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REVIEW PAPER

# Recent Progress in Microalgal Squalene Production and Its Cosmetic Application

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**Abstract** Squalene, [oxidized form squalane] is a terpenoid with biological activity that produced by animals and plants. In the human body, a significant excretion named as sebum includes squalene in 12 percent. This bioactive compound shows anti-inflammatory, detoxifying, moisturizing and antioxidant effects on the human body. In addition to having these properties, it is known that squalene production decreases as less sebum is produced with age. Because of that, the need for supplementation of squalene through products has arisen. As a result, squalene production has been drawn attention due to its many application possibilities by cosmetic, cosmeceutical and pharmaceutical fields. At this point, approximately 3,000 of sharks, the major and the most popular source of squalene must be killed to obtain 1 ton of squalene. These animals are on the verge of extinction. This situation has caused to focus on finding microalgae strains, which are sustainable producers of squalene as alternative to sharks. This review paper summarizes the recent progresses in the topic of squalene. For this purpose, it contains information on squalene producers, microalgal squalene production and cosmetic evaluation of squalene.

**Keywords:** squalene, microalgae, microalgal process, bioactive compound, cosmetics

## 1. Introduction

Squalene has been discovered in shark liver by Masumaru Tsujimoto at 1916 [1]. This natural product with the molecular formula of $C_{30}H_{50}$ (6 unsaturated bonds) consists of three terpene units (Fig. 1) [2,3]. As a result of its molecular structure, it shows hydrophobic effects. Therefore, its molecular weight and density are 410.5 g/mol and 0.858 g/cm$^3$, respectively [4].

Squalene is found in animal and plant cells [1,5]. Here, it is synthesized in the endoplasmic reticulum and stored in vesicles or transferred to the cell membrane by vesicles [1,6]. In human body, the skin is considered as the biggest organ and divided to three main layers as epidermis, dermis, and hypodermis [1]. The epidermis layer is a dynamic texture. The cells in the outermost layer of this tissue die as a result of their malnutrition [7,8]. At this point, a mechanism called keratinization comes into play and ensures the regeneration of cells. As a result of this, the color of the skin, smoothness of the skin surface, water retention and general appearance are determined [7]. The dermis consists of blood vessels, nerves, muscles, hair follicles, sweat glands, and sebaceous glands [9]. The sebaceous glands secrete the excretion which is called as sebum, which includes ~12-13% squalene (Fig. 2) [1,3]. The blockage and microbial contamination of these glands can cause acne. The last layer, the hypodermis, contains fat cells [9]. The skin is faced with aging as a result of internal and external effects in time. Internal effects cause the veins to appear more prominent, thinning the skin and decreasing its elasticity. Also, external effects can be defined as ultraviolet (UV) radiation and the associated irritation. As a result, wrinkles, sagging and color changes are occurred [9,10]. These appearance changes are usually due to the deterioration of the dermis layer. These degradations can be explained as a decrease in collagen gene expression, fibroblast activity, and reduced the moisture holding capacity of the skin caused by narrowing in the lipid layers [8]. At this point, in considering that UV radiation has important effect in collagen degradation [8,11,12]. Actually,

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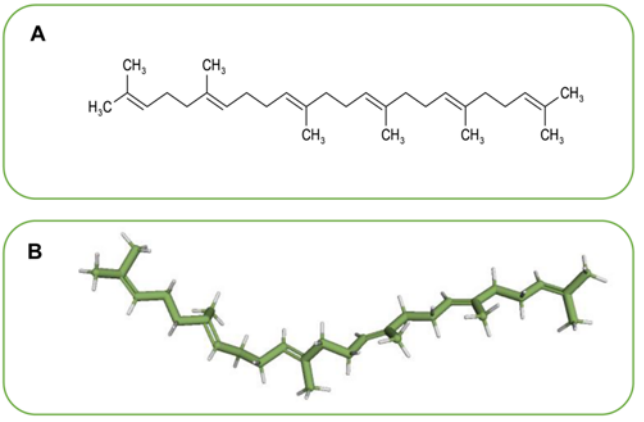


**Fig. 1.** (A) Chemical structure of squalene in two-dimensional. (B) Chemical structure of squalene in three-dimensional.

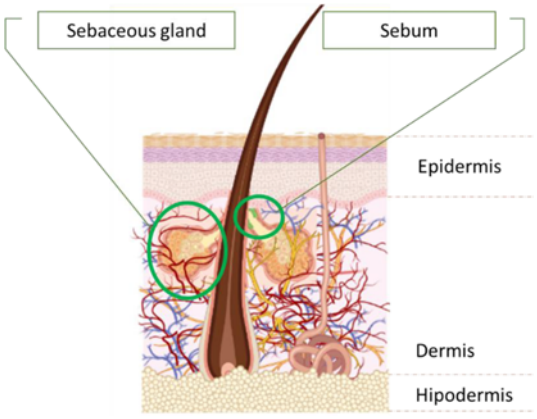


**Fig. 2.** Ultrastructure of skin.

the skin naturally produces antioxidant agents that have ability to inhibit reactive oxygen species caused by UV radiation. Unfortunately, this mechanism can be overcome by exposure to UV light for a long time and, thus oxidative stress can be occurred. Eventually, this stress can affect proteins, lipid membranes, and DNA breakdown to may cause cell death. As a natural consequence, skin dryness and wrinkles are ensued [9]. In this view, the use of antioxidant compounds in cosmetic products can reduce oxidative stress, which is the main mechanism in skin aging.

Squalene has ability to protect the skin from the damages caused by UV radiations. In addition to that, this compound shows important antioxidant, anticarcinogenic and antiinflammatory effects on the human body [1,3,6,13,14]. For example, it improves the skin texture via softening it [1]. Also, it reduces pores, wrinkles, tone difference caused by skin aging. Besides, it moisturizes the skin without showing toxic and irritant effects [1,10,15,16]. In this way, it prevents the formation of acne and even eczema on the skin [10]. Additionally, it also induces the immune system against several diseases like $H_1N_1$, leukemia, papilloma, and furuncle. Further, it shows antiproliferative effects for skin, lung, colon, and breast cancer cells. For this reason, it is used to wrap up the active ingredients in anticancer and antiviral drugs [2,3,14,17-20].

## 2. Squalene for Cosmetic Purposes

A cosmetic product can be defined as an any substance that implemented to human body for the purpose of hygienizing, perfuming, protecting, modifying its physiognomy and keeping it in good state [8,9,11,12,21,22]. Since the earliest times of history, these products (emulsion, lotion and cream formulations, *etc.*) have always been the focus of attention. In 2020, these products market value has stated as €200 billion by the company named L'Oréal. As a result of the statements made by the company, long-time leader of the cosmetics industry, even during the transition from 2019 to 2020, the sales speed of cosmetics over the internet increased by 40%. One of the reasons for this is that online shopping has become easier with the increase in technological developments. Another reason is that online shopping has become more popular with the COVID-19 epidemic. A more detailed examination on cosmetics market indicates that the most popular products are skin care products with 42%. Today, many companies use squalene as an ingredient in their skin care products (Table 1). For example, Amyris, the most prominent of these companies, sells squalene, which is obtained from sugarcane, as a cosmetic ingredient. This company offers products containing squalene under the Biossance brand and has won the Women's Health Beaty Awards (2020), Marie Claire Skin Awards (2020), Beauty Inc (2021), Allure Best of Beauty Awards (2020) with these products. As a result of market researches, the market value of this bioactive compound in 2020 has been determined as 129 million dollars. It has also been estimated that this value will grow by more than 6% annually and reach 184 million dollars in 2025. The leaders in this market are listed as follows: Asia, Europe, and North America. In additionally, it has been stated that its use for cosmetic purposes is increasing day by day in countries such as France, Germany, China, India, South Korea, Singapore, Indonesia, and Malaysia.

Nowadays, squalene has many utilization areas. For example, it is included in different cosmetic formulations such as moisturizers, face creams, and anti-aging creams. In addition to those, it is also added to shower oils, body powders, lotions, ointments, lipsticks, eye makeup, sunburns, and nail products. The reason of using squalene in such a variety of products can be explained as having anti-inflammatory, detoxifying, moisturizing and antioxidant

**Table 1.** The companies that include squalene/squalane in their cosmetic products, and their products

| Company | Product |
|---|---|
| Biossance (Amyris Inc.) | **Squalane** + Lactic Acid Resurfacing Night Serum (30 mL) |
| | **Squalane** + Vitamin C Dark Spot Serum (30 mL) |
| | **Squalane** + Vitamin C Rose Oil (30 mL) |
| | **Squalane** + Marine Algae Eye Cream (15 mL) |
| | **Squalane** + Omega Repair Cream (50 mL) |
| | **Squalane** + Probiotic Gel Moisturizer (50 mL) |
| | **Squalane** + Phyto-Retional Serum (30 mL) |
| | **Squalane** + Peptide Eye Gel (15 mL) |
| | **Squalane** + Zinc Sheer Mineral Sunscreen (100 mL) |
| | **Squalane** + Rose Vegan Lip Balm (15 g) |
| Omega Skin Lab | Granactive Retinoid 1,5 In **Squalene** Serum (30 mL) |
| | Retinol 0,2 Serum In **Squalene** (30 mL) |
| | Retinol 0,5 Serum In **Squalene** (30 mL) |
| | Retinol 1 Serum In **Squalene** (30 mL) |
| | **Squalene** Cleanser Gel (200 mL) |
| The Ordinary (Deciem Beaty Group Inc.) | Retinol 0.5% in **Squalane** (30 mL) |
| | %100 Plant-Derived **Squalane** (30 mL) |
| Jeuvenile Cosmetic | Retinol in **Squalene** %1 + Hyaluronic Acid %1.5 + Panthenol %3 Cilt Serumu (30 mL) |

**Table 2.** The list of companies that use squalene/squalane as an active ingredient in their products

| Company | Active ingredient | Source | Product |
|---|---|---|---|
| Amyris Inc., USA | Squalane | Sugarcane | Skin care product: "Biossance"<br>Adjuvant for COVID vaccines |
| Sophim, France | Squalene<br>Squalane | Shark liver oil<br>Olive oil | Skin care product: "Phytosqualane"<br>Vaccine surfactant: "Squapure" |
| Kishimoto Special Liver Oil Co. Ltd., Japan | Squalene<br>Squalane | Shark liver oil | Squalene<br>Squalane |
| Arista Industries Inc., USA | Squalene<br>Squalane | Shark liver oil<br>Olive oil | Squalene<br>Squalane |
| SeaDragon Marine Oils Limited, New Zealand | Squalene | Shark liver oil | Squalene |
| Nucelis LLC, USA | Squalane | Mutant yeast | Squalane |
| Empresa Figueirense De Pesca, Portegue | Squalane<br>Squalene/squalane | Shark liver oil<br>Olive oil | Squalene<br>Squalene/Squalane |
| Arbee Biomarine Extracts Pvt. Ltd., India | Squalene/squalane | Shark liver oil | Squalene/Squalane |
| New Zealand Green Health Ltd., New Zealand | Squalene | Shark liver oil | Squalene |
| Gracefruit Ltd., Scotland | Squalane | Olive oil | Squalane |

properties. Besides, it can be used in pharmaceutical formulations. It has ability to make the treatment more effective by enabling drugs to enter the cell more easily and stimulating the immune system. For this reason, it is currently used as an adjuvant in many cancer treatments. In addition, it has been added as an adjuvant to vaccines developed against the COVID-19 virus, which has caused the death of many people since 2019. Because of that, there are many companies that use squalene/squalane as an active ingredient in their products (Table 2).

As a result of using sharks as squalene sources by many companies, it has been reported that more than 100 million sharks have been killed just to obtain the amount of squalene required by currently available cosmetic and pharmaceutical products. As a result, it has been stated that their population has decreased by 70% in the last 50 years. Besides that, it has also been estimated that 500,000 more sharks will be needed annually for COVID-19 vaccines alone. As a result of considering this situation, it has concluded that sharks are very close to extinction. Therefore, researchers have been decided to find an alternative squalene source to sharks. After a few studies, it has been proven that plants can be sources of squalene. As it seen in Table 2, there are some companies that used plants as squalene producers. Unfortunately, although there are microalgae species with proven potential to produce

squalene, squalene obtained from any microalgae species has not been used for cosmetic or pharmaceutical purposes yet.

## 3. Squalene Producers

Nowadays, sharks are considered as major sources of squalene [14,23]. Their liver oil consist of squalene with vary wide range as 50-82% [23]. As a result of having high amount of squalene, sharks hunting has increased rapidly. At this point, cosmetic industry is the leader industry where squalene is used the most with 90 percent. Just to meet the squalene needs of cosmetic industry, 2.7 million shark livers have to be taken annually and, thus sharks are faced with the risk of extinction. Also, quality of the squalene derived from sharks is decreased with increasing sea pollution and chemical contaminants [2,24,25]. Also, their liver oil contents change according to climatic conditions [26]. In case of long-term productions, it is a problem that the quality and quantity of squalene is so uncontrollable. However, due to the bad odor of squalene produced by sharks, researchers have considered to find new sustainable sources for the production of squalene [24,26-28].

Researchers have proven that there are many plants (olive oil, amaranth, wheat germ, rice bran) with having ability to produce squalene [1,5,23,29]. These plants are considered as good alternatives to sharks for squalene production. For example, amaranth oil contains squalene as 69.5 mg/g. Also, olive tree, the most used plant as a squalene source, includes squalene as 7 mg/g in its leafs [23]. For plants cultivation processes, large lands are needed. Even if this need is met, they are easily affected by climatic changes, and thus they are not suitable for long-term productions [9,14]. For dealing with this significant problem, they can be cultivated in bioreactors which are a closed systems. Unfortunately, plant cells are not resistant to shear stress and tend to form aggregates while cultivating in bioreactors. As a result, their cultivation processes seem quite difficult due to low respiratory rates. Besides, they are inclusion in the food chain and, thus they can cause the ecosystem deterioration if they are used for squalene production. In addition to that, it has been mentioned that they are approximately 30% more expensive than shark-derived squalene [30]. Therefore, researches have been drawn attention about finding new squalene sources instead of sharks and plants [31].

Researches have been showed that microalgae have ability to produce squalene [14,31,32]. There are significant advantages for using these microorganisms as squalene sources. For example, their cultivation processes can be managed easily and successfully. Also, they have high growth rates in different environmental conditions and produce more amount of oils in the same land area compared to plants [10,16,25,33-38]. Thanks to these important features, they are seen as promising sources of squalene. However, the use of microalgae as squalene producers, both of marine animals, and plants consumed as food will be protected [30]. In addition to this, the remained part of microalgae, after squalene removing from their biomass, have high carbohydrate and protein contents. As a result, they have the potential to be used in biohydrogen, bioethanol, biogas, methane production and animal nutrition [38-46]. Consequently, microalgae are considered as the most advantageous squalene producers compared to other sources.

## 4. Microalgae as a Squalene Source

Microalgae that squalene producers are called *Thraustochytrids*. From a taxonomic view, these biotechnologically important microorganisms are in Thraustochytriacae family and characterized by their oval thallus and ectoplasmic threads that help them for attachment and feeding. Additionally, this family belongs to the Labyrinthulomycota class, which contains mostly marine, fungus-like, and plastid-free single-cell organisms. This class belongs to the phylum named Bigyra in Stramenopila kingdom, where non-plastidial and single-celled organisms exist. Stramenopila kingdom includes eukaryotic protists and algae which have two symmetric flagellata zoospores. For this reason, although *Thraustochytrids* are not able to photosynthesize and lack plastids, they are called microalgae in many scientific studies because they are in the Stramenopila kingdom [47-53]. However, in its most summarized form, *Thraustochytrids* can be considered as marine fungus-like Stramenopiles. More detailed, they are divided into different genera according to their life cycles. For this reason, after discovering new microalgae species as squalene producers, their phylogenetic analyzes should be completed by examining the life cycles of them. As a result of these analyzes, there are some patents numbered as US9249419B2 and US9476074B2 in which microalgae species are named. Also, it can be easily said that the most studied genera can be listed as *Schizochytrium*, *Aurantiochytrium* and *Thraustochytrium* (Fig. 3).

*Thraustochytrids* heterotropically growth and produce significant amount of lipid as 30% of their biomass. One of their lipid fragments is squalene and its synthetized via mevalonate pathway as shown in Fig. 4 [1,5,13,25,54,55].

In this mevolanet pathway, firstly two acetyl-CoA molecules are turned into acetoacetyl-CoA by thiolase enzyme. After that, one acetyl-CoA molecule is added to

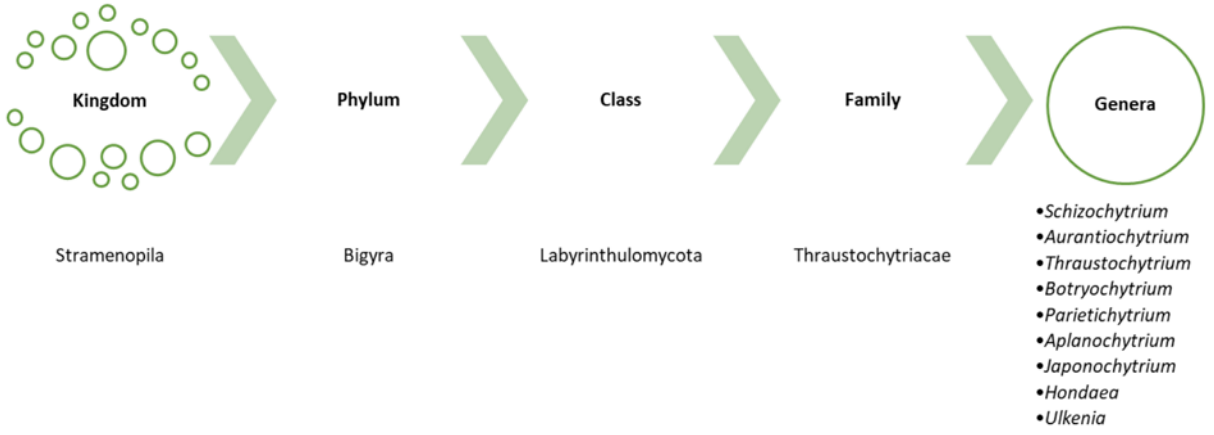


**Fig. 3.** Summarized taxonomic classification of *Thraustochytrids* [1,16,86-89].

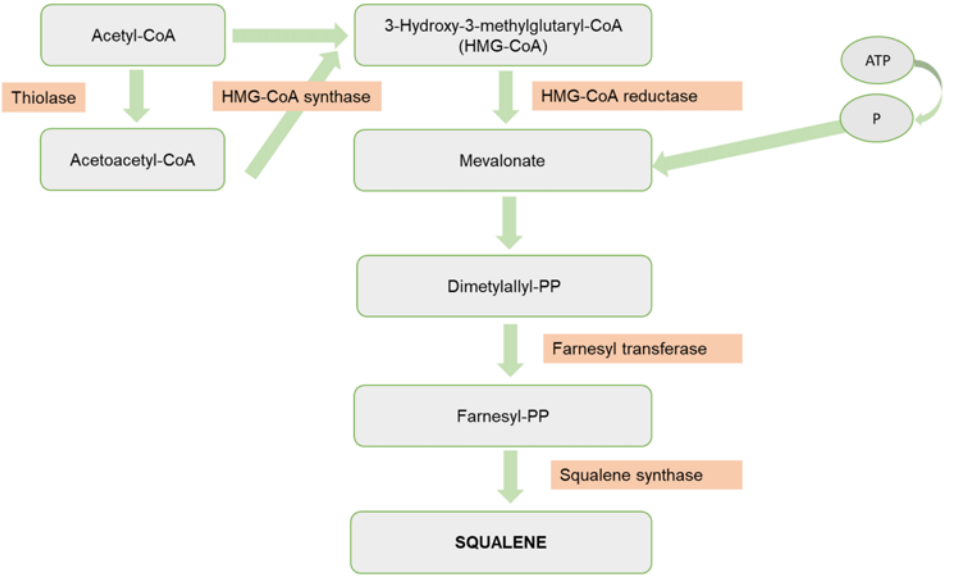


**Fig. 4.** Squalene synthesis in mevolanate pathway. ATP: adenosine triphosphate.

acetoacetyl-CoA via 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase enzyme. Following, HMG-CoA reductase enzyme converts HMG-CoA to mevalonate [54]. Released two phosphate molecules from adenosine triphosphate are applied to mevalonate and form dimetylallyl-diphosphate. At this point, farnesyl transferase converts this molecule to farnesyl-diphosphate. After then, this molecule is turned to squalene via squalene synthase enzyme. Thereafter, squalene is oxidized and named as 3S-squalene-2,3-oxide. Finally, after several sequential chemical reactions 3S-squalene-2,3-oxide is converted to sterol [13]. Sterol and squalene, an intermediate of sterol biosynthesis, are presented in the non-saponified fraction of lipids and both of them are included in the cell membrane and vesicles [1,5].

### 4.1. Microalgae cultivation for squalene production

Microalgal squalene production can be completed by going through different stages as shown in Fig. 5. Firstly, the selected microalgae strain should be made as a stock culture. The culture is then transferred into liquid nutrient medium. If the culture reaches the desired growth, it can be transferred to the Erlenmeyer flask and from Erlenmeyer flask to the bioreactor, respectively. After the targeted production of biomass has been achieved, extraction of squalene from the biomass can be performed. Finally, analyzes can be performed to determine the cosmetic use potential of obtained squalene.

Researches have been showed that *Thraustochytrids* have ability to survive until the carbon source in the nutrient medium is exhausted, and they switch to lipid accumulation then the nitrogen source starts to deplete [56]. Therefore, it is seen that the amount of carbon and nitrogen sources in the cultivation medium is highly effective on the growth curve of the culture. During the


**Fig. 5.** A flow chart of obtaining squalene from *Schizochytrium limacinum* for cosmetic analyses (courtesy of the authors). HPLC: high pressure liquid chromatography.

growth of the culture, they produce squalene in each their growth phases as lag, log, stationary, and death to ensure the cell integrity. However, as the culture progresses in the exponential phase, squalene production continues on the one hand and sterol production continues as squalene oxidizes on the other. As a result of that, the amount of this bioactive substance varies according to the phases they are in [57]. Since, squalene is very easily oxidized to sterol types, the amount of squalene undergoes a rapid decrease during the transition to the stationary phase [58,59]. For this reason, it is thought that the degradation of squalene should be stopped/slowed down by preventing this oxidation reaction in the exponential phase where squalene is mostly produced. There are some researches carried out about microalgal squalene production on the synthesis steps in the mevalonate pathway. As a result of detailed examination of this pathway, it has been reported that some of the chemicals can be used to increase squalene production (Table 3). For example, malic acid, one of these chemicals, activates the malic enzyme, increases the formation of acetyl-CoA, and as a result, provides more squalene production. In the case of exposing to nitrogen starvation caused by using a nitrogen source at a lower concentration than the culture needs, squalene production can be increased by converting carbon sources to acetoacetyl-CoA [56,60]. On the other hand, adding docosahexaenoic acid (DHA) decreases HMG-CoA enzyme activity, thus, causes to reduce in squalene production [4]. Besides these chemicals, terbinafine has also effect on this production process. It inhibits squalene monooxygenase enzyme and, by this way, it increases squalene production. There is a study that include *Aurantiochytrium mangrovei* FB3 cultivation with terbinafine. In this study, produced squalene concentration has increased nearly 36-40% and reached 0.53 mg/g [61]. In another study, *Aurantiochtyrium* sp. 18W-3a has cultivated with methyl jasmonate. As a result, the amount of squalene has been 1.17 mg/g and this amount 60% higher than in the production without this chemical [52]. Besides these studies, also there is a patent numbered as JP6265407B2 mentioned that about using Shochu, a distilled Japanese drink, as an alternative to the nutrient medium included glucose, tryptone and yeast extract during the cultivation of *Aurantiochytrium* sp. 18W-13a. In this patent, it has been proven that a higher amount of squalene can be produced by the wastewater utilization. In addition to these studies using different chemicals in the nutrient medium, there is a patent in which squalene is produced by making genetic changes on the mevalonate pathway. In this patent numbered WO2012159980A1, it has been proven that the mutant *Schizochytrium* sp. obtained by mutagenesis or gene transfer can produce more squalene.

### 4.2. Microalgal squalene production in bioreactor scale

Bioreactors are considered as closed systems that provide an opportunity for production processes in controlled conditions. Using these systems in microalgal cultivation enables to produce high amount of target biomass and bioactive compounds in a short span of time. There are different cultivation modes as batch, fed-batch, continuous and semi-continuous in cultivation at bioreactor. In batch process, both of the culture and fresh medium are added into the bioreactor at the same time and discharged end of

**Table 3.** The chemicals that affect squalene production potential [4,52,56,60,61]

| Chemical | Effect | Squalene production potential |
|---|---|---|
| Malic acid | Increases the acetyl-CoA formation | Increases |
| Nitrogen at low concentration | Converts carbon sources to acetoacetyl-CoA | |
| Terbinafine | Inhibits squalene monooxygenase | |
| Methyl jasmonate | Induces squalene synthase | |
| Shochu | Promotes mevalonate pathway | |
| DHA | Decreases HMG-CoA enzyme activity | Decreases |

DHA: docosahexaenoic acid, HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA.

the cultivation. In fed-batch cultivation, feeding is carried out with fresh medium which contains the culture or not while the culture in the bioreactor is not discharged until the end of the process. In continuous process, feed solution is added to the bioreactor while continuously draining the cultivation medium from this bioreactor until the cultivation is completed. In semi-continuous cultivation, this feeding and discharging processes are realized at intervals [62-64]. The number of the studies on *Thraustochytrids* cultivation in bioreactors for the purpose of squalene production is quite few. In one study, *Aurantiochytrium limacinum* SR21 cultivation has performed as batch process in 5.6 L bioreactor with 3.5 L working volume [65]. In another study, *Schizochytrium* sp. HX-308 as fed batch culture has performed in 50 L bioreactor with 30 L working volume [24]. Both of these studies have carried out just for biomass and DHA production, not for determining their squalene production potential [24,65]. Also, there are two different studies about *Aurantiochytrium* sp. T66 cultivation. One of these studies has performed as fed batch process in 2.8 L bioreactor, but its squalene production potential has not been evaluated [66]. In the other, this species has cultivated as batch culture in 1 L bioreactor and it has been proven to be a source of squalene [58]. In addition, it has been also noticed that *Schizochytrium limacinum* SR21 can be a squalene source as a result of the study on its cultivation in 1 L bioreactor [59]. However, there is no study about determining the effects of cultivation modes on squalene production from *Thraustochytrids*.

Researches have been proven that cultivation conditions have unique effect on squalene production by *Thraustochytrids*. Generally, cultivations have performed in a wide range of temperature as 20-30°C. The target temperature value has controlled by using an electric heating jacket or water jacket [57,67]. In addition, pH value has selected in a range of 6.5-7 and its control has provided by peristaltic acid and base pumps connected to the online control unit [67-70]. Most importantly, it has been noticed that these microalgae cultures produce higher amounts of squalene at low dissolved oxygen levels (nearly 10%) compared to their cultivation at high dissolved oxygen levels (higher than 50%). This cultivation parameter has arranged by controlling the agitation and aeration rates that in a range of 1-2 vvm and 200-300 rpm, respectively [57-59,66,71]. Anyway, the effects of these parameters on squalene production can be determined by performing cultivations while one of them applied in different values selected as a range, provided that others are kept constant in cultivation. However, in this case, the effects of the different parameters' interactions at different values on the production yield cannot be determined. For avoiding this situation, optimization process need to be done by using the programs (Design Expert, Statistical Package for The Social Sciences [SPSS], *etc*.) that show this interactions' effects. Since, this issue is very important, the study that about the squalene production by cultivating *Schizochytrium* sp. CNCM-I4469 in a 20 L bioreactor via optimization process has deemed suitable for patenting and has numbered US10087467B2.

### 4.3. Extraction and quantification of squalene from microalgal biomass

As mentioned before, squalene is an intermediate compound generated during sterol synthesize. Because of that, it is very sensitive to oxidation [57]. For this reason, the variety of squalene extraction, and quantification methods is quite limited. So, these methods can be summarized as mentioned in this section. Firstly, for the purpose to obtain squalene from biomass, the total lipids should be extracted from microalgal biomass. At this step, biomass has to be harvested for removing unwanted liquid content of culture medium. This process can be done by centrifugation, filtration, *etc*. Then, it should be completely dried by lyophilization and made ready for lipid extraction. This extraction process is usually done according to the Bligh and Dyer method or its modified versions. In this standard method, the dried biomass is transferred to glass vial or balloon and a chemical solution containing chloroform/methanol/water (2:1:0.8, v/v/v) is added to it. At this point, the lipid fractions are collected in the chloroform portion of this solution. Then, this chloroform layer can be transferred to new pre-weighted glass vial or balloons and evaporated to determine the mass of the lipid fractions [34,58,61,71,72]. Besides these methods, there is a patent numbered WO2012164211A1 in which the total lipid with higher squalene content has extracted by using protease enzyme instead of using organic solvents for cell disruption.

Squalene, one of the fractions in the total lipids, can be separated from the remaining fractions of the total lipids using the extraction method called as saponification. In this process, the selected chemical solution is added to the total lipids and the reaction is completed by keeping in at a certain temperature and time. At this point, it is ensured that the total lipids are separated into its saponifiable and non-saponifiable fractions. Henceforth, the non-saponifiable fraction which include squalene can be extracted by adding selected solution. For example, in one study, 10% KOH-ethanol solution has transferred into the total lipids and the sample was incubated at 60°C for 1 h. Thereafter, the non-saponifiable fraction has extracted by adding water/n-hexane (4:1, v/v) [4]. In another study, the saponification process has completed by adding 0.5 N KOH/methanol solution to the total lipids and waiting at 100°C for 10 min [73]. In additional other studies, this process has carried out by using KOH (10%, w/v)/ethanol (75%, v/v) at 50°C for

15 min, then the non-saponifiable fraction has extracted with n-hexane [74,75]. In the last example studies, 0.5 M ethanolic (75%, v/v) KOH solution as a solvent system has added to total lipids and the sample has incubated at 90°C for 1 h. The non-saponifiable fraction has then collected in n-hexane [58,59]. In addition to these studies, there is also a patent numbered WO2013156720A2 on increasing the squalene content of total lipids around 70-75% by counter flowing them with supercritical $CO_2$ in multiple stages and subjecting them to molecular distillation. After that, purification and quantification of squalene from the obtained non-saponifiable fraction can be handled by using high pressure liquid chromatography (HPLC) or gas chromatography [4,6,13,58,59,72,74-76]. At this point, HPLC is generally used for measurement. Some researchers have dried the non-saponifiable fraction under nitrogen gas and dissolved it in 100% acetonitrile. Then, they have injected the sample into a reversed-phase symmetry C18 column containing 100% acetonitrile with flow rate 1.5 mL/min at 30°C and detected it at 195 nm [72,74,75]. In the others, the sample has dissolved in same solution and transferred into the C-18 reverse-phase column. Then, it has washed with acetonitrile/water (9:1, v/v) solution as a mobile phase with 1.5 mL/min flow rate at 30°C and quantification has completed at UV detector at 210 nm [58,59].

### 4.4. Evaluation of squalene's potential for cosmetic use

Purified squalene has to be determined whether a usable bioactive compound in cosmetic products. For this purpose, its cytotoxicity can be evaluated by the cell viability assays which including the use of tetrazolium salts such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS), sodium 3'-[1-[phenylaminocarbonyl]-3,4-tetrazolium]-bis[4-methoxy6-nitro] benzene sulfonic acid hydrate (XTT) and 4-[3-[4-Iodophenyl]-2-[4-nitro-phenyl]-2H-5-tetrazolio]-1,3-benzene sulfonate (WST-1) [77,78]. In these assays, first step is cultivation of the selected animal cell culture to obtain enough amount of them for analyzation. At this point, cell lines named Vero, 4T1, MCF-7, MCF-10A, MDA-MB-231, HepG2 or B16F10 can be used [79-82]. After that, the cell culture is treated with different concentration of squalene and incubated. Then, the selected tetrazolium salt is applied to the sample and converted to dye formazan. For example, MTT and MTS are turned to purple and detected at 570 nm and 490 nm, respectively [81,83,84]. Additionally, XTT and WST-1 which are transformed to orange color are determined by UV detector at 450 nm [82,84,85]. Additionally, according to this plate read, their EC50 value (the effective concentration of it that gives half-maximal response) and IC50 value (the inhibition concentration of it as an inhibitor where the response is reduced by half) can be calculated. These concentrations help to determine the squalene concentration at which can be added to the formulations in order to avoid its toxic effects [80]. In addition, for determining the suitability of microalgal squalene for cosmetic use, its antioxidant property can be also measured. For this purpose, 2,2-diphenyl-1-picrylhydrazyl hydrate free radical method can be selected as a method which gives the information about its antioxidant activity. This simple method is based on detecting the sample's color changes by UV detector at 517 nm [79]. More detailed, it is known that microalgae produce antioxidants when exposed to stress conditions that caused by switching to an unsuitable range in the parameters required for their growth. Based on this information, it can be aimed to increase the production potential of squalene by exposing them to stress conditions as a strategy during the cultivation of microalgae. Additionally, for making sure that squalene is not change over time, both of squalene and squalene-contain cosmetic formulation should be subjected to stability tests. These tests can be completed by detecting their organoleptic character (appearance, color and odor), physicochemical properties (pH and viscosity), and microbial stability at certain intervals.

In our previous study, it has been proved that crude methanolic extract derived from *S. limacinum* has shown significant antioxidant efficiency with 90 ± 0.1% radical scavenging activity. Additionally, total phenolic content of this extract was measured as 129 ± 4 mg GAE/g extract which is really high value. Based on the fact that the content of phenolic substances in microalgae plays a unique role in the antioxidant response to UV light exposure, it can be concluded that the antioxidant activity of this microalgae strain is quite high [79]. In our other study, its cytotoxic effect was also evaluated. In the crude extract, IC50 and EC50 values were determined as 607.1 ± 282.6 μg/mL and 0.241 ± 0.152 μg/mL, respectively. The IC50 value of the crude extract is less than 100 μg/mL, suggesting that this extract is highly cytotoxic [80]. Based on this and also the EC50 value above 1 μg/mL, it can be safely said that this extract does not show a strong cytotoxic effect. It is noteworthy that even this mixed crude biomass extract, which contains many primary and secondary metabolites apart from squalene, that shows such a high antioxidant effect and does not have a toxic effect even at high concentrations. In addition, as mentioned above, it can be used as an additive in the nutrition of fish whose biomass is marine animals [40-44]. In addition, it is thought that there are changes that can be observed in the oil content obtained from sharks as a result of marine pollution. Therefore, given the potential safety of squalene produced from microalgae under controlled conditions, it is anticipated

that microalgal squalene may be more suitable for cosmetic use than squalene derived from sharks.

## 5. Conclusion

Squalene, a bioactive compound, with antioxidant activity has been drawn attention by cosmetic industry. In a case of using sharks as squalene sources causes the merciless animal hunting and adversely affects the ecosystem. At this point, microalgae are good candidate as squalene producers with easily cultivation potential. Also, the squalene removed microalgal biomass can be used as a raw material with the biorefinery approach. As a result of using microalgae as a squalene producer, the hunting of sea animals will be prevented and the plants in the food chain will be protected, as well as sustainable squalene production.

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## Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

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