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ANTIMICROBIAL AND ANTI-BIOFILM ACTIVITIES OF LIPIDS EXTRACTED FROM *SCHIZOCHYTRIUM* SP. S31 MICROALGAE

Doaa Abdullah Hammadi AL-OGAIDI

Master's Thesis

Supervisor

Asst. Prof. Dr. Nurcan Vardar Yel

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The thesis/dissertation titled ANTIMICROBIAL AND ANTI-BIOFILM ACTIVITIES OF LIPIDS EXTRACTED FROM *SCHİZOCHYTRİUM* SP. S31 MICROALGAE prepared by DOAA ABDULLAH HAMMADI AL-OGAIDI and submitted on 20/12/2022 has been **accepted unanimously** for the degree of Master of Science in Biomedical Science.

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I hereby declare that all information/data presented in this graduation project has been obtained in full accordance with academic rules and ethical conduct. I also declare all unoriginal materials and conclusions have been cited in the text and all references mentioned in the reference list have been cited in the text, and vice versa as required by the abovementioned rules and conduct.

Doaa Abdullah Hammadi AL-OGAIDI

Signature

DEDICATION

To my parents, for their endless love, support, and encouragement.

To my grandfather Hamed

To my brothers Taha and Yaseen.

To my sisters Hind, Huda, Sabaa, and Ghofran.

To my teachers

Doaa Abdullah Hammadi AL-OGAIDI

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ABSTRACT

ANTIMICROBIAL AND ANTI-BIOFILM ACTIVITIES OF LIPIDS EXTRACTED FROM *SCHIZOCHYTRIUM* SP. S31 MICROALGAE

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Biotechnology has been developing tools and procedures for the benefit of living things for many years. In most of these studies, the production of essential metabolic compounds is prioritized for human health. One of the crucial substances for human health that has gained popularity recently is omega-3 fatty acids. Alternative sources of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been searched since fish oils, the main commercial sources of omega-3 fatty acids, have heavy metal content, especially mercury, limited availability, and an unpleasant odor. *Schizochytrium* sp. S31 is a microalga with a high concentration of polyunsaturated fatty acids, particularly DHA. Because of its high DHA content and low heavy metal levels, heterotrophic cultivation of *Schizochytrium* sp. S31 provides an excellent opportunity to provide alternative omega-3 sources. It also contains a variety of bioactive substances that can be utilized as medicinal raw materials, food additives, animal feed, biodiesel and antimicrobial agents.

In this study, optimum conditions were applied to cultivate *Schizochytrium* sp. S31 heterotrophically and the nutritional composition of the dry biomass were analysed. According to the results, 45% protein, 5% total fat and 37% carbohydrate were obtained. Downstream processes, including cell lysis and lipid extraction, were adjusted in order to increase overall lipid content of *Schizochytrium* sp. S31. Five distinct solvent mixtures—hexane, hexane/ethanol (2:1), ethanol, methanol, and hexane/methanol (2:1)—as well as two different cell lysis techniques —mortar and pestle and ultrasonication—were examined and compared. Based on the results of cell lysis and lipid extraction, ultrasonication of hexane /methanol (2:1) enhanced the total lipid yields to 34% with a transparent appearance. According to the GCMS analysis results, 10.4% DHA and 16.11% EPA fatty acids were obtained. Total Omega-3 Fatty Acids and Total Omega-3 + Omega-6 Fatty Acids ratio were calculated as 27.42% and 32.15% respectively.

The antibacterial effects of *Schizochytrium* sp. S31 oil samples were also investigated on Gram-negative pathogens, *Pseudomonas aeruginosa*, *Escherichia coli* 35218 and *Klebsiella pneumonia*, which cause many different human infections, as well as the Gram-positive pathogen Streptococcus mutans. At the same time, their effects on biofilm formation in *Pseudomonas aeruginosa*, *Escherichia coli* 35218 bacteria were investigated. The *Schizochytrium* sp. S31 oil sample was found to have anti-pathogenic activity on pathogenic bacteria of *Pseudomonas aeruginosa*, *Escherichia coli* 35218, *Klebsiella pneumonia* and Streptococcus mutans. The surface adhesion ability and biofilm formation capability of *P. aeruginosa* and *Escherichia coli* 35218 strain, which are widely employed as model bacteria in biofilm research, were dramatically inhibited in the presence of a *Schizochytrium* sp. S31 oil sample.

Keywords: *Schizochytrium* sp. S31, antibacterial activity, antibiofilm, fatty acid, docosahexaenoic acid.

TABLE OF CONTENTS

Pages

LIST OF TABLES

Pages

LIST OF FIGURES

Pages

ABBREVIATIONS

ALA : Alpha linolenic acid

ARA : Arachidonic acid

ATCC : American Type Culture Collection

CDW : Cell Dry Weight

DHA : Docosahexaenoic Acid

DMSO : Dimethyl Sulfoxide

EPA : Eicosapentaenoic Acid

FAME : Fatty Acid Methyl Ester

FAO : Food and Agriculture Organization

FFA : Free Fatty Acid

GC : Gas Chromatography

GCMS : Gas Chromatography Mass Spectrometry

GLA : Gamma linolenik asit

HPLC : High Pressure Liquid Chromatography

MSG : Monosodium Glutamate

PUFA : Polyunsaturated Fatty Acids

TAG : Triacylglycerol

TFA : Total Fatty Acid

WHO : World Health Organization

1. INTRODUCTION AND PURPOSE

Biotechnological and multidisciplinary research are now underway in quest of new advances that may used for the benefit of all living things. The basic metabolic requirements for our metabolism are one of the most discussed topics globally since the human being takes precedence over all other considerations and health issues are given top importance. For instance, the percentage of DHA produced for supplemental feeding has dramatically increased during the early 2000s (Harun et al., 2010).

Algae are significant sources of a broad range of complex lipids that may be used in culinary, cosmetic, and pharmaceutical sectors (Hossain et al., 2005; Abd El-Baky & El-Baroty, 2013). It has been investigated whether microalgae can be utilized to create food supplements, lipids, enzymes, polymers, among other things. Some of these have been accomplished by the cultivation of microalgae in a variety of media and substrates (Perez-Garcia et al., 2011). Several studies have shown that marine macroalgae lipids have antibacterial, antiviral and cancer-fighting properties (Gerasimenko et al., 2010).

Long-chain polyunsaturated fatty acids (LC-PUFAs) are essential for human nutrition and brain development. Among all LC-PUFAs, DHA and EPA (Eicosapentaenoic acid) are vital for human growth. There is evidence that DHA helps the evolution of the human brain by regulating metabolisms such as sperm retina and brain lipids (Apt & Behrens, 1999; Chanda et al., 2018). Omega-3 fatty acids are essential for the immune system well as cardiovascular and neurological system issues (Peltomaa et al., 2018; Simopoulos, 2002). In addition, dietary DHA consumption affects amyloid precursor protein processing and reduces the risk of Alzheimer's disease by inhibiting amyloid formation (Lim et al., 2005). Furthermore, fatty acids have been investigated as possible therapeutic antimicrobial agents due to their potency, wide range of activity, and absence of typical resistance mechanisms (Desbois & Smith, 2010; Thormar, 2010; Mullen et al., 2010; P. Desbois, 2012; Feldlaufer et al., 1993; Zhang et al., 2012)

The aim of this study was to analyze the antibacterial and antibiofilm effect of lipid extracted from DHA-rich *Schizochytrium* sp. S31 microalgae. The antibacterial effects of *Schizochytrium* sp. S31 oil samples were studied on Gram-negative pathogens, *Pseudomonas aeruginosa*, *Escherichia coli* 35218 and *Klebsiella pneumonia*, which cause many different human infections, as well as the

Gram-positive pathogen *Streptococcus* mutans. At the same time, antibiofilm activity of the *Schizochytrium* sp. S31 oil on *Pseudomonas aeruginosa*, and *Escherichia coli* 35218 bacteria were investigated.

2. GENERAL INFORMATION

2.1 CLASSIFICATION OF ALGAE

Classification is the systematic grouping of organisms into groups that are based on evolutionary or structural links. The classification of algae into taxonomic groups may vary depending on many different factors. It is not possible to classify algae in terms of a single feature. Some algae live in a terrestrial environment, while others live in an aquatic environment. There are unicellular algae as well as multicellular algae. Algae can also be divided into prokaryotic and eukaryotic species. Nowadays, algae are commonly classified into three groups: macroalgae, cyanobacteria and microalgae (Baweja & Sahoo, 2015).

2.1.1 Macroalgae

Macroalgae (seaweed) are photosynthetic organisms that exist in aquatic habitats all over the globe, including the Arctic zone. Seaweeds have an important ecological and economic purpose. The highest variety is found on rocky seashores and coral reefs. Algae are thought to capture ten times the amount of solar energy as terrestrial plants. In food chains, they are the principal primary producers of organic molecules (Kaur et al., 2002).

Algae may be aquatic or subaerial, depending on whether they are on the surface or at the bottom of the water. Aquatic algae can tolerate high pH, temperature, turbidity, oxygen, and carbon dioxide concentrations. Subaerial (aerobic) algae are those that live in the air, soil, and litter, or water surface. There are several different types of algae, including epiphytic (found on living plants), epiphyllous (found on leaf surfaces), corticolous (found on bark, trunks, or stems), epizoic (found on animals), as well as lithophile (found in stone, brick, or cement), epixylous (found on dead wood, such as poles and posts), and epipetalous (found on metal surfaces). The growth habits of marine algae may be classified as supralittoral (above high tide), intertidal (onshores exposed to tidal cycles), or sublittoral (benthic environment from extreme low water to about 200 m deep in very clear water). The word "seaweed" typically refers to multicellular red, green, and brown marine algae. Each group is different in colour due to having different photosynthetic pigments. Red algae are known as Rhodophyta; brown algae are known as Ochrophyta; and green algae are

known as Chlorophyta. Marine plants, like terrestrial plants, are benign and should be preserved for future generations (Kaur et al., 2002).

2.1.2 Cyanobacteria

Cyanobacteria are naturally occurring photosynthetic bacteria found in all environments on Earth. However, although they may sometimes form colonies, they are normally unicellular (Figure 2.1). The oxygenation of the early earth's atmosphere and seas during the massive oxidation event 2.4 billion years ago is assumed to have been caused by cyanobacteria. They were previously known as Cyanophyta or blue-green algae because they are photosynthetic organism (Damatac & Cao, 2022). Iron is a crucial metal in the cosmos, and it plays a vital role in the development of cyanobacteria. The magnetic field created by the liquid iron in the earth's core allows life to exist on our planet. Ferredoxins (iron-sulfur proteins) are believed to have contributed to the development of fermentative bacteria (Rath et al., 2015). Certain archaea and bacteria share a common ancestor that can convert Fe (III) to Fe (II), which conserve energy and promotes growth. It is also thought that the ancestors of Proteobacteria and Cyanobacteria utilised Fe (OH+) as a significant electron donor for CO_2 fixation (Rath et al., 2015).

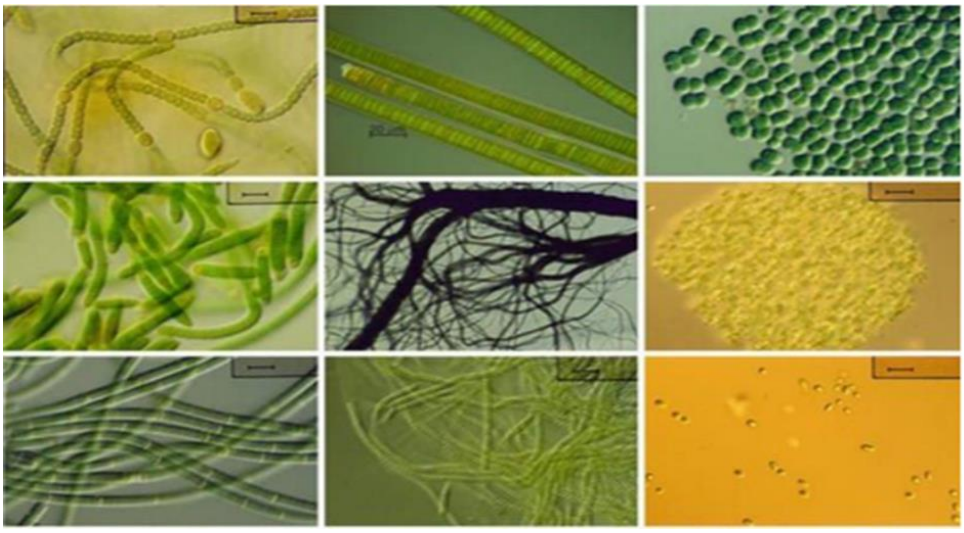

Figure 2.1: Images of different cyanobacteria species (Phiri, 2021).

The endosymbiotic theory states that endosymbiosis is the mechanism by which chloroplasts in eukaryotic plants and algae developed from cyanobacterial progenitors. Thermophilic Cyanobacteria can grow at over 45°C and were less common than mesophilic Cyanobacteria (Whitton, 2012). The maximum temperature limit for Cyanobacteria species in hot water is approximately 73°C, and *Synechococcus* sp. were discovered. (Damatac & Cao, 2022; Strunecký et al., 2019).

2.1.3 Microalgae

Microalgae are microscopic organisms that exist singly or in groups in the water and sediment (Figure 2.2). Similar to terrestrial plants, microalgae utilise the oxygen produced as a by-product of photosynthesis to produce organic molecules.

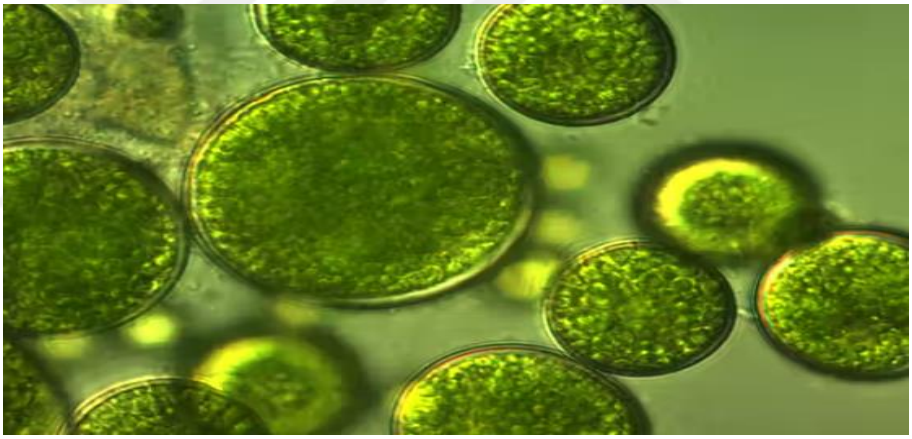

Figure 2.2: Microalgae under the microscope, unicellular green algae with large cells (Maltsev,2022).

Algae are utilized as food and fuel, and their extracts have significance in the medicinal business. These primitive plants have chlorophyll but no roots or leaves. Photosynthetic microalgae can use sunlight for energy to convert CO_2 into biomass and produce oxygen. Although microalgae develop in several ways, they can often double their biomass in 24 hours. There are around 50.000 microalgae species; however, only 30.000 have been examined (Handayania & Ariyantib, 2011).

2.2 IMPORTANT MICROALGAE SPECIES

The biotechnological use of microalgae has been concentrated on important species during the past two decades. Some of the important microalgae species are *Spirulina, Chlorella vulgaris, Dunaliella. salina, Haematococcus pluvialis,* and *Schizochytrium sp.* (McCann et al., 2014). The following subsections discuss the features, manufacturing method, and makeup of each of these microalgal species.

2.2.1 Spirulina

Spirulina is a symbiotic, filamentous, and multicellular blue-green microalgae that utilizes nitrogen from the surrounding environment. It is available in two separate shapes: spiral rod as shown in (Figure 2.3) or disk-shaped. The blue pigment phycocyanin serves as the principal photosynthetic pigment in Spirulina. Some include phycoerythrin, a pigment that gives microalgae their characteristic red or pink colour.

Figure 2.3: Spirulina meneghiniana algae under the microscope (Fox, 2022).

Spirulina is a well-studied algae for outdoor cultivation on a huge scale. It thrives in situations with high bicarbonate concentrations and pH levels (Paniagua-Michel et al., 2015; De Oliveira et al., 1999). Spirulina is cultured in raceway ponds powered by paddle wheels. The pond's water depth typically ranges between 300-500 mm, depending on the microalgal density and season. The water depth of a pond is also depended on its size as well as flow rate, and the algal culture's optimum light absorption. The temperature has a significant influence on spirulina production. It thrives at temperatures between 35°C and 37°C (De Oliveira et al., 1999).

2.2.2 Chlorella

Chlorella microalgae are photoautotrophic, single-celled, spherical (diameter: 5–10μm) and green in colour with no flagella (Figure 2.4) (Becker, 2007). It needs CO_2, water, light, and a few minerals in order to grow. Photobioreactors, large round tanks, mixed open ponds with paddle wheels, or circular open ponds have all been used commercially to cultivate Chlorella (Borowitzka & Larkum, 1987; Mobin et al., 2001). First, it is cultured in tiny containers and then transferred to a larger tank or pond.

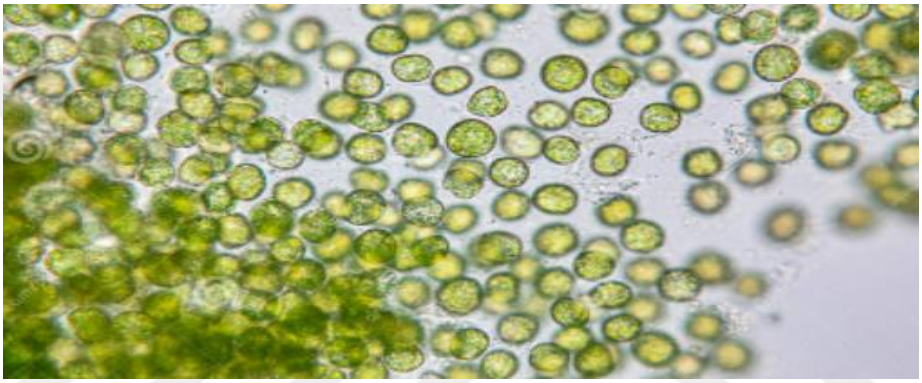

Figure 2.4: *Chlorella* under microscope (Thongdumhyu, 2018).

Circular tanks and ponds are two of the most frequent outdoor cultivation techniques. The biggest pond has a surface area of (500 $m^{2)}$ and a depth of (200 mm), respectively. Chlorella microalgae for aquaculture are often produced on a smaller scale, such as in 20–40 L carboys or enormous plastic bags. (Borowitzka & Larkum, 1987). Centrifugation and autoflocculation are used to extract chlorella. Several ways to process the biomass after harvesting, including spray drying or drum drying. In terms of dry weight, Chlorella includes 2-46 % of lipid, 12-28 % of carbohydrates, and 11–58 % of proteins (Zhu et al., 2014). Chlorella is rich in vitamins, including provitamin A, carotene, retinol, vitamin E, and vitamin C (Borowitzka, 1998).

2.2.3 Dunaliella

They are green microalgae that are edible, nutrient-dense, single-cell, and flagellated as shown under the microscope in Figure2.5. Additionally, it is found in a variety of marine and freshwater settings. Dunaliella salina has garnered considerable interest owing to its remarkable antioxidant activity. It is one the best-known sources of high-carotenoid-containing compounds compared to

other sources, as it has β-carotene concentrations of up to 14% of dry biomass. Although Dunaliella can grow at salinities above 30% NaCl saturation, the ideal growing salinity is 22% NaCl saturation. D. salina is often farmed in huge (5–200 ha) shallow, shallow paddlewheel raceway ponds or unstirred ponds with an average size of less than 1000 m^2. This species thrives on a semi-continuous cultivation procedure (Borowitzka & Vonshak, 2017). Borowitzka et al. (Borowitzka & Vonshak, 2017) showed that Dunaliella grows faster at lower salinity (less than roughly 22 percent (w/v). NaCl) saturation) but has the maximum beta-carotene concentration at greater salinity. (about 33 % of NaCl).

Figure 2.5: Dunaliella under microscope (Rappe, 2019).

Although Dunaliella grows more rapidly at low salinity, there is the potential for contamination by protozoa. Ben-Amotz studied numerous ways of producing Dunaliella (Borowitzka & Vonshak, 2017). A nitrogen deficiency, an increase in salt content, and high sun radiation are characteristics of intensive culture environments that encourage the growth of Dunaliella biomass and the production of carotene. (Borowitzka & Vonshak, 2017). Typically, Dunaliella is harvested using centrifugation, flocculation, or a process that utilizes the cell membrane's hydrophobic characteristics (Borowitzka & Larkum, 1987). Once harvested, biomass can be dried using a spray or tumble dryer, or carotene can be directly extracted using heated oil or other solvents. The biological composition of Dunaliella consists of 49–57% protein, 4–32% carbs, and 6–8% lipids. (Zhu et al., 2014).

2.2.4 Haematococcus

The unicellular Chlorophyte microalga *Haematococcus pluvialis* (H. *pluvialis*) is found in freshwater across the globe (Figure 2.6). It is well known that this species accumulates astaxanthin of up to 2–3 percent by dry weight under stressful conditions. the most significant commercial producer of astaxanthin is *H. pluvialis* (Ambati et al., 2014). *H. pluvialis* is grown in various

photobioreactors, including closed photobioreactors, open raceway ponds, and photobioreactors of various types (based on growth conditions) (Kang et al., 2010; Wang et al., 2013).

Figure 2.6: *Haematococcus* sp. under microscope (Fox, 2022).

The majority of *H. Pluvialis* photoautotrophic culture occurs in open raceway ponds or enclosed photobioreactors, where algae thrive. For commercial production, a two-stage cultivation approach is generally used (Shah et al., 2016). Environmental variables such as light, pH, temperature, salt content, and nutritional stress affect astaxanthin accumulation (Shah et al., 2016).

2.2.5 *Schizochytrium* sp.

Schizochytrium sp. species were formerly classified as fungi due to their heterotrophic nature and similarity to Chytrids. However, this organism was categorized as heterokont algae in the Thraustochytrids by using contemporary molecular biology methods (Cavalier-Smith, 1999). Schizochytrium sp. microalgae may be regarded as a component of the human food chain because it is consumed by fish and other marine organisms. The search for alternatives to fish oil has risen due to the increased focus on the benefits of including omega -3 fatty acids in both human and animal diets. As a result of the growing need for omega 3 fatty acids in human diets, the hunt for alternatives to fish oil has grown significantly. *Schizochytrium* sp. is one of a number of single-celled organisms that generate polyunsaturated fatty acid (PUFA)-rich oils, including EPA and DHA (Figure 2.7) (Vardar, 2016).

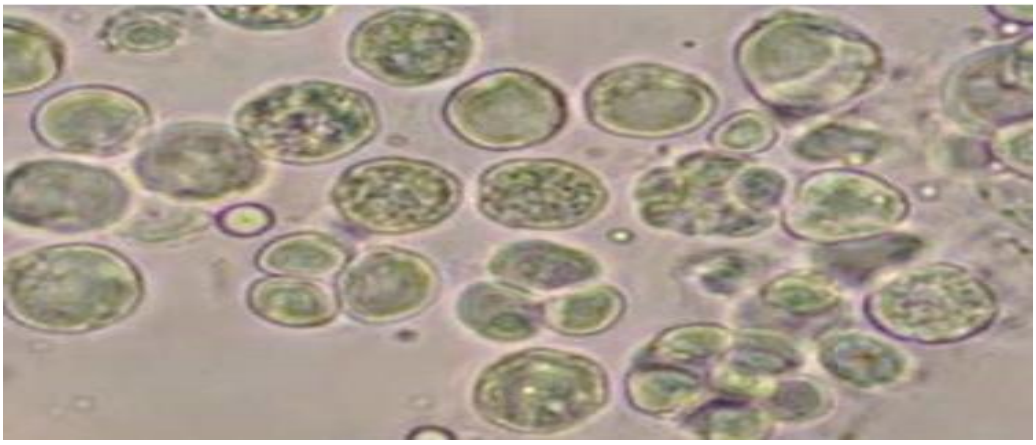

Figure 2.7: *Schizochytrium* sp. cells under microscope (Vardar, 2016).

Manufacturing *Schizochytrium* sp. has several advantages, such as improved oxidative stability, reduced purification costs, production from natural and sustainable ingredients, and consistent product quality. (Ratledge, 2004). *Schizochytrium* sp. is one of the most widely used commercial strains owing to its PUFA synthase complex's O2-independent activity (Jakobsen et al., 2008). Under oxygen-limiting circumstances, the lipid content of *Schizochytrium* sp. may rise, which may impact the activity of the PUFA synthase enzyme complex. The changing dissolved oxygen level in a shaking flask scale was also examined, and DHA content was found to be elevated, according to a two-stage growth experiment with *Schizochytrium* sp. (Chi et al., 2009).

2.3 COMPOSITION OF MICROALGAL BIOMASS

Microalgae are very adaptable to changing environmental circumstances, displaying exceptional flexibility and adaptability. As a consequence of their defensive and adaptation systems, they create a wide range of biosynthetic chemicals or molecules with distinct features (Guschina & Harwood, 2006). There is significant potential for some of these chemicals to be used as precursors or raw materials for value-added bioproducts in various industries. Components of microalgae (such as proteins, carbohydrates, and lipids) enable them to adapt to fluctuating environmental circumstances for growth (Mooij et al., 2016; Markou et al., 2015). Typical microalgae nutritional products include powders, pills, and capsules that provide between 12 to 35 % protein, 7.2 to 23 % fat, and 4.6 to 23 % carbohydrates (Borowitzka, 2013).

Microalgal biomass content, especially lipid production, is significantly affected by key abiotic variables such as light intensity, medium composition, temperature, pH, salt content, and nutrient restriction (especially nitrogen restriction) (Pal et al., 2011). The effects of these traits are species-specific; therefore, ideal conditions for one microalgae species may not necessarily be advantageous for another microalgae species (Robertson et al., 2013). Understanding how these parameters interact with metabolic pathways in different microalgae species is therefore crucial for making well-informed decisions.

2.3.1 Carbohydrates

Microalgae are the principal producers of biomass, such as carbohydrates, proteins, and lipids throughout the planet by photosynthesizing light energy and carbon dioxide (CO_2) (Becker, 2007). Microalgae synthesize carbohydrates for two primary functions: as a structural component of the cell wall and as a storage component inside the cell (Cheng et al., 2017; Schulze et al., 2016). As a storage molecule, carbohydrates supply the microalgae with the energy required for their metabolism and enable them to temporarily live in the dark when necessary (Fimbres-Olivarría et al., 2016). Carbohydrate concentration in biomass is dependent on microalgal species and growing circumstances (Patel et al., 2014). Certain microalgal species, such as *Porphyridium cruentum* (40–57%) and *Spirogyra* sp. (33–64%), contain a great deal of carbohydrates (Mühlroth et al., 2013; Dai et al., 2020). Microalgal polysaccharides are used in the generation of biofuels, particularly biohydrogen. To improve biofuel production, it is required to combine circumstances with a high carbohydrate content with microalgae's ability to generate substantial volumes of biomass. Therefore, culture conditions can be changed to increase the growth rate and carbohydrate content of microalgae (Blockx et al., 2018; Chng et al., 2017).

Microalgal carbohydrate concentration is primarily influenced by nutritional content, stress conditions, light intensity, and temperature. The technique of lowering or raising the quantity of nutrients is regarded as a cost-effective method for producing microalgae high in carbohydrates (Corrêa et al., 2019). This is feasible because it's easy to regulate the medium's nutritional content. (Corrêa et al., 2019; Gerchman et al., 2017).

2.3.2 Protein

Many species of microalgae have significantly variable protein compositions. For example, *Chaetoceros gracilis* has 12% protein, while *Arthrospira platensis* has between 53-70% protein (Wang et al., 2021). The protein content in the microalgal biomass is affected by many factors such as genetic characteristics, light intensity, temperature, pH, CO_2 content, medium content, and the culture system. These factors, affect the protein content of different microalgae species differently. The amount of protein obtained from microalgae can also vary according to the extraction method. Under the ideal circumstances of biomass concentration, ionic strength, temperature, pH, and extraction time, microalgae proteins can typically be extracted from acidic, aqueous, and/or alkaline media. After centrifuging the medium, proteins may be recovered using chromatography, ultrafiltration, and separation methods like precipitation. The isolation of crude protein concentrates using this method is straightforward, although there is no universally applicable pH value for the extraction of microalgae protein. For instance, *Chlorella vulgaris* and *Spirulina platensis* reported the maximum protein solubility achieved at pH 11 and pH 12, respectively (Ursu et al., 2014), but a higher value of protein was isolated from *Nannochloropsis* sp. up to pH 13.(Gerde et al., 2013).

2.3.3 Lipids

Microalgae cells use both organic and inorganic carbon sources for lipid production. The lipid content of microalgae generally ranges from 10-60% of dry biomass. However, under certain conditions, the microalgal lipid content can even reach 80%. Microalgal lipids can be divided into two different classes based on their carbon number. Polyunsaturated fatty acids (PUFA) with more than 20 carbons especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are frequently utilized as health food supplements, while fatty acids with 14–20 carbons are mostly employed for the manufacturing of biodiesel. It is known that the DHA range of *Schizochytrium* sp. ranges from 20% to 40% of the total amount of fatty acids.

Under stressful circumstances like nutrition restriction and low temperature, microalgae can synthesize significant levels of lipids. For example, lipid accumulation in *Dunaliella* sp. and *Chlorella vulgaris* increased significantly in high-salt media, reaching 70% and 21%, respectively.

In another study, observations revealed that the lipid ratio of *Schizochytrium* sp. microalgae increased 4-fold in a nitrogen-limited environment.

Industrial output is supported by the capacity of green algae to accumulate carotenoids, cell proliferation, and lipid content (Aguilar-Rosas et al., 2013). Microalgal lipid amounts can be increased by overexpression of key enzymes involved in lipid biosynthesis. Lipid synthesis may be improved by manipulating DGAT levels. For example, in *Arabidopsis thaliana*, DGAT overexpression resulted in a 10-70 % increase in lipid content (Jako et al., 2001). Another study found that the DGTT2 gene could be successfully transplanted into Arabidopsis after being extracted from *Chlamydomonas reinhardtii*. The quantity of lipids in transgenic plants increased by about ten times, and the newly generated lipids included exceptionally long-chain fatty acids. Sometimes, the overexpression of enzymes in fatty acid metabolism is not effective at all in increasing the total fat ratio. ACCase is an enzyme widely acknowledged as the building block of fatty acids, and hence it is critical to the metabolism of lipids (Dunahay et al., 1996). Excessive overexpression of ACCase in diatoms and other plants was ineffective in increasing lipid synthesis. Even in the case of the diatom *Cyclotella cryptica*, a study in 1996 found that overexpressing ACCase did not affect the diatom's ability to produce fat.

2.4 LONG CHAIN POLYUNSATURATED FATTY ACIDS (PUFA)

There are several metabolic processes in which fatty acids play an essential role (Robertson et al., 2013). Polyunsaturated fatty acids (PUFAs) are important for various functions such as enzyme interactions, membrane fluidity, and lipid peroxidation (Borowitzka & Vonshak, 2017). An important role of polyunsaturated fatty acids in cell signaling and inflammatory processes has been demonstrated in many studies (Mozaffarian and Wu, 2011). Some disorders, such as coronary artery disease, cardiac arrhythmias, diabetes, hyperlipidaemia, and atherogenesis, can be exacerbated by poor dietary habits. (Surette, 2008). Supplementing LC-PUFAs to diet has been demonstrated to lower the chance of getting Alzheimer's and serious depression. Lipids, namely triacylglycerol (TAGs), are regarded as universal energy storage molecules and play an essential role in cellular metabolism (Xin et al., 2019). TAGs may be exploited as nutritional supplements or biofuels, depending on their fatty acid makeup (number of carbons, chain length, and degree of

unsaturation). Polyunsaturated fatty acids (PUFAs) are necessary components of the human diet and are crucial for the maintenance of proper growth and health (Petrie et al., 2010).

Triacylglycerol (TAG) plays an important role in cellular metabolism and membrane structure. TAGs are also known universally as energy storage molecules (Xin et al., 2019). Depending on the fatty acid in the structure of TAGs, it can also be used as a nutritional supplement, mostly as a biofuel. PUFAs (polyunsaturated fatty acids), on the other hand, contain important components of the human diet with their long chain and unsaturated carbon structure and are very important for healthy growth and protection of health (Petrie et al., 2010). (LC-PUFAs) are classified according to the carbon where the first double bond is closest to the methyl group. Omega-3 fatty acids are called "ω-3 fatty acids" or "n-3 fatty acids" because they contain a double bond three atoms away from the terminal methyl group in their chemical structure. Alpha-linolenic acid (ALA), which is found in vegetable oils, EPA, and DHA, which are both often found in marine fish oils, are three different forms of omega-3 fatty acids that are important in human physiology.

Omega-6 fatty acids are called "ω-6 fatty acids " or "n-6 fatty acids " because they contain a double bond six atoms away from the terminal methyl group in their chemical structure. Fatty acids with omega-6 bonds cannot be produced in the human body, so they are called essential (essential) fatty acids. Examples of omega-6 fatty acids are linoleic acid (18:2, n−6) and arachidonic acid (20:4, n-6). Deficiency in essential fatty acids can cause diseases, especially since they are found in the brain.

Omega-9 fatty acids are called "ω-9 fatty acids" or "n-9 fatty acids" because they contain a double bond nine atoms away from the terminal methyl group in their chemical structure. The two most well-known types of omega 9, which are mostly found in products such as olive oil, are oleic acid and meadow acid. Unlike omega-3 and omega-6, it does not show essential fatty acid properties.

2.4.1 Omega-3

Alpha-linolenic acid (ALA), Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are omega-3 polyunsaturated fatty acids known to have beneficial effects on human health and nutrition. The omega-3 fatty acids important in the human diet are alpha-linolenic acid (18:3, ALA), (20:5, EPA eicosapentaenoic acid), and (22:6, DHA docosahexaenoic acid). Polyunsaturated fatty acids, which have beneficial effects on human health and nutrition, have

been the subject of study for many years. It guards against high blood pressure, lowers triglyceride levels, raises HDL, or "good cholesterol," which helps lower the risk of heart attack and stroke, and preserves cardiovascular health. It has an anti-inflammatory effect and has potential effects in the control of cell proliferation and the fight against tumors. It is known that it contributes to the reduction of symptoms of some neurological and psychiatric diseases and to the prevention of age-related vision loss. Omega-3 is also very important for the brain development, the development of the central nervous system, and the bodily development of babies in the womb. (Green et al., 2008; Chin et al., 1992; Mattson & Grundy, 1985).

Some studies have shown that DHA and EPA, two important omega-3 PUFAs, have antimicrobial activity (Sun et al., 2016). The antibacterial effects of EPA and DHA that appear to target the membrane of the bacterial cell include disruption of the electron transport chain and decoupling of oxygen-dependent phosphorylation, enzyme inhibition, cell necrosis, depletion of nutritional intake, and autooxidation (Sun et al., 2016; Desbois & Smith, 2010). Due to the amphipathic nature of EPA and DHA, they follow an action path that can make contact with the cell membrane and cause holes in the membrane. Both gram-negative and gram-positive types of bacteria are inhibited by EPA and DHA, while gram-positive bacteria are more successfully neutralized by EPA and DHA. (Desbois & Smith, 2010; J. Sun & Song, 2011).

2.4.1.1 Eicosapentaenoic acid (EPA)

Eicosapentaenoic acid is an omega-3 fatty acid that shown as 20:5 because it has twenty carbon chains and five cis double bonds in its chemical structure (Fig 2.8). EPA, an essential omega-3 fatty acid, is critical for the circulatory system in animals because it inhibits platelet aggregation (Parka & Harris, 2002). Additionally, the availability of the essential EPA dosage throughout the human body aids in inflammatory management and improves the immune system (Calder, 2013). Additionally, EPA is critical in preventing heart disease and reducing chronic and inflammatory processes (Swanson et al., 2012). DHA and EPA also play a significant role in the body's defensive system by lowering inflammation and eliminating reactive oxygen species at the cellular level. Since our body cannot produce enough EPA and DHA according to its own needs, it should be supplemented with nutrients in appropriate proportions. (Nichols et al., 2010).

2.4.1.2 Docosahexaenoic acid (DHA)

An omega-3 fatty acid called docosahexaenoic acid serves as the main structural element of the human brain, skin, cerebral cortex, and retina. Due to its twenty-two carbon chains and six cis double bonds in its chemical structure, it is represented as 22:6 (Figure 2.8). Human diseases like cancer, Alzheimer's disease, cardiovascular disease, and schizophrenia can all be prevented with DHA (Lee & Lip, 2003).

Animals, like humans, receive omega-3 fatty acids from dietary sources (Certik & Shimizu, 1999). Coldwater marine fish are the best-known commercial source of these fatty acids worldwide. Due to its high DHA content, its commercial value has increased during the last several decades. However, it is known that animals (fish) lack the enzymes required to produce DHA, which should be supplemented externally. In contrast, microalgae are the primary natural producers of omega-3 and omega-6 fatty acids (Nichols et al., 2014). Some microalgae species living in the aquatic environment are rich in DHA. *Schizochytrium* sp. microalgae have become more attractive as a dietary supplement due to their high DHA content. (Apt & Behrens, 1999). By directly ingesting microalgae and/or indirectly via other tiny fish and zooplanktons that consume microalgae, most fish may retain significant levels of DHA and EPA (Peltomaa et al., 2018; Nichols et al., 2014).

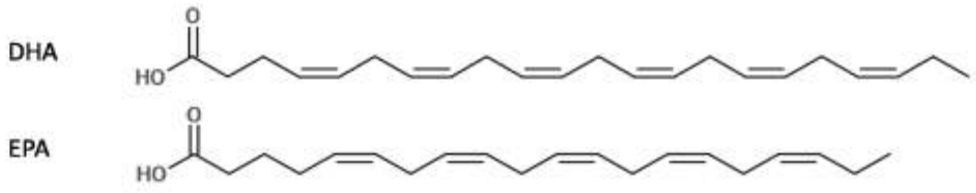

Figure 2.8: Molecular structures of DHA and EPA fatty acids

2.5 ANTIMICROBIAL ACTIVITY OF MICROALGAE

Although microalgae have been used for medicinal purposes for a long time, systematic screening for biologically active components began in the mid-1900s. However, microalgae have been the subject of intensive study aimed at discovering new chemicals that may lead to therapeutically effective medicines in the last decade (Mendes et al., 2003; Cardozo et al., 2007). Microalgae contain many extracts and/or extracellular products with proven antibacterial, antiprotozoal,

antifungal, and antiplasmodial properties (Kellam & Walker, 1989; Ghasemi et al., 2004). The antibacterial effect of microalgae has been associated with many chemical classes such as terpenes, phenolics, and amines. An organic extract of *Anabaena virabilis*, a blue-green algae species, obtained with hexane and methanol has been shown to be effective on vancomycin-resistant *S. aureus* strains (Mayer & Hamann, 2005; Cardozo et al., 2007). The bioactive components of marine algae species related to different divisions, such as Chlorophyta and Rhodophyta, were extracted using solvents such as acetone, ethanol, and methanol-toluene. The antibacterial and antifungal activities of the related extracts were evaluated. Studies in Rhodophyta spp. species such as *Laurencia okamurai, Grateloupia, and Plocamium telfairiae* have shown that these species have a broad spectrum of antibacterial activity. Some strains were found to show very strong antimicrobial activity against test organisms. Among these test organisms, it was found to be particularly sensitive to extracts of *Pseudomonas solancearum,* and *Penicilium citrinum* marine algae, and Rhodophyta spp. In these results, it was defined that marine algae and Rhodophyta spp. extracts have considerable potential for application in the creation of novel medications (Yi et al., 2001).

Another study examined the antibacterial activity of six different microalgae extracts of Chlorophyta and Rhodophyta using several solvents, such as ethanol, hexane, and chloroform. According to the findings, *Amansia multifida* had significant antibacterial activity against gram-negative pathogens including *Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae., Salmonella choleraesuis*, and *E. aerogenes* and gram-positive bacteria such as *Bacillus subtilis* and *S. aureus* (Lima-Filho et al., 2002). Similar findings were discovered by Bhagavathy et al. (2011) who assessed the presence of the phytochemicals and antibacterial properties of *Chlorococcum humicola*. The screening of *C. humicola* extracts revealed antibacterial efficacy against pathogenic strains including *K. pneumoniae, B. subtilis, P. aeruginosa, V. cholerae, Salmonella typhi, S. aureus, Aspergillus flavus, Candida albicans, Aspergillus niger,* and *E. coli*.

Interestingly, several studies indicate that algae antimicrobial compounds undergo seasonal and growth-related changes. For example, Hornsey and Hide (Bourgougnon, 2011) showed that seasonal fluctuations in antibacterial content in examined algae occurred in four major patterns:

a. The *Polysiphonia* kind, which produces antibiotics continuously throughout the year.

b. *Laurencia Pinnata, Chondrus crispus,* and *U. lactuca* also include the *Laminaria* type, which has the highest antibiotic production, especially in winter.

c. The *Dictyota* type has an activity peak mostly in the summer and is found in *Ascophyllum nodosum* and *Dilsea carnosa.*

d. The *Codium* type, characterized by a springtime activity peak, as shown by *Halidrys siliquosa*.

Today, new generation techniques such as environmentally friendly new extraction methods and modern analytical devices are used to screen new products and determine the biological activity of microorganism extracts. By using new generation devices such as GC-MS and HPLC-DAD, antioxidant and antimicrobial compounds that may show antimicrobial activity against pathogenic species such as *E. coli, S. aureus,* and *C. albicans* are being screened (Plaza et al., 2010).

2.5.1 Antibacterial Activity

The resistance of harmful bacteria to antibacterial drugs has increased significantly over the past decade. Therefore, new antimicrobial treatment options for diseases caused by resistant microorganisms have gained considerable interest in addition to our understanding of the process of antimicrobial resistance. Selected compounds from various microalgae and their antibacterial properties are shown in Table 2.1.

Table 2.1: Antibacterial properties of compounds from microalgae (Amaro et al., 2011).

Microalga	**Active compound**	**Target microorganism**
Phaeodactylum tricornutum	Eicosapentaenoic acid	MRSA, *Listonella anguillarum, Lactococcus garvieae, Vibrio spp.*
Haematococcus pluvialis	Short chain fatty acids	-
	Short-chain fatty acids (butanoic acid and methyl lactate)	*Escherichia coli, Staphylococcus aureus*
Skeletonema costatum	Unsaturated, saturated long chain fatty acids	*Vibrio spp.*
Euglena viridis	Organic extracts	*Pseudomonas, Aeromonas, Edwardsiella, Vibrio, E. coli*
S. costatum	Extra-metabolites	*Listeria monocytogenes*
Staurastrum gracile	Methanolic extracts	-
Pleurastrum terrestre		
Dictyosphaerium pulchellum		
Klebsormidium crenulatum		
Chlorococcum sp.	Aqueous extract	-
Chlorococcum HS-101	α-Linolenic acid	-
Chlorokybus atmophyticus	Acetone extract	-
Chlamydomonas reinhardtii	Methanolic and hexanolic extracts	*S. aureus, Staphylococcus epidermidis, Bacillus subtilis, E. coli, Salmonella typhi*
Chlorella vulgaris		

Antibiotic resistance in bacteria has been the focus of research for the following reasons: (i) The majority of community-acquired and hospital-acquired illnesses are caused by bacteria. (ii) An increase in the number and dose of antibacterial agents provides a wider variety of resistance. (iii)

The capacity to transfer bacterial resistance makes it possible to delve deeper into the processes underlying resistance formation (Amaro et al., 2011).

Meanwhile, research aimed at discovering the active principles of microalgal antibacterials has gained popularity. The first antibacterial chemical, chlorellin, was discovered in *Chlorella* microalgae and was found to be responsible for its inhibitory effect against Gr+ and Gr- bacteria (Pratt et al., 2022). Eicosapentaenoic acid, a compound produced by *P. tricornutum* diatoms, has been shown to be responsible for its antimicrobial activity against Gr - and Gr+ bacteria (including MRSA). However, two different antibacterial properties of free fatty acids (C16:1 n-7; palmitoleic acid) and hexadecatrienoic acid (HTA; C16:3 n-4) produced by *P. tricornutum* were also discovered. It has been shown to inhibit Gram-positive bacteria with HTA as well as the Gram-negative marine pathogen *Listonella anguillarum.*(Desbois et al., 2008). While free fatty acids can act on different cellular targets, cell membranes are the most plausible in terms of antibacterial activity. Because damage to the cell membrane will likely lead to disruption of cell integrity and reduced nutrient absorption in addition to limiting cellular respiration. For example, chemicals produced by *Scenedesmus costatum* and partly isolated from its organic extract displayed antibacterial action against aquaculture bacteria due to the presence of fatty acids with chain lengths greater than 10 carbon atoms, which trigger the lysis of bacterial protoplasts (Desbois et al., 2009). It has been known for some time that fatty acids have the potential to inhibit bacterial growth and survival, but new structure-function connection studies reveal that this ability depends on both the degree of unsaturation and the length of the chain (Benkendorff et al., 2005).

Among microalgal-derived oxylipins, polyunsaturated aldehydes stand out for their antibacterial properties. These chemicals are produced by some diatoms, including *Thalassiosira rotula* and S. *costatum*. There are studies in which (C20:4 n-3) arachidonic acid shows effective activity against significant human pathogens, such as *Haemophilus influenza*. In this study, the MIC values for MRSA and *H. influenza* were found to be 1.9 µg/mL and 7.8, respectively. It also showed that it impaired the growth of gram-negative bacteria such as *L. anguillarum, Photobacterium phosphoreum, Aeromonas hydrophila,* and *Alteromonas haloplankti*, and gram-positive marine bacteria such as *Micrococcus luteus* and *Planococcus citreus* (Smith et al., 2010).

2.5.2 Antiviral Activity

In recent years, various infectious disorders caused by several viruses have developed (and re-emerged). Despite the fact that a number of antiviral medications have been created, drug-resistant mutations are continually occurring, necessitating the development of novel antiviral active principles. As a result, microalgae have attracted considerable interest as possible sources of antiviral medicines. Selected compounds from various microalgae and their antiviral properties are shown in Table 2.2.

Table 2.2: Antiviral properties of compounds from microalgae (Amaro et al.,2011).

Microalga	**Active compound**	**Mechanism of action**	**Target virus**
Navicula directa	Polysaccharide	Inhibition of hyaluronidase	HSV1 & 2, Influenza A virus
Gyrodinium impudicum	p-KG03 exopolysaccharide	Inhibition (or slowing down) of cytopathic effect	Encephalomyocarditis virus
Dunaliella primolecta	Pheophorbide α-, β-like compounds	Inhibition of cytopathic effect	HSV1
Chlorella autotrophica	Sulfated polysaccharides	Replication inhibition *C. autotrophica*: 47.4-67.4 % *Ellipsoidon* sp.: up to 44 %	VHSV, ASFV
Ellipsoidon sp.			
Cryptomonads	Allophycocyanin	Inhibition of cytopathic effect, delay in synthesis of viral RNA	Enterovirus 71
Cochlodinium polykrikoide	Extracellular sulfated polysaccharides	Inhibition of cytopathic effect 1	Influenza virus A & B, RSV A & B, HSV-1

Antiviral activity may occur during a single or many phases of viral growth. Phase I is concerned with cell adsorption and invasion; Phase II, or the eclipse phase, is distinguished by the cell's compulsion to produce numerous copies of the virus; and Phase III, which is concerned with viral

particle maturation and release. Each antiviral compound can act at different stages. For example, the anti-HSV effect of an antiviral agent, acyclovir, occurs in phase II, while the anti-HSV component produced from *Dunaliella sp* inhibits viral function in phase I (Elion et al., 1977). It has been suggested that sulfated exopolysaccharides from marine microalgae interact with phase I of some enveloped viruses (Batinić & Robey, 1992). They provide a competitive advantage against viruses such as HSV and HIV due to their wide antiviral range (Veettil et al., 2022). Apparently, their inhibitory effect is due to their interaction with positive charges on the virus surface, preventing the virus from invading host cells (Ehresmann et al., 1979). The administration of such compounds appears to be an especially promising antiviral therapeutic option because their pleiotropic mode of action makes the development of resistance mutants less likely than with other antiviral drugs that demonstrate just a single target throughout the viral life cycle. Analyses of antiviral substances produced by *D. primolecta* were performed by NMR and MS. As a result of the study, the pheophorbide-like compounds that had the proton at position 21 substituted by a hydroxyl group that may account for their exceptional antiviral activity were detected (Ohta et al., 1998).

2.5.3 Antifungal Activity

Until the last few years, studies on the development of resistance to antifungal agents had not received as much attention as the study of antibacterial resistance. (Anaissie at el., 1989; Wey at el., 1988). This is because fungi are not seen as being just as pathogenic as bacteria. While the death rate from fungal infections was stable in the mid-1900s, it has increased in recent years. During this rise in fungal infections, researchers have begun to look for new and effective antifungal drugs to fight the growing number of fungal infections. Selected compounds from various microalgae and their antifungal properties are shown in Table 2.3.

Table 2.3: Antifungal properties of compounds from microalgae (Amaro et al.,2011)

Microalga	**Active compound**	**Target microorganism**
Chlamydomonas reinhardtii	Methanolic extracts	*Candida kefir, Aspergillus niger, Aspergillus fumigatus*
Chlorella vulgaris		
Oocystis sp.		
Scenedesmus obliquus		
Amphidinium sp.	Karatungiols	*A. niger, Trichomonas foetus*
Goniodoma pseudogoniaulax	Goniodomin A	-
Gambierdiscus toxicus	Polyether compounds (gambieric acids A and B)	-
Prorocentrum lima	Polyether compounds	-
Dinophysis fortii		-
Haematococcus pluvialis	Butanoic acid and methyl lactate	*Candida albicans*
Chlorella pyrenoidosa, Scenedesmus quadricauda	-	*A. niger, Aspergillus flavus, Penicillium herquei, Fusarium moniliforme, Helminthosporium sp., Alternaria brassicae, Saccharomyces cerevisiae, C. albicans*

Katırcıoğlu et al. (Katırcıoğlu et al., 2006) studied the antifungal properties of ten microalgae strains isolated from different fresh water sources in Turkey against *S. cerevisiae, Candida tropicalis, and C. albicans*. According to the results they obtained, *Oscillatoria* sp. and *Chlorococcus sp.* strains showed the best antifungal activity.

In another study by Santoyo et al. (Santoyo et al., 2009), extracts of *H. pluvialis* obtained with pressurized liquid and ethanol were tested on *C. albicans.* It has been suggested that two compounds with antifungal effects are butanoic acid and methyl lactate.

Abedin and Taha (Abedin & Taha, 2008) investigated the antifungal activities of *S. quadricauda* and *C. pyrenoidosa* microalgae extracts on eight different fungi. According to their results, all the extracts showed positive effects on almost all fungi. The results obtained from this study are in line with similar studies done before.

2.6 ALGAL FATTY ACIDS AS ANTIMICROBIAL COMPOUND

Free fatty acids are the lipids in human skin that are thought to be the most active. The most important of these fatty acids are lauric acid (C12:0), palmitic acid (C16:0), myristic acid (C14:0), and C18:1n-10 (cis-8-24 octadecenoic acid) (Stabili et al., 2012). Algae species such as *Haptophyta* and *Cryptomonas* are known to contain significant amounts of polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SFAs). These fatty acids are important for human health and also because they cannot be produced by humans and must be taken from outside (Ryckebosch et al., 2014). A list of bioactive molecules isolated from different algal species is summarized in Table 2.4.

Table 2.4: A list of bioactive molecules isolated from different algal species (Parsaeimehr et al.,2016).

Bioactive Molecule	**Algal Source**	**Mode of Action**	**Target**
3-Formylindole	*Botryocladia leptopoda*	Antiviral and anticancer activity	*Vaccinia* virus
Polyhydroxylated	*Fucus vesiculosus*	Bactericidal	Gram-positive and Gram-negative bacteria
Fucophlorethol	*Ascophyllum nodosum*	Antitumor activity	HT-29 and HCT-116 human colon cancer
Fucoidan	*Symphyocladia latiuscula*	Bactericidal	*Staphylococcus aureus, Staphylococcus epidermidis,* and *Pseudomonas aeruginosa*

Table 2.4 continued: A list of bioactive molecules isolated from different algal species (Parsaeimehr et al.,2016).

Bioactive Molecule	**Algal Source**	**Mode of Action**	**Target**
Sulfated galactans	*Botryocladia occidentalis*	Immunostimulatory activity, antiviral and anticoagulant activity	Herpes simplex virus, human metapneumovirus
Phycarine	*Laminaria digitata*	Antitumor activity	Stimulator of both hormonal and cellular immunity, and stimulator of phagocytosis by peripheral blood cells
Loliolide	*Undaria pinnatifida*	Antioxidant, bactericidal, and antitumor activity	*Bacillus subtilis, Escherichia coli, S. aureus,* and *Neisseria gonorrhoeae*
Bisebromoamide	*Lyngbya sp.*	Anticancer activity	Renal cell carcinoma
p-KG03 exopolysaccharide	*Gyrodinium impudicum*	Antifungal HSV-1 Antiprotozoan Antiviral	HSV-1, *Aspergillus niger, Trichomonas foetus Encephalomyocarditis virus*
Allophycocyanin	*Spirulina platensis*	Antiviral	*Enterovirus* 71
Calothrixin-A	*Calothrix sp.*	Antimalarial and anticancerous	Human myeloid leukemia K562 cells
Sulfoquinovosyldiacylglycerol, KM043	*Gigartina tenella*	Antibacterial	Inhibitor of eukaryotic DNA polymerases and HIV reverse transcriptase type 1
Deschloroelatol	*Laurencia rigida*	Antifungal	*Mycotypha microspora*

Many microalgae species have palmitic acid in their fatty acid composition, which is saturated fatty acids and has demonstrated antiviral effects. Santoyo et al. (Santoyo et al., 2012) reported that palmitic acid isolated from *Haematococcus pluvialis* and *Dunaliella salina* showed anti-HSV-1 activity.

The *Polysiphonia virgata's* extract which was obtained by dichloromethane, showed antimicrobial activity against *Mycobacterium tuberculosis* and *M. smegmatis.* The results obtained show that the fatty acids isolated from *Polysiphonia virgata,* especially oleic acid, lauric acid, linoleic acid, and myristic acid, have antimicrobial properties (Saravanakumar et al., 2008).

The antimicrobial activity of *Dunialella salina*, which is a halophilic green microalga and widely grown commercially for the production of β-carotene, on microorganisms such as *E. coli*, *C. albicans, S. aureus* was investigated. When *Dunaliella salina* extracts were tested with the GC-MS device, 15 different antimicrobial compounds were reported, including β-cyclocitral, phytol, fatty acid, and β-ionone (Herrero et al., 2006).

In another study, the antimicrobial activity of extracts of *Dunaliella primolekta* and *Chlorococcum* microalgae using methanol was tested on methicillin-resistant *S. aureus*. As a result, it was determined that the extracts showed antimicrobial activity against the resistant organism. In addition, it was observed that the antibiotic production of *Dunaliella primolekta* and *Chlorococcum* increased 1.8-2.3 times in the developed BG-11 medium (Ohta et al., 1995).

Rodríguez-Meizoso et al. (2010) studied the antimicrobial activity of *Haematococcus pluvialis*, a freshwater microalga that produces high levels of astaxanthin. They analyzed the extracts of *Haematococcus pluvialis* using analytical techniques such as HPLC-DAD, HPLC-MS, and GC. According to the results they obtained, it was determined that short-chain fatty acids were responsible for the antimicrobial activity.

When the studies are examined, it is possible to say that the hydroxyl groups of fatty acids have antimicrobial properties. Because it has been determined that antimicrobial properties of methylated fatty acids that do not contain hydroxyl groups are very low or none at all. Studies have shown that the antimicrobial activities of medium and long chain unsaturated fatty acids are higher than those of saturated fatty acids of the same length (Desbois et al., 2008). In general, there is a very important correlation between the number of the double bonds in the carbon chains of

unsaturated fatty acids and the antibacterial activity of fatty acids (Feldlaufe et al., 1993). On the other hand, it is known that saturated fatty acids with a length of 10-12 carbons also show very strong antimicrobial activity (Wille et al., 2003).

When the data obtained from the studies are examined, it has been revealed that fatty acids show antimicrobial activity behaviour on microorganisms in many different ways, such as disruption of the electron transport chain, cell lysis, peroxidation and autoxidation, and deterioration of food intake (Desbois et al., 2010).

2.7 ANTIBIOFILM ACTIVITY

The term "biofilm" refers to the microbial population that is adherent to abiotic or biotic surfaces. The process of forming a biofilm involves several steps, including the "first adhesion" stage, in which bacteria adhere to a surface, the "micro-colony formation" stage, in which bacteria divide to produce extracellular polymeric molecules (EPS) that position the cells and increase adhesion, the "biofilm maturation" stage, in which the EPS matrix forms a three-dimensional structure in the form of a multifunctional and protective skeleton and the "splitting" (also called dispersal) stages, in which bacteria can break away from the biofilm and recolonize new areas. There are two strategies for dealing with biofilms: preventing biofilm formation or eliminating the growing biofilm. Biofilm-forming bacteria differ from planktonic bacteria in that they have immune responses and extreme tolerance to antimicrobial therapy. Therefore, biofilm formation requires higher doses of antibiotics and longer duration of therapy in the treatment of chronic infections (Wannigama et al., 2020). The genetic variety of organisms that create biofilms demonstrates that biofilms are a primitive form of microbial human life (Uzundağ, 2020). Van Leeuwenhoek made the first discovery of biofilm on the tooth surface (Between & Concentration, 1940). Biofilms are believed to be a collection of adherent microorganisms (Hall-Stoodley & Stoodley, 2009). Bacterial cells are protected by biofilms from severe environmental conditions, and as a result, they are particularly resistant to antibiotics.

In industrial, ecological, and medicinal environments, biofilms are exceedingly dangerous. For instance, biofilms on the surface of medical equipment such as implants or catheters commonly result in infections that are difficult to cure. On the other hand, infections have been linked to the production of biofilms on the surfaces of the human urinary system, skin, and teeth (Romeo, 2008).

Dental plaque biofilms contain more than one species, and the disease-causing capacity of the community is often affected by the structure of the biofilm. (Kreth et al., 2008). *E. coli* has been warned that it is capable of forming biofilms in the human gut, posing a substantial danger to human health (Van Houdt & Michiels, 2005).

While the antimicrobial properties of many compounds produced by microalgae and blue-green algae have been examined, very few of them have been examined in terms of their antibiofilm properties. Selected compounds from various microalgae and their antibiofilm properties are shown in Table 2.5.

Table 2.5: Selected compounds from various microalgae and their antibiofilm properties (Nag et al.,2022)

Name of the active compound/extract	**Algae**	**Activity**
Galactoglycerolipids	*Sargassum muticum*	Exhibits antibiofilm activity
Mahorone, 5-bromomahorone	*Asparagopsis taxiformis*	Helps in the eradication of biofilm
1,1,3,3-Tetrabromo-2-heptanone	*Bonnemaisonia hamifera*	Helps in the eradication of biofilm
Linear alkadienes	*Botryococcus braunii*	Helps in the eradication of biofilm and are also responsible for the process of phycoremediation
Neophtadiene, topcopherol, palmitoleic acid, phycocyanin	*Spirulina platensis*	Acts as potent antimicrobial and antibiofilm agent
Astaxanthin	*Chlorella zofingiensis*	Has effective antibiofilm properties
Palmitoleic acid C-phycocyanin, oleic acid, PUFAs (n-3) fatty acids, phytol, phenolic acids, neophytadiene linolenic acid	*Spirulina platensis*	Helps in the eradication of biofilm

Methanol and ethanol extracts	*Ulva fasciata*	Helps in the eradication of biofilm
Chromanols	*Sargassum horneri*	Has effective antibiofilm properties
15,16-epoxythyrsiferol B; 15,16-epoxythyrsiferol A; 28-hydroxysaiyacenol B saiyacenol C	*Laurencia viridis*	Helps in the eradication of biofilm
Proline, fucoxanthin, dimethyl sulphopropionate	*Fucus vesiculosus*	Helps in the eradication of biofilm

It was determined that the extracts obtained from approximately 225 different microalgae and cyanobacteria species showed antibiofilm activity. Among these extracts, it is known that the components showing the most antibiofilm activity are polyunsaturated fatty acids (Nag et al.,2022). It has been determined that halogenated furanones produced by seaweeds such as *D. pulchra* have an antibiofilm effect on both gram-positive and gram-negative bacterial species (Vairappan, 2003). There are studies showing that halogenated furanones also inhibit the biofilms formed by *Escherichia coli and P. aeruginosa*. The mechanism of inhibition occurs when furanones inhibit *P. aeruginosa* elastase B enzyme production. However, sulfur and brominated furanones were also found to inhibit biofilm formation (Eom et al., 2012).

Cepas et al. (2021) investigated the antibiofilm activities of microalgae and cyanobacteria by analytical methods such as HPLC and NMR. As a result, it has been determined that lipids obtained from cyanobacteria and microalgae show antibiofilm properties against pathogens that may cause serious infection. These lipids were thought to be an alternative source for treatments. Another study examined the antibiofilm effects of lutein from *Chlorella pyrenoidosa*, especially on resistant *P. aeruginosa*. It has been determined that even very low concentrations of lytein extract show biofilm inhibition properties. It is also thought that this may be an antiquated sensing feature. For this reason, lutein obtained from *Chlorella pyrenoidosa* can be used in the treatment of diseases such as diabetic foot ulcers, which may pose a danger due to the high biofilm formation capacity of *P. aeruginosa* (Sampathkumar et al., 2019).

3. METHODOLOGY

3.1 MICROORGANISM AND MEDIUMS

Schizochytrium sp. S31 (ATCC 20888) was used in the current investigation. Experiments on microalgal growth were conducted in a complex medium containing 15 g/l glucose and 8 g/l protease peptone, 5 g/l yeast extract, 20 g/l NaCl, and 18 g/l MOPS. On an analytical scale, all medium components were weighed, and 1 liter of distilled water was added. A homogeneous mixture was made by stirring with a magnetic stirrer. 1 ml of NaOH was added to achieve a pH of 7. The prepared broth was autoclaved for 20 minutes at 121°C.

3.2 GROWTH EXPERIMENTS

The experiments were carried out in a 250 ml flask with complex media for 48 hours at 27 °C and 200 rpm orbital shaker incubator. 500 µl of samples were taken at regular intervals under sterile conditions in a laminar flow cabinet. Using a spectrophotometer, the optical density (OD600) of 1/10 diluted samples is determined.

Spectrophotometric analysis

a. The spectrophotometer must be prepared for use by setting and calibrating the instrument.

b. Input a value of 600 nm for the wavelength.

c. Resuspend the microalgal culture and take 500 µl of sample under sterile conditions. Dilute the sample 1:10 with distilled water.

d. Read and record the absorbance of the sample at 600 nm.

3.3 DOWNSTREAM PROCESS

3.3.1 Harvesting Microalgal Culture

A microalgae culture of *Schizochytrium* sp. S31 was harvested by centrifuging at 5000 rpm for 15 minutes. After 15 minutes of centrifugation at 5000 rpm, the supernatant was discarded carefully. The cell pellet was rinsed twice with deionized water to eliminate residual medium. The lipase

enzyme, which breaks down complex lipids, was inactivated by treating the pellet in a water bath at 65°C for an hour.

3.3.2 Lyophilization

For freeze-drying the samples, a Teknosem TRS-2 stainless steel lyophilizer with a condenser temperature of -65 °C and a vacuum capacity of 2.5 m^3 was employed. A harvested and cleaned microalgal cell pellet was dried at -55°C under vacuum for 70 hours.

Lyophilization Protocol:

a. Spread the cell pellet on the lyophilizer tray and incubate it at -80 °C for 90 min.
b. Freeze samples at -65 °C for 2 hours.
c. Vacuum is applied under 2,5 m^3 pressure. At this stage, most of the water in the sample sublimes with the heat.
d. Air is gently introduced into the chamber to reduce pressure before opening the lid.
e. Dried cell biomass is removed from the tray and weighted to determine CDW.

3.3.3 Cell Disruption

Different physical and chemical techniques of cell lysis were investigated to enhance the total lipid content. Two different cell lysis techniques—manual grinding and ultrasonication—were used, and the outcomes were contrasted.

3.3.3.1 Mortar and pestle

Usually, manual grinding is employed to destroy plant cells. For degradation, cell pellets were frozen at -80 °C overnight. After cooling the mortar and pestle at -20 °C for one hour, the frozen sample is pulverized using a cool mortar and pestle.

3.3.3.2 Sonication

The samples were sonicated in the extracting solvent at 50W for 15 minutes with several brief bursts (on/off every 30 seconds) by using ultrasonic homogenizer model3000. During sonication, the mixture of biomass and extraction solvent is always kept on ice to avoid overheating.

3.4 DRY BIOMASS ANALYSIS

The determination of total fat and oil (g/100g), nitrogen (protein) quantity (g/100g), energy (kcal/100g and kj/100g), carbohydrate (g/100g), moisture and/or volatile substance (g/100g), and total ash (g/100g) of the dry biomass were performed by Eurofins Izmir Food Control Laboratories, Republic of the Turkey Ministry of Agriculture and Forestry, according to Turkish Food Codex food labeling and consumer information regulation.

3.5 LIPID EXTRACTION

For the extraction of lipid from *Schizochytrium* sp. S31, solvent-based lipid extraction, which is the usual approach was applied. For lipid extraction, different solvents or solvent mixtures were used such as hexane only, ethanol only, hexane/ethanol (2:1), methanol only, and hexane/methanol (2:1). A mixture of 5 g of dried biomass and 100 ml of each solvent or solvent combination was incubated for 6 hours at room temperature and 225 rpm.

3.6 LIPID CHARACTERIZATION

3.6.1 Total Lipid Analysis

The combination of biomass and solvent was placed in a falcon tube and centrifuged to achieve a pure supernatant. Using a rotary evaporator, solvent was removed from a supernatant containing a solvent and lipid mixture. Solvent was evaporated at 95°C for 30 min. The amount of lipid was determined by subtracting the weight of the bottle before and after evaporation. The amount and proportion of lipid recovered were estimated as follows:

$$\boldsymbol{Mass\ of\ lipid} = (weight\ of\ the\ flask + extracted\ oil) - (weight\ of\ the\ flask)$$

$$\boldsymbol{Lipid\ content}\ (\%) = \frac{mass\ of\ lipid\ extracted(g)}{sample\ weight(g)} \times 100$$

3.6.2 Fatty Acid Analysis

Fatty acid methyl ester (FAME) analysis was performed to analyze the samples by gas chromatography and ultimately determine the total fatty acid composition. The total lipid sample was incubated in 5 ml of 0.5N methanolic NaOH for 10 minutes at 100°C. The mixture was then mixed with 5 ml of BF3-methanol for methylation. The extraction, evaporation, and addition of 5 ml of n-heptane, NaCl, and water resulted in the production of FAME in the top phase.

3.6.3 Gas Chromatography Analysis

FAMEs samples were transferred to the bottle containing Na_2SO_4/heptane. Gas chromatography analyses were performed by Eurofins Izmir Food Control Laboratories, Republic of Turkey Ministry of Agriculture and Forestry, according to these standards.

Conditions for the GC (Agilent Technologies 6890 N) analysis were as follows: Oven temperature was maintained at 140°C for 5 minutes then the temperature was increased to 240°C at a rate of 4°C/min. Temperatures for the detector (FID) and inlet were set at 240 to 280°C, respectively.

3.7 ANTIBACTERIAL ACTIVITY ASSAY

Overnight cultures of *Pseudomonas aeruginosa* 27853, *Escherichia coli* 35218, *Streptococcus* mutans, or *Klebsiella pneumonia*e 700603 (10^6 CFU/ml) were incubated with *Schizochytrium* sp. S31 oil sample or uninoculated TSB medium in 96-well flat-bottom sterile plates (Nest Scientific, Maryland, USA) at 37 °C for 8 or 24 hours without shaking. A multiwell plate reader (Multiskan FC, USA) was used to measure the rate of bacterial growth at a wavelength of 600 nm. The optical density of the uninoculated bacterial growth medium, and medium containing a *Schizochytrium* sp. S31 oil sample at 600 nm were used as blank. For the plate assays, *P. aeruginosa*, *Escherichia coli* 35218, *Streptococcus* mutans, or *K. pneumonia* was grown in *Schizochytrium* sp. S31 oil samples (or in control growth medium; TSB or medium containing *Schizochytrium* sp. S31 oil sample) in 96-well plates then, sample were serially diluted by using TSB medium. 3 µl of the diluted samples were taken and pipetted into agar plates and incubated at 37°C overnight. The spots were visualized using the G-BOX imaging system.

3.8 BIOFILM FORMATION ASSAY

The biofilm formed on the 96-well plate was evaluated with the crystal violet (CV) staining protocol, as described previously (Merritt et al. 2011; Tunçer & Karacam, 2020). *Schizochytrium sp.* S31 oil sample was inoculated with *P. aeruginosa* and *Escherichia coli* 35218 (10^6 CFU/ml) in 96-well f-bottom polystyrene plates (Nest Scientific). The plate samples were incubated for 24 hours at 37°C without shaking, then all the medium in the well was removed and washed three times to get rid of any remaining planktonic bacteria. For the fixation process, 150 µl of methanol were added to each well, and incubated at room temperature for 15 minutes. After methanol was removed, the plates were dried by air. The plates were incubated at room temperature for 15 minutes after being stained with 130 µl of a 0.1% crystal violet. The stain were removed and the plates were washed three times to remove any CV residues. 130 µl of 30% acetic acid was added to each well of plates for solubilization of the CV. Optical density at 550 nm was measured using a multi-well plate reader. The uninoculated culture was used as a control.

4. RESULTS

4.1 GROWTH EXPERIMENTS

Schizochytrium sp. S31, a stock culture, was stored at -80 °C with a mixture of cryoprotectant consisting of 5% DMSO and 5% glycerol. The viability checks of the stocks were carried out at regular intervals. Under sterile conditions, the sample taken from the stock with a loop was inoculated on a complex agar medium and incubated at 25 °C for 72 hours. Colonies were counted after the incubation. In cases of small numbers of colonies, stock cultures were re-prepared from fresh cultures. For microalgae growth curve analysis, a single colony was selected and inoculated into 50 ml of sterile complex medium in a 250 ml flask. The culture was incubated for 48 hours at 25°C at 225rpm. The growth curve of *Schizochytrium* sp. S31 was obtained by measuring the optical density of the samples taken from the culture at regular intervals at OD_{600} nm using a spectrophotometer. Figure 4.1 shows the growth curve of *Schizochytrium* sp. S31 in a complex medium.

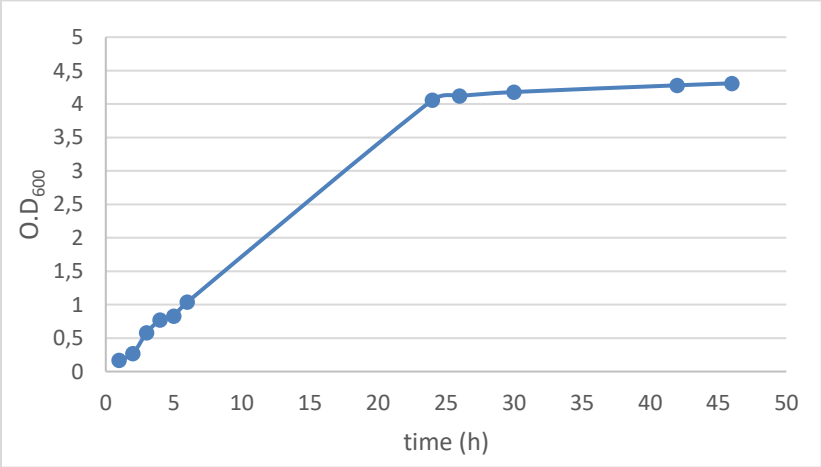

Figure 4.1: Growth curve of *Schizochytrium* sp. S31

Figure 4.1 shows that *Schizochytrium* sp. S31 grows logarithmically between 4-24 hours before entering the stationary phase at 24 hours due to the decrease in the amount of nutrients.

4.2 DRY BIOMASS CALCULATION AND ANALYSIS

Microalgae cell culture was harvested by centrifuging at 5000 rpm for 15 minutes, and the supernatant was discarded. The cell pellet was washed twice with sterile deionized water. The pellet was placed in a 65°C water bath for one hour to inactivate the lipase enzyme. The harvested and washed cell pellet was lyophilized at -55°C under vacuum for 70 hours by following the freeze-dry protocol. The lyophilized biomass was scraped off the tray and powdered using a simple homogenizer. Cell dry weight was calculated as the amount of weighed biomass per liter (g/L) (Table 4.1).

Table 4.1: Chemical characteristics of the of *Schizochytrium* sp. S31 dry biomass

Analyses	**Result**
Cell Dry Weight (g/L)	57.1
Determination of Total Fat and Oil) (g/100g)	5.31
Determination of Nitrogen (Protein) Quantity) (g/100g(Nx6,25)	44.62
Determination of Energy (kcal / 100g)	374.95
Determination of Energy (kj/100g)	1568.79
(Determination of Moisture and / or Volatile Substance, Drying loss, Moisture content) (g/100g)	7.19
(Total ash) (g/100g)	5.71

Table 4.1 shows the amount of total fat and oil (g/100g), nitrogen (protein) quantity (g/100g), energy (kcal/100g and kj/100g), carbohydrate (g/100g), moisture and / or volatile substance (g/100g) ant total ash (g/100g) of the dry biomass determined by Eurofins Izmir Food Control Laboratories, Republic of Turkey Ministry of Agriculture and Forestry. The analysis report is placed in Appendix A.1. According to the results, the weight of dry biomass with a moisture content of 7.19% was calculated as 57.1 g/l. Dry biomass is composed of 44.62% of protein and 5.31% of total fat.

Total amino acid and vitamin amounts of dry biomass were shown in Tables 4.2 and 4.3, respectively. Analysis report is placed in Appendix A.2.

Table 4.2: Total amino acid content of the dry biomass

Amino Acid	g/100g protein
Aspartic acid	10.5 ± 0.09
Threonine	5.24 ± 0.12
Serine	5.08 ± 0.07
Glutamic acid	10.7 ± 0.03
Proline	6.45 ± 0.26
Glycine	5.13 ± 0.07
Alanine	8.44 ± 0.19
Valine	6.44 ± 0.12
Methionine	1.52 ± 0.13
Isoleucine	5.01 ± 0.14
Leucine	6.84 ± 0.17
Tyrosine	5.21 ± 0.21
Phenylalanine	4.26 ± 0.04
Histidine	5.02 ± 0.10
Lysine	5.63 ± 0.08
Arginine	8.25 ± 0.26

According to the amino acid content of the proteins in dry biomass, the amino acids with the highest ratio are responsible for aspartic acid and glutamic acid, with the ratios of 10.5g/ 100g and 10.7g/ 100g, respectively.

Table 4.3: Total vitamin content of the dry biomass

Vitamin	Concentration
Iron, mg/kg	869.51 ± 38.96
Niacin, mg/kg	245 ± 16.58
Potassium, mg/kg	1360 ± 241.74
Calcium, mg/kg	100.08 ± 9.62
Lutein, mg/kg	368.65 ± 56.72
Vitamin A, IU/kg	216.41 ± 72.13
Vitamin B 1, mg/kg	4.48 ± 0.12
Vitamin B 2, mg/kg	14.26 ± 3.04
Vitamin B 6, mg/kg	9.62 ± 2.84
Vitamin C, mg/kg	74.16 ± 1.85
Vitamin B 12, µg/kg	725.17 ± 156.20

Potassium (1360 mg/kg), iron (869.51 mg/kg), vitamin B12 (725.17 mg/kg), lutein (368.65 mg/kg), niacin (245 mg/kg), vitamin A (216.41 mg/kg), and calcium (100.08 mg/kg) were found to have the highest quantities of vitamins and elements in dry biomass.

4.3 COMPARISON OF SEVERAL CELL LYSIS AND LIPID EXTRACTION TECHNIQUES

Cell lysis and lipid extraction are operations that are a part of the downstream process. For cell lysis, two distinct techniques—mortar and pestle and ultrasonication—were evaluated, and the results were then compared. Five different solvent mixtures, hexane only, ethanol only, and hexane/ethanol (2:1), methanol only, hexane/methanol (2:1), were examined for lipid extraction.

When hexane/methanol (2:1) was used among five different solvents, the best result was obtained with a 34% lipid ratio. The outcomes were averaged after each experiment was carried out three

times. Error bars show the standard deviation. Comparative results of the cell lysis and lipids were shown in Figure 4.2.

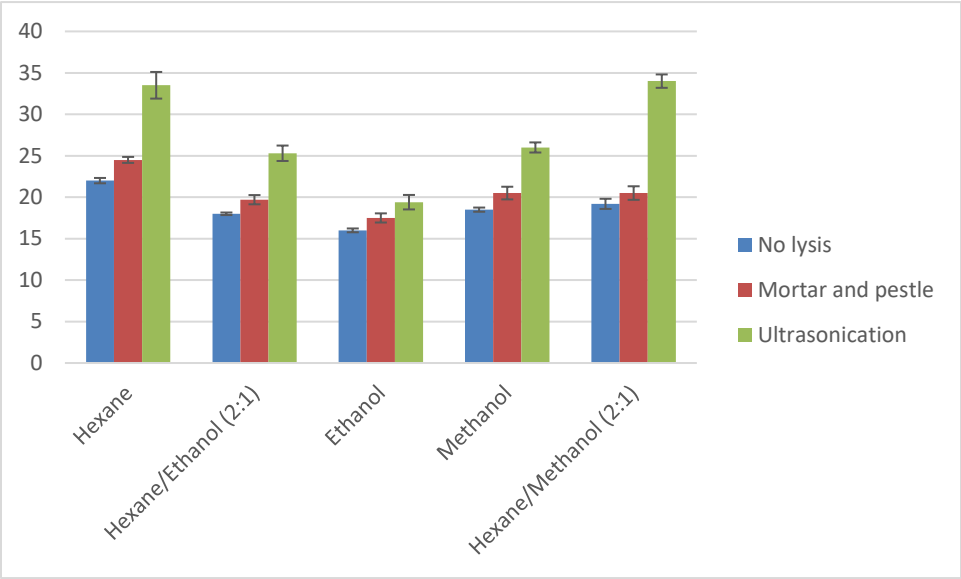

Figure 4.2: Comparative results of different cell lysis and lipid extraction methods

4.4 GAS CHROMATOGRAPHY ANALYSIS

Gas chromatography analyses were performed by Eurofins Izmir Food Control Laboratories, Republic of Turkey Ministry of Agriculture and Forestry, according to standards. The analysis report is placed in Appendix A.3. The fatty acid profile results obtained from GCMS analysis were shown in Table 4.4.

Table 4.4: Fatty acid composition of *Schizochytrium* sp. S31

Analyses	Result
Arachidic Acid (C20:0)	0.41
Arachidonic Acid (C20:4)	1.07
Behenic acid(C22:0)	0.25
Eicosenoic Acid (C20: 1)	1.15
Eicosatrienoic Acid (C20: 3 (n3)	0.61
Eicosatrienoic Acid (C20: 3(n6)	0
Eicosadienoic Acid (C20:2)	0.17
Docosahexaenoic Acid (DHA) (C22: 6)	10.47
Eicosapentaenoic Acid (C22:5)	1.84
Eicosapentaenoic Acid (EPA) (C20:5)	16.11
Gamma Linolenic Acid (C18:3 (n6)	0
Heneicosanoic Acid (C21:0)	2.93
Heptadecanoic Acid (C17:0)	0.76
Heptadecenoic Acid (C17:1)	0.05
Capric Acid (C10:0)	0
Caprylic Acid (C8:0)	0
Lauric acid (C12:0)	0.17
Lignoceric Acid (C24:0)	0.78
Erucic Acid (C22:1)	0.11
Linoleic Acid (C18:2)	1.49
Myristic Acid (C14:0)	7.92
Myristoleic Acid (C14:1)	0.23
Nervonic Acid (C24:1)	0.33
Oleic Acid (C18:1)	16.8
Palmitic Acid(C16:0)	14.38
Penta-Decanoic Acid (C15:0)	0.49
Palmitoleic Acid (C16:1)	10.11
Stearic Acid (C18:0) Total	2.84
Omega-3 + Omega-6 (Fatty Acids)	32.15
Total Omega-3 Fatty Acids	27.42
Total Omega-6 Fatty Acids	4.73
Trans Linolenic Acid (tC18:3)	0.13
Trans-9-Octadecenoic Acid (Trans Oleic Acid) (tC18:1)	0.15
Trans-9,12- Octadecenoic Acid (Trans Linoleic Acid) (tC18:2)	0.06
Tricosanoic Acid (C23:0)	0.75
Unidentified Fatty Acids	6.38

Omega-3 + Omega-6 (Fatty Acids) lipid ratio had the highest rate at 32.15%, according to the results. The ratio of total omega-3 fatty acids, which is the second-highest lipid ratio, was found to be 27.42%. The ratios of Oleic acid (C18:1), Eicosapentaenoic Acid (EPA) (C20:5), Palmitic Acid (C16:0), Docosahexaenoic acid (DHA) (C22:6), which are the fatty acids with the highest values in the total fatty acid profile, were obtained as 16.8%, 16.11%, 14.38%, and 10.47%, respectively. Palmitoleic acid (C16:1) and Myristic Acid (C14:0) fatty acids are intermediate-level fatty acids obtained at 10.11% and 7.92%, respectively. Heneicosanoic Acid (C21:0), Stearic Acid (C18:0), Eicosapentaenoic Acid (C22:5), and Linoleic Acid (C18:2) are low-level fatty acids obtained at 2.93%, 2.84%, 1.84%, and 1.49%, respectively. Apart from these, Arachidic Acid (C20:0), Arachidonic Acid (C20:4), Behenic acid (C22:0), Eicosenoic Acid (C20:1), Eicosatrienoic Acid (C20: 3), Eicosadienoic Acid (C20:2), Gamma Linolenic Acid (C18:3), Heptadecanoic Acid (C17:0), Heptadecenoic Acid (C17:1), Capric Acid (C10:0), Caprylic Acid (C8:0), Lauric acid (C12:0), Lignoceric Acid (C24:0), Erucic Acid (C22:1), Myristoleic Acid (C14:1), Nervonic Acid (C24:1), Penta-Decanoic Acid (C15:0), Trans Linolenic Acid (tC18:3), Trans-9-Octadecenoic Acid (Trans Oleic Acid) (tC18:1), Trans-9,12- Octadecenoic Acid (Trans Linoleic Acid) (tC18:2) and Tricosanoic Acid (C23:0) are in the class of fatty acids that are almost absent in their fatty acid composition (Table 4.4).

4.5 ANTIBACTERIAL ACTIVITY

Schizochytrium sp. S31 oil sample was incubated with 10^6 CFU/ml of three different gram-negative pathogen bacteria, *Escherichia coli* 35218, *Pseudomonas aeruginosa* 27853, *Klebsiella pneumoniae* 700603, as well as the Gram-positive pathogen *Streptococcus mutans*, at 37 °C for 8 or 24 hours without shaking. Uninoculated TSB medium was used as a negative control. The bacterial growth was determined by using a multi-well plate reader at OD_{600}. For spot plate assays, undiluted and serially diluted pathogenic bacterial samples were incubated separately for 8 and 24 hours with *Schizochytrium* sp. oil samples at various rates. The spots were captured on camera using the G: Box imaging technology. The experiments were repeated three times, and the results from each trial are shown as SEM. The t test was used for the analysis of the results. The antibacterial effect of a *Schizochytrium* sp. S31 oil sample on *Pseudomonas aeruginosa* 27853, *Escherichia coli* 35218, *Streptococcus mutans,* and *Klebsiella pneumoniae* 700603 were shown in Figure 4.3, Figure 4.4, Figure 4.5, and Figure 4.6, respectively.

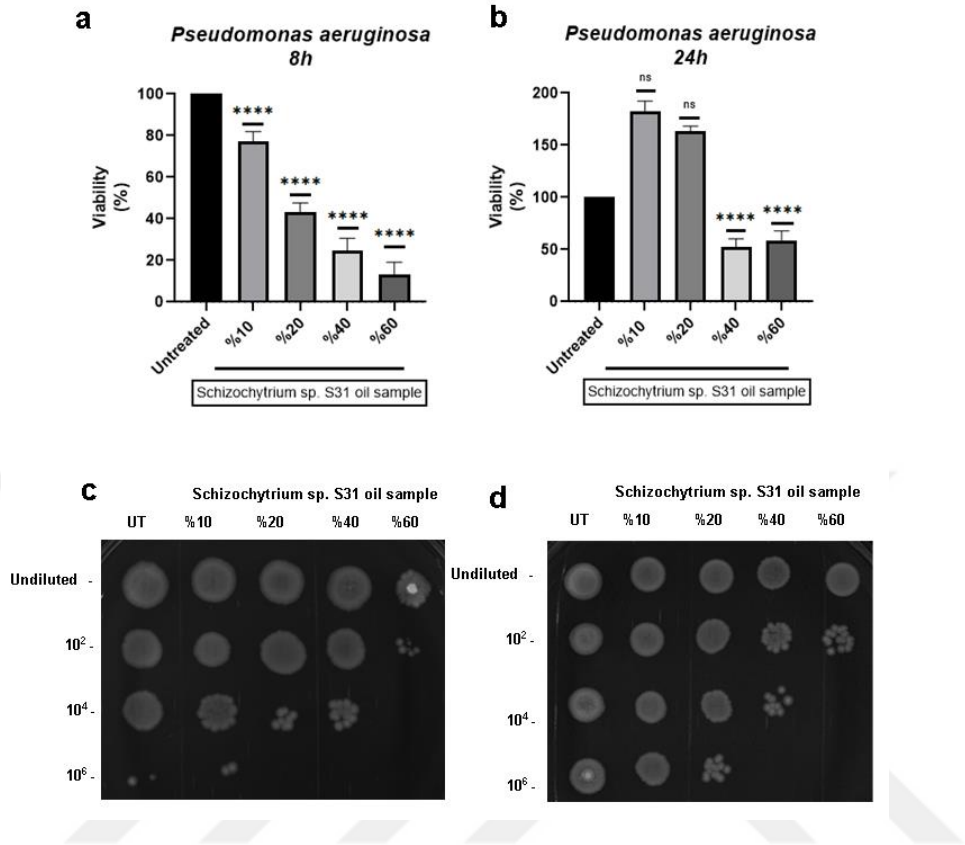

Figure 4.3*:* Investigation of antibacterial effect of *Schizochytrium* sp. S31 oil sample on *P. aeruginosa* bacteria. *P. aeruginosa* was incubated with oil or with control medium, for **a.** 8h and **b.** 24h. The bacterial growth was determined at OD600. Spot plate assays for **c.** 8h and **d.** 24h were used to visualizing cell growth. Then incubated at 37°C. Experiments were repeated three times and results from each trial are shown as ± SEM. The t test was used for the analysis of the results (ns:no significant**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

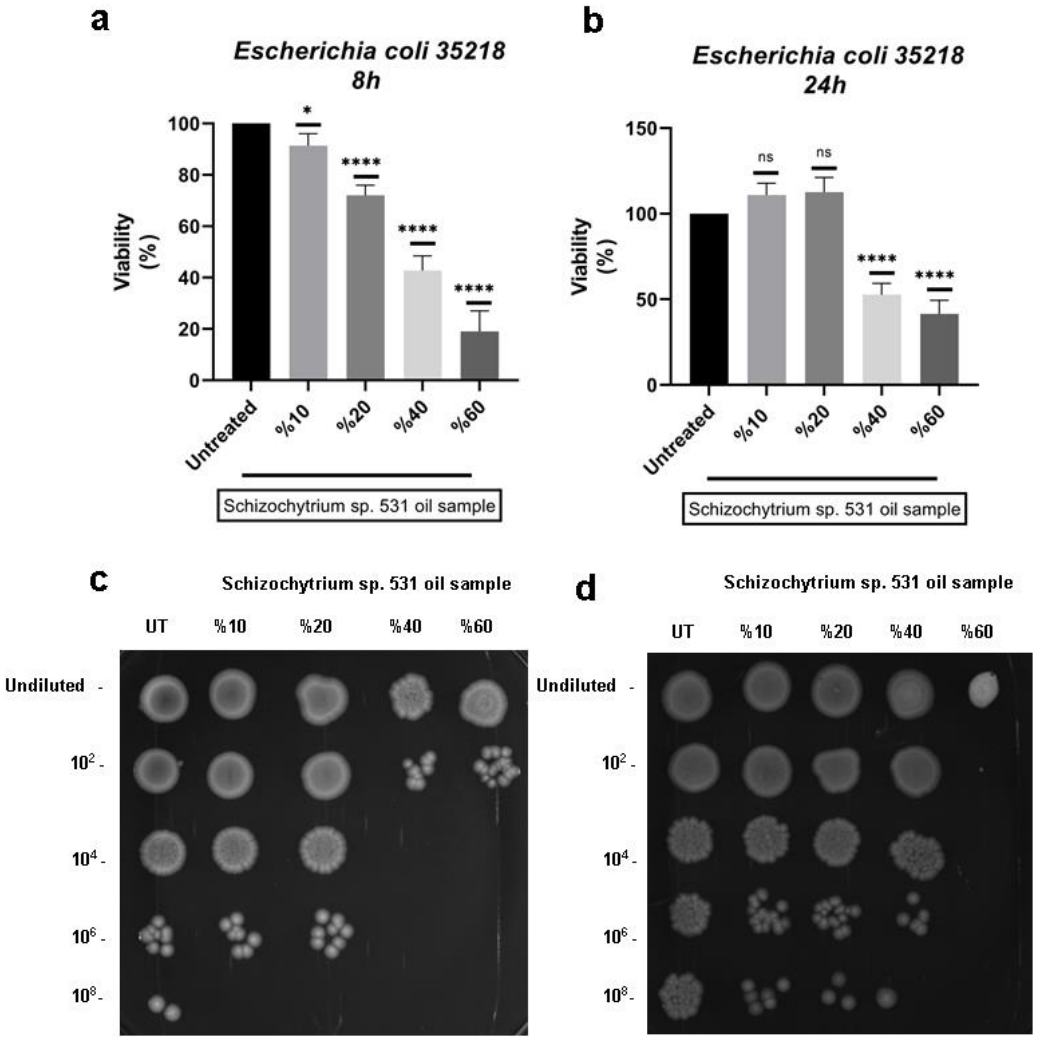

Figure 4.4: Investigation of antibacterial effect of *Schizochytrium* sp. S31 oil sample on *Escherichia coli* 35218. *E. coli* was incubated with oil or with control medium, for a. 8h and b. 24h. The bacterial growth was determined at OD600. Spot plate assays for c. 8h and d. 24h were used to visualizing cell growth. Then incubated at 37. Experiments were repeated three times and results from each trial are shown as ± SEM. The t test was used for the analysis of the results (ns:no significant**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$)

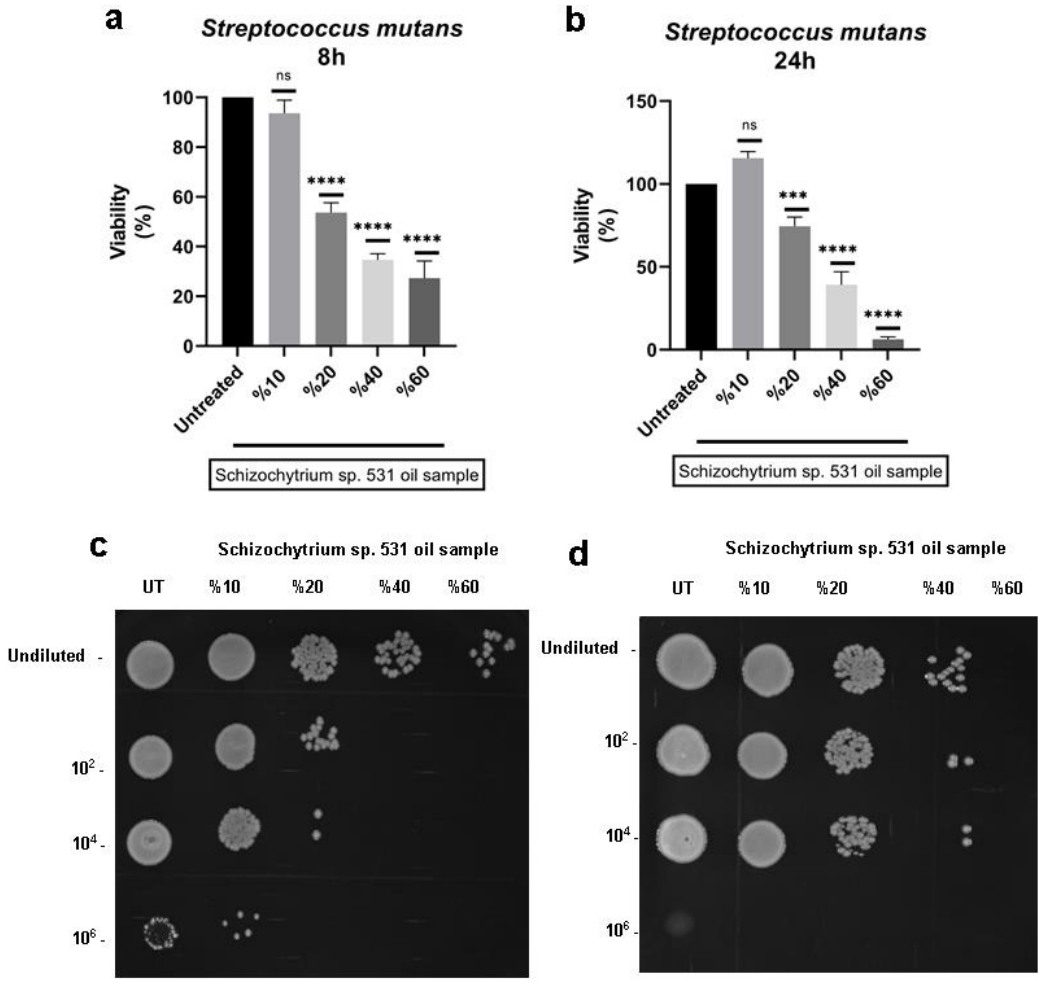

Figure 4.5: Investigation of antibacterial effect of *Schizochytrium* sp. S31 oil sample on Streptococcus mutans. S. mutans was incubated with oil or with. control medium, for a. 8h and b. 24h. The bacterial growth was determined at OD600. Spot plate assays for c. 8h and d. 24h were used to visualizing cell growth. Then incubated at 37. Experiments were repeated three times and results from each trial are shown as ± SEM. The t test was used for the analysis of the results (ns:no significant**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

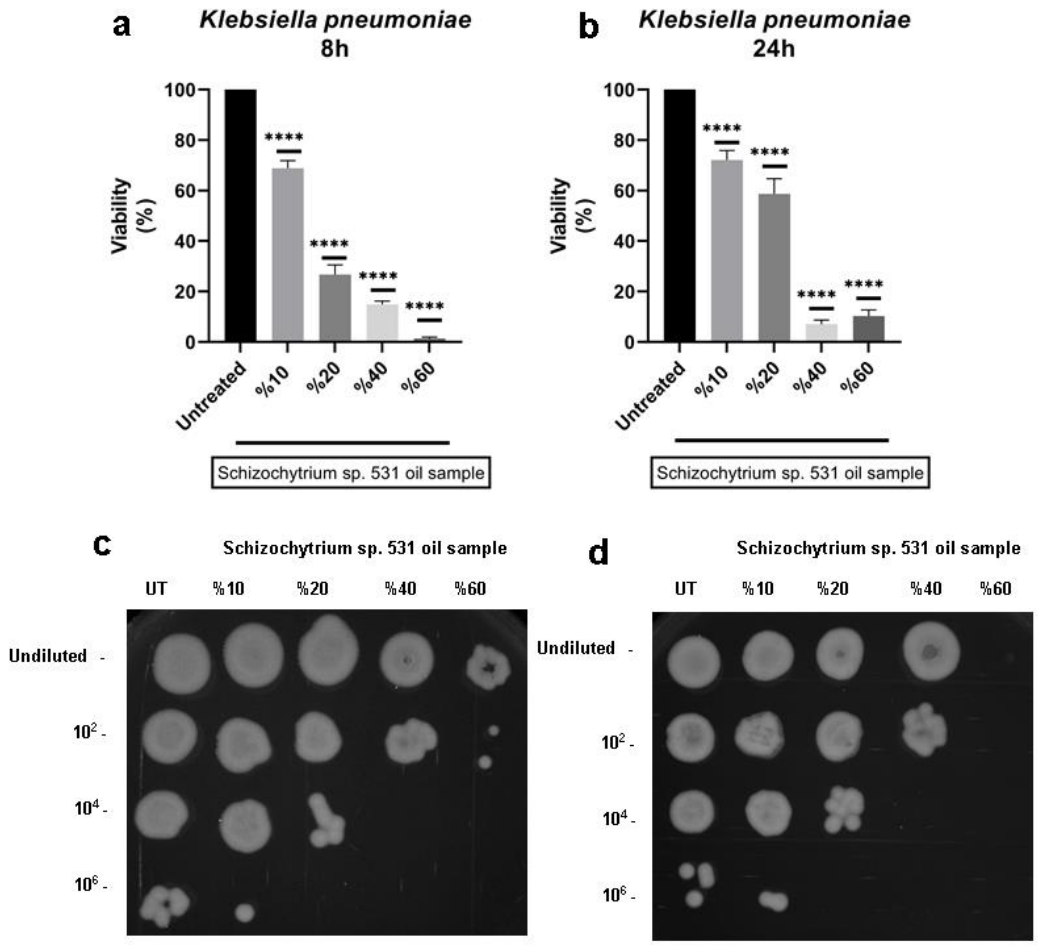

Figure 2.6: Investigation of antibacterial effect of *Schizochytrium* sp. S31 oil sample on *Klebsiella pneumonia*e. *K. pneumoniae* was incubated with oil or with control medium, for **a**. 8h and **b.** 24h. The bacterial growth was determined at OD600. Spot plate assays for **c**. 8h and **d.** 24h were used to visualizing cell growth. Then incubated at 37. Experiments were repeated three times and results from each trial are shown as ± SEM. The t test was used for the analysis of the results (ns:no significant**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

According to results, the *Schizochytrium* sp. S31 oil sample was found to have antibacterial activity on pathogenic bacteria of *Pseudomonas aeruginosa*, *Escherichia coli* 35218, *Klebsiella pneumonia,* and *Streptococcus mutans.*

Antibacterial activity study on *Pseudomonas aeruginosa* showed that cell viability decreased by 1.3, 2.3, 4.0, and 7.7 times, when the bacterial culture was incubated for 8 hours with 10%, 20%,

40%, and 60% concentration of *Schizochytrium* sp. S31 oil respectively. Cell viability was determined to decrease by 1.9 and 1.7 times, at 40% and 60% concentration of *Schizochytrium* sp. S31 oil respectively, when the bacterial culture was treated with for 24 hours.

Antibacterial activity study on *Escherichia coli* 35218 showed that cell viability decreased by 1.1, 1.4, 2.3, and 13.1 times, when the bacterial culture was incubated for 8 hours with 10%, 20%, 40%, and 60% concentration of *Schizochytrium* sp. S31 oil respectively. When the bacterial culture was incubated with *Schizochytrium* sp. S31 oil at 40% and 60% concentration for 24 hours, cell viability was observed to decrease by 1.9 and 2.4 times, respectively.

Antibacterial activity study on *Klebsiella pneumonia* showed that cell viability decreased by 1.4, 3.7, 6.7, and 72.7 times, when the bacterial culture was incubated for 8 hours with 10%, 20%, 40%, and 60% concentration of *Schizochytrium* sp. S31 oil respectively. Cell viability was shown to decrease by 1.4, 1.7, 14.3, and 9.7 times, at 10%, 20%, 40%, and 60% concentration of *Schizochytrium* sp. S31 oil when the bacterial culture was treated with for 24 hours respectively.

Antibacterial activity study on *Streptococcus mutans* showed that cell viability decreased by 1.1, 1.8, 2.9, and 6.4 times, when the bacterial culture was incubated for 8 hours with 10%, 20%, 40%, and 60% concentration of *Schizochytrium* sp. S31 oil respectively. When the bacterial culture was incubated for 24 hours, cell viability was observed to decrease by 1.3, 2.5, and 15.8 times, at 20%, 40%, and 60% concentration of *Schizochytrium* sp. S31 oil respectively.

4.6 ANTIBIOFILM ACTIVITY

Schizochytrium sp. S31 oil sample was incubated with 10^6 CFU/ml of two different gram-negative pathogen bacteria, *Escherichia coli* 35218 and *Pseudomonas aeruginosa* 27853 in 96-well flat-bottom polystyrene cell culture plates at 37 °C for 8 or 24 hours without shaking. The samples were washed, dried and then stained with crystal violet. After staining, plates were washed three times to remove any residual CV. Optical density at 550nm was measured using a multi-well plate reader. The uninoculated culture was used as a control. Effect of *Schizochytrium* sp. S31 oil sample on biofilm formation in *P. aeruginosa* and *Escherichia coli* 35218, were shown in Figure 4.6 and Figure 4.7 respectively. According to results, surface adhesion ability and biofilm forming capacity of *P. aeruginosa*, a widely used model bacterium in biofilm studies, were severely inhibited in the

presence of *Schizochytrium* sp. S31 oil sample. The same results were observed with the known biofilm-forming gram-negative bacteria, *Escherichia coli* 35218.

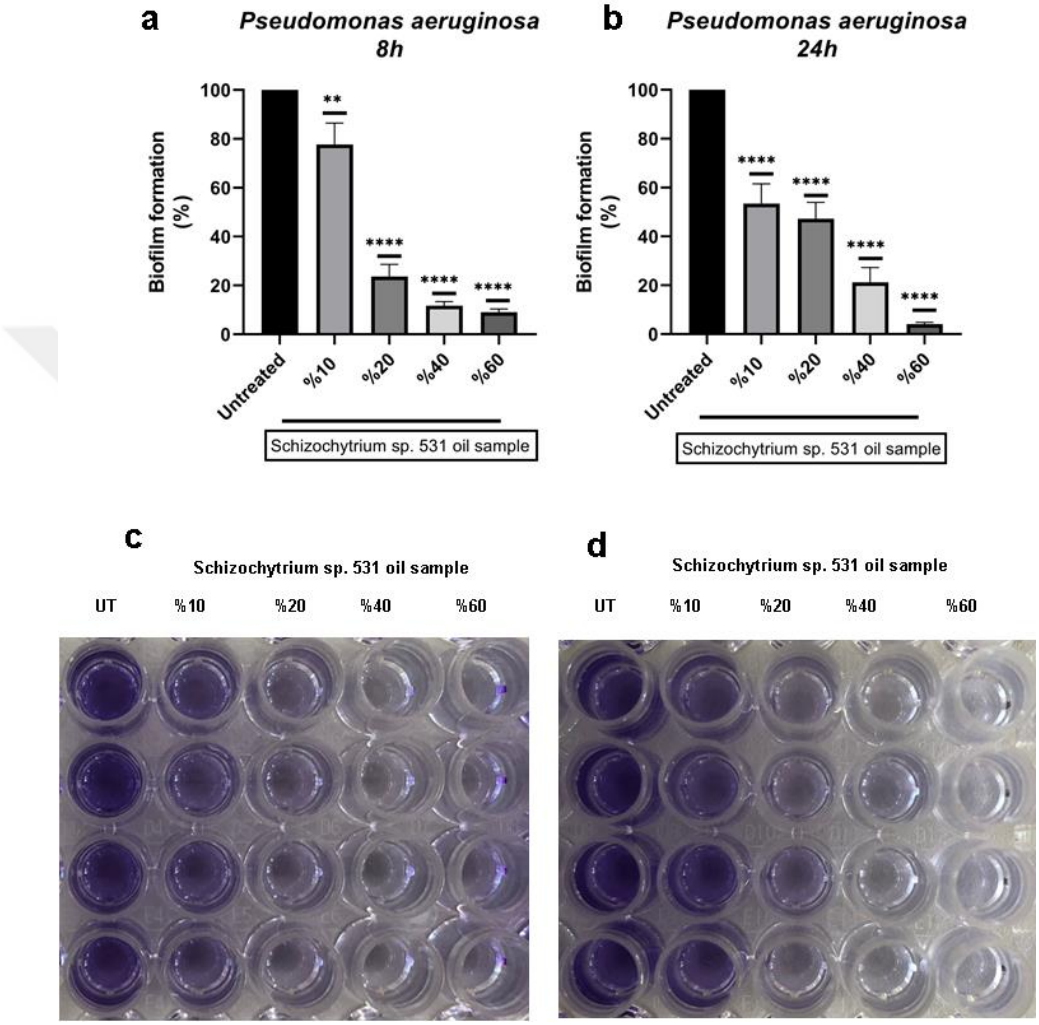

Figure 4.7: Effect of *Schizochytrium* sp. S31 oil sample on biofilm formation in *P. aeruginosa*. Biofilm development in *P. aeruginosa*, which was incubated with the oil used in the specified amounts for a. 8h and b. 24h, c. 8h d. 24h was investigated by CV staining. Quantitative results are given in percent (%) compared to *P. aeruginosa* grown in control medium. Well staining are representative experimental results. Experiments were repeated three times and results from each trial are shown as ± SEM. The t test was used for the analysis of the results (ns:no significant**p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

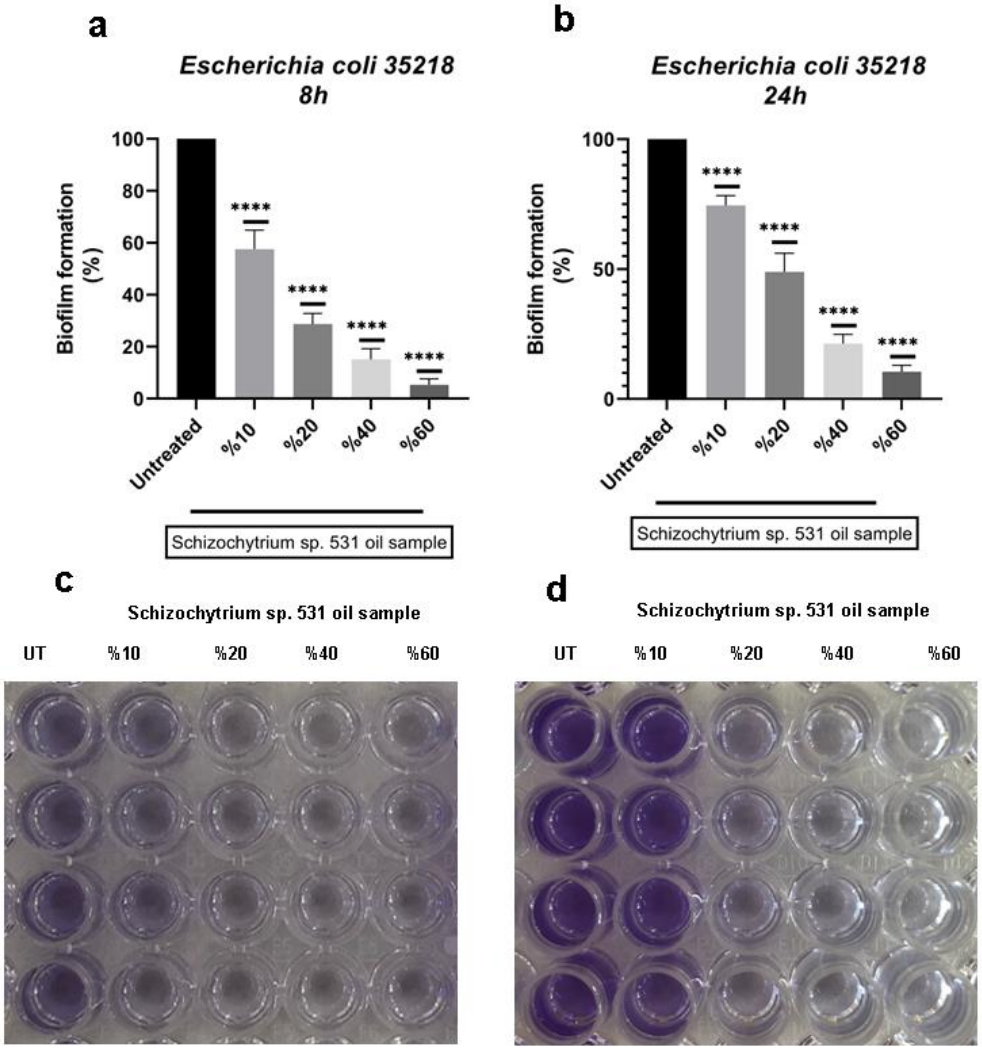

Figure 4.8: Effect of *Schizochytrium* sp. S31 oil sample on biofilm formation in *Escherichia coli* 35218. Biofilm development in E. coli, which was incubated with the oil used in the specified amounts for a. 8h and b. 24h, c. 8h d. 24h was investigated by CV staining. Quantitative results are given in percent (%) compared to *Escherichia coli* grown in control medium. Well staining are representative experimental results. Experiments were repeated three times and results from each trial are shown as ± SEM. The t test was used for the analysis of the results (ns:no significant**p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

The antibiofilm activity investigation on *P. aeruginosa* revealed that biofilm formation was decreased by 22.3%, 76.3%, 88.3%, and 91% when the bacterial culture was treated for 8 hours and by 46.5%, 52.7%, 78.8%, and 96% when it was treated for 24 hours with 10%, 20%, 40%, and 60% concentration of *Schizochytrium* sp. S31 oil respectively.

According to the results of the antibiofilm activity study on *Escherichia coli,* biofilm formation decreased by 42.5%, 71.3%, 84.9%, and 94.7%, when the bacterial culture was treated for 8 hours and by 25.4%, 51%, 78.7%, and 89.5%, when it was treated for 24 hours with 10%, 20%, 40%, and 60% concentration of *Schizochytrium* sp. S31 oil respectively.

5. DISCUSSION AND CONCLUSION

Algae are harmless and effective sources of bioactive molecules with a wide range of uses. Algae themselves or their extracts have the potential to be used in many different areas. Food additive, animal feed, aquaculture, biofertilizer, drug precursor, anti-inflammatory, anti-allergic, and supplement drugs, as well as antimicrobial and anti-biofilm products, waste treatment, bioenergy, and CO_2 emission (thus a potential global warming prevention agent) are a few examples of applications. (Concas et al., 2013; Ji et al., 2014; Axelsson et al., 2012). Algae can be used as a bio-based compound or as biodiesel from raw materials or refined products (Bahadar & Khan, 2013). Also, it has been demonstrated that chemical compounds derived from algae, such as lipids, proteins, enzymes, vitamins, and antioxidant chemicals, are beneficial for human health (Parsaeimehr et al., 2015). Biological compounds such as EPA, DHA, astaxanthin, and protein pigments produced by algae are widely used in the health, pharmaceutical, and food sectors. With all these features and the widespread use of algae, many private companies from all over the world have started to invest much more in algae-based bioproducts. UAS facilities and research programs provide potential for sustainable energy, sustainable fuel, food, feed, and many other emerging algae-based businesses. Solazyme Roquette Company, located in San Francisco, America produces microalgae and products. This company has recently launched a product called Almagine HL. This product is a natural, environmentally friendly, nutritious, rich food containing micronutrients including carbohydrates (simple sugars and polysaccharides), protein, lipids, fiber and lutein. Commercialization of algae products has accelerated in recent years, and many new products will be produced in the coming years. Moreover, numerous species of algae and microalgae have been studied recently for their antibacterial, antifungal, antiviral, and antibiofilm properties, and remarkable results have been obtained (Desbois et al., 2008; Smith et al., 2010; Veettil et al., 2022; Santoyo et al., 2009; Abedin & Taha, 2008; Katircioğlu et al., 2006). Due to the increasing need and the insufficient effect of existing antimicrobial agents, microalgae extract can also be used in treatment due to its antimicrobial activities.

In this study, the antimicrobial and antibiofilm activity of heterotrophic microalgae *Schizochytrium* sp. S31 microalgae oil were investigated. For this purpose, *Schizochytrium* sp. S31 was first cultivated heterotrophically under optimum conditions. Microalgae can grow rapidly under controlled conditions that provides great advantages. The level of some macromolecules in the

biomass can also be enhanced when the growth conditions are changed as desired. For example, it is known that the polar lipid content of microalgae increases under the low temperature and nitrogen-limiting environment (Vardar et al.,2016). Microalgae cell culture was then harvested and dried by lyophilization. Cell dry weight (CDW) was calculated as 57.1 g/l. In the downstream processing stage, various cell lysis and lipid extraction procedures were examined. Five different solvent mixtures—hexane, hexane/ethanol (2:1), ethanol, methanol, and hexane/methanol (2:1)—as well as two different cell disruption techniques—mortar and pestle and ultrasonication—were examined and compared. The total lipid yields were increased up to 34% with a clear appearance using hexane/methanol (2:1) together with ultrasonication. The extraction of high yield lipid from *Schizochytrium* sp. microalgae has previously been studied using a variety of cell lysis and lipid extraction procedures. Vardar-Yel et al. (2017) examined various cell lysis (French press, ultrasonication, CTAB, SDS, alcalase) and lipid extraction (hexane, isopropanol, ethanol, and supercritical CO_2) methods on *Schizochytrium* sp. and discovered that when hexane solvent with sonication and/or French press was used, a high content of lipid with a high TAG content was obtained. In another study, acid treatment, enzyme digestion, osmotic shock, and homogenizer methods were combined with the Bligh and Dyer or Soxhlet methods to obtain high lipid content from *Schizochytrium* sp. It was stated that the most efficient result was obtained by increasing the lipid ratio above 21% and the %DHA to 19.25% as a result of the application of the Bligh and Dyer method together with the enzyme application (Hac Isa et al., 2022). Byreddy et al., (2015) carried out a similar study with *Schizochytrium* sp. S31 and *Thraustochytrium* sp. AMCQS5-5 strains using various solvent combinations. The author also tried different cell lysis methods such as grinding with liquid nitrogen, a water bath, osmotic shock, bead vortexing, and sonication. They obtained the highest lipid ration (48.7%) by using an osmotic shock method together with chloroform:methanol (2:1). Gas chromatography was used to assess the fatty acid content following lipid extraction. According to the GCMS analysis results, 10.4% DHA and 16.11% EPA fatty acids were obtained. Apart from these, other fatty acids with high levels were determined as 16.8% oleic acid, 14.8% palmitic acid and 10.11% palmitoleic acid. Total Omega-3 Fatty Acids and Total Omega-3 + Omega-6 Fatty Acids ratio were calculated as 27.42% and 32.15% respectively. *Schizochytrium* sp. S31 is once again confirmed to be rich in omega-3 fatty acids. Numerous other research in the literature demonstrate that *Schizochytrium* sp. contains a high concentration of omega-3 fatty acids. (Hakim, 2012; Ward et al., 2005; Byreddy, 2016).

P. aeruginosa is a pathogenic bacterium that can form biofilms on biotic and abiotic surfaces and is used as a model for biofilm formation (Pericolini et al., 2018). *P. aeruginosa*, an opportunistic pathogenic bacterium, can cause serious infections, including pneumonia, urinary tract infections, otitis externa, otitis media, soft tissue infections (keratitis), and bacteremia (Lyczak et al., 2000). Infections caused by *P. aeruginosa* often lead to high morbidity and mortality due to resistance to antibiotics and antimicrobial treatments. *P. aeruginosa* infections are generally characterized by eliciting an intense neutrophilic response (Gellatly & Hancock, 2013). *K. pneumonia* also causes many human infections. These include various infections such as bacteremia, pneumonia, urinary tract infections, meningitis, bloodstream infection, and intra-abdominal infection (Caneiras et al., 2019). These bacteria can be commonly found in raw fish, meat, milk, and dairy products, as well as in street foods (Zhang et al., 2018). *Escherichia coli (E. coli)* is a pathogenic bacterium known to cause gastrointestinal diseases such as diarrhea in humans and animals. *E. coli* has the capacity to easily form biofilms on the surface of living or non-living carriers, which can lead to cross-contamination in foods (Zhou et al., 2022). *Streptococcus mutans* (*S. mutans*) are gram-positive bacteria found in the human mouth and more specifically on tooth surfaces. *S. mutans* are able to produce large quantities of glucans and acids, exceeding their saliva buffering capacity, giving bacteria the advantage to outcompete non-cariogenic common species in low pH environments (Metwalli et al., 2013).

In this study, the antibacterial effects of *Schizochytrium* sp. S31 oil samples were investigated on Gram-negative pathogens, which cause many different human infections, as well as the Gram-positive pathogen Streptococcus mutans. *Schizochytrium* sp. S31 oil sample was found to have antibacterial activity on pathogenic bacteria including *Klebsiella pneumonia, Pseudomonas aeruginosa*, and *Escherichia coli* 35218. It can be concluded that the best results in terms of antibacterial activity were obtained under the condition of 60% concentration of *Schizochytrium* sp. S31 oil for 8 hours for *Pseudomonas aeruginosa*, *Escherichia coli* 35218 and *Klebsiella pneumonia* with a decrease of 7.7%, 13.1% and 72.7% in cell viability respectively. In the study with Streptococcus mutans, the highest antibacterial activity was obtained with a decrease of 15.8% in cell viability under 60% concentration of *Schizochytrium* sp oil for 24 hours. Although it was clear from all of the results that the oil sample had an antibacterial effect on all the tested pathogenic organisms, the most striking outcome came from the treatment of the 60% concentration *Schizochytrium* sp. S31 oil sample with *Klebsiella pneumonia* for 8 hours.

Antibiofilm activity of the *Schizochytrium* sp S31 oil on *Pseudomonas aeruginosa*, and *Escherichia coli* 35218 bacteria were also investigated. Both pathogens are also known for their biofilm-forming potential. It is possible to conclude that the conditions for the optimum antibiofilm activity were 60% *Schizochytrium* sp. S31 oil concentration for 24 hours for *Pseudomonas aeruginosa* and 8 hours for *Escherichia coli* 35218 with a decrease of 96% and 94.7% in terms of biofilm formation, respectively.

As a conclusion, the *Schizochytrium* sp. S31 oil sample was found to have anti-pathogenic activity on pathogenic bacteria of *Pseudomonas aeruginosa*, *Escherichia coli* 35218, *Klebsiella pneumonia* and *Streptococcus mutans.* In addition, the surface adhesion ability and biofilm formation capability of *P. aeruginosa*, a frequently used model bacteria in biofilm research, were investigated (Ryder et al., 2007), and were dramatically inhibited in the presence of a *Schizochytrium* sp. S31 oil sample. The same results were obtained with the gram-negative bacteria *Escherichia coli* 35218 strain, which forms biofilms (Mariscal et al., 2007). Although the active compound in the *Schizochytrium* sp. S31 oil sample with antibacterial and antibiotic capabilities is not yet known, it is assumed to be DHA and EPA, which have the highest ratio in fatty acid composition. In future studies, it can be determined which active substance with antibacterial and antibiofilm properties in *Schizochytrium* sp. S31 oil sample. This study highlights that *Schizochytrium* sp. S31 oil can potentially be a natural antibacterial and antibiofilm agent that could be applied in the pharmaceutical and food industries.

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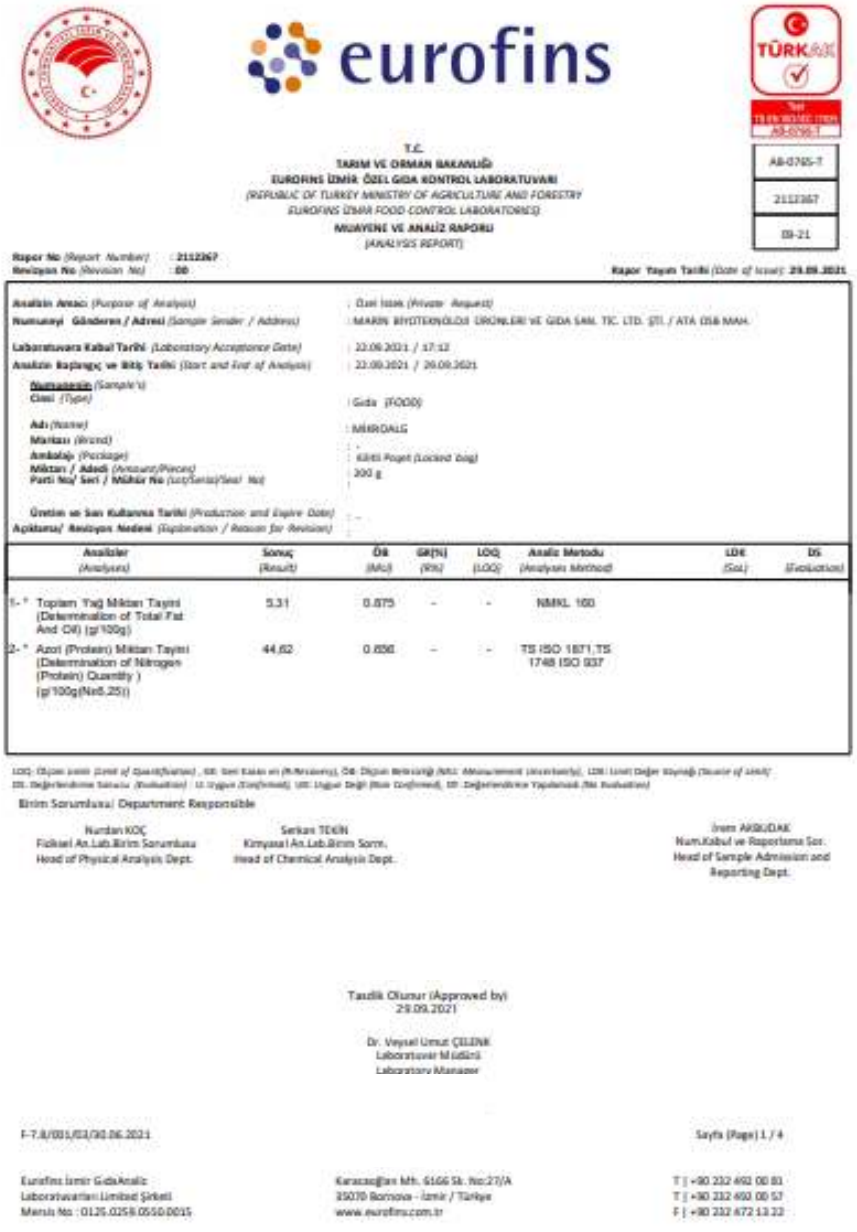
APPENDIX A

A.1 Chemical Characteristics of the of *Schizochytrium* sp. S31 Dry Biomass

A.2 Total Amino Acid and Vitamin Content of the Dry Biomass

A.3 Gas Chromatography Analysis Report

A.1 Chemical Characteristics of the of *Schizochytrium* sp. S31 Dry Biomass



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EUROFINS İZMİR ÖZEL GIDA KONTROL LABORATUVARI
(REPUBLIC OF TURKEY MINISTRY OF AGRICULTURE AND FORESTRY
EUROFINS İZMİR FOOD CONTROL LABORATORIES)
MUAYENE VE ANALİZ RAPORU
(ANALYSIS REPORT)

AB-0765-T
2112367
00-21

Rapor No (Report Number) : 2112367
Revizyon No (Revision No) : 00
Rapor Yayım Tarihi (Date of Issue): 29.09.2021

Analizin Amacı (Purpose of Analysis) : Özel İstek (Private Request)
Numuneyi Gönderen / Adresi (Sample Sender / Address) : MARİN BİYOTEKNOLOJİ ÜRÜNLERİ VE GIDA SAN. TİC. LTD. ŞTİ. / ATA OSB MAH.

Laboratuvara Kabul Tarihi (Laboratory Acceptance Date) : 22.09.2021 / 17:12
Analizin Başlangıç ve Bitiş Tarihi (Start and End of Analysis) : 22.09.2021 / 26.09.2021

Numune(ler) (Sample's)
Cinsi (Type) : Gıda (FOOD)
Adı (Name) : MİKROALG
Markası (Brand) : -
Ambalajı (Package) : Kilitli Poşet (Locked bag)
Miktarı / Adedi (Amount/Pieces) : 200 g
Parti No/ Seri / Mühür No (Lot/Serial/Seal No) :

Üretim ve Son Kullanma Tarihi (Production and Expire Date) : -
Açıklama/ Revizyon Nedeni (Explanation / Reason for Revision) :

	Analizler (Analyses)	Sonuç (Result)	ÖB (MU)	GR(%) (RSt)	LOQ (LOQ)	Analiz Metodu (Analysis Method)	LDK (Sou)	DS (Evaluation)
1- *	Toplam Yağ Miktarı Tayini (Determination of Total Fat And Oil) (g/100g)	5.31	0.875	-	-	NMKL 160		
2- *	Azot (Protein) Miktarı Tayini (Determination of Nitrogen (Protein) Quantity) (g/100g(Nx6.25))	44,62	0.656	-	-	TS ISO 1871,TS 1748 ISO 937		

LOQ: Ölçüm sonu (Limit of Quantification) , GR: Geri Kazanım (% Recovery), ÖB: Ölçüm Belirsizliği (MU): Measurement Uncertainty), LDK: Limit Değer Kaynağı (Source of Limit)
DS: Değerlendirme Sonucu (Evaluation) : U: Uygun (Conformed), UD: Uygun Değil (Non Conformed), DY: Değerlendirme Yapılamadı (No Evaluation)

Birim Sorumlusu/ Department Responsible

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Head of Physical Analysis Dept.

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Num.Kabul ve Raporlama Sor.
Head of Sample Admission and Reporting Dept.

Tasdik Olunur (Approved by)
29.09.2021

Dr. Veysel Umut ÇELİNK
Laboratuvar Müdürü
Laboratory Manager

F-7.8/001/02/30.06.2021

Sayfa (Page) 1 / 4

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MUAYENE VE ANALİZ RAPORU
(ANALYSIS REPORT)

AB-0765-T
2112367
09-21

Rapor No *(Report Number)* : **2112367**
Revizyon No *(Revision No)* : **00**

Rapor Yayım Tarihi *(Date of Issue)*: **29.09.2021**

	Analizler *(Analyses)*	Sonuç *(Result)*	ÖB *(MU)*	GK(%) *(Rik)*	LOQ *(LOQ)*	Analiz Metodu *(Analyses Method)*	LDK *(Sol.)*	DS *(Evaluation)*
3.- *	Enerji tayini (kcal/100g) (Determination of Energy (kcal / 100g)) (kcal/100g)	374,95	36.22	-	-	26.01.2017 29660 Sayılı Resmi Gazete Türk Gıda Kodeksi Gıda Etiketleme ve Tüketicileri Bilgilendirme Yönetmeliği		
4.- *	Karbonhidrat Tayini (Determination of Carbohydrate) (g/100g)	37,17	3.591	-	-	26.01.2017 29660 Sayılı Resmi Gazete Türk Gıda Kodeksi Gıda Etiketleme ve Tüketicileri Bilgilendirme Yönetmeliği		
5.- *	Enerji tayini (kj/100g) (Determination of Energy (kj/100g)) (kj/100g)	1568,79	151.545	-	-	26.01.2017 29660 Sayılı Resmi Gazete Türk Gıda Kodeksi Gıda Etiketleme ve Tüketicileri Bilgilendirme Yönetmeliği		

LOQ: Ölçüm Limiti *(Limit of Quantification)* , GK: Geri Kazan im *(R-Recovery)*, ÖB: Ölçüm Belirsizliği *(MU: Measurement Uncertainty)*, LDK: Limit Değer Kaynağı *(Source of Limit)*
DS: Değerlendirme Sonucu *(Evaluation)* : U: Uygun *(Conformal)*, UD: Uygun Değil *(Not Conformal)*, DY: Değerlendirme Yapılamadı *(No Evaluation)*

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29.09.2021

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09-21

Rapor No *(Report Number)* : **2112367**
Revizyon No *(Revision No)* : **00**

Rapor Yayın Tarihi *(Date of Issue)*: **29.09.2021**

	Analizler *(Analyses)*	Sonuç *(Result)*	ÖB *(MU)*	GK[%] *(R%)*	LOQ *(LOQ)*	Analiz Metodu *(Analysis Method)*	LDK *(SoL)*	DS *(Evaluation)*
6- *	Rutubet ve/veya Uçucu Madde, Kurutma Kaybı, Nem Miktarı Tayini (Determination of Moisture and / or Volatile Substance,Drying loss,Moisture content) (g/100g)	7,19	0.829	-	-	TS EN ISO 712		
7- *	Kül / Ham Kül Tayini (Total ash) (g/100g)	5,71	0.818	-	-	TS EN ISO 2171		

LOQ: Ölçüm Limiti *(Limit of Quantification)* , GK: Geri Kazanım *(R:Recovery)*, ÖB: Ölçüm Belirsizliği *(MU: Measurement Uncertainty)*, LDK: Limit Değer Kaynağı *(Source of Limit)*
DS: Değerlendirme Sonucu *(Evaluation)* - U: Uygun *(Conformed)*, UD: Uygun Değil *(Non Conformed)*, DY: Değerlendirme Yapılamadı *(No Evaluation)*

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Head of Sample Admission and Reporting Dept.

Tasdik Olunur (Approved by)
29.09.2021

Dr. Veysel Umut ÇELENK
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TARIM VE ORMAN BAKANLIĞI
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(REPUBLIC OF TURKEY MINISTRY OF AGRICULTURE AND FORESTRY
EUROFINS İZMİR FOOD CONTROL LABORATORIES)

MUAYENE VE ANALİZ RAPORU
(ANALYSIS REPORT)

AB-0765-T
2112367
09-21

Rapor No *(Report Number)* : **2112367**
Revizyon No *(Revision No)* : **00**

Rapor Yayın Tarihi *(Date of Issue)*: **29.09.2021**

Analizler *(Analyses)*	Sonuç *(Result)*	ÖB *(MU)*	GK(%) *(95)*	LOQ *(LOQ)*	Analiz Metodu *(Analysis Method)*	LDK *(Stat.)*	DS *(Evaluation)*

Notlar (Notes)
1. Yapılan muayene ve analiz sonucunda yukarıda belirtilen değerler tespit edilmiştir. *(Above mentioned values have been determined from the analytical work performed.)*
2. Bu analiz raporu adli olan işlemlerde ve reklam amacıyla kullanılamaz. *(This report shall not be used in the executive-judicial processes and for advertising purposes.)*
3. Bu analiz raporunun hiçbir bölümü tek başına veya ayrı ayrı kullanılamaz. *(This report with all parts is a whole, no part of this report can be used separately.)*
4. Analiz sonuçları yukarıda belirtilen numune için geçerlidir. *(Results of analysis belong to sample mentioned above.)*
5.* işaretli analizler akreditasyon kapsamındadır. *(* Analysis is covered by our accreditation scope.)*
6.Raporlar laboratuvarın yazılı izni olmadan kısmen kopyalanıp çoğaltılamaz. İncelesi ve mahkemeyi raporlar geçerlidir. *(Reports may not be copied or reproduced in part without the written permission of the laboratory. Unsigned and unsealed reports are invalid)*
7. * ile işaretlenmiş analizlerin kapsamında, hakkında yorum yapılan sonuçlar akredite edilmiş kapsam ile ilgili değildir. *(Excepting marked with *, the commented results are not associated with scope of accreditation.)*
8.Laboratuvar bilgisine müşteri tarafından sağlanağı durumlar dışında, raporlarda verilen bilgilerden sorumludur. Bilgisine müşteri tarafından sağlanağı verilen bilginin sonuçlarını geçerliliğini etkileyebilecek olması durumunda ilgili sonuçlardan oluşabilecek sapmalardan sorumlu değildir. *(The laboratory is responsible for the information provided in the reports, except where the information is provided by the customer. The laboratory can not be held responsible for any deviations in the relevant results if the sample information is provided by the customer and this information may affect the validity of the results.)*
9.Numune alımı laboratuvar tarafından yapılmamakta olup müşterinin sorumluluğundadır. Analiz raporunda belirtilen sonuçlar gönderilen numunenin teslim alındığı hali için geçerlidir. *(Sampling is not performed by the laboratory and sampling is the responsibility of the customer. The analysis results are valid for the sample that has been delivered to the laboratory.)*
10.Kısaltmalar: U: Uygun, UD: Uygun Değil, DY: Değerlendirme Yapılamadı, DS: Değerlendirme Sonucu *(Abbreviations: C: Confirmed, NC: Not Confirmed, NE : No Evaluation)*
11.Türk Akreditasyon Kurumu (Türkak) ilmiey raporlarının tanınırlığı konusunda Avrupa Akreditasyon Birliği (EA) ile Çok Taraflı Anlaşma ve Uluslararası Laboratuvar Akreditasyon Birliği (ILAC) ile karşılıklı tanıma anlaşması imzalamıştır. *(Turkish Accreditation Agency (TURKAK) is a signatory to the European co-operation for Accreditation (EA) Multilateral Agreement (MLA) and to the International Laboratory Accreditation Cooperation (ILAC) Mutual Recognition Arrangement (MRA) for the recognition of test reports.)*
12.Uygunluk beyanı analiz sonucundan ölçüm belirsizliğinin çıkarılması ile elde edilen sonuca göre yapılmıştır. *(The declaration of conformity was made according to the result obtained by subtracting the measurement uncertainty from the analysis result.)*
13.Beyan edilen genişletilmiş ölçüm belirsizliği, standart belirsizliğin k=2 olan genişletme katsayısı ile çarpımı sonucunda %95 civarında güvenilirlik seviyesi sağlanmaktadır. *(The declared expanded measurement uncertainty provides a 95% confidence level as a result of multiplying the standard uncertainty by the expansion coefficient k = 2.)*

LOQ: Ölçüm Limiti *(Limit of Quantification)* , GK: Geri Kazanım *(% Recovery)*, ÖB: Ölçüm Belirsizliği *(MU: Measurement Uncertainty)*, LDK: Limit Değer Kaynağı *(Source of Limit)*
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F-7.8/001/03/30.06.2021

Sayfa (Page) 4 / 4

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A.2 Total Amino Acid and Vitamin Content of the Dry Biomass

MarinBio

Marin Biyoteknoloji Ürünleri ve Gıda San. Tic. Ltd. Şti.

Amino Acid	g/100g protein
Aspartik asit	10.5 ± 0.09
Tireonin	5.24 ± 0.12
Serin	5.08 ± 0.07
Glutamik asit	10.7 ± 0.03
Prolin	6.45 ± 0.26
Glisin	5.13 ± 0.07
Alanin	8.44 ± 0.19
Valin	6.44 ± 0.12
Methionin	1.52 ± 0.13
İzolösin	5.01 ± 0.14
Lösin	6.84 ± 0.17
Tirosin	5.21 ± 0.21
Fenilalanin	4.26 ± 0.04
Histidin	5.02 ± 0.10
Lisin	5.63 ± 0.08
Arjinin	8.25 ± 0.26

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Vitamin	Concentration
Demir, mg/kg	869.51 ± 38.96
Niyasin, mg/kg	245 ± 16.58
Potasyum, mg/kg	1360 ± 241.74
Kalsiyum, mg/kg	100.08 ± 9.62
Lutein, mg/kg	368.65 ± 56.72
A vitamini, IU/kg	216.41 ± 72.13
B_1 vitamini, mg/kg	4.48 ± 0.12
B_2 vitamini, mg/kg	14.26 ± 3.04
B_6 vitamini, mg/kg	9.62 ± 2.84
C vitamini, mg/kg	74.16 ± 1.85
B_{12} vitamini, µg/kg	725.17 ± 156.20

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A.3 Gas Chromatography Analysis Report

T.C.
TARIM VE ORMAN BAKANLIĞI
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EUROFINS IZMIR FOOD CONTROL LABORATORIES)
MUAYENE VE ANALİZ RAPORU
(ANALYSIS REPORT)

TÜRKAK
AB-0765-T
211373?
10-21

Rapor No (Report Number) : 211373?
Revizyon No (Revision No) : 00
Rapor Yayım Tarihi (Date of issue): 20.10.2021

Analizin Amacı (Purpose of Analysis) : Özel İstek (Private Request)
Numuneyi Gönderen (Sample Sender) : Altınbaş Üniversitesi

Laboratuvara Kabul Tarihi (Laboratory Acceptance Date) : 18.10.2021 / 13:26
Analizin Başlangıç ve Bitiş Tarihi (Start and End of Analysis) : 18.10.2021 / 20.10.2021

Numunenin (Sample's)
Cinsi (Type) : Bitkisel / Hayvansal Yağ
Adı (Name) : MERCIMEK YAĞI
Markası (Brand) : -
Ambalajı (Package) : Plastik Kap (Plastic package)
Miktarı / Adedi (Amount/Pieces) : 5 ml.
Parti No/ Seri / Mühür No (Lot/Serial/Seal No) : 10/2021

Üretim ve Son Kullanma Tarihi (Production and Expire Date) : -
Açıklama/ Revizyon Nedeni (Explanation / Reason for Revision) : -

Analizler (Analyses)	Sonuç (Result)	ÖB (MU)	GK(%) (Rk)	LOQ (LOQ)	Analiz Metodu (Analysis Method)	LDK (Loc.)	DS (Evaluation)
) - * Yağ Asitleri Komposizyonu Tayini (Fatty Acids Composition) (%)	-	-	-	-	COI/T.20/Doc No:33		
* -Bütrik Asit (C4:0) (-Butyric acid C4:0) (%)	Tespit Edilemedi (Not Detected)	0	-	-			
* -Alfa Linolenik Asit (C18:3 (n3)) (-Alfa Linolenic Acid (C18:3 (n3))) (%)	0.84	0.044	-	-			

LOQ: Ölçüm Limiti (Limit of Quantification) , GK :Geri Kazanım (Recovery) ÖB: Ölçüm Belirsizliği (MU: Measurement Uncertainty), LDK: Limit Değer Kaynağı (Source of Limit)
DS: Değerlendirme Sonucu (Evaluation) - U: Uygun (Confirmed), UD: Uygun Değil (Not Confirmed), DY: Değerlendirme Yapılamadı (No Evaluation)

Birim Sorumlusu/ Department Responsible

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Head of Chemical Analysis Dept.

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Num.Kabul ve Raporlama Sor.
Head of Sample Admission and Reporting Dept.

Tasdik Olunur (Approved by)
20.10.2021

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Sayfa (Page) 1 / 6

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EUROFINS İZMİR FOOD CONTROL LABORATORIES)

MUAYENE VE ANALİZ RAPORU
(ANALYSIS REPORT)

AB-0765-T
2113737
10-21

Rapor No *(Report Number)*: **2113737**
Revizyon No *(Revision No)*: **00**

Rapor Yayın Tarihi *(Date of Issue)*: **20.10.2021**

Analizler *(Analyses)*	Sonuç *(Result)*	ÖB *(MU)*	GK(%) *(Rec)*	LOQ *(LOQ)*	Analiz Metodu *(Analyses Method)*	LOK *(Loc.)*	DS *(Evaluation)*
* -Araşidik Asit (C20:0) (-Arachidic Acid (C20:0)) (%)	0,41	0,050	-	-			
* -Araşidonik Asit (C20:4) (-Arachidonic Acid (C20:4)) (%)	1,07	0,098	-	-			
* -Behenik Asit (C22:0) (%)	0,25	0,049	-	-			
* -Cis-11 Eikosenoik Asit (C20:1) (-Cis-11 Eicosenoic Acid (C20: 1)) (%)	1,15	0,129	-	-			
* -Cis-11,14,17 Eikosatrienoik Asit (C20:3 (n3)) (-Cis-11,14,17 Eicosatrienoic Acid (C20: 3 (n3)) (%)	0,61	0,105	-	-			
* -Cis-8,11,14 Eikosatrienoik Asit (C20:3 (n6)) (-Cis-8,11,14 Eicosatrienoic Acid (C20: 3 (n6)) (%)	Tespit Edilemedi (Not Detected)	0	-	-			
* -Cis-11,14 Eikosadienoik Asit (C20:2) (-Cis-11,14 Eicosadienoic Acid (C20: 2)) (%)	0,17	0,023	-	-			
* -Dekosaheksanoik Asit (DHA) (C22:6) (-Decosahexanoic Acid (DHA) (C22: 6)) (%)	10,47	0,550	-	-			
* -Dekosapentanoik Asit (C22:5) (-Decosapentanoic Acid (C22: 5)) (%)	1,84	0,116	-	-			

(LOQ: Ölçüm Limiti (Limit of Quantification) , GK: Geri Kazanım (% Recovery), ÖB: Ölçüm Belirsizliği (MU: Measurement Uncertainty), LOK: Limit Değer Sayısı) (Source of Limit)
(DS: Değerlendirme Sonucu (Evaluation) , U: Uygun (Conformed), UD: Uygun Değil (Non Conformed), D/: Değerlendirme Yapılamadı (No Evaluation)

Birim Sorumlusu/ Department Responsible

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MUAYENE VE ANALİZ RAPORU
(ANALYSIS REPORT)

AB-0765-T	
2113737	
10-21	

Rapor No *(Report Number)* : **2113737**
Revizyon No *(Revision No)* : **00**

Rapor Yayın Tarihi *(Date of Issue)*: **20.10.2021**

Analizler *(Analyses)*	Sonuç *(Result)*	ÖB *(MU)*	GK(%) *(R%)*	LOQ *(LOQ)*	Analiz Metodu *(Analysis Method)*	LOK *(LOL)*	DS *(Evaluation)*
* -Eikosapentenoik Asit (EPA) (C20:5) (-Eicosapentenoic Acid (EPA) (C20:5)) (%)	16,11	0,470	-	-			
* -Gama Linolenik Asit (C18:3 (n6)) (-Gama Linolenic Acid (C18:3 (n6))) (%)	Tespit Edilemedi (Not Detected)	0	-	-			
* -Henekosanoik Asit (C21:0) (-Henekosanoic Acid (C21:0)) (%)	2,93	0,135	-	-			
* -Margarik (C17:0) (-Heptadecanoic Acid (C17:0)) (%)	0,76	0,067	-	-			
* -Heptadesenoik (C17:1) (-Heptadecanoic Acid (C17:1)) (%)	0,05	0,006	-	-			
* -Kaprik Asit (C10:0) (-Capric Acid (C10:0)) (%)	Tespit Edilemedi (Not Detected)	0	-	-			
* -Kaprilik Asit (C8:0) (-Caprilic Acid (C8:0)) (%)	Tespit Edilemedi (Not Detected)	0	-	-			
* -Kaproik Asit (C6:0) (-Caproic Acid (C6:0)) (%)	Tespit Edilemedi (Not Detected)	0	-	-			
* -Lauric Asit (C12:0) (-Lauric acid (C12:0)) (%)	0,17	0,029	-	-			

LOQ: Ölçüm Limiti (*Limit of Quantification*) , GK: Geri Kazanım (*% Recovery*) ÖB: Ölçüm Belirsizliği (*MU: Measurement Uncertainty*), LOK: Limit Değer Kaynağı (*Source of Limit*)
DS: Değerlendirme Sonucu (*Evaluation*) - U: Uygun (*Confirmed*), UD: Uygun Değil (*Non Confirmed*), DY: Değerlendirmeye Yapılamadı (*Not Evaluation*)

Birim Sorumlusu/ Department Responsible

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Head of Sample Admission and Reporting Dept.

Tasdik Olunur (*Approved by*)
20.10.2021

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MUAYENE VE ANALİZ RAPORU
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AB-0765-T
211373?
03-21

Rapor No *(Report Number)*: 211373?
Revizyon No *(Revision No)*: 00
Rapor Yayın Tarihi *(Date of issue)*: 20.10.2021

Analizler *(Analyses)*	Sonuç *(Result)*	ÖB *(MU)*	ÖB(%) *(RSD)*	LOQ *(LOQ)*	Analiz Metodu *(Analysis Method)*	LOK *(LoL)*	DE *(Evaluation)*
* -Lignoserik Asit (C24:0) (-Lignoseric Acid (C24:0)) (%)	0,78	0,211	-	-			
* -Erusik asit C22:1 (-Erusic Acid (C22:1)) (%)	0,11	0,009	-	-			
* -Linolek Asit (C18:2) (-Linoleic Acid (C18:2)) (%)	1,49	0,035	-	-			
* -Miristik Asit (C14:0) (-Miristic Acid (C14:0)) (%)	7,92	1,797	-	-			
* -Miristoleik Asit (C14:1) (-Miristoleic Acid (C14:1)) (%)	0,23	0,031	-	-			
* -Nervonik Asit (C24:1) (-Nervonic Acid (C24:1)) (%)	0,33	0,059	-	-			
* -Oleik Asit (C18:1) (-Oleic Acid (C18:1)) (%)	16,80	0,339	-	-			
* -Palmitik Asit (C16:0) (-Palmitic Acid(C16:0)) (%)	14,38	1,171	-	-			
* -Pentadekanoik Asit (C15:0) (-Penta-Decanoic Acid (C15:0)) (%)	0,49	0,038	-	-			
* -Palmitoleik Asit (C16:1) (-Palmitoleic Acid (C16:1)) (%)	10,11	0,976	-	-			
* -Pentadekenoik Asit (C15:1) (-Penta-Decanoic acid (C15:1)) (%)	0,15	0,012	-	-			
* -Stearik Asit (C18:0) (-Stearic Acid (C18:0)) (%)	2,84	0,097	-	-			

LOQ: Ölçüm Limiti (*Limit of Quantification*) , SD: Geri Kazanım (*Recovery*), ÖB: Ölçüm Belirsizliği (*MU: Measurement Uncertainty*), LOK: Limit Değer Kaynağı (*Source of Limit*)
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Birim Sorumlusu/ *Department Responsible*

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20.10.2021

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F-7.8/001/03/10.06.2021

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MUAYENE VE ANALİZ RAPORU
(ANALYSIS REPORT)

AB-0765-T	
2113737	
10-21	

Rapor No *(Report Number)* : **2113737**
Revizyon No *(Revision No)* : **00**

Rapor Yayın Tarihi *(Date of issue)*: **20.10.2021**

Analizler *(Analyses)*	Sonuç *(Result)*	ÖB *(MU)*	GK(%) *(Rec)*	LOQ *(LOQ)*	Analiz Metodu *(Analyses Method)*	LDK *(Sol.)*	DS *(Evaluation)*
* -Toplam Omega - 3 + Omega - 6 Yağ Asitleri (-Total Omega-3 + Omega-6 Fatty Acids) (%)	32,15	2,569	-	-			
* -Toplam Omega - 3 Yağ Asitleri (-Total Omega-3 Fatty Acids) (%)	27,42	2,191	-	-			
* -Toplam Omega - 6 Yağ Asitleri (-Total Omega-6 Fatty Acids) (%)	4,73	0,175	-	-			
* -Trans Linolenik Asit (tC18:3) (-Trans Linolenic Acid (tC18:3)) (%)	0,13	0,038	-	-			
* -Trans-9-Oktadekenoik Asit (Trans Oleik Asit) (tC18:1) (-Trans-9-Octadecenoic Acid (Trans Oleic Acid) (tC18:1)) (%)	0,15	0,063	-	-			
* -Trans-9,12-Oktadekadienoik Asit (Trans Linoleik Asit) (tC18:2) (-Trans-9,12-Octadecenoic Acid (Trans Linoleic Acid) (tC18:2)) (%)	0,06	0,009	-	-			
* -Trikosanoik Asit (C23:0) (-Tricosanoic Acid (C23:0)) (%)	0,75	0,060	-	-			
* -Tanımlanamayan Yağ Asitleri (Unidentified Fatty Acids) (%)	8,38	1,404	-	-			

LOQ: Ölçüm Limiti (*Limit of Quantification*) , GK: Geri Kazanım (*Recovery*) ÖB: Ölçüm Belirsizliği (MU: *Measurement Uncertainty*), LDK: Limit Değer Kaynağı (*Source of Limit*)
DS: Değerlendirme Sonucu (*Evaluation*) - U: Uygun (*Confirmed*), UD: Uygun Değil (*Not Confirmed*), DY: Değerlendirme Yapılamadı (*No Evaluation*)

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Tasdik Olunur (*Approved by*)
20.10.2021

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(REPUBLIC OF TURKEY MINISTRY OF AGRICULTURE AND FORESTRY
EUROFINS İZMİR FOOD CONTROL LABORATORIES)

MUAYENE VE ANALİZ RAPORU
(ANALYSIS REPORT)

AB-0765-T
2113737
10-21

Rapor No *(Report Number)* : **2113737**
Revizyon No *(Revision No)* : **00**

Rapor Yayın Tarihi *(Date of Issue)*: **20.10.2021**

Analizler *(Analyses)*	Sonuç *(Result)*	ÖB *(MU)*	GR(%) *(Wb)*	LOQ *(LOQ)*	Analiz Metodu *(Analyses Method)*	LDK *(Unit)*	DS *(Evaluation)*

Notlar (Notes);
1. Yapılan muayene ve analiz sonucunda yukarıda belirtilen değerler tespit edilmiştir. *(Above mentioned values have been determined from the analytical work performed.)*
2. Bu analiz raporu adli olan işlemlerde ve reklam amacıyla kullanılamaz. *(This report shall not be used in the executive-judicial processes and for advertising purposes.)*
3. Bu analiz raporunun hiçbir bölümü tek başına veya ayrı ayrı kullanılamaz. *(This report with all parts is a whole, no part of this report can be used separately.)*
4. Analiz sonuçları yukarıda belirtilen numune için geçerlidir. *(Results of analysis belong to sample mentioned above.)*
5. * işaretli analizler akreditasyon kapsamındadır. *(* Analysis is covered by our accreditation scope.)*
6. Raporlar laboratuvarımız yazılı izni olmadan kısmen kopyalanıp çoğaltılamaz. İmzasız ve mührüz raporlar geçersizdir. *(Reports may not be copied or reproduced in part without the written permission of the laboratory. Unsigned and unsealed reports are invalid)*
7. * ile işaretlenmiş analizlerin haricinde, herkesin yorum yaptığı sonuçlar akredite edilmiş kapsam dışındadır. *(Except marked with *, the commented results are not associated with scope of accreditation.)*
8. Laboratuvar bilgisi müşteri tarafından sağlandığı durumlar dışında, raporlarda verilen bilgilerden sorumludur. Bilgisi müşteri tarafından sağlandığı ve bu bilginin sonuçların geçerliliğini etkileyebilecek olması durumunda ilgili sonuçlardan oluşabilecek sapmalardan sorumlu değildir. *(The laboratory is responsible for the information provided in the reports, except where the information is provided by the customer. The laboratory can not be held responsible for any deviations in the relevant results if the sample information is provided by the customer and this information may affect the validity of the results.)*
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11. Türk Akreditasyon Kurumu (Türkak) deney raporlarının tanınırlığı konusunda Avrupa Akreditasyon Birliği (EA) ile Çok Taraflı Anlaşma ve Uluslararası Laboratuvar Akreditasyon Birliği (ILAC) ile karşılıklı tanıma anlaşması imzalamıştır. *(Turkish Accreditation Agency (TURKAK) is a signatory to the European co-operation for Accreditation (EA) Multilateral Agreement (MLA) and to the International Laboratory Accreditation Cooperation (ILAC) Mutual Recognition Arrangement (MRA) for the recognition of test reports.)*
11. Uygunluk beyanı analiz sonuçları ölçüm belirsizliğinin çıkarılması ile elde edilen sonuca göre yapılmıştır. *(The declaration of conformity was made according to the result obtained by subtracting the measurement uncertainty from the analysis result.)*
11. Beyan edilen genişletilmiş ölçüm belirsizliği, standart belirsizliğin k=2 olan genişletme katsayısı ile çarpımı sonucunda %95 civarında güvenilirlik seviyesi sağlanmaktadır. *(The declared expanded measurement uncertainty provides a 95% confidence level as a result of multiplying the standard uncertainty by the expansion coefficient k = 2.)*

LOQ: Ölçüm Limiti *(Limit of Quantification)* , GR: Geri Kazan en *(% Recovery)*, ÖB: Ölçüm Belirsizliği *(MU: Measurement Uncertainty)*, LDK: Limit Değer Kaynağı *(Source of Limit)*
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