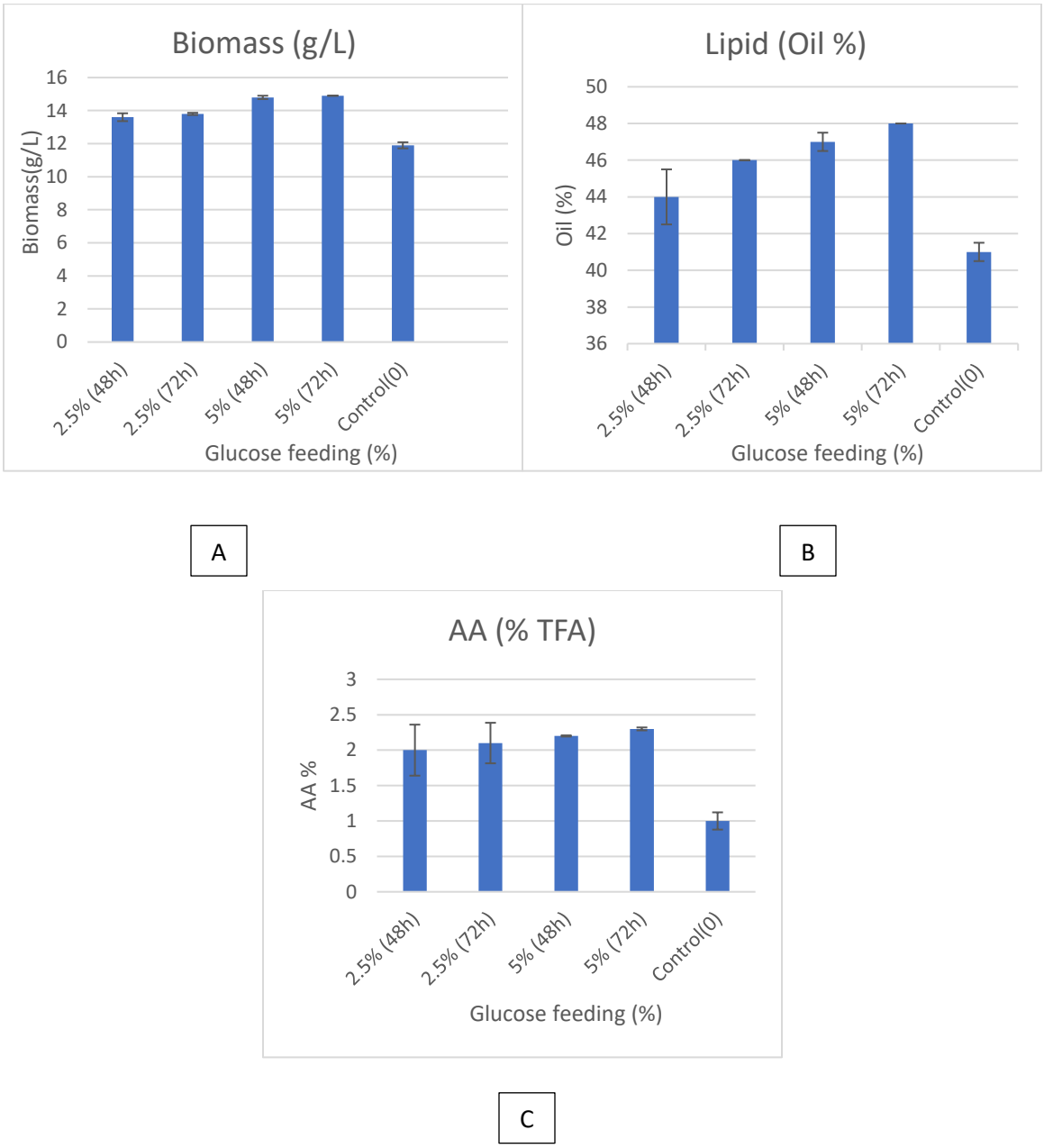
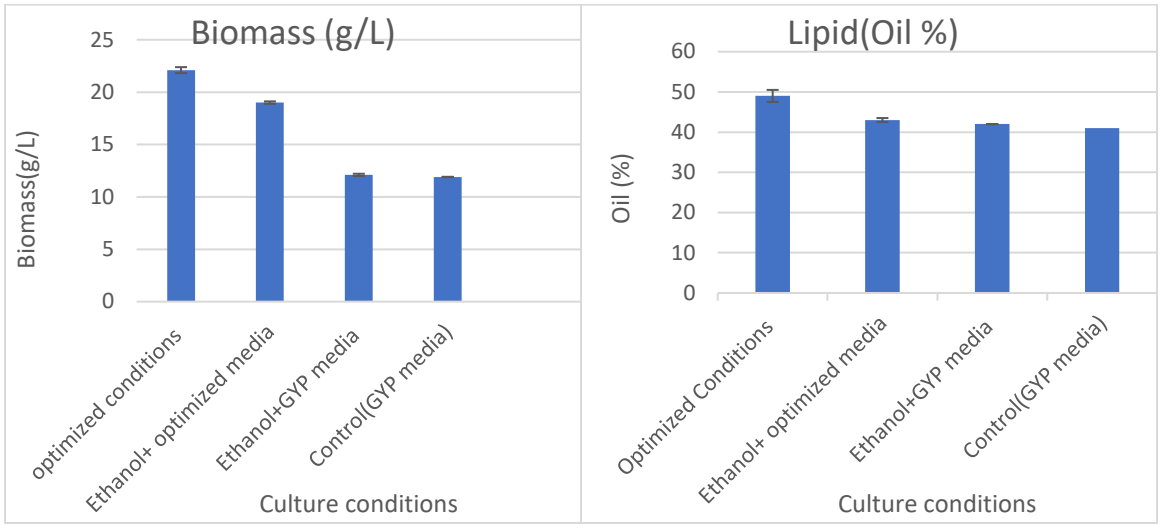


Fig 18: Effect glucose feeding on dry cell weight (DCW) (A), lipid content (B), and AA composition (C) of thraustochytrid strain S2. Data are presented as mean ± SD of duplicates.

It was observed during fermentation that glucose concentration reduced after 24 hours (Fig 8). Therefore, it was decided to feed glucose after 24 hours and then again at 72 hours of fermentation once the first glucose feeding gets exhausted. The feeding concentration of glucose ranged from 2.5% to 5%. The highest increase in DCW (14.9 g/L), lipid (48%) and AA (2.3 % of TFA) was observed on 5% glucose feeding at 72h of fermentation. A slight increase in biomass, lipid and AA were recorded at 5% feeding between 48h and 72h (Fig .18).

It was observed that cell growth, total lipid production, and AA synthesized more rapidly in fed-batch cultures, which was in agreement with a previous study (Wu et al. 2017). Higher growth rate led to increased biomass from 3.3 g/L to 13 g/L, indicating that the rate of the accumulation of lipid was high during that period (Ji et al. 2014).

3.8 AA production under the optimized parameters:



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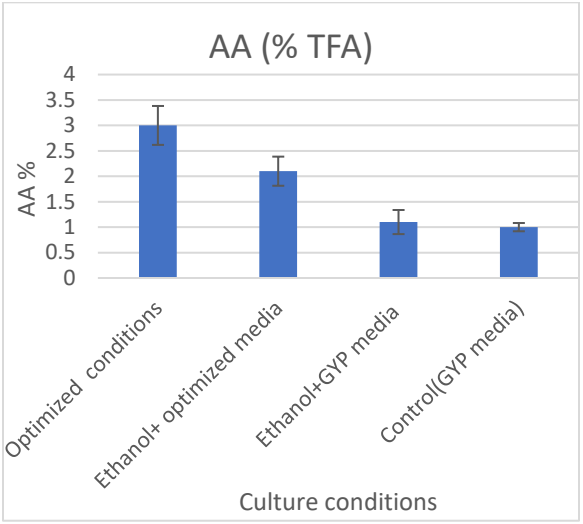


C

Fig 19: Production of AA under optimized conditions of thraustochytrid strain S2. Data are presented as mean ± SD of duplicates.

The thraustochytrid strain S2 was grown under optimal culture conditions (20 ml fermentation medium containing 5% glucose, 0.04% bacteriological peptone, 20 g/L yeast extract, 20 g/L NaCl, 50% ASW and 15 mg/L BA pH 6.5, was taken in a 100-ml shaking flask and incubated at 22^0C and 150 rpm) to investigate AA production. Maximum biomass (22.1 g/L), lipid content (49%) and AA content (3% of TFA) were obtained when compared to a control (Fig .19). To the best of our knowledge, the highest AA yields were demonstrated by thraustochytrid S2 strain.

Physical factors and nutrient supplementation are considered significant factors for both microbial cell growth and FA production (Saelao et al. 2011). The improvement in AA production was facilitated using phytohormones and carbon feeding. The cell growth rate and lipid accumulation of thraustochytrid S2 were strongly related to carbon feeding.

3.8.1 Investigation on other Fatty acids produced during fermentation.

Prominent fatty acids such as methyl palmitate (C16:0), methyl docosahexaenoate (C22:6) and methyl palmitoleate (C16:1 n7) were identified as part of the fatty acid profile. The percentage of TFA changed as per the optimized culture conditions, AA was observed at a lower proportion. These results were in agreement with the previous study where the FA profile of Thraustochytrid showed mainly 33.52% of PA (C16:0) and 39.92% of DHA (C22:6n-3) as a proportion of TFA (Ludevese-Pascual et al. 2016). In addition, EPA (C20:5n-3, 1.01%) and AA (C20:4n-3, 0.90%) were observed at low proportion when compared to other FAs (Ludevese-Pascual et al. 2016).

Linoleic acid (LA) acts as the main precursor of AA in human dietary intakes (Brenna 2016). LA was considered as a precursor of ω-6 FA synthesis (Pratiwi et al. 2009). It was concluded that linolenic, AA and eicosatrienoic acids have played a major role in the metabolic pathway of Thraustochytrid. In this study, (C18:2n6c) methyl linolelaidate/ (C18:2n6t) methyl linoleate was observed to decrease proportionally when AA increased. According to the FA profiles (Table .9),

it was concluded that linolenic acid was converted into AA in the fermentation medium, because it was observed that C18:2n6c/ C18:2n6t was decreasing with increasing AA percentages in the fermentation medium. Thus, it can be concluded that the metabolic pathways of fungi and microalgae (Thraustochytrid strain) has some similarities. In *Pythium* sp., a high percentage of myristic acid and a low percentage of linolenic and linoleic acid (when comparing with other tested fungal strains) were observed (Haskins et al. 1964). When the AA % TFA increased, the precursors, such as dihomo-gamma-linolenic (C20:3) and gamma-linolenic (-C18:3) acids decreased. It was concluded that the above acids were converted rapidly to AA (Mironov et al. 2018).

Previous researchers have investigated that lipid synthesis related with fish oil consisted with AA (ω-6) and DHA (ω-3) and also it contained less quantities of linoleic acid (LA, ω-6) and alpha-linolenic acid (ALA, ω-3) (Le et al. 2009). According to recent studies, marine fish are considered as a major resource of EPA as well as DHA rich oils (Paredes et al. 2020)

According to the WHO, processed meat (such as red meat) has been identified as carcinogenic to human being (Hielkema et al. 2021). This led considerable number of Europeans to show their willingness to replace meat products with vegetables (Hielkema et al. 2021). Thus, the demand for plant-based meat products is surging. Therefore, it is imperative to explore other sustainable AA sources that can serve the rising nutritional demand. The main outcome of this research is the prospect of using thraustochytrid strain S2 for AA oil production; a microbe acquired a GRAS status thus suitable to meet both vegan and non-vegan populations daily nutritional needs. Moreover, plant-based products can reduce the possibility of cardiovascular disease as well as provides more health benefits (Hielkema et al. 2021).

Conclusion

PUFAs, also known as Omega-3/6 fatty acids, are commercially developed as nutraceuticals and dietary supplements since clinical studies have endorsed their role in promoting brain and cardiovascular health. Omega-3 fatty acids such as DHA and EPA are produced by single-cell marine microbes, such as thraustochytrids, by utilizing glucose as a carbon source (Scott et al. 2011). The present study investigated fatty acid production through novel microalgae to meet the rising demand for Arachidonic acid (Omega-6 fatty acid) and other PUFAs for infant nutrition. In this study, the effects of physical and chemical factors on growth, lipid content, and Arachidonic Acid production were investigated. The growth of thraustochytrid S2 strain demonstrated a significant relationship with a high concentration of yeast, salt (NaCl) and AA feeding to the fermentation medium. Phytohormones such as BA, KT, IAA and IBA, when added to the fermentation medium for 5 days, supported the AA (1.7 % of TFA) production with 15 mg/L of BA. Maximum AA content (3 % of TFA) was achieved under optimized conditions, compared to a control (1 % of AA).

Feeding of a precursor rich in unsaturated fatty acids enhanced the production of AA, DCW, and lipid in a fermentation medium using thraustochytrid S2 strain. This concluded that AA percentage could be increased by applying fermentation strategies, including medium manipulation. During fermentation, the medium manipulation could have stimulated the expression of Diacylglycerol acyltransferase 2 (DGAT2), resulting in increased biomass. Consecutively, FA synthesis also increased, especially AA, because AA is distributed mainly in TAG (Triacylglycerol). The increase in TAG is associated with the expression of DGAT2 (Ho et al. 2008).

In the literature, thraustochytrids have been documented for Omega-3 production. Whereas, in this study, we have demonstrated the suitability of thraustochytrids for Omega-6 fatty acids production.

Thus, thraustochytrid can be further investigated to meet the increasing demands of AA for infant nutrition. However, additional investigations involving FA distribution, gene expression, and lipid downstream processing in thraustochytrid S2 strain should be studied to improve AA levels. The genes involved in the metabolism of lipids should be investigated to ascertain the effect of culture conditions on AA production.

Future prospects

AA can apply as an ingredient in plant-based proteins. It is important to screen more organisms that can produce high AA and explore various methods to produce more yield of AA by using environment-friendly processes.

There are a smaller number of studies with respect to the potential benefits of fatty acids, especially conjugated fatty acids, and their sources. Also, more studies are required to recognize the applications of AA as a treatment of diseases, such as cancers and various inflammatory diseases. For that, more studies should focus on understanding the molecular mechanisms of the biosynthesis of PUFAs.

It is necessary to understand the biochemical pathway of AA that is essential for developing a high yield AA manufacturing process. The genes involved in the metabolism of lipids in thraustochytrid should be investigated to find out the effect of culture conditions on AA production.

The results from this study regarded as proof-of-concept and can be applied for the development of a large-scale cultivation of Thraustochytrids for ensuring commercial production of AA.

Limitations

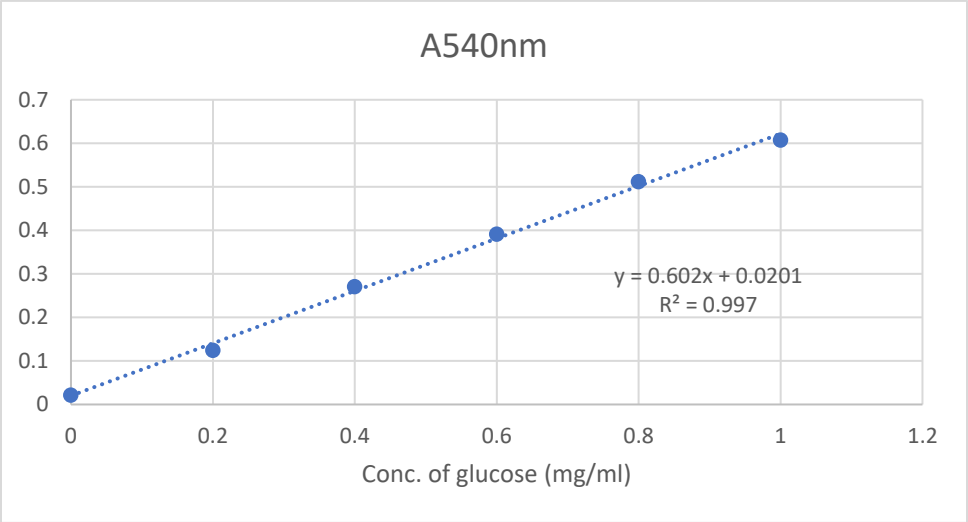
- Adhering to the proposed timeline as mentioned in the research project was the major limitation during the whole study. On two occasions, due to the Covid 19 lock down, I had to remove my fermentation flasks and halt experiments thus compromising project progress.
- Due to the solubility issues of some substrates (grinded dates seeds and fruits) could not be tried.
- Contamination issues encountered during inoculum development delayed fermentation experiments.

Appendix

Appendices A-1 Concentrations of glucose solutions used for standard curve preparation

Glucose concentration (mg/mL)	**Glucose stock solution (ug/mL) (µl)**	**Milli.Q. water (µl)**
0.00	0	1000
0.20	200	800
0.40	400	600
0.60	600	400
0.80	800	200

Appendices A-2 Glucose standard curve



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