Polyunsaturated Fatty Acid Production from Novel Thraustochytrid Strain Using Hemp Seeds and Its Antioxidative Potential

By

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DECLARATION

I declare that this work is an original research paper, written by myself, and not submitted for a previous degree. The experimental work is almost entirely my own; collaborative contributions have been clearly identified and acknowledged.

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ABBREVIATIONS

°C	Celcius degree
µg	microgram
µL	microlitre
µM	micromolar
AA	arachidonic acid
AA	Ascorbic acid
ACP	acyl carrier protein
AHA	American Heart Association
ALA	alpha-linolenic acid
AMR101	Eicosapentaenoic acid ethyl ester
ARA	arachidonic acid
ASW	artificial seawater
BHT	butylated hydroxytoluene
C19:0	methyl nonadecanoate
C. sativa	*Cannabis sativa*
CDW	Cell dry weight
CHSO	Commercial Hemp Seed Oil
CO_2	carbon dioxide
CoA	Coenzyme A
CVD	cardiovascular disease
DGLA	dihomo-γ-linoleic acid
DHA	Docosa Hexaenoic Acid
DNS	3,5-dinitrosalicylic acid
DPA	docosapentaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTA	docosatetraenoic acid
DW	distilled water
EFAs	Essential Fatty Acids
EFSA	European Food Safety Authority
ELOVL	Elongation of very-long-chain fatty acids
EPA	eicosapentaenoic acid
Eq.	Equation
ETA	eicosatetraenoic acid
FAMEs	Fatty Acid Methyl Esters
FAs	Fatty acids
FAS	Fatty Acid Synthase
g	universal gravitational constant
g/L	grams per liter
GA	gibberellic acid
GC	gas chromatography

GC-FID	gas chromatography-flame ionization detection
GHYP	Glucose Hemp Seed Yeast Peptone
GLA	Gamma-linolenic acid
GMYP	Glucose Market hemp seed Yeast Peptone
GOYP	Glucose Oil Yeast Peptone
GYP	Glucose Yeast Peptone
h	hour
HDL	high-density lipoprotein cholesterol
HS	Hemp Seed
HSO	Hemp Seed Oil
HYP	Hemp Yeast Peptone
JA	Jasmonic Acid
JELIS	Japan EPA Lipid Intervention Study
KIN	Kinetin
L	litre
LA	linoleic acid
LA	linoleic acid
LCB	Lignocellulose biomass
LC-PUFAs	long chain polyunsaturated fatty acids
LDL	low-density lipoprotein cholesterol
LHW	liquid hot water
M	Molarity
MAE	Microwave-assisted extractions
MAT	Malonyl-CoA:ACP transacylase
MeOH	methanol
mg	milligram
MHS	Market Hemp Seed
min	minutes
mL	millilitre
MQ	Milli-Q
MSG	Monosodium Glutamate
MUFA	Monounsaturated fatty acid
MYP	Market Hemp Seed Yeast Peptone
n-3	omega-3
n-6	omega-6
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
nm	nanometer
OD	Optical density
OYP	Oil Yeast Peptone
pH	potential of hydrogen

PIRSA	Primary Industries and Regions South Australia
PKS	Polyketide Synthase
PUFA	polyunsaturated fatty acid
rpm	revolutions per minute
SCFA	short-chain fatty acid
SD	Standard deviation
SDA	stearidonic acid
SFA	saturated fatty acids
SFE	supercritical fluid extraction
SH	sugar hydrolysate
SSF	solid state fermentation
TFA	Total Fatty Acid
THC	tetrahydrocannabinol
UV	ultraviolet
v/v	volume per volume
VFA	Volatile fatty acids
VLC-PUFAs	Very long chain polyunsaturated fatty acids
w/v	weight per volume
WHO	World Health Organization
X	mole fraction
ω-3	omega-3
ω-6	omega-6

ABSTRACT

The utilization of microorganisms to produce sustainable bioactive compounds or biofuels has received recent attention due to its potential benefits for human health. It is expected that the modification of microbial growth conditions could induce a higher production of desired bioactive compounds. In this study, a newly isolated marine Thraustochytrid strain S2 was used for polyunsaturated fatty acid (PUFA) production by growing on hemp seed (*Cannabis sativa*) powder. Considered as functional foods, PUFAs have been demonstrated to assist in maintaining biological functions, improving conditions of health, and effectively reducing risks from severe diseases. The Thraustochytrid strain was able to grow on the study (HS) and market hemp seed (MHS) powder and accumulated polyunsaturated fatty acids. As a result, biomass and total lipid production were remarkably increased after fermentation, particularly when glucose was combined in the fermentation medium. Fatty acid profile analysis of HS, MHS, and commercial hemp seed oil (CHSO) after fermentation resulted in higher production of some essential fatty acids. In addition, the lipid profile of HS and MHS presented significant alpha-linolenic acid (ALA) production, which was not observed in a control study. Several biostimulants applied to the hemp seed crop showed minimal effect on lipid production, but some remarkable changes in some specific FAs were noticed, especially in hemp seeds collected from crops added with complex biostimulants. In addition, HS performed low antioxidant activity when compared to Trolox as a positive control. However, the free radical scavenging effect was doubled after fermentation due to the inherent presence of antioxidant biomolecules in Thraustochytrid. The highest antioxidant activity was observed with the combination of HS and glucose at a high concentration. This study demonstrated that HS is a potential carbon source for enhancing fermentation products of Thraustochytrid in terms of biomass, essential PUFAs, and antioxidative activity.

Chapter I. GENERAL INTRODUCTION AND LITERATURE REVIEW

1. Introduction

1.1. Polyunsaturated fatty acids (PUFAs)

1.1.1. PUFAs and their relevance to dietary nutrition

1.1.1.1. Definition, structure, and applications

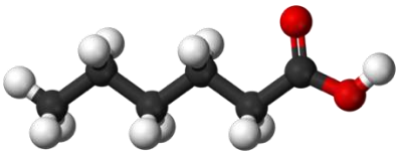
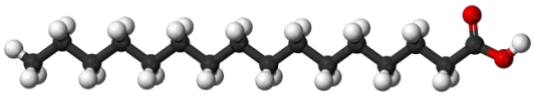
In addition to the typical healthy diet needs, a balanced diet involving consuming a diverse range of foods in the proper amounts is regarded as a healthier diet that will help human health (Rao & Singh 2021). Furthermore, malnutrition and undernutrition are serious concerns all throughout the world, particularly in developing countries (Muller 2005). Recently, "functional foods" have been defined as foods with a clear claim of health advantages, including boosting the immune system (López-Varela, González-Gross & Marcos 2002) as they provide the external critical nourishment for our bodies to function because the body cannot produce them by itself. Globalization has been a favorable chance for food companies to develop and upgrade the functional nutrition industry (Cuevas García-Dorado et al. 2019). However, the manufacturing of these dietary supplements confers various issues related to climate change, sustainability, and market pricing that must be addressed by substituting with cheaper sources, utilizing the industrial by-products, and more efficient procedures for the traditional sources or methods (Meléndez-Martínez et al. 2021).

Recently, the understanding of the public about the health benefits of nutritional intake like fatty acids has risen dramatically (He et al. 2020). One of the most promising compounds is polyunsaturated fatty acids (PUFAs). The carbon chain of fatty acid (FA) molecules is variable in length, having a methyl terminus and a carboxylic acid head group. They can be classified based on how saturated their carbon chains are (Timilsena et al. 2017). Monounsaturated FAs and PUFAs have one, two, or more double bonds, respectively, while the saturated FAs have the most hydrogen

atoms, as shown in **Table 1**. PUFAs normally consist of one or more double bonds in their backbone structure. They can be found in a variety of plant and animal foods, including salmon, vegetable oils, and certain nuts and seeds (Ander et al. 2003). On the other hand, the generation of PUFAs by these species is completely reliant on climatic circumstances, aquatic environment, nutrition, age, maturity, migration pattern, and spawning season (Meï et al. 2015; Taşbozan & Gökçe 2017). Due to their special structures, they have exceptional physiological roles that are crucial for human health. Skin irritation and dryness, sadness, joint discomfort and stiffness, dry eyes, and hair changes in texture, integrity, and density can all be caused by a lack of essential fatty acids like PUFAs (Messamore & McNamara 2016). It is popularly known that PUFAs are a kind of fat that includes "good" fats for human health such as unsaturated fats (monounsaturated and polyunsaturated fats) and "bad" fats such as trans fats and saturated fats that are required for the survival of all living things (Harvard Health 2019). Saturated and trans fats have been identified as possibly damaging to our health because they raise blood cholesterol and low-density lipoprotein cholesterol (LDL), which increases the risk of heart disease (Siri-Tarino et al. 2010). Trans fat is one of the worst types of fat. Trans fat, like saturated fat, can boost LDL cholesterol while simultaneously suppressing healthy high-density lipoprotein cholesterol (HDL) levels. Trans fats have also been linked to an increased risk of inflammation in the body, according to doctors (Lopez-Garcia et al. 2005; Mozaffarian et al. 2004). This inflammation can have negative health consequences, such as heart disease, diabetes, and stroke (Kummerow 2009). Essential fats are polyunsaturated fats that the body cannot produce on its own and must obtain from food. According to the American Heart Association, polyunsaturated fat, like monounsaturated fat, can reduce the risk of heart disease by lowering blood cholesterol levels (American Heart 2015). Consuming polyunsaturated and monounsaturated fats in moderation instead of saturated and trans fats can

improve our health (Hongu, Wise & Gallaway 2014). Because of advantages in long-term adherence and potentially favorable effects on lipids and lipoproteins, a moderate-fat diet can be as effective as, if not more effective than, a lower-fat diet in terms of weight control (Kris-Etherton et al. 2002).

Table 1: Example structures of three types of fatty acid

Type of fatty acids	Shorthand Notation	Typical name and formula	Chemical structure
Saturated	C4:0	Butyric acid $C_4H_8O_2$	Figure removed due to copyright restriction
	C6:0	Caproic acid $C_6H_{12}O_2$	
	C16:0	Palmitic acid	
Monounsaturated	18:1n-9	Oleic acid $C_{18}H_{34}O_2$ (ω-9)	Figure removed due to copyright restriction
Polyunsaturated	18:2n-6	Linoleic acid (LA) $C_{18}H_{32}O_2$ (ω-6)	Figure removed due to copyright restriction
	18:3n-3	Alpha-linolenic acid (ALA) $C_{18}H_{30}O_2$ (ω-3)	Figure removed due to copyright restriction

1.1.1.2. Omega-3 and omega-6

The placement of the first double bond in relation to the methyl terminus of the chain can further separate PUFAs (Cicero et al. 2012). Omega-3 (ω-3) and omega-6 (ω-6) FAs, also known as n-3 and n-6, respectively, depending on their first double bond on the third or sixth carbon from the chain terminus. They are among two of the most biologically important PUFA types as they are cell membrane components and precursors to many other substances that regulate metabolisms in the body (Hayashi et al. 2020; Jovanovic et al. 2021). The final carbon in the FA chain is referred to as the ω carbon, which is why these FAs are commonly referred to as ω-3 or ω-6 PUFAs. These two types of PUFAs should be differentiated because they have opposing physiological activities and are metabolically and functionally unique; their balance is critical for homeostasis and normal development. The essential FAs (EFAs) such as alpha-linolenic acid (ALA) and linoleic acid (LA) are used to make long-chain ω-3 and ω-6 PUFAs (LC-PUFAs) (Balić et al. 2020). They are not produced by the body and must be received through diet and could be converted to long-chain derivatives by both animals and humans (Ghiffary, Kim & Chang 2019). Gamma-linolenic acid (GLA) and arachidonic acid (ARA) are common ω-6 PUFA compounds and eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) are popular ω-3 PUFA compounds. Positive benefits of ω-3 PUFAs are popularly known as anti-inflammatory and anti-blood clotting effects, and they decrease triglyceride levels, reduce stress, and minimize the risks of some severe diseases such as diabetes and some kinds of cancer (Ren et al. 2010; Xie, D, Jackson & Zhu 2015). Besides, ω-6 PUFAs are popularly admitted as essential precursors that could produce a variety of biologically and clinically significant eicosanoid hormone compounds such as leukotrienes, prostaglandins, and thromboxane via the metabolism of some enzymes for tackling or avoiding a variety of human diseases (Fabien De Meester 2013). Eicosanoid metabolism gene expression

(Emam et al. 2020), and intercellular cell-to-cell communication are all influenced by ω-6 and ω-3 PUFAs (Luetić et al. 2020).

Dietary intake influences the PUFAs content of cell membranes to a large extent (Abbott et al. 2012). As a result, while developing dietary recommendations, suitable levels of dietary ω-6 and ω-3 PUFAs must be addressed (Simopoulos 2000). The ratio of ω-6 to ω-3 PUFAs is particularly crucial to human health because the ω-6 and ω-3 pathways compete for enzyme activity (Pratap Singh et al. 2020). As one of the essential ω-3 PUFAs compounds, DHA appears to be required for the correct functional development of the retina and brain, particularly in premature infants, according to studies with nonhuman primates and human newborns (Brenna & Carlson 2014). It is thought to enhance infant cognitive development, brain function, and eye health, whereas EPA is thought to improve cardiovascular health, mental health, and immunological function (Matsuzaki et al. 2009). Furthermore, EPA is a promising medication for preventing major coronary events, according to the Japan EPA Lipid Intervention Study (JELIS) (Kodera et al. 2018). Ballantyne et al. (2012) found that pure EPA fatty acid dramatically lowered triglyceride levels in adult patients with severe hypertriglyceridemia, according to research on eicosapentaenoic acid ethyl ester (AMR101). The human body can only inefficiently synthesize EPA and DHA from ω-3 ALA (C18:3) and cannot create them from scratch (Carlson et al. 2013). A healthy ω-6/ω-3 ratio (recommended at 4:1) in the diet is important for normal growth and development, as well as lowering the risk of cardiovascular disease and other chronic diseases, as well as improving mental health. An excess of FAs from one family will stifle the metabolic formation of longer-chain products from the other (Ander et al. 2003).

Although there is an increasing demand for EPA and DHA, the majority of commercially available EPA and DHA are derived from wild-caught ocean fish (Adarme-Vega, Thomas-Hall & Schenk

2014). Overfishing has devastated ocean fisheries, making them an unsustainable source of food to meet rising demand. However, the strong taste, odor, and stability issues associated with fish oil as a source of ω-3 PUFAs further complicate reliance on this type of oil (Tocher et al. 2019). Purifying these FAs from fish oil is a difficult process in and of itself (Ji, X-J, Ren & Huang 2015). Furthermore, the quality of fish oil-derived products is largely based on the season and location, and it can be influenced by pollution in the ocean (Hongu, Wise & Gallaway 2014). The environmental toxins identified in fish oils include methylmercury, polychlorinated biphenyls, dioxins, and a variety of other halogenated, persistent organic pollutants, necessitating the search for an alternative source of ω-3 PUFAs (Gribble et al. 2016). All these issues make it difficult to employ fish oil as a dietary addition or supplement. A land-based, long-term source is required. Finally, using non-animal sources for fish oils could make such food supplements suitable for vegetarians and people allergic to shellfish or fish (Scott, Srirama & Carani 2007). Some microorganisms can be employed to turn low-cost, high-nutrient agroindustry by-products into more valuable goods (Oliver et al. 2020). They are, however, traditionally synthesized from animal and plant-based sources, which have traditionally been unable to meet rising demand. A more dependable and long-term supply of ω-3 PUFAs is required due to rising demand for PUFAs and a deteriorating marine environment (Tocher 2009). Hence, the need for alternative sources has encouraged researchers to discover other kinds of inputs, especially from nature, for achieving an output that is high quality and can be produced on a large scale in a way that is efficient in cost and time (Quilodrán et al. 2020). If economic incentives are present, other sources can be substituted for PUFA accumulation. Biotechnology engineering is expected to improve PUFA synthesis in the future generation by utilizing systems and synthetic biology technologies to produce PUFAs from

a wider range of sources with greater efficiency, such as plant-based, microorganism-based, etc. (Béligon et al. 2016; Tocher et al. 2019), as discussed in the following section.

1.1.2. Using of carbon source in enhancing omega-3 production

Lipid biosynthesis in microorganisms is influenced by abiotic parameters such as temperature, pH, nitrogen, carbon sources, and their concentration (Anjum et al. 2015). Under several cultivation substrates, either heterotrophic (Zhang et al. 2019), autotrophic (Amjad Khan et al. 2017), or mixotrophic (Kannan, Rao & Nair 2021), several microorganisms created considerable amounts of PUFAs, even up to nearly 80% of total fatty acids produced (**Table 2**). However, this raised the overall cost of the process, so those substrates must be replaced with inexpensive alternatives. Efforts to achieve this goal have included the use of agricultural waste or by-products. As a result of this method, both the cost and the environmental impact can be minimized while FA production efficiency is increased (Tripathi et al. 2019). Furthermore, abundant carbon sources in the medium, nutrient-limiting circumstances, and a constant supply of nicotinamide adenine dinucleotide phosphate (NADPH) and acetyl-CoA trigger lipid synthesis in oleaginous bacteria (Ochsenreither et al. 2016). Under stressful conditions, DHA and EPA production is increased, and they take the place of GLA and arachidonic acid (AA; 20:4n-6). For the formation of some types of lipids, carbon sources other than glucose are desired. **Table 2** represents the recent strategies for alternative sources of carbon to traditional fish sources for improving the DHA yield, coming from several sources such as microalgae, transgenic plants, precursor fatty acids, phytohormones, and small molecules.

Table 2 : Use of alternative carbon sources and phytohormones for improving PUFA production

Raw material	**Strain**	**DHA (%)**	**Ref**
Marine diatoms	*Phaeodactylum tricornutum*	36.5	(Hamilton et al. 2016)
Plant (transgenic)	*Camelina sativa*	18.0 ± 4.0 (DHA + EPA)	(Betancor et al. 2016)
Volatile fatty acids (VFA)	*Crypthecodinium cohnii*	29.8	(Chalima et al. 2019)
Hemp hurd (*Cannabis sativa*)	*Schizochytrium* sp. DT3	38	(Gupta et al. 2015)
Glycerol (crude)	*Aurantiochytrium* sp. strains	27.4–37.8	(Lee Chang et al. 2015)
Glycerol	*Aurantiochytrium* sp. strains	54.3–55.2	
Rapeseed Meal Hydrolysate and Waste Molasses	*Crypthecodinium cohnii*	22–34	(Gong et al. 2015)
Phytohormones	*Thraustochytrium roseum*	982 mg/L	(Wu, Chai & Yang 2003)
Gibberellin	*Aurantiochytrium* sp. YLH70	79.1	(Yu et al. 2016)
Kinetin (KIN)	*Aurantiochytrium* sp. SW1	66-84	(Nazir et al. 2020)
Jasmonic acid (JA)			
Gibberellic acid (GA)			

Propyl gallate or butylated hydroxytoluene (BHT)	*Schizochytrium* sp. S31	35% (propyl gallate) and 51% BHT	(Singh et al. 2015)

The microbial production of sustainable biochemicals or biofuels from lignocellulose biomass derived from agricultural feedstock, industrial and urban bio-residue, or woody biomass has received significant interest (Kumar et al. 2020; Putro et al. 2016; Xu et al. 2019). Lignocellulose biomass (LCB) can be used as a cheap carbon source for growing microbes, such as oleaginous microorganisms, lowering the overall cost of producing biofuels, value-added metabolites, and coproducts (Wei et al. 2017). The primary components of LCB are lignin, cellulose, and hemicellulose, which require processing to disrupt cross-linked bonds and alter the compact structure. By expanding the cellulose surface area, pretreatment increases the hydrolysability of complex materials and maximizes enzyme penetration, resulting in sugar hydrolysate (SH) that can be used to produce ethanol and lipids (Kucharska et al. 2018). The primary ingredients of the sugar hydrolysate include reducing sugars such as glucose, xylose, mannose, arabinose, glucan, rhamnose, and cellobiose, and their concentration varies depending on the biomass composition. LCB can be used to produce nutraceuticals and other industrial chemicals in addition to biofuels (Li, X et al.). Nutraceuticals are food-derived supplements that have both nutritional and therapeutic properties (Nasri et al. 2014). Flavonoids, ginseng, Echinacea, green tea, glucosamine, omega-3, lutein, folic acid, and cod liver oil are all popular nutraceuticals. Nutraceuticals have recently attracted a lot of attention due to their potential nutritional, safety, and therapeutic benefits (Tapas, Sakarkar & Kakde 2008). Most nutraceuticals have antioxidant activity, which can help to prevent serious diseases such as diabetes and infection (Nasri et al. 2014). The mechanisms of action of nutraceuticals are not fully understood. They could, however, be engaged in a wide range of biological processes, including activation of signal transduction pathways, antioxidant defenses,

gene expression, cell proliferation, differentiation, and mitochondrial integrity preservation (Leena et al. 2020). The ability of LCB to synthesize critical fatty acids and their derivatives has been described in several recent papers (Gupta et al. 2015; Sawatdeenarunat, Sung & Khanal 2017; Zhang et al. 2019).

1.1.3. PUFAs in Hemp seed

Plants are valuable sources of a few PUFAs, such as oleic acid, linoleic acid (LA), GLA, ALA, and octadecatetraenoic acid, but they lack the desaturase and elongases needed to synthesize EPA and DHA (Abedi & Sahari 2014). Generally, ω-3 PUFAs can be found in flaxseed, chia, and walnuts, while ω-6 PUFAs may be found in safflower oil, corn oil, and soybean oil. Several seed companies have succeeded in improving conventional crops to produce more nutritional yields through the application of modern plant breeding (Qaim 2020), alternative organic nutrients (Timsina 2018), etc. However, some disadvantages still remain, including lower yields or higher consumer prices. The FA content of hemp contributes significantly to its nutritional benefits (Callaway 2004). The oil, which makes up half the weight of the seeds, contains 75% EFA, including 20% ω-3 ALA, 3% GLA, and 1% stearidonic acid (SDA), a rising star of ω-3 PUFAs (Small & Marcus 2003). LA (18:2 ω-6) and ALA (18:3 ω-3) are the two-primary ω-6 and ω-3 PUFAs found in hemp seed oil (HSO), respectively. It is generally known that the human body can create DHA and EPA from ALA and LA (Domenichiello, Kitson & Bazinet 2015). Furthermore, the high polyphenol content of HSO makes it a prospective source of bioactive components for use in functional foods and pharmaceuticals (Smeriglio et al. 2016). Flavonoids, phenolic acids, phenolic amides, and lignanamides are polyphenolic components found in HSO (Leonard et al. 2021). Phytochemicals produced from flavonoids account for 80% of phenolic acids. HSO has strong oxidative activity, with phenolic acids acting as antioxidants (Pratap Singh

et al. 2020). The DPPH assay is extensively utilized to predict antioxidant activity based on the process by which antioxidants limit lipid oxidation due to its rapid and simple procedure, resulting in DPPH radical scavenging and thereby determining free radical scavenging capacity (Garcia et al. 2012). To form a stable molecule, DPPH free radical absorbs an electron or hydrogen radical. The highest UV-vis absorption of this radical is between 515 and 519 nm (Rahman et al. 2015). The ability of a sample to donate hydrogen atoms is usually assessed by the decolorization of the DPPH solution. In methanol, DPPH creates a violet/purple color, which fades the yellow shades in the presence of antioxidants. The decrease in absorbance can be used to track the progress of the reaction until it hits a steady-state (Hartwig et al. 2012). HS is a comprehensive nutritional food because it provides all the required amino acids, as well as proteins, carbohydrates, and fiber (Leonard et al. 2020). The viability of producing ω-3 LC-PUFAs in higher plants has been investigated in recent years (Amjad Khan et al. 2017), and significant progress has been achieved in the effective production of EPA and DHA in oilseeds (Tocher et al. 2019). Due to its high oil yield and high biomass content, HSO generated from hemp seed (HS) using various processes is regarded as a low-cost triacyl glyceride feedstock and thus could be used in enhancing ω-3 fatty acid production in this work.

1.2. Hemp seed and hemp market status

1.2.1. Hemp plant– origin and benefits

Hemp, also known as *Cannabis sativa* L., which belongs to the *Cannabaceae* family, is a herbaceous, wind-pollinating plant originating in Central Asia (Farinon et al. 2020). Recently, there are two existing types of cannabis plants: domesticated plants that were selected based on their beneficial characteristics for humans and ruderal ones that are grown outside of the field. Independently of its origin, the modern-domesticated form of *C. sativa* L. is widely utilized and

farmed not only in Asian countries but also in Canada, the United States, Europe, and Africa (Russo 2007). It is popular as a multifunctioning, low-impacting crop that is utilized in a variety of disciplines, including agriculture and phytoremediation, as well as the food, cosmetic, construction, and pharmaceutical industries (Irakli et al. 2019). Indeed, several industrial products like fiber and shives, bio-based building and thermal insulating materials, seeds, flour, and oil with essential nutritional and functional properties, and bioactive substances of pharmaceutical relevance can be obtained from this highly adaptable plant (Abedi & Sahari 2014).

1.2.2. Hemp market - potential and challenges

Cannabis sativa is divided into two types: drug-type (marijuana) and non-drug-type (cannabis) (hemp) (Atakan 2012). The former is commonly used for medical and recreational purposes, while the latter is crucial to the food and fiber sectors (Crini et al. 2020). Hemp seed has experienced a resurgence of attention in the last 20 years because of its nutritional and medicinal benefits. Hempseed cultivation at low tetrahydrocannabinol (THC) levels (less than 0.3% w/w) has been permitted in Australia, Canada, and, most recently, the United States (Leonard et al. 2020). Demographic trends and their impact on social and health spending are one of the main drivers for the predicted growth of the ω-3 market (Oliver et al. 2020). According to the World Health Organization (WHO), the world's population is aging as a result of the continuous drop in birth rates and higher life expectancies, as well as the world's population's increasing age. As a result of this demographic shift, the number and proportion of people over 60 have increased. Eurostat updated population forecasts for the period 2018 to 2100 in July 2019: the EU-270s population is expected to peak at 449.1 million around 2030, then steadily fall to 416.9 million by 2100. The need for nutritional foods from the community, especially the elderly, which help them prevent and reduce co-morbidities, has increased. The worldwide market for microbial DHA was expected

to be valued at around $350 million in 2012, but that figure was raised to approximately $4,212 million in 2017, demonstrating a considerable increase in demand for high-quality microbial DHA (Bannenberg et al. 2017; Subhadra & Edwards 2011; Vigani et al. 2015). Correct nutrition must be addressed throughout the life of each person to give everyone the chance to live a long and healthy life. According to worldwide statistical estimates, there will be an increase in demand for specific substances that can help people age well. In 2016, the American Heart Association (AHA) predicted that by 2035, 45.1 percent of the US population would be suffering from cardiovascular disease (CVD), with a cost of $1.1 trillion (McClellan et al. 2019). Since numerous studies have demonstrated the effectiveness of ω-3 PUFA in preventing CVD, several international authoritative bodies and organizations, including WHO, the European Food Safety Authority (EFSA), AHA, and others, recommend and promote regular ω-3 PUFAs intake (Oliver et al. 2020). The growing frequency of ocular disorders presents another important opportunity for ω-3 PUFAs (Downie et al. 2019). Changes in the stratospheric ozone layer and variations in solar ultraviolet (UV) radiation are linked to climate change. However, the emergence of modern technology associated with the widespread use of blue light screens in our daily lives is hastening the onset of vision disorders in people of all ages (Zhao, Z-C et al. 2018). According to medical data, DHA support for retinal cells is a critical ingredient for healthy vision (Johnson et al. 2008). However, even if the market for ω-3 PUFAs components is anticipated to expand as a growing industry within the market for functional ingredients, there are other societal and environmental factors to consider. The Australian definition of HS is the seeds obtained from a plant of the genus Cannabis that contain no more than 1% total THC content and can be legally used for human consumption (The Office of Drug 2019). Despite their great nutritional content, the seeds of this plant were once

thought to be a waste product from the fiber industry and were therefore mostly used as animal feed, making them illegal for human use until 2017 (Food Standards & New 2017).

Cultivation costs make up a significant component of the cost of mass-producing microorganism products (Pham et al. 2019). Before their economic potential can be fully realized, more effort must be put into lowering production costs in an environmentally sustainable way. Producing enough biomass with a high yield of the target chemicals is part of this. Adjusting growth circumstances has been suggested to alter growth rates and cellular composition (Adadi et al. 2012). The use of bio-stimulants during plant growth—in this case, hemp—has recently piqued interest, as it may lead to changes in seed oil concentrations and/or composition, as well as changes in the phytochemical profile of these oils. As a result of this, the composition of hemp seeds has been tweaked or changed (Patel et al. 2014). Cultivation conditions should be chosen to maximize the accumulation of the principal product as lipids for biofuel production in the biorefinery (Alishah Aratboni et al. 2019). Furthermore, methods for increasing secondary metabolite production for the nutraceutical and pharmaceutical industries would considerably increase the value of the biomass (Isah et al. 2018).

1.2.3. Enhancing the quality of hemp seed by biostimulant treatment

Biostimulants have recently attracted increased interest in the agriculture industry due to their capacity to boost plant fitness and tolerance to biotic and abiotic challenges. Hormones, peptides, phenolic compounds, saccharides, and other chemical components contribute to their biological activity (Yakhin et al. 2017). On the other hand, commercial bio-stimulating preparations are mostly made up of marine algae (Kapoore, Wood & Llewellyn 2021), protein hydrolysates (Di Mola et al. 2021), free amino acids (Irani, ValizadehKaji & Naeini 2021), or humic compounds (Conselvan et al. 2017; Guerriero et al. 2021). Several studies have demonstrated the beneficial

effects of bio-stimulants derived from animals or plants on plant growth and metabolism, even when the environment is stressful, such as salt. Plants treated with bio-stimulating preparations containing active chemicals may reap a slew of unmistakable benefits, such as minimizing the use of fertilizers, contributing to enhanced plant growth, and increasing the resistance to water and abiotic stresses (du Jardin 2015). Their use not only promotes plant growth and development, resulting in improved seed quality but also lowers costs and improves crop efficiency (Rajabi Hamedani et al. 2020). Furthermore, the use of such seeds in Thraustochytrid fermentation may lead to interesting outcomes (Jorobekova & Kydralieva 2019). For instance, in a recent work, the organosolv-pretreated spruce hydrolysate was employed as a carbon source in the fermentation of *Schizochytrium limacinum* SR21 strain to create DHA at a maximum of 66.72% w/w total lipids and antioxidative squalene compounds after 72 h (Pratap Singh et al. 2020). Additionally, treatment of hemp during plant growth with biostimulants is hypothesized to alter functional food phytochemicals, which may further enhance the desirability of hemp as a carbon source for fermentation to produce FA products (Di Mola et al. 2021).

1.2.4. Hemp seed and hemp seed oil

Due to expanded information on the high nutritional value and potential utility of the seeds of the *C. sativa* L. plant, usually known as HS, there has now been a growing interest over the past 20 years (Leonard et al. 2020). In terms of appearance, hemp seed has a round shape, a dark red-brown color, and a diameter that varies in the range of 3.0 to 5.0 mm. Each seed is covered by two thin layers of the pericarp; the layer of tube celled outside and the layer of spongy parenchyma celled inside, along with endosperm and two inner cotyledons (Chandra et al. 2014). It is reported that depending on the hemp cultivar, the nutritional proportion in HS can be different, specifically oil (25–30%), protein (25–30%), fiber (30–40%), and moisture (6–7%) (Callaway 2004;

Rodriguez-Leyva & Pierce 2010). The HS also only contains a minimal amount of THC, which satisfies the threshold of the Food Standard laws (less than 0.005%) (The Office of Drug 2019). As a result, differences in the HS cultivar chemical composition contribute to the wide range of dietary, physicochemical, and perceptual aspects of hemp-added food products. HS is becoming more popular due to more than 90 percentages of unsaturated FAs amount and a favorable ratio between ω-6 and ω-3 PUFAs is 4:1 (Leizer et al. 2000).

Hemp seed oil can be extracted using a variety of methods, including cold pressing, solvent extraction, Soxhlet extraction, microwave-assisted extraction, etc. (Devi & Khanam 2019; Rezvankhah et al. 2019). Various thermolabile components in hemp seed oil are maintained at low temperatures. As a result, the extraction technique has a significant impact on the quality of oil in terms of its physico-chemical characteristics (Mwaurah et al. 2020). Soxhlet extraction is well-known and commonly used by researchers for extracting oils from seeds, but the method has the disadvantage of being time-consuming and non-eco-friendly due to the massive amounts of organic solvent emitted into the atmosphere or discharged into water resources (Danlami et al. 2014). In the batch process, it runs on a continuous cycle of solvent, leaving the extracts in the extract chamber and contacting the biomass. This equipment accommodates the strongest extraction driving force among all extraction procedures since it exposes the biomass to a fresh stream of the organic solvent throughout the operation (Valizadehderakhshan et al. 2021). Another approach is cold pressing, which is a convenient and common procedure with some benefits and drawbacks (Çakaloğlu, Özyurt & Ötleş 2018). Two of these advantages are the ability to extract oil without using high temperatures, which may affect the nutritional quality of the final product, and the absence of any organic solvent that is detrimental to human health and the environment (Mwaurah et al. 2020). However, as a negative, it should be noted that a significant amount of oil

is left in the seed cake, resulting in low extraction efficiency and, as a result, a predictable economic disadvantage (Çakaloğlu, Özyurt & Ötleş 2018). Some innovative solutions have arisen to overcome the above-mentioned issues related to cold-press and solvent methods. Microwave and ultrasound-assisted extractions (MAE and UAE), as well as supercritical fluid extraction (SFE), have all lately gained popularity as alternate ways of extracting hemp seed and other seed oils. In comparison to traditional Soxhlet and solvent extraction procedures, these approaches provide a high level of oil yield (particularly in terms of microwave and ultrasonicators) and a substantially shorter extraction time (Danlami et al. 2014; Rezvankhah et al. 2019). By improving the mass transfer rate, virtually all mechanical pretreatment procedures, including macro-scale grinding and micro-scale ultrasonication, may improve extraction yield and save operation time (Kaseke, Opara & Fawole 2021a). However, the mechanical energy applied to the system eventually converts to thermal energy, which can lead to transformation. As a result, caution is necessary when extracting essential fatty acids from hemp seeds, since the generated heat has the potential to decarboxylate the natural cannabinoids (Valizadehderakhshan et al. 2021). On the other hand, microwaving, enzymatic digestion, pulsed electric field, and ultrasonication are examples of innovative seed pretreatment procedures that not only increase oil yield and quality parameters, but also minimize seed oil extraction time, solvent, and energy consumption (Khadhraoui et al. 2021; Tamborrino et al. 2019; Wang, Y-H et al. 2020). Oil from pretreated seeds has more phenolic chemicals, carotenoids, tocopherols, phytosterols, and antioxidant qualities, which can help prevent serious diseases (Kaseke, Opara & Fawole 2021b). The potential of seed pretreatments to enhance the extractability of bioactive compounds from plant material has led to the application of novel pretreatment techniques to a variety of oilseeds, owing to increased consumer interest in functional

foods and the potential of seed pretreatments to enhance the extractability of bioactive compounds from plant material (Kaseke, Opara & Fawole 2021a).

In terms of biomass, biomass from hemp plants is largely polysaccharide-rich, with cellulose (30–50%), hemicellulose (10–20%), and lignin (10–20%), as well as ash and extractives comparable to those found in other herbaceous or woody plants (Ji, A et al. 2021). Hemp biomass has more cellulose components and less lignin, according to several studies, making it suitable for the generation of fermentable sugars and vehicle biofuel (Semhaoui et al. 2018). Pretreatment is required to remove/reduce hemicellulose and/or lignin and boost cellulose accessibility to alleviate biomass recalcitrance variables, such as crystallinity of cellulose in hemp tissues (Kim 2018). Dilute acid, hydrothermal-mechanical, liquid hot water (LHW), steam explosion, deep eutectic solvents, and ionic liquid are some of the pretreatment methods used to boost sugar yields (Roy, Rahman & Raynie 2020). The pretreatment condition and subsequent cellulolytic saccharification procedure determine the increase in sugar production. In a prior study, the authors discovered that pretreatment with sodium hydroxide (NaOH) increased glucose production by up to 88.9%, which was substantially greater than pretreatment with acid (41.7–58.7%) or LHW (59.1–71.7%) due to higher glucan composition and lignin removal (Zhao, J et al. 2020). The high severity of NaOH pretreatment and its swelling effect in alkaline solutions, which keeps the exterior surface of the particles in contact with the solution, contribute more to lignin removal and glucose conversion than other pretreatment methods (Modenbach & Sue 2014). Pretreatment of lignocellulosic materials enhances the internal components of cellulose by solubilizing hemicellulose and lignin as well as increasing surface area and porosity, which helps cellulolytic enzymes decompose cellulose (Kucharska et al. 2018). While pretreatment is required for the utilization of

lignocellulosic biomass, it also results in the production of toxic compounds that prevent enzyme exposure to cellulose and microbial cell viability during the fermentation process (Kim et al. 2021).

1.3. Thraustochytrid fermentation

1.3.1. Thraustochytrid and its application

It is essential to utilize biomass more efficiently for a sustainable food sector. Biomass conversion to biofuels and chemicals such as fuel, biogas, bioethanol, and biodiesel enhances global moderate energy supplies while reducing global warming (Haryana 2018). Our reliance on fossil fuels will be lessened as a result of these biofuels, and there will be fewer greenhouse gases and pollutants in the environment as a result. The bioeconomy can help with waste management by producing biofuels, energy, and other high-value goods from municipal solid trash, vegetable and market waste, agricultural residue, slaughterhouse wastes, and industrial wastes. In addition, the bioeconomy creates new job opportunities in industries such as biofuel manufacturing and processing (Liu et al. 2017). One strategy for reducing biomass loss is to use food industry by-products as raw materials to make other products of interest, such as methane (Kakuk et al. 2021), ethanol (Qarri & Israel 2020), or nutraceuticals (Majumdar, Mandal & Dasgupta Mandal 2020), to name but a few. In marine microorganisms and phytoplankton, EPA and DHA are generated *de novo*, and they accumulate in fish through the food chain (Sprague, Betancor & Tocher 2017). Some ocean fish, like Pacific sardines, can acquire considerable levels of EPA and DHA by eating microalgae cells in the ocean. Aquaculture, medicine, human nutritional supplements, terrestrial animal feed, pet food, and personal care all use EPA and DHA (Ji, X-J, Ren & Huang 2015). Novel sources of ω-3 PUFAs can also be produced from marine algal or algae-like microbial oils, which could alleviate many of the taste and odor issues associated with fish while also avoiding the flaws of fish oil-based processes (Harwood 2019). However, because EPA productivity is too low to

achieve commercial requirements, there was no large-scale land-based EPA production from wild-type species (Xie, D, Jackson & Zhu 2015). Microorganisms found in the sea, such as bacteria, fungi, and thraustochytrid, have been offered as a PUFA alternative to fish oil, which is now the most extensively utilized source of LC-PUFAs (Pérez et al. 2021). Thraustochytrids are oleaginous protists belonging to the Phylum Heterokonts (Labyrinthulomycota), Class Thraustochytridae (Labyrinthulomycetes), Order Thraustochytriales, Family Thraustochytriaceae, which includes the genera *Thraustochytrium, Ulkenia,* and *Aurantiochytrium* (Raghukumar 2002). Except in the biflagellate spore stage, Thraustochytrid has a globose sporangium wall with a multi-layer laminate that is stationary. Thraustochytrid is well-known for its high levels of LC-PUFAs, DHA, and docosapentaenoic acid (DPA). Their lipid accumulation is a complex process that is influenced by the organism, culture circumstances, and growth phase (Orozco Colonia, Vinícius de Melo Pereira & Soccol 2020). Thraustochytrids are natural producers of ω-3 PUFAs because they may manufacture up to 70% of total lipids as DHA (Patel et al. 2021). Some Thraustochytrids can be grown to produce a large amount of biomass with a significant amount of lipids rich in LC-PUFAs. However, the production of LC-PUFAs can be manipulated by adjusting the physical and chemical parameters of the growth medium (Quilodrán et al. 2020). Furthermore, the high expense of carbon substrates for Thraustochytrid development limits the economic and long-term synthesis of microbial DHA. Low-cost renewable substrates can be used to solve this challenge (Lopes da Silva et al. 2019). Thraustochytrid are promising marine protists to increase oil yield due to their potential for producing phospholipids. This novel marine strain has been utilised in a series of studies for ω-3 quality and quantity determination conducted by Gupta and his co-workers (Gupta et al. 2015). Moreover, the application of metabolic engineering promises to enhance the yield of PUFAs, DHA, EPA, and many other compounds (Morabito et al. 2019). Thraustochytrids, in particular DHA and

DPA, are a promising source of PUFAs (Quilodrán et al. 2020). Bioengineering tools must be used to adjust fermentation settings in order to improve biomass growth and PUFA content. Temperature, dissolved oxygen, pH, salinity, and the composition of the medium nutrition have all been shown to influence PUFA synthesis in Thraustochytrid (Patel et al. 2021). Hence, it is necessary to optimize settings for maximal production efficiency or investigate the implications of altered conditions.

1.3.2. Biosynthesis pathway

FA production occurs in Thraustochytrid via two metabolic pathways (Morabito et al. 2019). The first pathway, which creates FAs chains of 16C, requires a type I Fatty Acid Synthase (FAS) system identical to that seen in mammals. In the second pathway, polyketide synthase-like (PKS-like) machinery, or PUFA synthase, is used to produce very long-chain polyunsaturated fatty acids (VLC-PUFAs) of 20C and 22C. In oleaginous microorganisms, two different routes for LC-PUFA synthesis exist: the aerobic desaturase and elongase pathway and the anaerobic pathway (Jovanovic et al. 2021). All microalgae, as well as most fungi, bacteria, and plants, have an aerobic desaturase and elongase pathway (Monroig & Kabeya 2018). In the absence of molecular oxygen and with only a small quantity of reducing power (NADPH), the anaerobic pathway occurs in Thraustochytrid and bacteria (Morabito et al. 2019). Standard FA synthesis produces saturated FAs, while DHA is synthesized by a PKS. Despite this, the molecular pathway and regulatory network for PUFA synthesis, as well as the molecular mechanisms driving Thraustochytrid ecological activities, are largely unknown. Recently, the lipid synthesis pathway in Thraustochytrid and distinguishing it from that of other microorganisms, including proper microalgae, was described (Patel et al. 2021). Both eukaryotes and prokaryotes, including microalgae, fungi, thraustochytrids, bacteria, mammals, and plants, use the traditional aerobic

desaturase and elongase pathways for PUFA synthesis (Remize et al. 2021). As shown in **Figure 1**, in the endoplasmic reticulum, palmitic acid (C16:0) is converted to stearic acid (C18:0), which is then desaturated to oleic acid (C18:1) by 9-fatty acid desaturase and then to LA (C18:2) by 12-fatty acid desaturase. 15-fatty acid desaturase converts LA (one of ω-6 PUFAs) to ALA (C18:3), which initiates the ω-3 PUFA production pathway. All ω-3 PUFA-producing microorganisms and plants have desaturases 12 and 15, but humans and animals lack them. Elongase enzymes, also known as elongation of very-long-chain fatty acids (ELOVL6), catalyze the initial and rate-controlling condensation step. ALA is desaturated to stearidonic acid (C18:4) by 6-fatty acid desaturase, then elongated to eicosatetraenoic acid (C20:4, ETA) by ELOVL6 and becomes ETA (C20:4 or C18:2) before being introduced to another double bond by 5-fatty acid desaturase to form EPA (C20:5 or C18:2). The next step is the ELOVL5-mediated elongation of EPA to DPA (C22:5), followed by the 4 desaturase-mediated desaturation of DPA to create DHA (C22:6). The ω-6 PUFA production route begins with the conversion of LA to GLA (C18:3) by 6-fatty acid desaturase, followed by the activity of ELOVL6 on GLA to generate dihomo-γ-linoleic acid (C20:3, DGLA). 5-fatty acid desaturase converts the latter to AA (C20:4), which is then transformed into docosatetraenoic acid (C22:4, DTA) and DPA by ELOVL5 and 4-fatty acid desaturase, respectively (**Figure 1**).

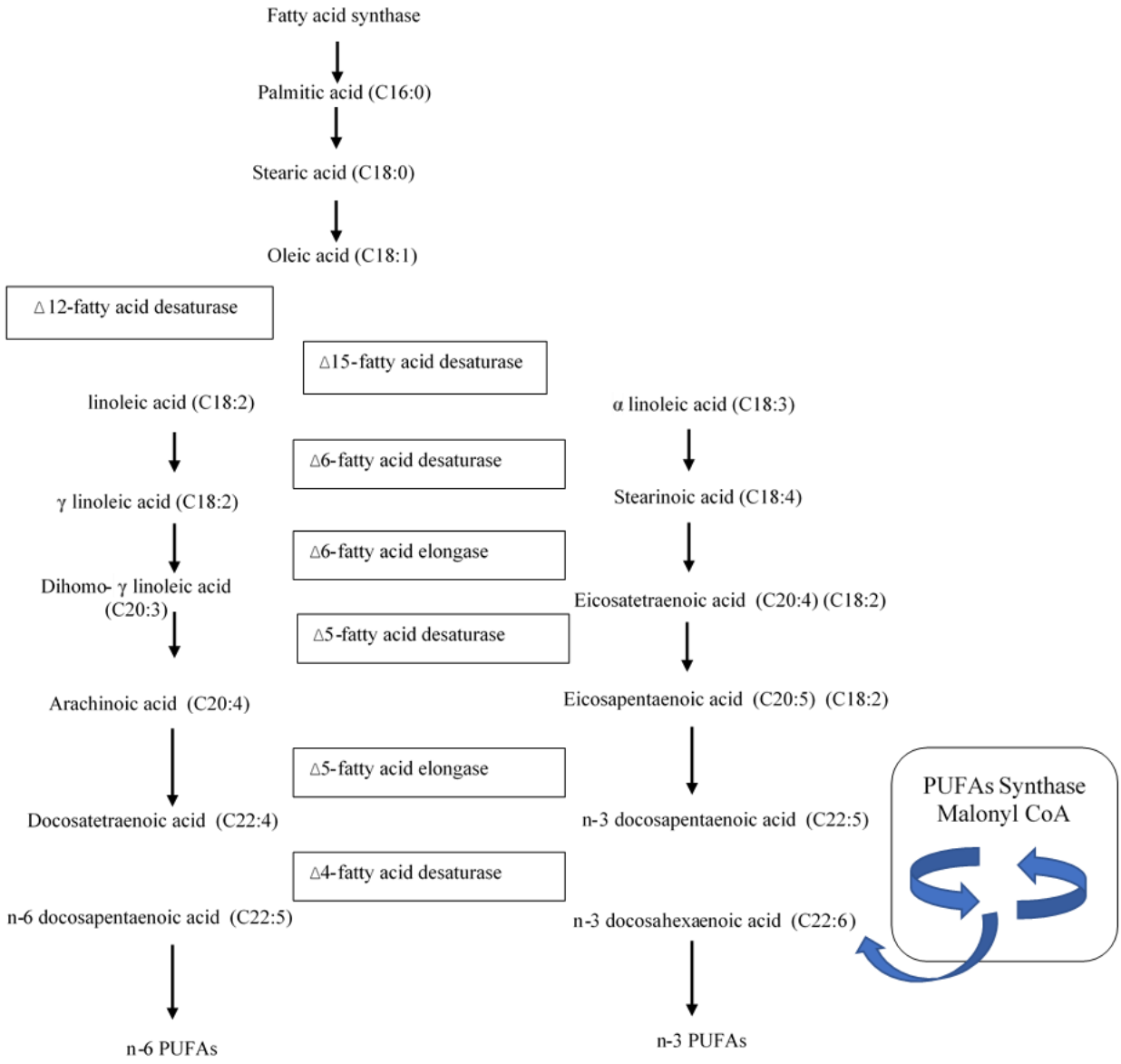


Figure 1: Biosynthesis pathway of PUFAs in Thraustochytrids (This figure has been adapted from Morabito (2019).

The anaerobic PKS pathway, which is found in both eukaryotes and prokaryotes, is an alternative to the desaturase or elongase systems for PUFA synthesis (Meesapyodsuk & Qiu 2016). The 3-ketoacylsynthase enzyme catalyzes the condensation of acyl-acyl carrier protein as an acyl carrier protein (ACP) and malonyl-ACP to create 3-ketoacyl-ACP, which is the first step in the PUFA

synthesis of the PKS system. The ketoreduction of ketoacyl-ACP to hydroxy-acyl-ACP is then mediated by 3-ketoacyl-ACP reductase. When hydroxy-acyl-ACP is dehydrated, unsaturated enoyl-ACP is formed, which is then reduced to a saturated acyl chain. The dehydratase-isomerase module of the PKS pathway for double bond insertion during elongation of FAs differs significantly from traditional oxygen-dependent desaturation.

Malonyl-CoA:ACP transacylase (MAT) is an important enzyme that can alter DHA and EPA synthesis. Overexpression of acetyl-CoA synthase has previously been shown to increase biomass and total lipid synthesis (Li, Z et al. 2018). Malonyl is transformed into malonyl-ACP, a fundamental building component of the FAS pathway, via MAT. The similar role that MAT plays in the PKS pathway, which is more active in Thraustochytrid, has resulted in increased DHA and EPA production (Wang, S et al. 2020). In a previous study, a novel marine Thraustochytrid, which is a large-cell marine microorganism belonging to the group of eukaryotic protists, can be considered as a promising candidate to substitute microalgal and plant species (Nham Tran et al. 2020). According to the phylogenetic analysis, Thraustochytrid is classified as oleaginous microbes capable of producing oils rich in PUFAs and a high yield of DHA and EPA through fermentation (Ghiffary, Kim & Chang 2019). Accordingly, the utilization of Thraustochytrid species could be a potential strategy for improving the value of low-waste hemp-based materials into highly valued functional and medicinal foods. However, the process has not been optimized yet, conventional inputs produce insufficient yield, and sustainability is the remaining issue that needs to be solved (Rupasinghe et al. 2020).

Recently, it has been suggested that the growth conditions of microorganisms could be modified for the possible enhancement of the desired product. Herein, a study was conducted in which hemp seed was evaluated for its potential to be utilized as an alternative carbon source/inducer for the

thraustochytrid fermentation. It was hypothesized since the hemp seed contain specific unsaturated fatty acid thus may enhance PUFA production including biomass and lipid content. Also, it was hypothesized if Thraustochytrid fermentation process can lead to antioxidative activity.

2. Aim and objectives

This project aimed to investigate the potential of hemp seed (HS) as an alternative carbon source in Thraustochytrid fermentation to enhance biomass and lipid production (**Figure 2**). The work was achieved by the completion of the following detailed objectives:

- Characterization of the impact of HS on the fermentation of the novel Thraustochytrid S2 strain to enhance biomass and PUFA yield
- Comparison between hemp seed, market hemp seed, and commercial hemp seed oil on PUFA content after Thraustochytrid fermentation
- Investigated the antioxidative potential of HS and fermentation products of Thraustochytrid via DPPH assay.

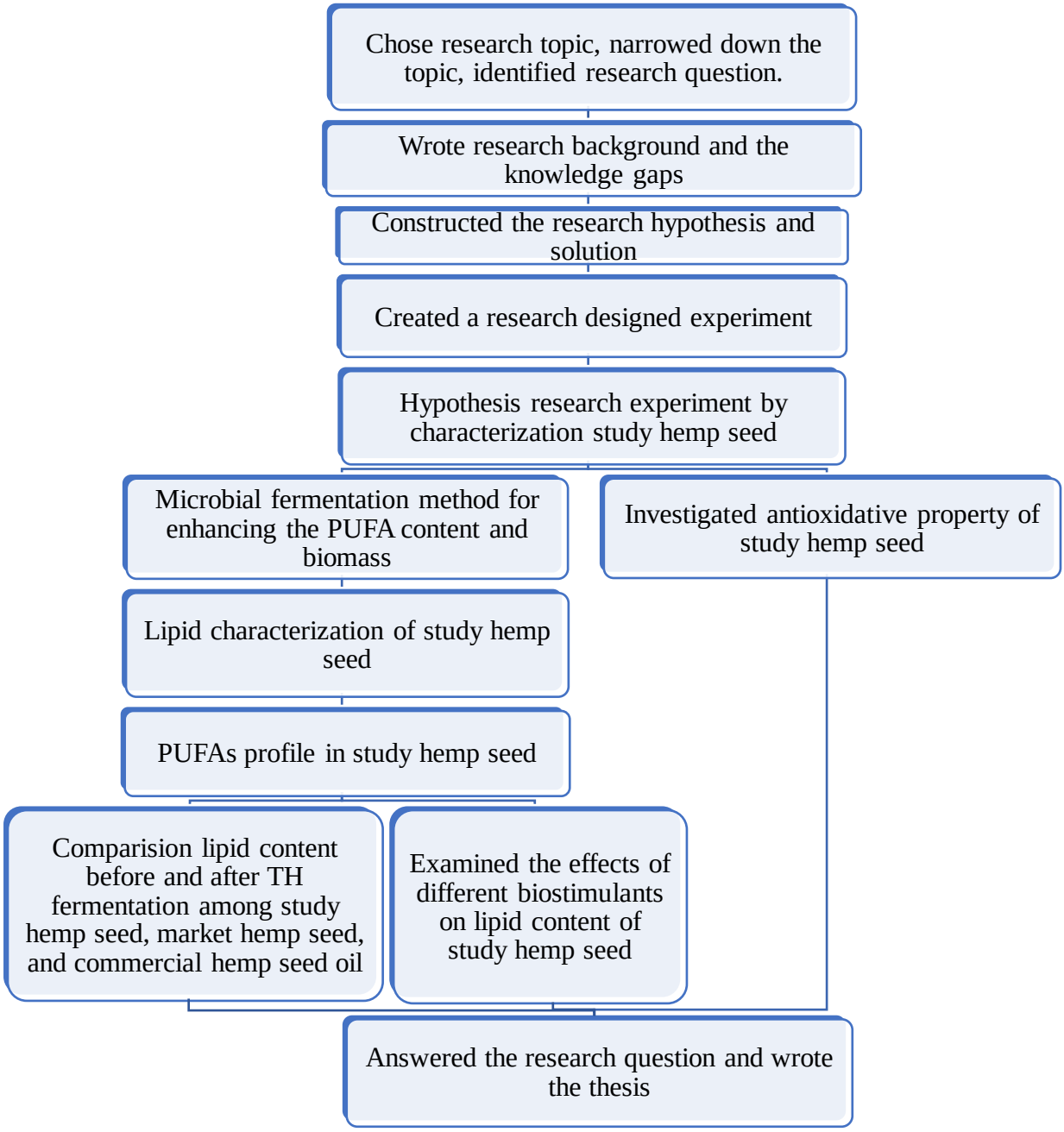


Figure 2: Overall scheme of the carried study

Chapter II. MATERIALS AND METHODS

1. Materials, Strain and Instruments

1.1. Materials

Glucose, yeast extract, peptone, artificial seawater (ASW), agar, NaOH, distilled water (DW), Milli-Q (MQ) water, chloroform, methanol (MeOH), sodium sulfate (Na_2SO_4), hexane, toluene, methyl nonadecanoate (C19:0), butylated hydroxytoluene (BHT), acetyl chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, hydrochloric acid (HCl, 0.02M), 3,5-dinitrosalicylic acid (DNS), and other chemicals used in assays were sourced from Sigma-Aldrich, USA. Acidic MeOH was prepared by drop-wise acetyl chloride (5 mL) to MeOH on ice (50 mL). To prepare 1L of ASW (50%) stock, Instant Ocean sea salts (33.33g) was dissolved into distilled water, then underwent vacuum-filter. Glucose stock 40 % (w/v) was prepared as follows: 40g of glucose was stirred in 50 mL of MQ water with heat (50°C) until completely dissolved. The final volume was made up to 100 mL by adding MQ water, then stock solution was filtered by syringe with filter. All the steps were performed under laminar flow.

Hemp seed (HS) was kindly provided by Nutrifield Pty, Ltd (Melbourne, Victoria). The requisite permission for using hemp seeds in this study was secured from Primary Industries and Regions South Australia (PIRSA) for implementing this work.

Market hemp seeds (MHS) were bought from an online store (Melbourne,Victoria) which is said to be comprising of 100% hemp seed powder originating from Farnsfield—an Australian hemp cultivar from Hemp Farms Australia (Queensland, Australia). Both types of hemp seeds were totally crushed into powder and kept in a refrigerator for further use. Lipids were extracted following Gupta et al. (2013)methodology.

Commercial hemp seed oil (CHSO) sample preparation: Organic Hemp Gold™ Seed Oil (500 ml, v/v) was purchased (Adelaide, SA) and diluted for later use.

Fermentation medium: To grow microbes, Glucose Yeast Peptone (GYP) medium was prepared containing glucose (50 g/L), yeast extract (4 g/L), $MgSO_4$ (10 g/L), Monosodium Glutamate (MSG, 20 g/L) and peptone (0.4 g/L) in 50% ASW (500 mL). For each experiment, 20 ml of the medium was transferred to 100 ml flask and sterilized in autoclave at 121℃ for 20 min. Similarly, GYP agar medium was made by further adding agar powder (12 g/L). Fermentation medium consisting of 0.1% yeast extract, 0.1% mycological peptone, 50% ASW and either glucose or HS powder or CHSO was also prepared at pH 7.

Microbial strain: Thraustochytrid strain S2 (GenBank accession number KF682125) was isolated and kindly provided by Dr. Adarsha Gupta (Medical Biotechnology, College of Medicine and Public Health, Flinders University), was maintained in a GYP agar medium and incubated at 25°C with sterilized conditions for further use.

1.2. Instruments

Freeze-drier, centrifuge, spectrophotometer, capillary column (SGE, BPX70, 30m x 0.25mm, 0.25lm thickness), ultrasonic bath, shaking incubator, vortex, and a mass balance were used in this study. A Shimadzu GC 2090N (FAMEWAX column, 30 m x 0.32 mm ID (inner diameter) equipped with a flame ionization detector (FID) and coupled to a BID 2030 unit (Shimadzu) for detection of fatty acid methyl esters (FAME) (split injection, 1/100) was used for fatty acid analysis.

2. Methodology

2.1. Experimental Design

2.1.1. Extraction and Analysis of PUFAs Content

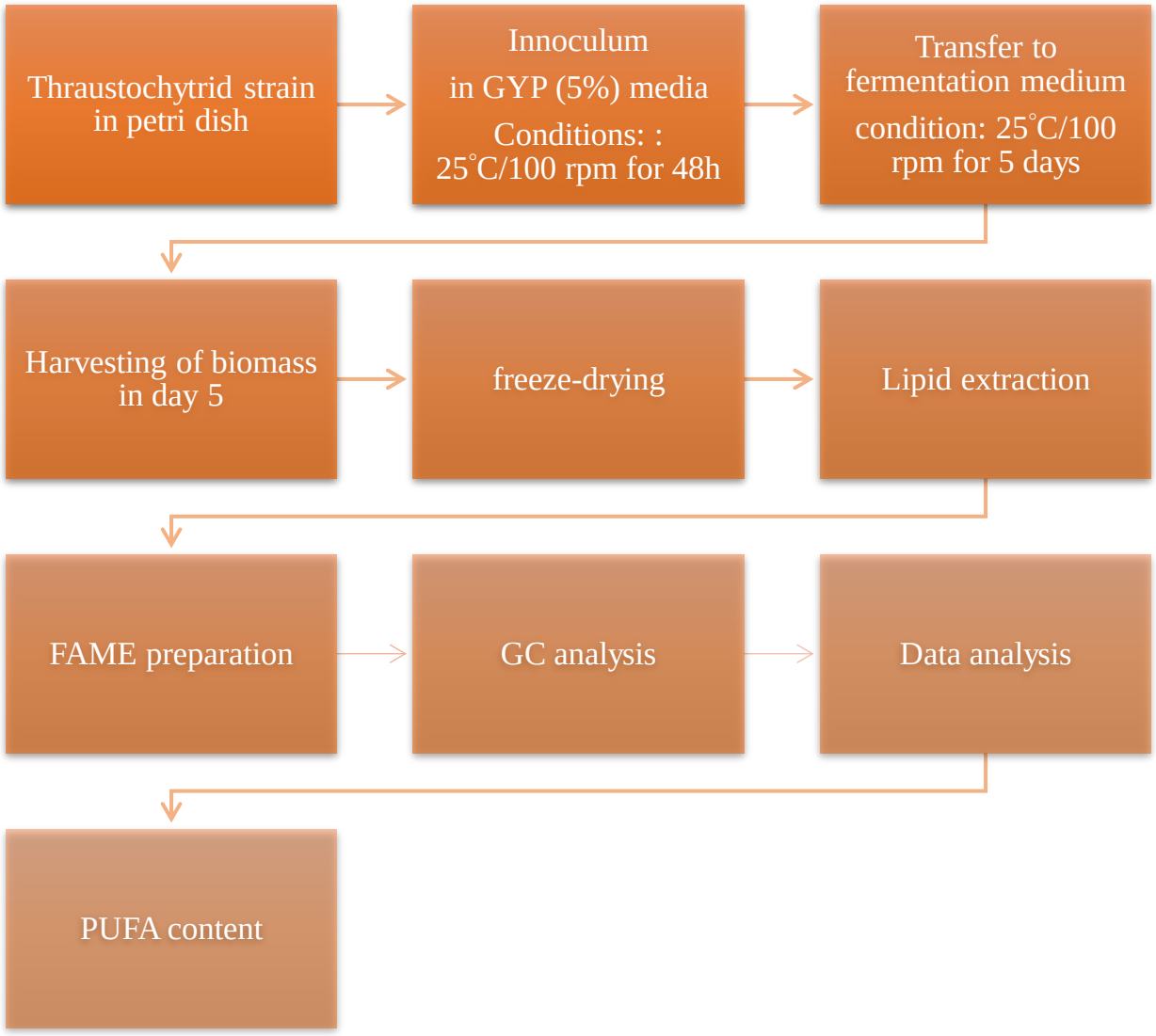


Figure 3: Overview of PUFA extraction and analysis procedure

2.1.2. Investigation of antioxidative property of HS and fermentation products

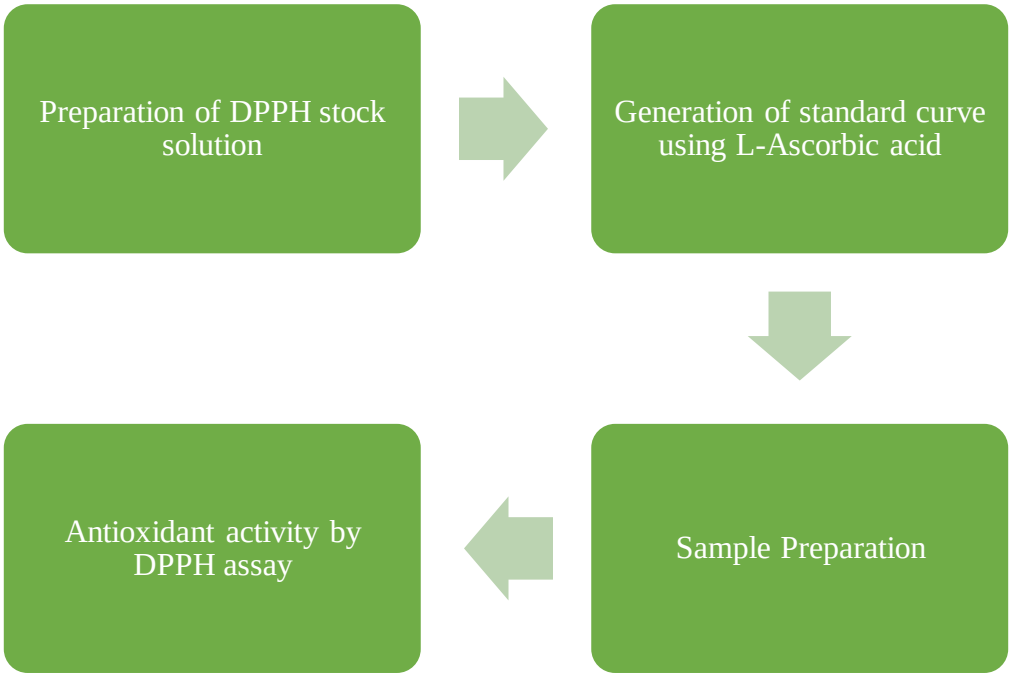


Figure 4: Investigation of antioxidant activities of hemp seed and fermentation product by DPPH assay

2.2. Procedure

2.2.1. Biomass growth and fermentation

According to the previous project carried out by Gupta et al. (2015), biomass growth and fermentation of Thraustochytrid cells were performed with minor modifications. Briefly, the Thraustochytrid cells were inoculated in GYP medium (5%) and incubated at 25°C with 150 rpm of shaking speed for 48 h at pH 7. Following that, 2 ml of subculture medium was transferred into fermentation medium and incubated for 5 days. To investigate the change in biomass, the biomass after each 24 h was harvested by withdrawing 20 mL of culture solution to measure optical density (OD) at the wavelength of 660 nm (OD660 nm) and cell dry weight (CDW). The growth of biomass was expressed as an increase in OD value at different incubation times. Biomass obtained

from fermented HS samples were diluted with DW (ratio 9:1), and the absorbance was measured at 660nm by using a spectrometer. The biomass was collected, and the supernatant was discarded by kill-cycle.

In this work, Thraustochytrid cells were grown on different fermentation medium as the following:

- **GYP 5%** (Glucose 5% - Yeast – Peptone – MSG – $MgSO_4$ – Artificial Seawater 50% strength),
- **GHYP 2.5%** (Glucose 2.5% - HS 2.5%- Yeast – Peptone – MSG – $MgSO_4$ - Artificial Seawater 50% strength),
- **GHYP 5%** (Glucose 5% - HS 5%- Yeast – Peptone – MSG – $MgSO_4$ - Artificial Seawater 50% strength),
- **HYP 5%** (HS 5%- Yeast – Peptone – MSG – MgSO4 - Artificial Seawater 50% strength),
- **GMYP 2.5%** (Glucose 2.5% - MHS 2.5%- Yeast – Peptone – MSG – MgSO4 - Artificial Seawater 50% strength),
- **GMYP 5%** (Glucose 5% - MHS 5%- Yeast – Peptone – MSG – MgSO4 - Artificial Seawater 50% strength),
- **MYP 5%** (MHS 5%- Yeast – Peptone – MSG – MgSO4 - Artificial Seawater 50% strength),
- **GOYP 2.5%** (Glucose 2.5% - CHSO 2.5%- Yeast – Peptone – MSG – $MgSO_4$ - Artificial Seawater 50% strength),
- **GOYP 5%** (Glucose 5% - CHSO 5%- Yeast – Peptone – MSG – $MgSO_4$ - Artificial Seawater 50% strength),
- **OYP 5%** (CHSO 5%- Yeast – Peptone – MSG – MgSO4 - Artificial Seawater 50% strength).

- GYP 5% was considered as control.

2.2.2. Cell dry weight (CDW) determination

The isolate was grown with different types of HS or MHS powder or CHSO added in 50 mL of fermentation medium. After 5 days, the biomass was harvested and centrifuged at 10,000 g for 10 min. The pellet was collected and washed three times with DW and freeze dried. Thraustochytrid cell pellet was weighed and recorded as CDW. The freeze-dried sample was stored at -20°C before proceeding with lipid extraction. Results are presented as mean ± standard deviation (SD) of samples prepared in triplicates.

2.2.3. Lipid extraction

The lipid extraction was conducted according to Gupta et al. (2013). In more details, 600 µL of solvent mixture of chloroform and MeOH (ratio 2:1, respectively) was added to 10 mg of freeze-dried biomass or 50 mg of powdered HS or MHS; and the mixture was vortexed for 2 min, followed by centrifugation at 10,000 g for 10 min. These steps were repeated three times to obtain three resultant supernatants in clear 12 mL pre-weighed glass vials after filtration with a 0.22 µm filter. Then they were all dried in an oven at 50°C or in a rotavap (R-300, Buchi) and performed for the measurement of lipid percentage gravimetrically. Two types of lipid production were collected and labelled as: HS and MHS.

2.2.4. Fatty acid ester methys (FAMEs) of HS and MHS powder

This step was conducted following the previous project carried out by Gupta et al. (2015). The extracted FAs were converted to methyl esters by acid-catalyzed transesterification as follows. First, 1mL of toluene containing 10mg of sample was added into a test tube, then 10 µL of C19:0 stock as an internal standard (50 mg of C19:0 in 10 mL of toluene) and 200 µL BHT as an

antioxidant (100 mg of BHT in 100 mL of toluene) were subsequently added. Next, 500 µL of acidic MeOH (prepared by adding dropwise 1 mL of acetyl chloride to 10 mL of methanol on ice and stirring for at least 1 hour) was added to the tube, and the solution was incubated overnight at 50°C. On the following day, the solution was cooled down to room temperature, then 1 mL of sodium chloride stock solution (5% w/v in MQ water) was added. The fatty acid methyl esters were extracted into hexane or heptane (1 mL of hexane, 2 times). The hexane layer was collected and washed with a 1 mL potassium bicarbonate stock solution (2% w/v in MQ water). The FAMEs sample was then extracted into hexane. The hexane layer was collected after three repetitions and dried over sodium sulfate. Hexane or heptane was evaporated to concentrate the FAME if needed. The final product was transferred to a 1.5 mL glass vial for GC analysis.

2.2.5. Gas chromatography (GC) analysis of FAMEs

According to previous study by Gupta et al. (2015), FAME samples were collected under a nitrogen stream and analysed using GC-FID system equipped with capillary column. In this case, helium gas was utilized to carry the sample, and the flow rate was kept at 1.5 mL/min. The rate of discharge gas was fixed at 50 mL/min. The oven program was set to 170°C, then ramped to 200°C (at a rate of 5°C per minute) and finally to 240°C (at a rate of 10°C per minute), with a 10-minute hold at 240°C. The temperature of the detector was kept at 240°C. The program ran for 20 minutes in total. A 1-liter sample volume was injected. The peak regions of authentic standards are compared to the retention times of external standards to determine the amount of fatty acids (Sigma Aldrich CRM47885). The stock of external standards was prepared by adding 1 mL of hexane to the FAME standard mix and kept frozen for further use. For the working solution, depending on the concentration of the FAMEs standard, the stock was diluted 5 times in hexane. 1 µL of the

standard dilution was injected into the GC. Total fatty acid was calculated as the sum of all FAMEs, and each FA was expressed as a percentage of total fatty acid (%TFA).

2.2.6. Effects of biostimulants on lipid content of HS

In detail, different hemp seed samples (12 samples provided by Nutrifield) were harvested from the crops which were supplemented with several types of biostimulants such as molasses, complex, and control (numbered from 1–12). The complex was a blend of biostimulants containing molasses as the most abundant component (10% w/v), but also fish emulsion-derived amino acids (5% v/v), aloe vera extract (2.5% v/v), beeswax-derived triacontanol (0.01% w/v), kelp (seaweed) (0.5% w/v), and fulvic acid (0.2% w/v). Lipids were extracted from procured samples and analyzed for PUFA composition. All experiments were performed in triplicate.

2.2.7. Antioxidant activity by DPPH assay

To investigate the antioxidant activity of HS and Thraustochytrid fermentation products with HS as a carbon source, 100 mg of each sample was dissolved in 2 mL of MeOH to make the stock solution (50 mg/mL). A series of dilutions of samples was prepared at 6.2, 12.5, 25, and 50 mg/mL. A stock solution of DPPH was prepared by dissolving 10 mg of DPPH (1M) in 100 mL of MeOH (99%, v/v) solvent. The DPPH solution was freshly made before every experiment and stored in a dark and cool place. At the same time, the stock of L-Ascorbic acid solution was also prepared by dissolving 1 mg of L-Ascorbic acid in 1 ml of MeOH. Antioxidant activity was measured by the DPPH assay according to the study by Chandra et al. (2014).

2.2.7.1.DPPH assay

A standard curve was prepared by measuring the absorbance of serial dilutions of L-Ascorbic solution in MeOH (final concentrations at 12, 6, 3, and 1.5 µg/mL) with reference to DPPH reagent. The assay was performed in a 96-well plate with the reaction mixtures containing 100 µL of stock DPPH solution and 1 mL of samples. The reaction was allowed to react by an incubation at 37°C for 30 min. The mixtures were measured for absorbance at 517/520 nm, and Trolox was used as a positive control. The results were expressed as percentage inhibition of the DPPH, as shown in the following equation:

(Eq. 1) $$\%\ \text{DPPH Radical Scavenging Activity} = \frac{\text{Abc}-\text{Abs}}{\text{Abc}} \times 100$$

where Abc is the absorbance control (DPPH in methanol) and Abs is the absorbance sample.

Chapter III. RESULTS AND DISCUSSION

1. The use of HS on Thraustochytrid growth and its biomass production and lipid content

Hemp seeds (*Cannabis sativa* L.) are considered a nutritional powerhouse, rich in protein and unsaturated fatty acids (Leizer et al. 2000). Protein recovery from by-products can be achieved by various techniques, such as chemical extraction, liquid fermentation by microorganisms, and solid-state fermentation (SSF) by fungi (Verduzco-Oliva & Gutierrez-Uribe 2020). Converting this material into value-added products through a biorefinery approach could meet the ever-increasing demand for sustainable protein sources while reducing food waste (Tsegaye, Jaiswal & Jaiswal 2021). It is popularly known that supplementation with sugars could accelerate microbial growth by inducing metabolic activities (Nikolic et al. 2017). For single-cell microorganisms like Thraustochytrid, glucose is the most common carbon source for producing microbial PUFAs

(Quilodrán et al. 2020; Shene et al. 2010). As compared to glucose, the HS herein was investigated for its ability to be used as an alternative carbon source, which could help minimize the fermentation cost and improve biomass and lipid production in Thraustochytrid.

Due to low carbohydrate concentration, HS was crushed and directly added into fermentation medium (data not shown). Variable amounts of HS (2.5 to 5%, w/v) were used and also combined with glucose to observe any changes in biomass and lipid content in Thraustochytrid during course of fermentation. There was a significant interaction between incubation time and different supplement treatments for biomass, lipid production, and microbial growth, indicating that the incubation time for fermentation will have differential effects on different fermentation products. The longer incubation duration resulted in higher production of biomass, lipid content, and accelerated cell growth. As shown in **Figure 5**, **Figure 6** and **Figure 7**, HS enhanced the biomass (6 times, $p<0.001$, **Appendix 1**) and lipid production (7 times, $p<0.001$, **Appendix 2**) after 24 h as compared to those from glucose-supplemented fermentation medium. During 120 h of experiment, a noteworthy enhancement in both biomass and lipid content, indicating high utilization of the sugars in HS as compared to glucose, was observed.

As shown in **Figures 5** and **6**, the microbial growth reached 16.3 g/L of biomass after the first day of incubation with the supplement of only HS (HYP 5%), which was 6-times increased as compared to the control, which was supplied with only glucose 5% (GYP 5%) with 2.5 g/L of biomass. The Thraustochytrid cells were recognized to grow 3 times faster in the HYP 5% in the first 24 hours. However, fermentation medium supplemented with only HS (HYP 5%) had the highest log phase after 96 h (29.5 g/L of biomass), while the cells continued growing slightly in the glucose medium to gain 8.47 ± 0.35 g/L of biomass after 120 h of experiment. After that, the cell density in HYP 5% medium started declining and was estimated at 22.67 ± 3.47 g/L at 120 h.

This can be explained by following the death phase of microbial cells. Furthermore, it is noticed that the CDW was found to be strongly affected by the HS and its concentration. In the presence of the low concentration of HS of 2.5% along with 2.5% of glucose (GHYP 2.5%), biomass production was 13.7 g/L after 24 h, which was five times higher than GYP 5%, even though the amount of glucose was replaced half by HS. The stationary phase was reached after 48h and proceeded to the death phase after 72h with a small decrease in biomass production (21.46 ± 1.29 g/L) after 120 h. Moreover, as the amount of sugar, including HS and glucose, increased to 5% each (GHYP 5%), the growth rate was slightly lower than in GHYP 2.5%, but biomass was the highest (35.22 ± 2.22 g/L of biomass) compared to all conditions after 120 h. The growth rate was slower in GHYP 5%, which could be due to the inhibition of microbial growth by high concentrations of sugar (Mizzi et al. 2020), but the harvested biomass was much improved.

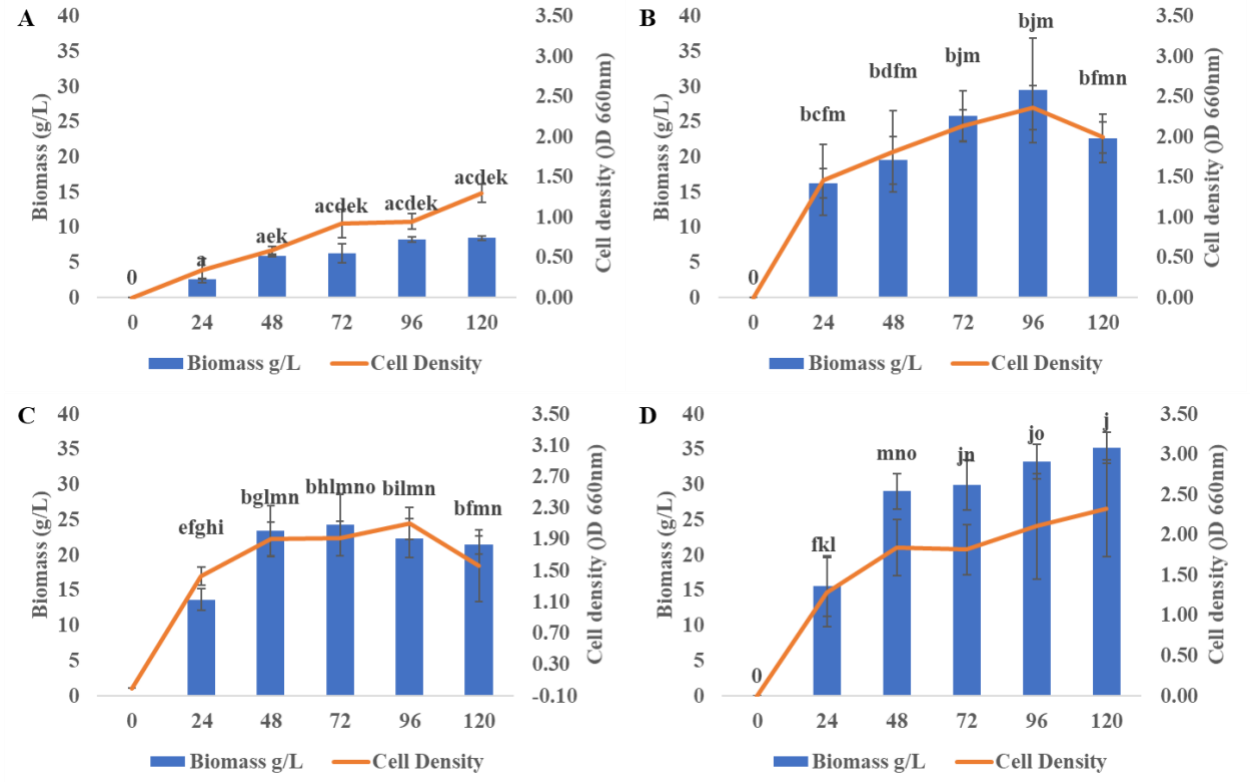


Figure 5: Time-dependent biomass (column, blue) and cell density (line, orange) of the Thraustochytrid S2 strain grown in various fermentation media: (A) GYP 5%, (B) HYP 5%, (C) GHYP 2.5%, and (D) GHYP 5%, measured at 660nm after specific incubation time (0–120 h) at 25°C with 150-rpm shaking, where GYP 5% was the control. All experiments were performed in triplicate. Error bars represent the standard deviation of the mean. Different lowercase letters represent significant differences (Tukey's test, $p < 0.001$, **Appendix 1**).

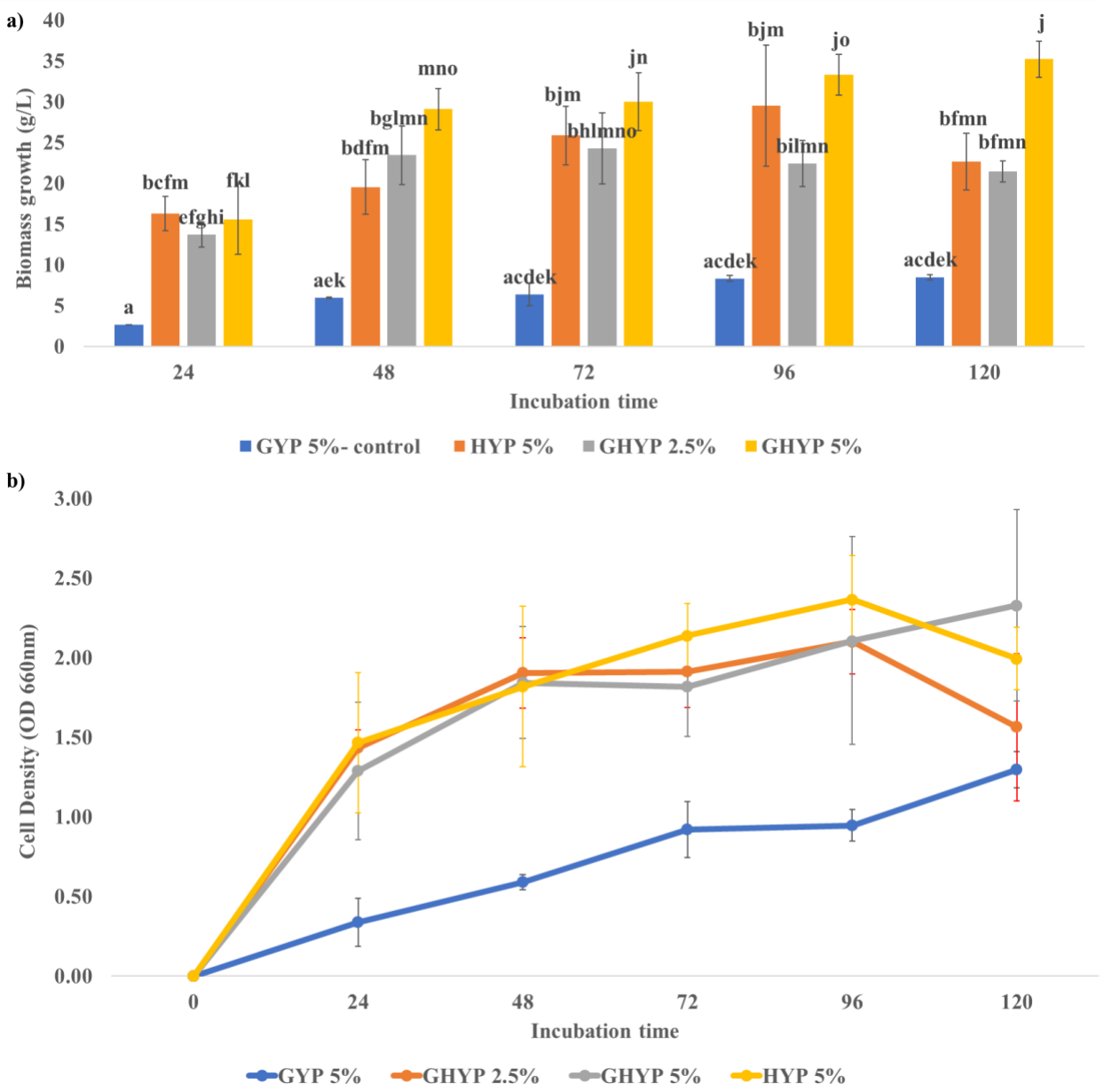


Figure 6: The biomass and cell density of Thraustochytrid S2 grown in different types of fermentation mediums (GYP 5%, GHYP 2.5%, GHYP 5%, and HYP 5%) were measured at 660nm at specific incubation times during 120 hours of experiment at 25°C with 150-rpm shaking, where GYP 5% was used as a control. Error bars represent the standard deviation of the mean of three measurements. Different lowercase letters represent significant differences (Tukey's test, $p < 0.001$, **Appendix 1**)

In terms of lipid content, HS also showed high effectiveness in improving the amount of lipid production. However, the fatty acids profile in all conditions demonstrated a small difference as compared to biomass (**Figure 7**). In detail, the lipid production in the strain grown in the control medium of only glucose marginally increased by the time it resulted in around 3.1 g/L at 120 h. At the same time, the lipid content of the S2 strain grown in HYP-supplemented medium, including GHYP 2.5% (2.8 g/L), GHYP 5% (3.6 g/L), and HYP 5% (2.9 g/L), was significantly higher than that of glucose medium (0.4 g/L) after 24 h. In the medium with the absence of glucose, the lipid production did not increase much after that until the end of the experiment at 120 h, estimating a similar amount to the control condition of GYP 5% (2.91 g/L). When both glucose and HS were added to the fermentation medium (GHYP 2.5% and GHYP 5%), a significant lipid content (7.3 g/L at 48 h) was observed. The lipid content was recognized to be proportional to the HS levels. The higher the concentration of HS added, the higher the lipid production was observed. After 120 h, the TFA in the GHYP 5% medium had doubled (6.1 g/L), whereas the TFA in the GHYP 2.5% medium was approximately 3.2 g/L.

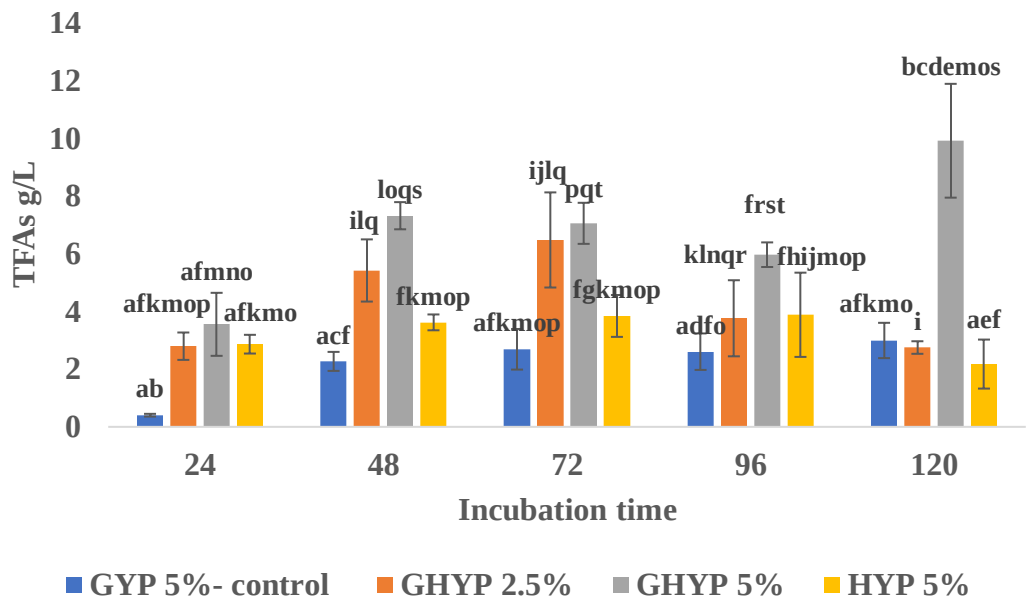


Figure 7: Incubation time-dependent lipid production in Thraustochytrid S2 strain incubated in different fermentation mediums, namely GYP 5% (blue), GHYP 2.5% (orange), GHYP 5% (gray), and HYP 5% (yellow). GYP 5% media was used as a control. All experiments were performed in triplicate. The error bars represent the standard deviation. Different lowercase letters represent significant differences (Tukey's test, $p < 0.05$, **Appendix 2**).

2. FAMEs profile of HS and Thraustochytrid S2 strain when grown in fermentation mediums supplemented with HS

To further investigate the composition of HS inside the medium, gas chromatography was performed to analyze the fatty acid profile of 50 mg of HS. Several studies on the fatty acid composition of hemp seed have shown that the seed oil is mostly made up of essential unsaturated fatty acids, with linoleic and α-linolenic acids being the most abundant (Galasso et al. 2016; Leizer et al. 2000). Linoleic acid (C18:2; n-6) and α-linolenic acid (C18:3; n-3) can be found in hemp seed oil in a 2:1 ratio (Callaway 2004). As shown in **Figure 8**, HS in the study was mainly

comprised of unsaturated fatty acids (approximately 74.5% of TFA) such as methyl linolelaidate (C18:2n6c) or methyl linoleate (C18:2n6t) with around 46% of TFA, followed by methyl oleate (C18:1n9c, 15.8% of TFA), linolenic acid (C18:3 n-3, 9.6% of TFA), and a small amount of saturated fatty acids (around 19.8% of TFA) such as palmitic acid (C16:0, 8.5% of TFA), stearic acid (C18:0, 3.9% of TFA). According to Kenar, Moser and List (2017), linoleic acid (LA) is the most common PUFA in plant oils and has the shortest chain of all the n-6 fatty acids. Desaturase and elongation enzymes in the liver transform linoleic acid in the human diet into other longer-chain n-6 PUFAs such as arachidonic (5c, 8c, 11c, 14c-20:4) and docosapentaenoic (4c, 7c, 10c, 13, 16c-22:5) acids (Kaur et al. 2011; Tallima & El Ridi 2018). Aside from those PUFAs, γ-linolenic (C18:3 n-6), docosahexaenoic (C22:6 n-3), and arachidonic (C20:4 n-6) acids were also found. Only traces of each of them were found. Other PUFAs were not detected, such as eicosatrienoic acid (C20:3 n-6), and eicosapentaenoic acid (C20:5 n-3).

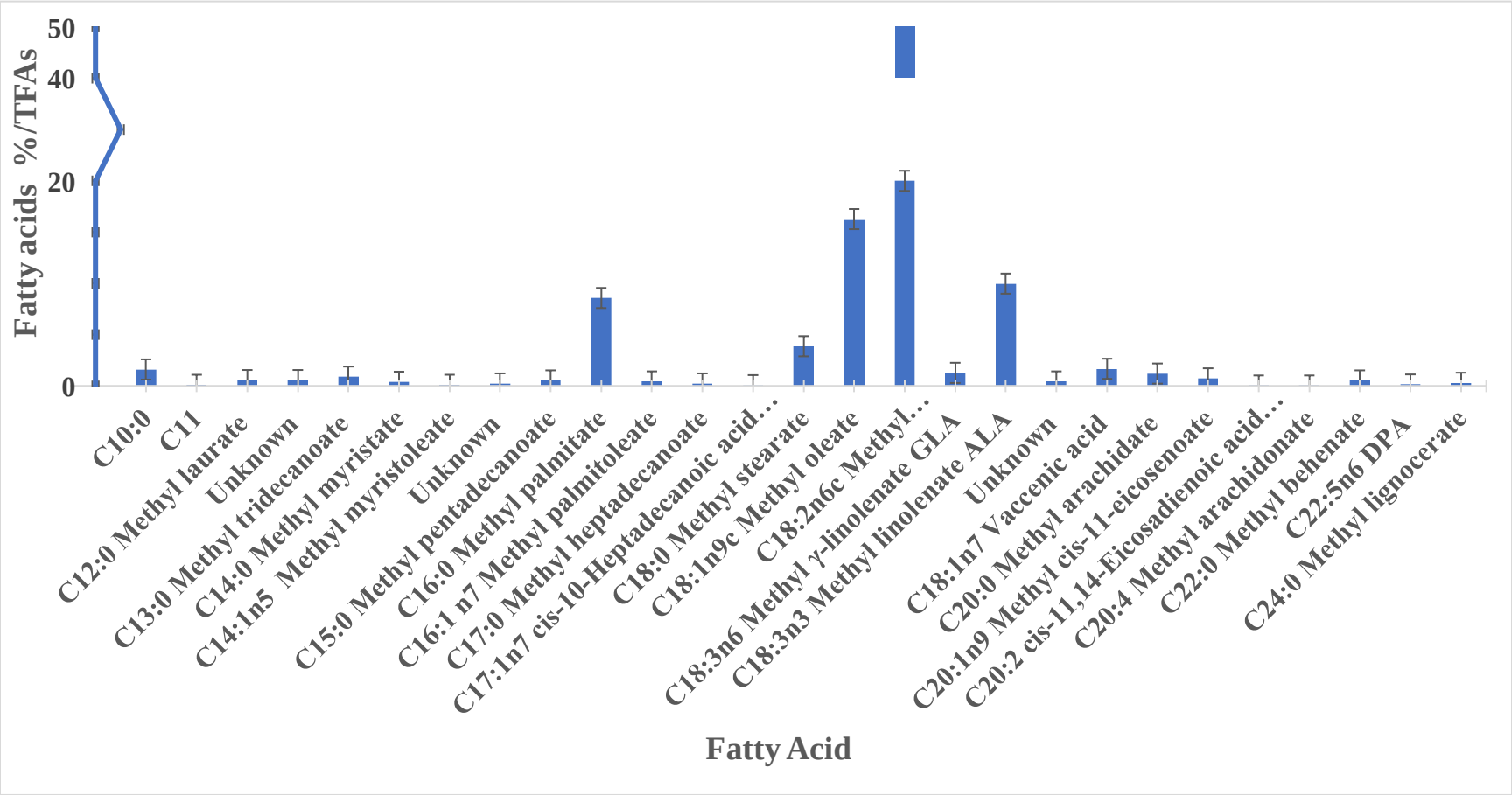


Figure 8: FAME analysis of HS. All experiments were performed in triplicate. Error bars represent the standard deviation ($p < 0.05$).

Omega-3 produced by marine Thraustochytrid has emerged as a fish oil substitute and an efficient and environmentally friendly alternative to overfishing (Xie, Y & Wang 2015). In this work, HS powder was supplemented into the fermentation medium of Thraustochytrid without any pretreatment to investigate the impact of Thraustochytrid fermentation on lipid production, especially PUFA products. This phenomenon was observed in all hempseed fermentation products since the medium has an excess carbon supply and a limiting nitrogen amount, which resulted in lipid accummulation in oleaginous microbes. When the organism grows, the nitrogen source is quickly depleted, but it continues to assimilate the carbon source, which is routed directly into lipid synthesis (Colin Ratledge & Wynn 2002). The accumulated lipid was extracted from the dried biomass in each medium before and after fermentation, and its composition was recorded and analyzed. After supplementing with HS, the oil content of dried collected biomass was found to be different. It is noteworthy that the level of saturated fatty acids (SFA) was reduced. The accumulation of polyunsaturated and mono-unsaturated fatty acids (MUFAs) was observed to be three times lower than SFA (51.6% of total fatty acids) in the fermentation medium, including glucose (GYP 5%). Otherwise, the obtained biomass in the medium comprising only 5% of HS (HYP 5%) demonstrated a slight decrease in the highest saturated fatty acid accumulation at 42.6%, and the level of unsaturated fatty acids was particularly high at 45.8% of total fatty acids. In the combined medium (glucose and HS), the level of SFA was two-thirds of that in the control medium (GYP 5%), and the production of saturated fatty acids was enhanced significantly in the medium of GHYP 5% and GHYP 2.5%, which added up to 52.1% of TFA and 50.8% of TFA, respectively, corresponding to the amount of supplemented HS. The highest levels of unsaturated FAs were accumulated at the higher HS concentration in the combined medium, indicating that the production of PUFA and MUFA is potentially more efficient in medium containing HS than

glucose, while the production of SFA is preferred in the presence of glucose in the fermentation medium. In this study, in terms of PUFA production, the main extracted FA product was linoleic acid methyl ester of methyl linolelaidate (C18:2n6c) or methyl linoleate (C18:2n6t), ranging from 22.6% to 24.8% of TFA. After Thraustochytrid fermentation, the contents of some fatty acids were elevated, such as pentadecanoic acid (C15:0), palmitic acid (C16:0), stearic acid (C18:0), DPA, and DHA. Docosahexaenoic acid (C22:6 n-3) or DHA was determined by FAMEs to be 24.7% in 5% glucose and only 2.7% in HYP 5%. As the carbon concentration increased with both glucose and HS, the proportion of DHA was increased but was not comparable to the glucose medium. Pentadecanoic acid is recently considered an essential fatty acid due to its health benefits for humans (Venn-Watson, Lumpkin & Dennis 2020). It is a dietary fatty acid that reduces inflammation, anemia, dyslipidemia, and fibrosis in animals, possibly by binding to key metabolic regulators and restoring mitochondrial function. Without HS addition, pentadecanoic acid presented 5.2% of TFA after Thraustochytrid fermentation in the use of glucose as a carbon source. That was much higher than other medium conditions containing HS, indicating that the presence of HS inhibited the production of this FA. Palmitic and stearic acids are common long-chain saturated FAs in plants and animals (Anushree et al. 2017; Rico, Allen & Lock 2014). Palmitic acid was produced at a higher rate in the medium free of HS (GYP 5%), accounting for 27.3% of TFA, whereas the fermentation medium supplemented with only HS (HYP 5%) or both HS and glucose (GHYP 2.5% and GHYP 5%) accumulated amounts ranging from 14.8% to 17.1% of TFA. However, in terms of stearic acid, the pattern was different, where stearic acid production was higher (8.8% of TFA) in the presence of only HS (HYP 5%), whereas the value was only 2% of TFA in the carbon source using glucose. This indicates that HS is more compatible for the synthesis of stearic acid than glucose.

3. Comparision of the use of HS and MHS on fatty acid production

Due to the regulatory environment, hemp is farmed primarily for food or industrial reasons in Australia. It is a high-yielding, hardy, and fast-growing annual crop that can be planted anytime between early spring and late summer or early fall. Hemp is primarily farmed in Victoria for the production of hemp seed, which can now be legally sold for human use (Department of Jobs & Regions 2020). In another context, the hemp seed used in the study (referred as "HS") was compared to hemp seed bought on the online market (market hemp seed; MHS). The same amounts of both seed types were used for lipid extraction and resulting lipid profile analysis following established procedures (Gupta et al. 2015). The lipid content (% dry wt. basis) was comparable at 35.7% in HS than at 18.9% in MHS (two-sided t-test assuming equal variances, $p<0.05$, **Appendix 3**) (**Figure 9a**). This could further indicate the specificity of HS characteristics and associated implications for thraustochytrid fermentation (Pascoalino et al. 2021). Compared to normal hemp seed in the market, this type of seed had better quality with higher lipid content and had previously shown its positive effectiveness in improving biomass and lipid production during thraustochytrid fermentation. As shown in **Figure 9b**, the compositions in both types of seeds were similar and the levels of most of the FA types in the HS were higher, especially induced higher percentage of 9% in C18:2n6c methyl linolelaidate and 5% in methyl oleate (C18:1n9c). HS also contained more essential FAs than MHS and CHSO (**Table 3**), including methyl myristoleate (C14:1n5) and methyl arachidonate (C20:4). Methyl myristoleate, also known as myristoleic acid, has been studied elsewhere because it has the ability to eliminate cancer and inhibit microbial germination (Clément et al. 2007; Iguchi et al. 2001). Arachidonic acid is considered as a starting material in the synthesis of two kinds of important unsaturated carboxylic acids in our body, namely prostaglandins and leukotrienes (Tallima & El Ridi 2018). Some fatty acids, such as C10:0, methyl

palmitate (C16:0), methyl stearate (C18:0), and some PUFAs, such as methyl linolenate ALA (C18:3n3) or methyl γ-linolenate GLA (C18:3n3), were found in higher concentrations in MHS than HS.

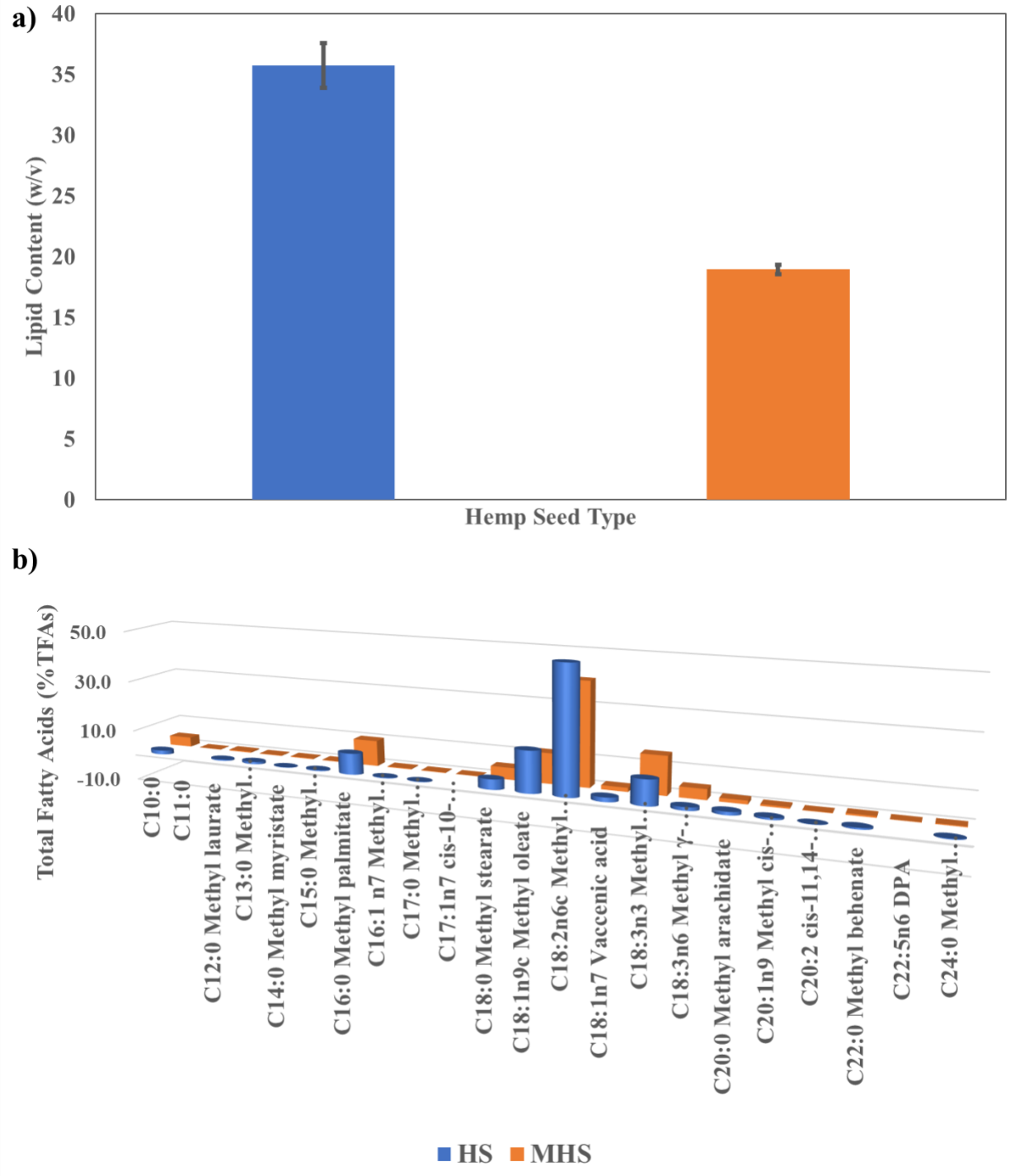


Figure 9: Comparison of a) total lipid content and b) lipid profile between HS (blue) and MHS (orange). All experiments were performed in triplicate. The standard deviation is represented by the error bars (t-test, $p < 0.05$, **Appendix 3**).

Furthermore, a commercial hemp seed oil (CHSO) was bought and three samples, including HS (crushed-seed), MHS (fined powder), and CHSO (cold-press oil), were compared in terms of lipid content. Generally, HS and MHS had mostly similar lipid profiles with some differences in the levels of some types of fatty acids, while the number of fatty acid components in CHSO was lower (statistical analysis by ANOVA was done with specific fatty acids, presented as **Appendix 4** and **5**). As shown in **Table 3**, the most common component present in CHSO was C18:2n6c methyl linolelaidate or C18:2n6t methyl linoleate, accounting for 48.36% ± 1.05 of the total fatty acid. This value was higher than that of fatty acid in HS (46.06% ± 1.30 of TFA) and MHS (39.76% ± 0.20 of TFA). Some fatty acids were only found in CHSO with small amount, such as cis-8,11,14-eicosatrienoic acid methyl ester (C20:3n6), cis-11,14,17-eicosatrienoic acid methyl ester (C20:3n3), and methyl nervonate (C24:1). In all three samples, C18:2n6c Methyl linolelaidate/C18:2n6t Methyl linoleate (LA), C18:3n3 Methyl linolenate (ALA), C16:0 Methyl palmitate, C18:1n9c Methyl oleate, and C18:0 Methyl stearate were all recognized with noticeable concentrations, where LA was dominant. HS included the highest amount of C18:1n9c Methyl oleate with 16.20 ± 0.47% of TFA. Similar fatty acid composition ratios have been shown in other studies for other kinds of hemp seed (Vodolazska & Lauridsen 2020). In the next step, the lipid production after fermentation with each supplement was done to evaluate the effect of utilizing the S2 strain on improving FA content.

Table 3: Comparison of composition of fatty acids in different lipid extracts from HS, MHS, and CHSO.

FAMEs (% W/V)	STUDY HEMP SEED (HS)	MARKET HEMP SEED (MHS)	COMMERCIAL HEMP SEED OIL (CHSO)
C10:0	1.60 ± 0.05	3.76 ± 0.13	3.03 ± 0.40
C11:0	0.10 ± 0.05	0.10 ± 9.81E-18	
C12:0 Methyl laurate	0.57 ± 0.12	0.43 ± 0.12	
C13:0 Methyl tridecanoate	0.90 ± 0.08	0.06 ± 0.06	
C14:0 Methyl myristate	0.40 ± 0.20	0.30 ± 0.10	0.10 ± 9.81308E-18
C14:1n5 Methyl myristoleate	0.10 ± 0.05		
C15:0 Methyl pentadecanoate	0.53 ± 0.38	0.23 ± 0.06	
C15:1 n5 Methyl cis-10-pentadecenoate			
C16:0 Methyl palmitate	8.56 ± 0.18	9.93 ± 0.06	9.37 ± 0.20
C16:1 n7 Methyl palmitoleate	0.43 ± 0.13	0.30 ± 0.00	0.20 ± 1.96E-17
C17:0 Methyl heptadecanoate	0.23 ± 0.08	0.20 ± 1.96262E-17	0.10 ± 9.81308E-18
C17:1n7 cis-10-Heptadecanoic acid methyl ester	0.10 ± 0.03	0.10 ± 9.81308E-18	0.10 ± 9.81308E-18
C18:0 Methyl stearate	3.87 ± 0.06	4.63 ± 0.03	4.40 ± 0.05
C18:1n9c Methyl oleate	16.20 ± 0.47	11.70 ± 0.05	13.10 ± 0.32
C18:1n7 Vaccenic acid	1.60 ± 0.08	1.56 ± 0.03	1.20 ± 0.00
C18:1n9t Elaidic acid methyl ester			

C18:2n6c Methyl linolelaidate/C18:2n6t Methyl linoleate	46.06 ± 1.30	39.76 ± 0.20	48.36 ± 1.05
C18:3n6 Methyl γ-linolenate GLA	1.26 ± 0.03	4.06 ± 0.03	0.80 ± 7.85E-17
C18:3n3 Methyl linolenate ALA	9.97 ± 0.36	14.83 ± 0.18	15.73 ± 0.20
C20:0 Methyl arachidate	1.20 ± 0.00	1.40 ± 1.57E-16	1.03 ± 0.03
C20:1n9 Methyl cis-11-eicosenoate	0.73 ± 0.03	0.63 ± 0.03	0.60 ± 0.00
C20:2 cis-11,14-Eicosadienoic acid methyl ester	0.03 ± 0.03	0.16 ± 0.03	0.10 ± 9.81308E-18
C20:3n6 cis-8,11,14-Eicosatrienoic acid methyl ester			0.03 ± 0.03
C21:0 Methyl heneicosanoate 200 μg/mL			
C20:3n3 cis-11,14,17-Eicosatrienoic acid methyl ester			0.03 ± 0.03
C20:4 Methyl arachidonate	0.03 ± 0.03		
C20:5 Methyl eicosapentaenoate EPA			
C22:0 Methyl behenate	0.53 ± 0.03	0.60 ± 0.00	0.40 ± 3.92523E-17
C22:1 Methyl erucate			
C22:2 cis-13,16-Docosadienoic acid methyl ester			
C23:0 Methyl tricosanoate			
C22:5n6 DPA	0.13 ± 0.03	0.13 ± 0.03	0.26 ± 0.03
C24:0 Methyl lignocerate	0.30 ± 0.03	0.33 ± 0.03	0.10 ± 9.81308E-18
C22:6 Methyl docosahexaenoate			
C24:1 Methyl nervonate			0.26 ± 0.20

All experiments were performed in triplicate. Data presented as mean ± standard deviation.

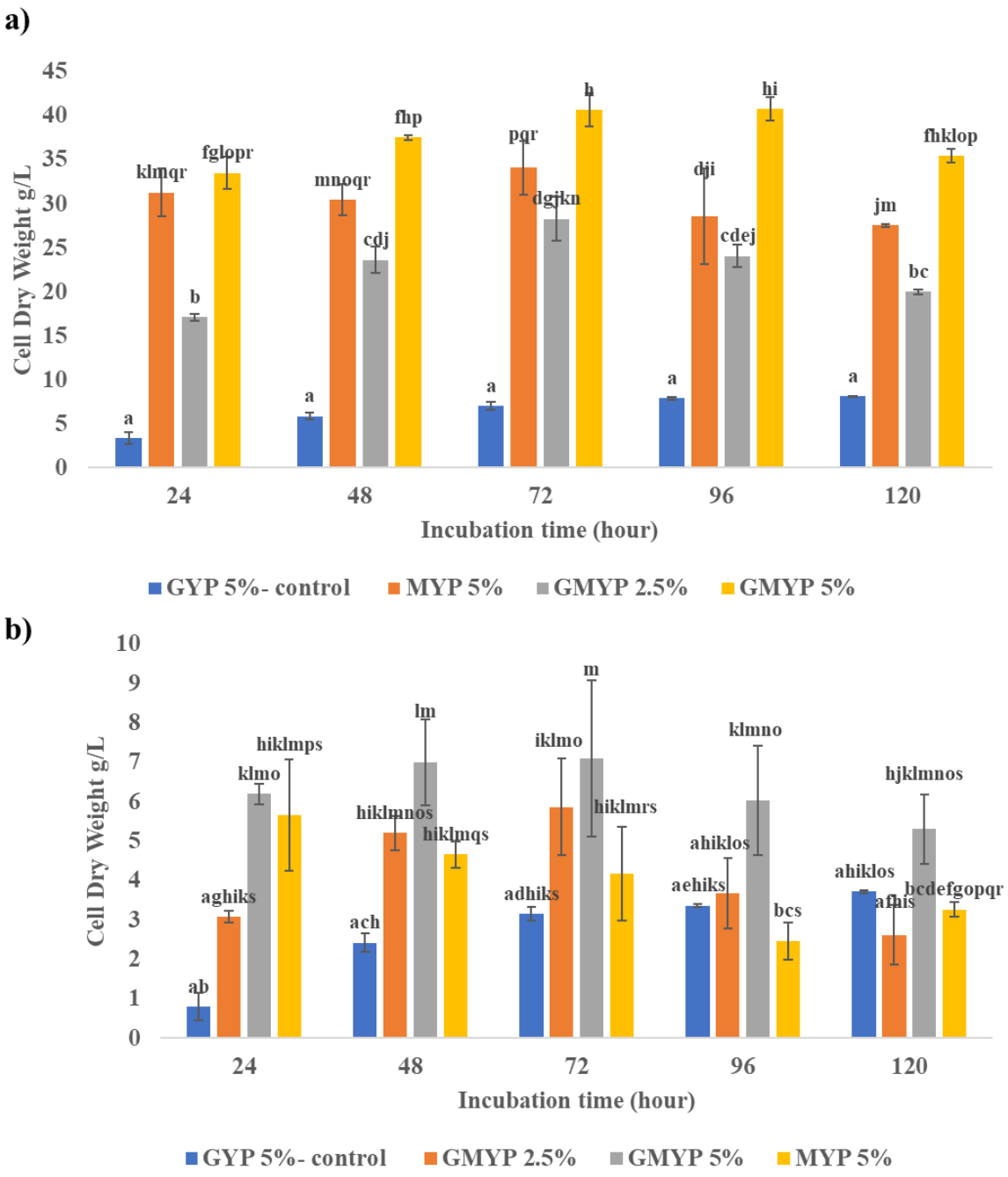


Figure 10: Effect of incubation time on a) biomass and b) lipid production from Thraustochytrid S2 strain in different fermentation medium supplemented with MHS, namely GYP 5%, GMYP 2.5%, GMYP 5%, and MYP 5%. GYP 5% media was considered as control. All experiments were performed in triplicate. Error bars represent standard deviation. Different lowercase letters represent significant differences (Tukey's test, $p < 0.001$, **Appendix 6**).

Besides that, MHS was also added into the fermentation medium under the same conditions as HS for a better comparison of the effects on biomass and lipid production from the Thraustochytrid S2 strain (**Figure 10**). Overall, higher amount of biomass was obtained as the incubation time was extended until the highest biomass production was recorded after 72 h, then reduced until the end of the experiment. The highest biomass (40.64 g/L at 72 h) was recognized in the MHS along with glucose (GMYP 5%). Lower concentrations of hemp seed and glucose did not improve biomass yield. **Figure 10a** demonstrated that the biomass obtained in the presence of MHS in the fermentation medium was much more than that in HS (Tukey's test, $p<0.001$, **Appendix 6**). In detail, after the first day of incubation, biomass in MYP 5% and GMYP 5% was doubled (31.277 g/L and 33.478 g/L, respectively) as compared to HYP % and GHYP 5% in the HS containing medium (16.27 g/L and 15.5 g/L, respectively). This indicated the faster growth of microorganisms in the presence of MHS powder.

Considering the lipid production in the case of MHS, **Figure 10b** showed the highest value of lipid at 6.188 g/L in the presence of a high amount of both MHS and glucose (GMYP 5%) after 24 h of incubation. The biomass was doubled when compared to HS (3.55 TFA g/L).

Table 4: Comparison of the effect of the supplement of HS, MHS, and CHSO on the lipid production in different fermentation mediums after 120 h of fermentation.

	MARKET HEMP SEED (MHS) SUPPLEMENT				STUDY HEMP SEED (HS) SUPPLEMENT				COMMERCIAL HEMP SEED OIL (CHSO) SUPPLEMENT			
FAMEs	**GYP 5%**	**GMYP 2.5%**	**GMYP 5%**	**MYP 5%**	**GYP 5%**	**GHYP 2.5%**	**GHYP 5%**	**HYP 5%**	**GYP 5%**	**GOYP 2.5%**	**GOYP 5%**	**OYP 5%**
C10:0	3.00 ± 0.49	16.33 ± 1.70	8.20 ± 0.70	11.87 ± 0.93	2.90 ± 0.46	7.00 ± 1.22	5.10 ± 1.53	8.33 ± 2.22	0.63 ± 0.03	0.50 ± 0.00	0.70 ± 0.00	0.47 ± 0.09
C11:0	0.03 ± 0.03	0.30 ± 0.17	0.17 ± 0.03	0.33 ± 0.12	0.23 ± 0.23	0.56 ± 0.29	0.33 ± 0.23	0.30 ± 0.21	0.03 ± 0.03	–	–	–
C12:0 Methyl laurate	0.30 ± 0.00	0.07 ± 0.07	0.13 ± 0.03	0.10 ± 0.00	0.33 ± 0.03	0.10 ± 0.00	0.16 ± 0.03	–	0.30 ± 0.00	0.13 ± 0.03	0.10 ± 0.00	–
C13:0 Methyl tridecanoate	–	–	–	–	0.20 ± 0.20	0.43 ± 0.26	0.20 ± 0.20	–	–	–	–	–
C14:0 Methyl myristate	8.00 ± 0.15	1.47 ± 0.09	3.67 ± 0.64	0.23 ± 0.03	8.40 ± 0.40	2.80 ± 0.86	3.36 ± 1.18	0.97 ± 0.67	8.30 ± 0.12	5.30 ± 0.17	3.23 ± 0.35	0.10 ± 0.00
C14:1n5 Methyl myristoleate	–	–	–	–	0.10 ± 0.10	0.13 ± 0.09	0.13 ± 0.07	0.15 ± 0.04	–	0.10 ± 0.00	0.03 ± 0.03	
C15:0 Methyl pentadecanoate	5.53 ± 0.15	0.97 ± 0.27	3.37 ± 0.07	0.27 ± 0.07	5.17 ± 0.27	1.16 ± 0.15	2.80 ± 0.56	0.70 ± 0.29	4.50 ± 0.06	2.83 ± 0.09	2.10 ± 0.10	0.20 ± 0.06
C15:1 n5 Methyl cis-10-pentadecenoate	–	–	–	–	0.03 ± 0.03	–	–	–	–	–	–	–

C16:0 Methyl palmitate	29.60 ± 0.15	11.47 ± 0.87	16.47 ± 0.71	11.13 ± 0.20	27.30 ± 0.55	15.50 ± 1.21	14.80 ± 1.04	17.07 ± 0.70	31.47 ± 0.26	22.20 ± 0.65	14.67 ± 0.79	9.23 ± 0.73
C16:1 n7 Methyl palmitoleate	1.47 ± 0.03	0.03 ± 0.03	0.57 ± 0.07	0.33 ± 0.03	2.50 ± 0.68	0.73 ± 0.34	1.07 ± 0.58	0.27 ± 0.03	1.43 ± 0.03	1.16 ± 0.09	0.94 ± 0.07	0.16 ± 0.03
C17:0 Methyl heptadecanoate	2.07 ± 0.09	0.43 ± 0.09	1.33 ± 0.19	0.23 ± 0.03	1.87 ± 0.12	0.36 ± 0.09	1.16 ± 0.27	0.30 ± 0.06	1.67 ± 0.03	1.10 ± 0.06	0.76 ± 0.03	0.20 ± 0.00
C17:1n7 cis-10-Heptadecanoic acid methyl ester	0.10 ± 0.00	–	0.10 ± 0.00	0.13 ± 0.03	0.20 ± 0.05	0.06 ± 0.03	0.16 ± 0.03	0.07 ± 0.07	0.10 ± 0.00	0.20 ± 0.00	0.13 ± 0.03	0.10 ± 0.00
C18:0 Methyl stearate	1.87 ± 0.03	4.03 ± 0.13	3.13 ± 0.03	5.47 ± 0.33	2.03 ± 0.03	4.90 ± 0.26	3.76 ± 0.07	8.83 ± 0.64	1.83 ± 0.03	1.97 ± 0.09	3.36 ± 0.13	4.30 ± 0.35
C18:1n9c Methyl oleate	0.14 ± 0.03	7.70 ± 0.31	7.10 ± 0.06	11.30 ± 1.10	0.17 ± 0.06	12.96 ± 1.77	12.90 ± 1.20	14.24 ± 0.68	0.90 ± 0.40	4.03 ± 0.38	9.27 ± 0.42	13.06 ± 0.07
C18:1n7 Vaccenic acid	1.44 ± 0.07	0.63 ± 0.07	0.80 ± 0.10	1.20 ± 0.00	2.33 ± 0.61	1.60 ± 0.55	1.70 ± 0.49	1.20 ± 0.10	0.83 ± 0.43	1.23 ± 0.03	1.16 ± 0.03	1.16 ± 0.03
C18:1n9t Elaidic acid methyl ester	–	–	–	–	–	–	–	–	–	–	–	–
C18:2n6c Methyl linolelaidate/C18:2n6t Methyl linoleate	0.03 ± 0.03	26.90 ± 1.04	25.03 ± 0.79	29.50 ± 0.55	0.13 ± 0.13	23.16 ± 2.24	24.83 ± 3.49	22.57 ± 1.51		17.30 ± 1.08	34.90 ± 1.63	50.33 ± 0.74
C18:3n6 Methyl γ-linolenate GLA	0.10 ± 0.00	2.20 ± 0.15	1.67 ± 0.33	2.53 ± 0.03	0.10 ± 9.81308E-18	0.56 ± 0.12	0.66 ± 0.07	0.46 ± 0.03	0.10 ± 0.00	0.30 ± 0.00	0.56 ± 0.03	0.76 ± 0.03
C18:3n3 Methyl linolenate ALA	–	8.37 ± 0.57	8.93 ± 0.23	9.90 ± 0.06	0.1	6.73 ± 1.16	8.10 ± 0.80	5.63 ± 0.55		7.83 ± 0.42	12.06 ± 0.52	14.90 ± 0.21

C20:0 Methyl arachidate	0.20 ± 0.00	1.10 ± 0.10	0.70 ± 0.00	1.47 ± 0.17	0.20 ±1.96262 E-17	1.30 ± 0.10	0.90 ± 0.00	2.46 ± 0.29	0.13 ± 0.03	0.27 ± 0.03	0.76 ± 0.03	1.06 ± 0.07
C20:1n9 Methyl cis-11-eicosenoate	–	0.13 ± 0.13	0.27 ± 0.07	0.50 ± 0.15	–	0.46 ± 0.03	0.43 ± 0.03	0.66 ± 0.12		0.13 ± 0.03	0.33 ± 0.03	0.63 ± 0.03
C20:2 cis-11,14-Eicosadienoic acid methyl ester	0.23 ± 0.03	–	0.20 ± 0.10	0.47 ± 0.03	0.17 ± 0.03	0.16 ± 0.17	0.20 ± 0.00	0.06 ± 0.07	0.20 ± 0.00	0.13 0.03	0.13 ± 0.03	0.10 ± 0.00
C20:3n6 cis-8,11,14-Eicosatrienoic acid methyl ester	0.30 ± 0.00	–	0.37 ± 0.03	0.40 ± 0.20	0.20 ± 0.10	0.06 ± 0.07	0.26 ± 0.03	0.23 ± 0.19	0.86 ± 0.28	0.23 ± 0.03	0.06 ± 0.03	–
C21:0 Methyl heneicosanoate 200 µg/mL	0.03 ± 0.03	–	0.10 ± 0.10	–	0.43 ± 0.43	–	–	–		0.63 ± 0.03	0.16 ± 0.03	–
C20:3n3 cis-11,14,17-Eicosatrienoic acid methyl ester	1.2 ± 0.00	0.10 ± 0.08	0.17 ± 0.03	0.13 ± 0.07	0.70 ± 0.351188 458	0.13 ± 0.13	0.30 ± 0.12	–	0.40 ± 0.40	0.13 ± 0.03	–	–
C20:4 Methyl arachidonate	0.17 ± 0.17	–	0.07 ± 0.03	–	0.30 ± 0.15	0.30 ± 0.17	0.23 ± 0.15	–	–	–	–	–
C20:5 Methyl eicosapentaenoate EPA	3.47 ± 0.03	0.70 ± 0.16	0.67 ± 0.03	0.47 ± 0.03	3.23 ± 0.30	–	0.30 ± 0.15	0.33 ± 0.17	1.80 ± 1.04	1.83 ± 0.09	0.53 ± 0.07	–
C22:0 Methyl behenate	0.03 ± 0.03	0.53 ± 0.03	0.30 ± 0.00	0.83 ± 0.07	0.10 ± 9.81308E-18	0.60 ± 0.06	0.40 ± 0.00	1.06 ± 0.12	0.10 ± 0.00	0.10 ± 0.00	0.23 ± 0.03	0.33 ± 0.03
C22:1 Methyl erucate	–	–	–	–	–	–	–	–	–	–	–	–
C22:2 cis-13,16-Docosadienoic acid methyl ester	–	–	–	–	–	–	–	–	–	–	–	–

C23:0 Methyl tricosanoate	–	–	–	–	–	–	–	–	–	–	–	–
C22:5n6 DPA	11.50 ± 0.06	2.23 ± 0.18	3.13 ± 0.33	1.57 ± 0.07	11.67 ± 0.43	2.73 ± 0.91	2.66 ± 0.47	1.13 ± 0.09	12.73 ± 0.43	7.97 ± 0.33	3.13 ± 0.35	0.17 ± 0.07
C24:0 Methyl lignocerate	0.40 ± 0.00	–	0.40 ± 0.00	–	0.50 ± 0.00	0.46 ± 0.07	0.46 ± 0.03	0.60 ± 0.10	0.40 ± 0.00	19.83 ± 0.61	8.56 ± 0.87	0.60 ± 0.25
C22:6 Methyl docosahexaenoate	26.17 ± 0.19	3.97 ± 0.29	8.87 ± 0.49	3.30 ± 0.21	24.73 ± 1.30	6.53 ± 2.41	7.60 ± 0.74	2.66 ± 0.22	27.83 ± 0.47	–	–	0.33 ± 0.24
C24:1 Methyl nervonate	–	–	–	–	–	–	–	–	–	–	–	–

Data presented as mean ± standard deviation. All experiments were performed in triplicate ($p < 0.05$)

FAME analyses of the fatty acids produced by Thraustochytrid S2 strain in the presence of HS, ground MHS, or CHSO have been performed. In general, the results of FA products by MHS were not that much different as compared to the FA results recorded by the supplement of HS, while the use of CHSO produced some difference ($p<0.05$, **Appendix 7** and **8**). C18:2n6c methyl linolelaidate or C18:2n6t methyl linoleate was the most common fatty acid produced in MHS and CHSO supplements, accounting for 29.50 ± 0.55 of the total fatty acid with only MHS powder (MYP 5%), slightly higher than 22.57 ± 1.51% for HYP 5% of the HS. Furthermore, the C18:2n6c level was remarkably reached at 50.33 ± 0.74 in the use of CHSO (**Table 4**). Depending on the use of carbon sources, the ratio of each FA was also altered. As a control, Thraustochytrid fermentation with only glucose as a carbon source produced a high amount of FAs such as methyl palmitate (C16:0, 31% TFA), methyl docosahexaenoate (C22:6, 27% TFA), DPA (C22:5n6, 12% TFA), methyl myristate (C14:0, 8% TFA), and methyl pentadecanoate (C15:0, 5% TFA) which are regarded as signature fatty acid in Thraustochytrid (Gupta et al., 2021). Exceptionally, only the utilization of HS in all states produced methyl cis-11-eicosenoate (C20:1n9), whereas glucose did not. It can be noticed that the levels of some fatty acids were significantly increased after Thraustochytrid fermentation with the supplement of HS, especially methyl oleate (C18:1n9c, 14.24 ± 0.68), and methyl stearate (C18:0, 8.83 ± 0.64). In the solid forms of HS and MHS, the fatty acid profile after fermentation was similar, while more types of FAs were produced than in the liquid form of CHSO. Interestingly, methyl docosahexaenoate (C22:6) level was reduced after fermentation in the presence of CHSO, whereas a small amount of CHSO and glucose supplementation increased the production of methyl lignocerate (C24:0) up to nearly 20% of TFA after fermentation. At this condition, CHSO contributed to the high production of methyl palmitate (C16:0, 22% of TFA) and DPA (C22:5n6, 8% of TFA) levels as well. The reduction in DHA levels

upon HS and CHSO addition in the fermentation medium needs further investigation, This could be due to some minor alkaloids present in the hemp seed powder that may have inhibited the key enzyme (delta-4 fatty acid desaturase) involved in DHA synthesis, however, this would be a follow-up study.

4. Effects of different biostimulants on fatty acid composition of hemp seed

Hemp is known as a versatile plant that can adapt to a variety of environments. Although plant genotype can influence hemp seed composition, additional factors such as agronomic and climatic circumstances can influence the presence of nutraceutical chemicals (Cattaneo et al. 2021). Nutraceuticals are products that have been extracted from food and are sold as medicine. Nutraceuticals have recently drawn a lot of interest due to their potential to be consumed for nutritional, safety, and therapeutic uses (Crescente et al. 2018). Nutraceuticals can be used to promote health, slow down the aging process, prevent chronic diseases, prolong life, and support the function and structure of the body (Nasri et al. 2014). As a result, it is critical to select a variety (cultivar) that is optimized for the specific application as well as adapted to the growing environment. A variety of microbial biofertilizers have been available for agricultural applications (Rajabi Hamedani et al. 2020; Schenck zu Schweinsberg-Mickan & Müller 2009). They are expected to add beneficial microbial inoculants to soil and stimulate soil microorganisms, as well as promote plant development and yield while further improving soil conditions (Rouphael & Colla 2020). Effective organisms, biostimulators, Bactofil-A, and Bactofil-B are some of the most popular modern biofertilizers (Agrinova GmbH). Kuglarz and Grübel (2018) described how molasses is one of the most regularly used biostimulants and is a

key tool in the armory of many organic cannabis farmers. Carbohydrates are well-known for their importance in all phases of plant growth (Arnao et al. 2021; Trouvelot et al. 2014). Plants capture light energy and convert it into oxygen and carbohydrates through photosynthesis. These carbohydrates supply the chemical energy that allows plants to grow and produce strong, healthy structures from root to bud. Sugar is involved in energy metabolism as well as serving as a form of chemical messenger that aids in the promotion of health throughout the cannabis life cycle. In this study, we further investigated the effect of applying different biostimulant treatments on the hemp crop and sampling those hemp seeds for fatty acid composition analysis. It was intended to evaluate potential changes in lipid profiles as a result of treating hemp crops with different biostimulants. The samples were labeled from 1 to 12 as sample numbers with different types of treatments, including molasses, complex, and control. Under the supplementation of different types of biostimulants, lipid content in harvested hemp seed samples was detected with a slight difference (ranging from 31 to 39% w/v; condensed data is shown in **Figure 11** and full data provided in an **Appendix 9**). However, further investigation by FAME analysis revealed changes in fatty acids profiles. The data for all 12 samples was condensed into three major groups: molasses, complex, and control (**Table 5** and full data provided in an **Appendix 10**). Similar fatty acid compositions were observed in all hemp seed groups, with unsaturated FAs accounting for more than 50% of TFA. The major fatty acids existing in all groups with small differences in mean values were arranged from high to low in amount as the order: Linoleic acid (C18:2n6, LNA), alpha-linolenic acid (C18:3n3, ALA), oleic acid (C18:19c), palmitic acid (C16:0), and stearic acid (C18:0). Other fatty acids were also detected in trace percentages. Likewise, it is noticed that the use of complex biostimulants resulted in higher levels of some essential PUFAs in hemp seed products such as linoleic acid (C18:2n6, LNA) or Alpha-linolenic acid (C18:3n3,

ALA), and only the hemp seeds in this group contained a small amount of docosapentaenoic acid (C22:5n6, DPA) detected (statistical SPSS analysis and ANOVA test were shown in **Appendix 11 and 12, respectively**). These findings may be useful in determining the appropriate plant biostimulants to use in improving the quality of hemp seeds with the goal of increasing the expression of a specific fatty acid of interest and increasing the commercial value of these products.

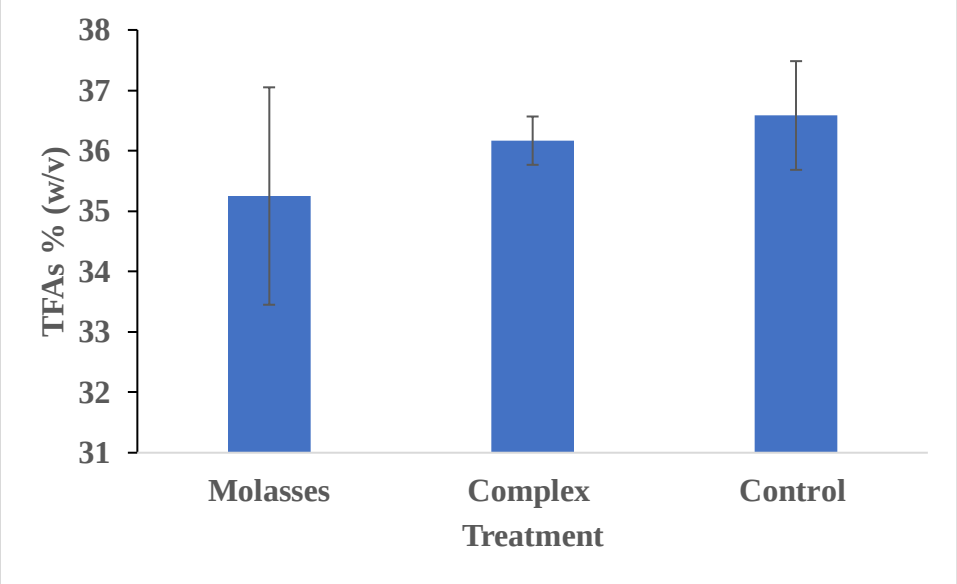


Figure 11: Summarized lipid contents (% w/v) extracted from 12 hemp seed samples grown in the different treatment of several types of biostimulants. Bars are treatment means (± SD). ($p<0.05$). Detailed data for each sample can be seen in **Appendix 9**.

Table 5: Major fatty acid compositions (% TFA) of 12 samples of hemp seeds grown on different biostimulants.

Fatty Acids	Name	Molasses treated-hemp seeds	Complex treated-hemp seeds	Control treated-hemp seeds
SFAs	C16:0 Palmitic acid	$9.27_{a} \pm 0.84$	$12.05_{a} \pm 0.38$	$10.48_{a} \pm 0.97$
	C18:0 Stearic acid	$3.47_{a} \pm 0.33$	$3.40_{a} \pm 0.17$	$3.40_{a} \pm 0.29$
MUFAs	C18:1n9c Oleic acid	$10.45_{a} \pm 0.99$	$11.95_{a} \pm 0.80$	$10.96_{a} \pm 0.94$
PUFAs	C18:3n3 Alpha-linolenic acid ALA	$14.64_{a} \pm 1.50$	$16.31_{a} \pm 0.55$	$15.97_{a} \pm 1.52$
	C20:5n3 Eicosatetraenoic acid EPA	–	–	–
	C22:6n3 Docosahexaenoic acid DHA	–	–	–
	C18:2n6 Linoleic acid LNA	$36.76_{a} \pm 3.10$	$41.75_{a} \pm 0.67$	$38.40_{a} \pm 3.41$
	C20:4n6 Arachidonic acid ARA	–	–	–
	C22:5n6 Docosapentaenoic acid DPA	–	$0.09_{a} \pm 0.09$	–

The values were means of 4 samples in the same group. Detailed data for each sample can be seen in **Appendix 9**. Bars are treatment means (± SD). ($p<0.0$

5. Antioxidant property

Plants contain a variety of natural bioactives that are gaining attraction due to their pharmacological potential, which comprises antioxidant, anticancer, anti-inflammatory, and antibacterial properties (Vitorović, 2021 #213). Due to their nutritional and pharmacological qualities, oils derived from the seeds of various plants have received a lot of interest in recent years (K et al. 2021; Martin et al. 2020). In this assay, gallic acid is the most widely used commercially, but it has a variety of negative side effects on humans, therefore several natural alternatives have been discovered to provide more or similar antioxidant protection at a lower cost (Bai et al. 2021). Alternatively, ascorbic acid (as known as Vitamin C) was utilized as the standard in this study to determine antioxidant activity using the DPPH technique. Because it is the strongest natural water-soluble antioxidant that found in a variety of foods, commonly in citrus fruit extracts. In the aqueous phases of cells and the circulatory system, ascorbic acid (AA) has been proven to directly scavenge free radicals (Guo et al. 2021). By renewing the antioxidant form of vitamin E, AA has been shown to protect membranes and other hydrophobic compartments against such damage (Beyer 1994). Herein, samples including HS and Thraustochytrid fermentation products by supplemented with HS were undergone the examination for its antioxidant characteristics by DPPH assay. Standard Curve of L-Ascorbic Acid by DPPH Assay is presented in **Appendix 13**.

Along with ascorbic acid, Trolox was used as a positive control in this work to compare the antioxidant potential. Trolox is an artificial water-soluble analog of Vitamin E that is generally used as a reference due to its strong antioxidant property (Giordano, Caricato & Lionetto 2020). As presented in **Figure 12**, the higher concentration of Trolox showed higher antioxidant activity and reached nearly 50% of scavenging effect of DPPH free radical from the concentration of 12 µg/mL.

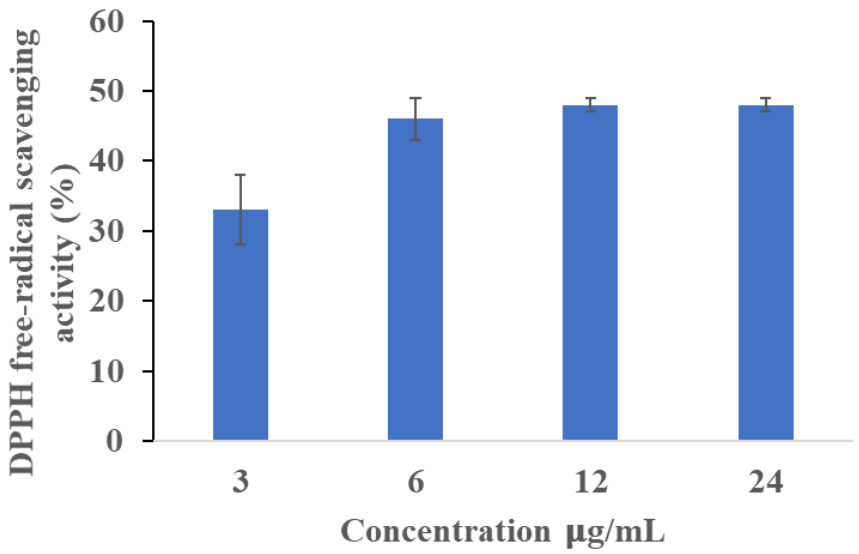


Figure 12: Scavenging activity of the DPPH free radical by Trolox (concentration in the range of 3 – 24 µg/ml). Error bars indicate standard deviation (n = 3, **Appendix 14**)

Recent studies have identified a variety of natural antioxidant compounds in hemp seed, such as phenolic compounds, tocopherols, and phytosterols (Andre, Hausman & Guerriero 2016). These compounds can scavenge free radicals, and many of them have therapeutic potential for disorders caused by oxidative stress (Kitamura et al. 2020). Herein, the antioxidant activity of HS and the fermentation products after HS addition were investigated. **Figure 13** shows the percentage of antioxidant activity of extracts from HS and Thraustochytrid fermentation products with HS or glucose supplement (GYP 5%, GHYP 2.5%, GHYP 5%, HYP 5%), determined as a function of concentration. Overall, all the samples performed their antioxidant effects according to concentration; that is, higher concentrations showed a higher percentage of scavenging. The antioxidant activity of HS in this work was low when compared to the positive control of Trolox, with values of 6%, 14%, 25%, and 32% obtained for concentrations of 6.2 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL, respectively. Instead of glucose, HS as a carbon source for

Thraustochytrid fermentation also contributed similar values in all concentrations, which revealed the maximum antioxidant activity (48%) at the concentration of 25 mg/mL. This indicated that after Thraustochytrid fermentation, with the same concentration of HS, the antioxidant activity was doubled, which could possibly be explained by the presence of carotenoids and xanthophyll pigments, antioxidant compounds produced by Thraustochytrid (Gupta, Barrow & Puri 2021). Among the fermentation conditions analyzed in this study, the combination of HS and glucose as carbon sources (GHYP 5%) showed 59% scavenging ability at a concentration of 50 mg/mL, suggesting the best option for the nutritional supplement for Thraustochytrid fermentation to enhance the production of bioactive compounds. Moreover, further work on analyzing and quantifying bioactive compounds presented in each condition after Thraustochytrid fermentation should be done for detailed clarification.

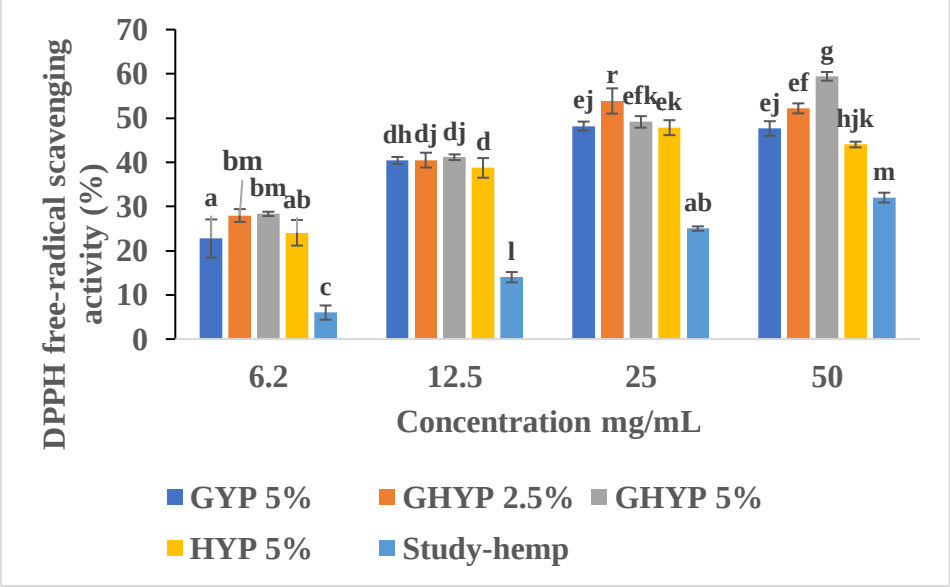


Figure 13: Antioxidant activity in different concentrations of extracts from HS and Thraustochytrid fermentation products supplemented with HS. Error bars indicate the standard deviation (n = 3). Different lowercase letters (a-m) represent significant differences (Tukey's test, $p < 0.001$, **Appendix 15**)

Chapter IV. CONCLUSION

In this work, the hemp seed in the study (HS) was evaluated for its ability to be used as an alternative carbon source in the fermentation of Thraustochytrid S2 strain for enhancing biomass and lipid content in general, especially PUFA production. First, the biomass and lipid content obtained after Thraustochytrid fermentation with the addition of HS showed more than 6 times the biomass and 7 times the total lipid content after the first day of fermentation as combined with glucose. Secondly, HS was compared to market hemp seed (MHS) and commercial hemp seed oil (CHSO) by FAME analysis before and after Thraustochytrid fermentation. After the fermentation process, several essential FAs were produced, specifically based on the carbon sources. The results suggested that significant ALA production in the supplement of HS was observed. Furthermore, the impact of different biostimulants on the FA composition of hemp seed was investigated. Twelve samples of hemp seeds collected from each crop showed an insignificant difference in FA profile but some noticeable variance in specific FAs. In terms of antioxidant activity, HS itself had a low antioxidant effect, however doubled (48% of DPPH free radical scavenging) after Thraustochytrid fermentation. Moreover, the combination with glucose exhibited the highest antioxidant activity in all concentrations and across all conditions. Hence, in this study, HS is possibly used as an extra supplement along with glucose for the improvement of the Thraustochytrid fermentation product, while further analyses on antioxidant biomolecules in the HS and after fermentation need to be addressed.

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APPENDIX 1: SPSS Analysis of Some Specific Fatty Acids Produced in Different Fermentation Mediums (ANOVA test)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	1.615	3	.538	30.647	.000
	Within Groups	.984	56	.018		
	Total	2.599	59			
OD	Between Groups	12.413	3	4.138	10.589	.000
	Within Groups	21.883	56	.391		
	Total	34.296	59			
Lipid	Between Groups	171.392	3	57.131	14.740	.000
	Within Groups	217.054	56	3.876		
	Total	388.446	59			

APPENDIX 2: SPSS Analysis of Lipid Production from Thraustochytrid S2 Strain Incubating in Different Fermentation Mediums Supplemented with HS (Tukey's test)

ANOVA

lipid

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	171.400	3	57.133	14.739	.000
Within Groups	217.077	56	3.876		
Total	388.477	59			

APPENDIX 3: Two-sided t-test Analysis for Comparison of Extracted Lipid Content between HS and MHS

	media			
	1		2	
	(A)		(B)	
	Mean	Standard Error of Mean	Mean	Standard Error of Mean
Lipid content	.02B	.00	.01	.00

Results are based on two-sided tests assuming equal variances. For each significant pair, the key of the smaller category appears in the category with the larger mean.

Significance level for upper case letters (A, B, C): .05[1]

1. Tests are adjusted for all pairwise comparisons within a row of each innermost sub-table using the Benjamini-Hochberg correction.

APPENDIX 4: SPSS ANALYSIS OF SOME SPECIFIC FATTY ACIDS PRESENTED IN HS, MHS, OR CHSO

Fatty acids	HS	MHS	CHSO
C16:0 Methyl palmitate	18.67 ± 1.57a	17.18 ± 2.27a	19.39 ± 2.53a
C18:0 Methyl stearate	4.88 ± 0.77a	3.63 ± 0.40ab*	2.87 ± 0.32b
C18:2n6c Methyl linolelaidate/C18:2n6t Methyl linoleate	34.3 ± 17.00a	20.4 ± 3.60a	25.6 ± 5.70a
C18:3n3 Methyl linolenate ALA	5.14 ± 0.97a	6.8 ± 1.20a	8.70 ± 1.70a
C22:5n6 DPA	4.55 ± 1.28a	4.61 ± 1.21a	6.00 ± 1.45a
C22:6 Methyl docosahexaenoate	10.38 ± 2.63a	10.58 ± 2.79a	7.04 ± 3.62a

Data presented as mean ± standard error.

* Different letters indicate statistical significance at the P = 0.05

APPENDIX 5: SPSS ANALYSIS OF SOME SPECIFIC FATTY ACIDS PRESENTED IN HS, MHS, OR CHSO (ANOVA TEST)

		Sum of Squares	df	Mean Square	F	Sig.
c16	Between Groups	30.657	2	15.329	0.273	0.763
	Within Groups	1852.038	33	56.122		
	Total	1882.696	35			
c18	Between Groups	24.902	2	12.451	3.646	0.037
	Within Groups	112.686	33	3.415		
	Total	137.587	35			
c18_2n6	Between Groups	1194.212	2	597.106	0.447	0.644
	Within Groups	44104.038	33	1336.486		
	Total	45298.25	35			
c18_3n3	Between Groups	76.087	2	38.044	1.801	0.181
	Within Groups	697.009	33	21.121		
	Total	773.096	35			
c22_5	Between Groups	16.171	2	8.085	0.389	0.681
	Within Groups	686.439	33	20.801		
	Total	702.61	35			
c22_6	Between Groups	94.752	2	47.376	0.426	0.657
	Within Groups	3673.748	33	111.326		
	Total	3768.5	35			

APPENDIX 6: SPSS Analysis of Biomass and Lipid Production from Thraustochytrid S2 Strain Incubating in Different Fermentation Mediums Supplemented with MHS (Tukey's test)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Result biomass	Between Groups	3.203	3	1.068	169.019	.000
	Within Groups	.354	56	.006		
	Total	3.557	59			
Result OD	Between Groups	40.801	3	13.600	54.438	.000
	Within Groups	13.990	56	.250		
	Total	54.791	59			
lipid	Between Groups	101.919	3	33.973	12.350	.000
	Within Groups	154.049	56	2.751		
	Total	255.969	59			

APPENDIX 7: SPSS ANALYSIS OF SOME SPECIFIC FATTY ACIDS PRODUCED IN DIFFERENT FERMENTATION MEDIUMS

Fatty acids	GYP 5% - Control	GHYP 2.5%/GOYP2.5%	GHYP 5%/GOYP 5%	HYP 5%/OYP 5%
C16:0 Methyl palmitate	29.46 ± 0.63a	16.4 ± 1.63b	15.31 ± 0.52bc*	12.48 ± 1.22c
C18:0 Methyl stearate	1.91 ± 0.4a	3.63 ± 0.44b	3.42 ± 0.1b	6.20 ± 0.72c
C18:2n6c Methyl linolelaidate/C18:2n6t Methyl linoleate	0.10 ± 0.0a	22.5 ± 1.6ab	50.50 ± 21.00b	34.10 ± 4.20ab
C18:3n3 Methyl linolenate ALA	0.03 ± 0.02a	7.64 ± 0.46b	9.70 ± 0.67bc	10.14 ± 1.35c
C22:5n6 DPA	11.97 ± 0.26a	4.31 ± 0.96b	2.98 ± 0.21b	0.96 ± 0.21c
C22:6 Methyl docosahexaenoate	26.24 ± 0.60a	3.50 ± 1.18bc	5.49 ± 1.41b	2.10 0.46c

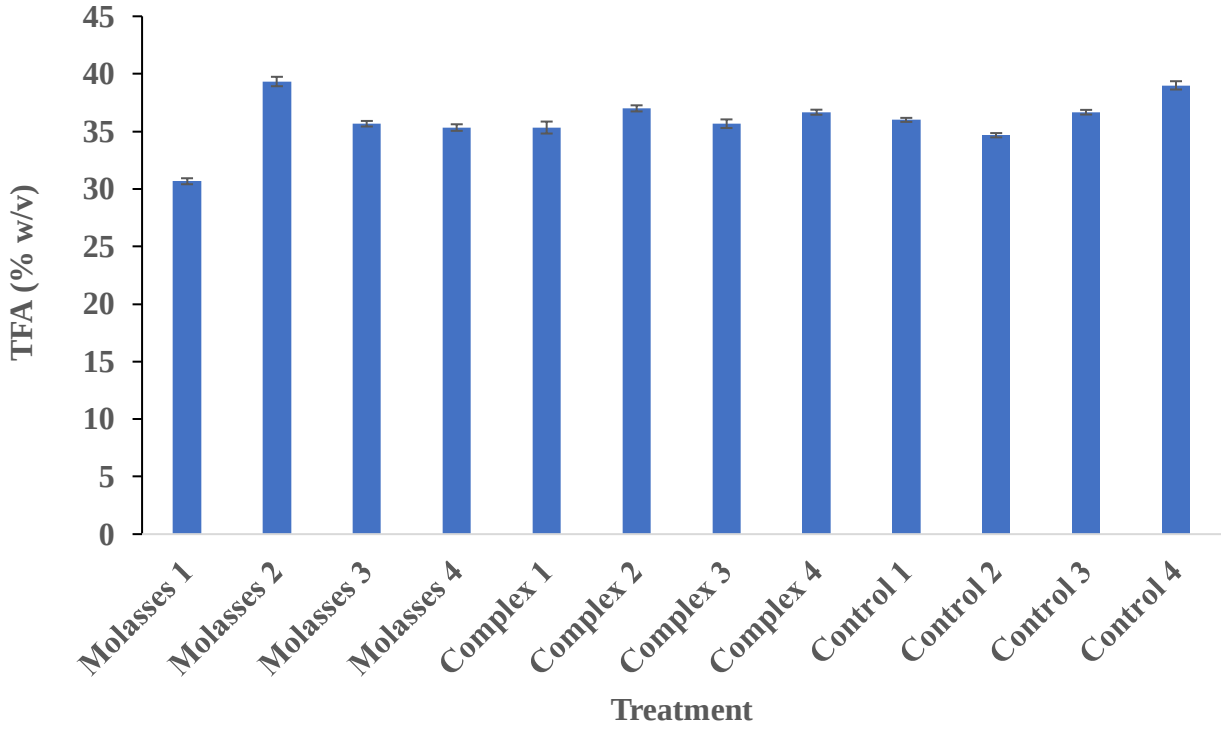
Data presented as mean ± standard error.

* Different letters indicate statistical significance at the P = 0.05

APPENDIX 8: SPSS analysis of some specific fatty acids produced in different fermentation mediums (ANOVA test)

		Sum of Squares	df	Mean Square	F	Sig.
c16	Between Groups	1537.55	3.00	512.52	47.52	0.00
	Within Groups	345.15	32.00	10.79		
	Total	1882.70	35.00			
c18	Between Groups	85.48	3.00	28.49	17.50	0.00
	Within Groups	52.10	32.00	1.63		
	Total	137.59	35.00			
c18_2n6	Between Groups	12136.11	3.00	4045.37	3.90	0.02
	Within Groups	33162.14	32.00	1036.32		
	Total	45298.25	35.00			
c18_3n3	Between Groups	594.63	3.00	198.21	35.54	0.00
	Within Groups	178.46	32.00	5.58		
	Total	773.10	35.00			
c22_5	Between Groups	625.00	3.00	208.33	85.90	0.00
	Within Groups	77.61	32.00	2.43		
	Total	702.61	35.00			
c22_6	Between Groups	3484.03	3.00	1161.34	130.64	0.00
	Within Groups	284.47	32.00	8.89		
	Total	3768.50	35.00			

APPENDIX 9: TOTAL LIPID EXTRACTED FROM COLLECTED 12 HEMP SEED SAMPLES



Bars present standard deviation

APPENDIX 10: FATTY ACID COMPOSITIONS (% TFA) OF 12 SAMPLES OF HEMP SEEDS GROWN WITH ADDED DIFFERENT BIOSTIMULANTS.

	1	2	3	4	5	6	7	8	9	10	11	12
FA	Molasses	Molasses	Complex	Complex	Control	Control	Control	Control	Complex	Complex	Molasses	Molasses
C10:0	2.7 ± 0.3	2.93 ± 0.28	3.76 ± 0.57	3.90 ± 0.10	3.60 ± 0.05	3.47 ± 0.06	4.17 ± 0.42	4.37 ± 0.60	4.00 ± 0.26	4.00 ± 0.20	3.70 ± 0.15	3.73 ± 0.20
C11:0	0.066 ± 0.1	0.16 ± 0.08	0.33 ± 0.13	0.20 ± 1.96262 E-17	0.20 ± 1.96262 E-17	0.17 ± 0.03	0.23 ± 0.03	0.30 ± 0.05	0.30 ± 0.05	0.13 ± 0.03	0.43 ± 0.06	0.57 ± 0.29
C12:0 Methyl laurate						0.03 ± 0.03		0.07 ± 0.06			0.03 ± 0.03	
C14:0 Methyl myristate	0.26 ± 0.03	0.33 ± 0.03	1.04 ± 0.40	0.33 ± 0.03	0.33 ± 0.03	0.33 ± 0.03	0.33 ± 0.03	0.40 ± 1.96262 E-17	0.37 ± 0.03	0.40 ± 3.92523 E-17	0.33 ± 0.03	0.87 ± 0.20
C15:0 Methyl pentadecanoate	0.03 ± 0.033	0.03 ± 0.03	0.03 ± 0.03									
C16:0 Methyl palmitate	8.80 ± 0.40	10.06 ± 0.4	13.2 ± 1.00	11.63 ± 0.21	11.20 ± 0.11	11.43 ± 0.12	11.43 ± 0.26	11.73 ± 0.37	11.83 ± 0.31	11.53 ± 0.17	11.03 ± 0.16	11.23 ± 0.40
C16:1 n7 Methyl palmitoleate	0.20 ± 0.00	0.20 ± 0.10	0.43 ± 0.08	0.36 ± 0.03	0.30 ± 0.00	0.30 ± 0.00	0.33 ± 0.03	0.33 ± 0.03	0.23 ± 0.12	0.37 ± 0.03	0.30 ± 0.00	0.27 ± 0.03
C17:0 Methyl heptadecanoate	0.07 ± 0.03	0.10 ± 9.81308 E-18	0.10 ± 0.05	0.07 ± 0.03	0.16 ± 0.03	0.13 ± 0.03	0.10 ± 0.05	0.17 ± 0.03	0.20 ±	0.13 ± 0.03	0.17 ± 0.03	0.17 ± 0.03

C17:1n7 cis-10-Heptadecanoic acid methyl ester		0.10 ± 9.81308 E-18	0.06 ± 0.03	0.03 ± 0.03	0.10 ± 9.81308 E-18	0.10 ± 9.81308 E-18	0.07 ± 0.03	0.10 ±. .00	0.07 ± 0.03	0.10 ± 9.81308 E-18	0.10 ± 9.81308E -18	0.10 ± 9.81308E -18
C18:0 Methyl stearate	3.13 ± 0.18	3.83 ± 0.06	3.46 ± 0.03	2.93 ± 0.03	3.80 ± 0.10	3.63 ± 0.03	4.20 ± 0.10	3.67 ± 0.03	3.77 ± 0.20	3.43 ± 0.08	4.17 ± 0.17	4.13 ± 0.03
C18:1n9c Methyl oleate	9.40 ± 0.36	12.16 ± 0.57	10.16 ± 0.3	12.06 ± 0.14	12.67 ± 0.54	11.57 ± 0.18	14 ± 0.15	11.37 ± 0.23	11.53 ± 0.20	14.03 ± 0.14	12.03 ± 0.14	12.87 ± 0.12
C18:1n7 Vaccenic acid	1.46 ± 0.03	1.83 ± 0.13	1.93 ± 0.03	2.27 ± 0.03	2.03 ± 0.03	1.97 ± 0.03	2.03 ± 0.06	1.93 ± 0.08	2.03 ± 0.03	2.17 ± 0.03	1.93 ± 0.03	1.90 ± 0.10
C18:1n9t Elaidic acid methyl ester												
C18:2n6c Methyl linolelaidate/C18:2n6t Methyl linoleate	37.3 ± 1.66	41.4 ± 0.8	40.36 ± 1.36	43.30 ± 0.26	42.77 ± 0.34	41.50 ± 0.47	42.43 ± 0.47	41.17 ± 0.57	40.93 ± 0.20	42.43 ± 0.26	40.50 ± 0.17	40.27 ± 1.10
C18:3n6 Methyl γ-linolenate GLA	0.87 ± 0.03	0.86 ± 0.03	1.10 ± 0.05	1.17 ± 0.03	1.13 ± 0.03	0.97 ± 0.03	1.00 ± 0.05	1.30 ± 0.40	1.00 ± 0.00	1.10 ± 0.00	1.00 ± 0.00	0.93 ± 0.03
C18:3n3 Methyl linolenate ALA	12.87 ± 0.51	15.93 ± 0.41	16.76 ± 0.80	16.10 ± 0.49	16.07 ± 0.29	18.70 ± 0.20	13.73 ± 0.03	17.43 ± 0.26	17.50 ± 0.37	14.90 ± 0.55	18.20 ± 0.05	14.97 ± 0.29
C20:0 Methyl arachidate	0.53 ± 0.03	0.60 ± 0.00	0.56 ± 0.03	0.67 ± 0.03	0.60 ± 0.00	0.60 ± 0.00	0.67 ± 0.03	0.63 ± 0.03	0.60 ± 0.00	0.67 ± 0.03	0.67 ± 0.03	0.70 ± 7.85046E -17

C20:1n9 Methyl cis-11-eicosenoate	0.36 ± 0.03	0.30 ± 0.00	0.33 ± 0.03	0.40 ± 3.92523 E-17	0.30 ± 0.00	0.30 ± 0.00	0.33 ± 0.03	0.33 ± 0.03	0.30 ± 0.00	0.37 ± 0.03	0.37 ± 0.06	0.33 ± 0.03
C22:0 Methyl behenate	0.20 ± 0.00	0.20 ± 1.96262 E-17	0.27 ± 0.03	0.30 ± 0.00	0.20 ± 1.96262 E-17	0.20 ± 1.96262 E-17	0.30 ± 0.00	0.27 ± 0.03	0.23 ± 0.03	0.30 ± 0.00	0.23 ± 0.03	0.30 ± 0.00
C23:0 Methyl tricosanoate	0.10 ± 0.10		0.10 ± 0.10									0.10 ± 0.10
C22:5n6 DPA			0.36 ± 0.23									
C24:0 Methyl lignocerate	1.20 ± 1.20	0.07 ± 0.06	0.76 ± 0.57	0.17 ± 0.06			0.07 ± 0.06				0.10 ± 0.10	0.13 ± 0.06
C22:6 Methyl docosahexaenoate												

Data presented as mean ± standard deviation

APPENDIX 11: SPSS ANALYSIS BETWEEN MAJOR FATTY ACID COMPOSITIONS (% TFA) OF 12 SAMPLES OF HEMP SEEDS GROWN ON DIFFERENT BIOSTIMULANTS.

	Treatment					
	Molasses		Complex		Control	
	Mean	Standard Error of Mean	Mean	Standard Error of Mean	Mean	Standard Error of Mean
c16	9.26[a]	.84	12.18[a]	.35	10.49[a]	.97
c18	3.46[a]	.33	3.40[a]	.17	3.40[a]	.30
c18_1n9c	10.44[a]	.99	11.95[a]	.80	10.96[a]	.95
c18_2n6	36.76[a]	3.10	41.76[a]	.68	38.41[a]	3.42
c18_3n3	14.64[a]	1.50	16.31[a]	.55	15.97[a]	1.53
c22_5	.00[a]	.00	.09[a]	.09	.00[a]	.00

Note: Values in the same row and sub-table not sharing the same subscript are significantly different at p< .05 in the two-sided test of equality for column means. Cells with no subscript are not included in the test. Tests assume equal variances.1

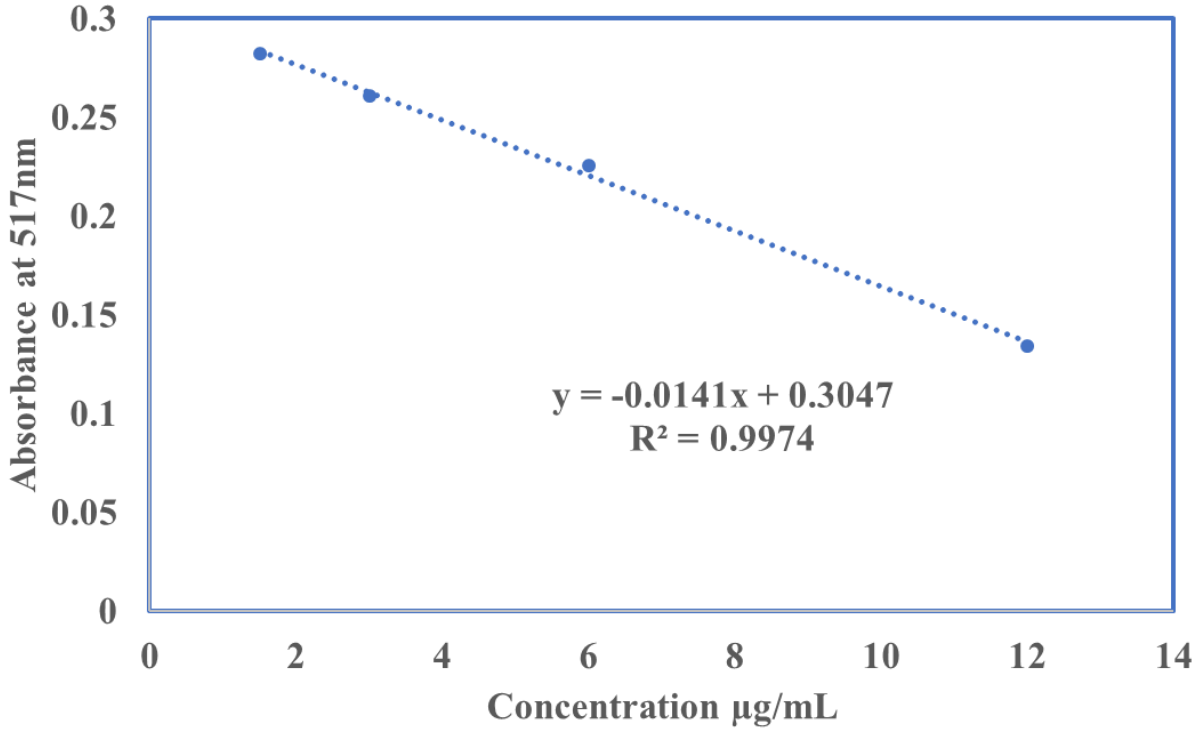
Tests are adjusted for all pairwise comparisons within a row of each innermost sub-table using the Benjamini-Hochberg correction

APPENDIX 12: SPSS ANALYSIS BETWEEN MAJOR FATTY ACID COMPOSITIONS (% TFA) OF 12 SAMPLES OF HEMP SEEDS GROWN ON DIFFERENT BIOSTIMULANTS (ANOVA TEST)

		Sum of Squares	df	Mean Square	F	Sig.
c16	Between Groups	17.142	2	8.571	3.627	.070
	Within Groups	21.270	9	2.363		
	Total	38.412	11			
c18	Between Groups	.010	2	.005	.017	.983
	Within Groups	2.711	9	.301		
	Total	2.721	11			
c18_1n9c	Between Groups	4.658	2	2.329	.696	.524
	Within Groups	30.129	9	3.348		
	Total	34.786	11			
c18_2n6	Between Groups	51.794	2	25.897	.893	.443
	Within Groups	260.943	9	28.994		

	Total	312.737	11			
c18_3n3	Between Groups	6.278	2	3.139	.481	.633
	Within Groups	58.693	9	6.521		
	Total	64.971	11			
c22_5	Between Groups	.022	2	.011	1.000	.405
	Within Groups	.097	9	.011		
	Total	.119	11			

APPENDIX 13: STANDARD CURVE OF L-ASCORBIC ACID BY DPPH ASSAY



APPENDIX 14: STATISTICAL ANALYSIS OF ANTIOXIDANT ACTIVITY OF TROLOX VIA DPPH ASSAY

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	74.667	2	37.333	.569	.585
Within Groups	590.250	9	65.583		
Total	664.917	11			

APPENDIX 15: STATISTICAL ANALYSIS OF ANTIOXIDANT ACTIVITY OF HS AND FERMENTATION PRODUCTS IN THE HS SUPPLEMENT VIA DPPH ASSAY

% DPPH inhibition	GYP 5%	GHYP 2.5%	GHYP 5%	HYP %	HS
Mean	40e ± 3	44e ± 3	45e ± 3	39e ± 3	19 ± 3

Data presented as mean ± standard error

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5067.075	4	1266.769	10.484	.000
Within Groups	6645.696	55	120.831		
Total	11712.770	59			