Microbial genetic engineering approach to replace shark livering for squalene

Trends in Biotechnology

CellPress OPEN ACCESS

Review

Microbial genetic engineering approach to replace shark livering for squalene

Alok Patel [1,*] Maurizio Bettiga,[2,3] Ulrika Rova,[1] Paul Christakopoulos,[1] and Leonidas Matsakas[1]

Squalene is generally sourced from the liver oil of deep sea sharks (*Squalus* spp.), in which it accounts for 40–70% of liver mass. To meet the growing demand for squalene because of its beneficial effects for human health, three to six million deep sea sharks are slaughtered each year, profoundly endangering marine ecosystems. To overcome this unsustainable practice, microbial sources of squalene might offer a viable alternative to plant- or animal-based squalene, although only a few microorganisms have been found that are capable of synthesizing up to 30% squalene of dry biomass by native biosynthetic pathways. These squalene biosynthetic pathways, on the other hand, can be genetically manipulated to transform microorganisms into 'cellular factories' for squalene overproduction.

Highlights

Squalene is an isoprenoid hydrocarbon naturally synthesized via the mevalonate (MVA) or methyl-D-erythritol 4-phosphate (MEP) pathways and is the key precursor for cholesterol biosynthesis.

Microbial sources are alternatives to plant and animal sources; however, genetic manipulation of the native squalene biosynthesis pathway can turn microorganisms into a cell factories for squalene production at industrial scale.

Overexpression of MEP or MVA pathway structural genes has been used to boost metabolic flux towards squalene synthesis.

Downregulation or knockout of genes responsible for the conversion of squalene to sterols can enhance the overall titer of squalene in yeast.

Squalene synthesis is higher in thraustochtrids than in any other known microbe; however, further improvement in squalene titer in these marine protists by genetic manipulation of native biochemical pathways remains a challenge.

Boosting the squalene content in microbial sources to replace the shark-based squalene

Squalene (see Glossary) is a crucial intermediary and precursor for the production of all steroid hormones as well as cholesterol in both plants and animals [1]. Squalene serves as a precursor to numerous bioactive compounds including sterols and hopanoids [2]. In microorganisms, such as bacteria, fungi, and protists, it plays an important role in the synthesis of hopanoids, ergosterol, 24-methylenecholesterol, and several other sterols [3–6] (Figure S1 in the supplementary material online). Squalene is in liquid form at ambient temperature and is secreted through human skin [7]. It is a strong natural antioxidant that can protect cells from harmful free radicals and reactive oxygen species by preventing lipid peroxidation [8]. Moreover, it has been demonstrated to inhibit tumorigenesis in colon, lungs, and skin, as well as to exert chemoprotective activity [9,10] and immunostimulatory properties, attracting the interest of the medical and pharmaceutical industries [11]. Squalene is the most prominent component together with polyunsaturated fatty acids in the skin, and is commonly used for its emollient, antioxidant, and hydrating properties in various skincare products [2]. A recent study on rabbits suggested that dietary squalene can enhance plasma total cholesterol (non-esterified cholesterol in intermediate-density lipoprotein and large low-density lipoprotein) without increasing the concentration of triacylglycerols [12]. According to Allied Market Research, the global market for squalene was US$110 million in 2015 and is expected to reach US$214 million by 2022[i]. According to another report, the squalene global market size was US$129 million in 2020 and is expected to grow at a CAGR of 7.3 percent to US$184 million by 2025[ii].

Currently, the primary commercial source of squalene is the liver of deep sea sharks [13]. Squalene typically accounts for 40–60% of the shark liver mass. In sharks such as *Centrophorus moluccensis* (smallfin gulper shark), almost 83% of the liver mass is composed of oils (**shark liver oil**), of which about 95% is squalene [14]. Given the growing demand for squalene (Figure 1), its rising cost because of diminishing supply, and concerns about environmental sustainability, there is a need to develop alternative sources of this high-value product. Squalene

[1]Biochemical Process Engineering, Division of Chemical Engineering, Department of Civil, Environmental, and Natural Resources Engineering, Luleå University of Technology, SE-971 87 Luleå, Sweden
[2]Department of Biology and Biological Engineering, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden
[3]Bioeconomy Division, EviKrets Biobased Processes Consultants, Landvetter, Sweden

*Correspondence:
alok.kumar.patel@ltu.se (A. Patel).

content is negligible in most foods except some plant species. Olive oil contains a higher amount of squalene, 200–700 mg/100 g of biomass, than other crops. Amaranth seeds are another good source of squalene (6–8% of oil), but oil accounts for only 4–6 % of seed biomass [15]. Furthermore, plant and animal sources must complete their life cycle before being harvested, and this can take 6 months to 1 year. Therefore, the productivity of squalene (in mg/g/day) from plant sources is very low. Indeed, the amount of squalene obtained from plants is insufficient to meet worldwide industrial or food-supplement demands [16]. The Thraustochytriaceae comprise a family of marine protists which could compete with plants and animals as a sustainable source of squalene owing to their fast growth and elevated toxin-free squalene content (Figure 1). In addition, squalene biosynthesis has been reported in some yeasts and fungi (Figure 1), although their output is lower than of marine thraustochytrids. The yeast and fungal strains *Saccharomyces cerevisiae* [17], *Torulaspora delbrueckii* [18], *Aspergillus nidulans*, and *Saccharomyces uvarum* [19] have been shown to accumulate 1.6, 0.24, 0.3, and 14.3 mg/g squalene on a per cell dry weight (CDW) basis, respectively. Thraustochytrids accumulate squalene at up to 316.64 mg/g CDW using sugar substrates [20]. Taken together, previous studies suggest that bioprocess optimization including media (e.g., nitrogen sources) and cultivation parameters (e.g., temperature) can remarkably enhance cell growth and squalene content/yield by thraustochytrid strains. Nevertheless, the experimental designs for bioprocess optimization towards enhancing squalene production in thraustochytrids remain poorly described. Furthermore, **genetic engineering of microbes** seems to be the only potential way to produce squalene at a large scale. Although metabolic engineering aimed at improving squalene content has been attempted in yeast and bacterial strains, genetic approaches seeking to boost squalene production by thraustochytrids remain scarce. Recent attempts to improve squalene production through genetic modification of these microorganisms have focused on (i) overexpression of the native rate-limiting enzyme, **3-hydroxy-3-methylglutaryl-CoA reductase (HMGR),** encoded by the *HMG1* gene [21], (ii) optimization of the native metabolic pathway [22], (iii) improving NADPH and acetyl-CoA supply [21], (iv) co-compartmentation engineering, and (v) bioprocess engineering including C/N ratio optimization and terbinafine supplementation [21]. To build strains that produce valuable chemicals at significantly higher rates and titers, genetic engineering tools have become essential for understanding specific metabolic processes [23]. A lack of genetic tools and an inadequate understanding of the physiology of the target organism are two main barriers to using non-model species for metabolic engineering [23]. To date, only a few strains of the thraustochytrid *Aurantiochytrium* (*Schizochytrium*), such as CCAP_4062/1, SR21, T66, CCTCC M209059, and KH105, have been explored for their genomic sequences [24–28]. Genomic sequences and gene targets for genetic manipulation are reasonably straightforward to obtain because of advances in sequencing technology and declining costs. However, the fact that genetic-alteration tools are only accessible for a restricted number of species is a major hindrance to understanding the cell biology of these diverse eukaryotes [29]. No universal protocol has been developed so far that can be used for marine thraustochytrids across different taxa [30]. In this review we summarize possible ways of tailoring the metabolic network of bacteria, yeasts, and thraustochytrids to improve squalene synthesis.

Shark livering: an inhuman practice

More than a quarter of shark species are overexploited for economic reasons, according to the Food and Agriculture Organization (FAO) [31]. The swim bladder is a gas-filled organ in most fish that helps them retain buoyancy in the ocean and prevents them from sinking. Deep sea sharks, on the other hand, lack a swim bladder and live at depths of 300 to 1500 m in the ocean. Shark liver is filled with oils that make it less dense than water to support neutral buoyancy without the expenditure of high amount of energy. A shark liver can account for up to 20% of its total body weight, depending on the species. For squalene production, **shark livering** is a cruel

Glossary

Genetic engineering: the process of manipulating one or more genes in the genetic constitution of an organism by using recombinant DNA technology.
3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR): the rate-limiting enzyme of the MVA pathway which is responsible for the conversion of HMG-CoA to mevalonic acid in cholesterol biosynthesis.
Methyl-D-erythritol 4-phosphate pathway (MEP) pathway: an alternative non-MVA pathway for the synthesis of isoprenoid precursors that is predominantly present in bacteria and plants.
Mevalonate (MVA) pathway: also known as the HMGR pathway, this is usually present in eukaryotes and some bacteria for the biosynthesis of isoprenoid precursors such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) that are further converted into terpenoids.
Shark finning: similarly to shark livering, fins are extracted, and the remaining body is discarded into the sea.
Shark livering: an inhuman and cruel practice in which the liver is removed from sharks and the remainder of the body is discarded into the water.
Shark liver oil: swim bladders help many fish to stay afloat. Because sharks lack swim bladders, they rely on their large oil-filled livers to keep them afloat.
Squalene: a triterpene; the term squalene derives from the genus of shark *Squalus* liver oil from where it was originally extracted.
Squalene synthase: EC 2.5.1.21; SQS, is the primary rate-limiting enzyme for squalene synthesis by thraustochytrids.
Sustainable development goals (SDGs): 17 SDGs have been adopted by all United Nations (UN) member states in 2015 to fulfill the 2030 Agenda for Sustainable Developments.

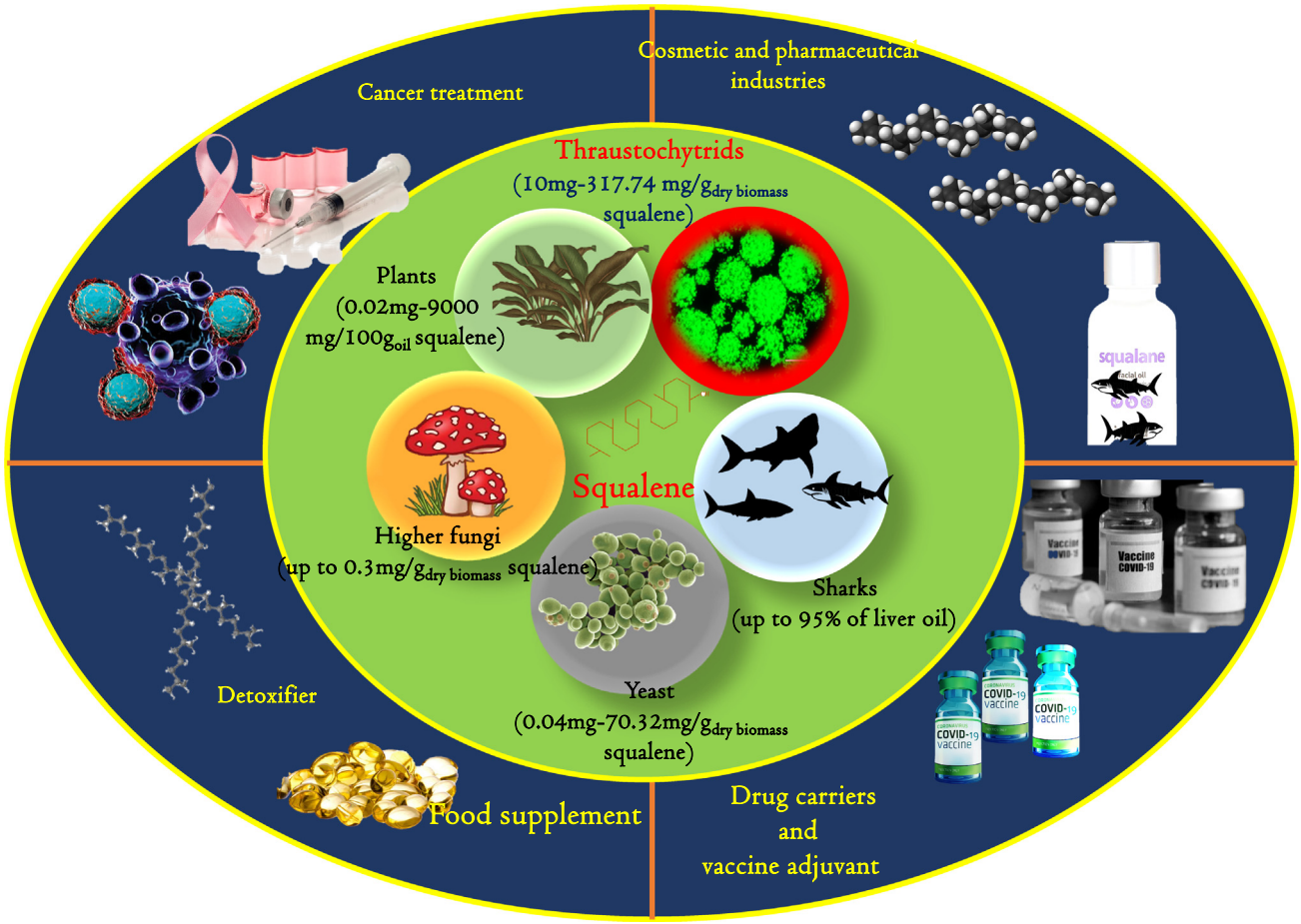

Figure 1. Squalene content in sharks [14], plants [35], fungi and yeasts [45], and protists [58], as well as its uses for medical, food, and cosmetics applications.

practice, similar to **shark finning**, whereby fishermen frequently take only the liver of the animal and discard the remainder of the body into the sea. Sharks are prone to overexploitation because they are slow-growing, mature late in life, and have extended periods between reproductive cycles. As top predators, they are crucial for the integrity of marine ecosystems [32], and many shark species are now listed as vulnerable to extinction by the International Union for the Conservation of Nature [33].

Alternatives to shark liver

Plant sources

Numerous plants have proved to be suitable for the extraction of squalene. Olive oil (486 mg/100 g oil) [34] and amaranth oil (9.87 g/100 g oil) [35] have the highest levels of squalene, with lesser quantities found also in soybean oil (9.9 mg/100 g), grape seed oil (14.1 mg/100 g), palm oil (20–50 mg/100 g), wheat germ oil, peanut oil (27.4 mg/100 g), and rice bran oil (320 mg/100 g) (Table S1). In addition, sunflower seeds (0–19 mg/100 g), sesame seeds (57.2–60.7 mg/100 g), pumpkin seeds (260–523 mg/100 g), and flaxseed (1.0–4.2 mg/100 g) are promising sources of squalene.

Microbial sources

Despite the fact that microorganisms do not accumulate as much squalene as sharks and plants, their rapid growth and amenability to metabolic engineering make them an attractive choice for squalene synthesis. Yeast, bacteria, thraustochytrids, and some species of green microalgae are natural producers of squalene. The oleaginous yeast *Yarrowia lipolytica* [36], the non-oleaginous yeast *Saccharomyces cerevisiae* [37], and the bacterium *Escherichia coli* [38] have been genetically engineered and exploited extensively for the production of squalene. Although only a few strains of microalgae and cyanobacteria produce squalene, the green microalga *Botryococcus braunii* has been targeted for hydrocarbon production [39,40]. *B. braunii* strain Abt02 was reported to contain 43.75% hydrocarbons based on dry biomass, of which 87.54% were squalene and its derivatives [41]. The cyanobacterial strain *Phormidium autumnale* synthesized 0.2–0.6 mg/kg of squalene when cultivated heterotrophically on a mixture of carbon sources from the slaughterhouse, cassava, and brewery wastewaters [42].

Boosting microbial squalene content via genetic engineering

Squalene can be synthesized by two different pathways depending on the type of microorganism. In bacteria and cyanobacteria, squalene is synthesized via the **2-C-methyl-D-erythritol 4-phosphate (MEP)** pathway, whereas eukaryotes including yeast, fungi, microalgae, thraustochytrids, plants, and animals employ the **mevalonate (MVA) pathway**. The first key enzyme of the MVA pathway is 3-hydroxy-3-methylglutaryl-CoA synthase, which is responsible for the conversion of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA. The latter is then reduced to mevalonate by HMGR (Figure 2, Key figure). Mevalonate is subsequently phosphorylated into mevalonate-5-phosphate and then mevalonate-5-pyrophosphate by mevalonate kinase and mevalonate-5-phosphate-kinase, respectively. Next, decarboxylation of mevalonate-5-pyrophosphate into isopentyl diphosphate (IPP) is carried out by IPP synthase, followed by isomerization into dimethylallyl diphosphate (DMAPP) by IPP isomerase. IPP and DMAPP are condensed first into geranyl diphosphate and then farnesyl pyrophosphate (FPP) by FPP synthase. Finally, squalene is formed from two molecules of FPP by **squalene synthase** using NADPH as the cofactor.

Overexpression of the *HMGR* gene

HMGR overexpression can stimulate the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate and finally squalene (Figure 2). Hueng and colleagues suggested that HMGR was a rate-limiting enzyme in *Y. lipolytica* squalene biosynthesis. Indeed, its overexpression led to a 3.2-fold increase in squalene production. Furthermore, overexpression of acetyl-CoA synthetase and ATP citrate lyase together with HMGR increased by 50% the amount of cytosolic acetyl-CoA, which consequently increased squalene content by 16.4-fold [43]. Moreover, after supplementation with 10 mM citrate, the genetically modified strain achieved a maximum squalene yield of 10 mg/g_{CDW} [43]. In another study, overexpression of *HMG1* in *Y. lipolytica* enhanced the squalene titer to 208.88 mg/l, which doubled to 439.14 mg/l after coexpression of diacylglycerol acyltransferase and increased even further to 514.34 mg/l through optimization of the fermentation medium [44]. Finally, after the addition of terbinafine as inhibitor of squalene epoxidase, the yield of squalene was 731.18 mg/l (49.6 mg/g_{CDW}) [44]. When *HMG1* overexpression in *Y. lipolytica* was coupled to NADPH from the mannitol cycle, 188.2 mg/l squalene was obtained using acetate-based medium, increasing further to 502.7 mg/l upon optimization of the C/N ratio, pH, and acetyl-CoA flux [21].

In *S. cerevisiae*, overexpression of the truncated HMG-CoA (*t*)*HMG1* gene in a dual-gene expression system improved squalene content by 16.8-fold compared to a single-gene expression system. Dual expression was limited by NADPH as a cofactor. Overexpression of the NADH-

Key figure

Microbial biosynthesis of squalene via the mevalonate (MVA) pathway

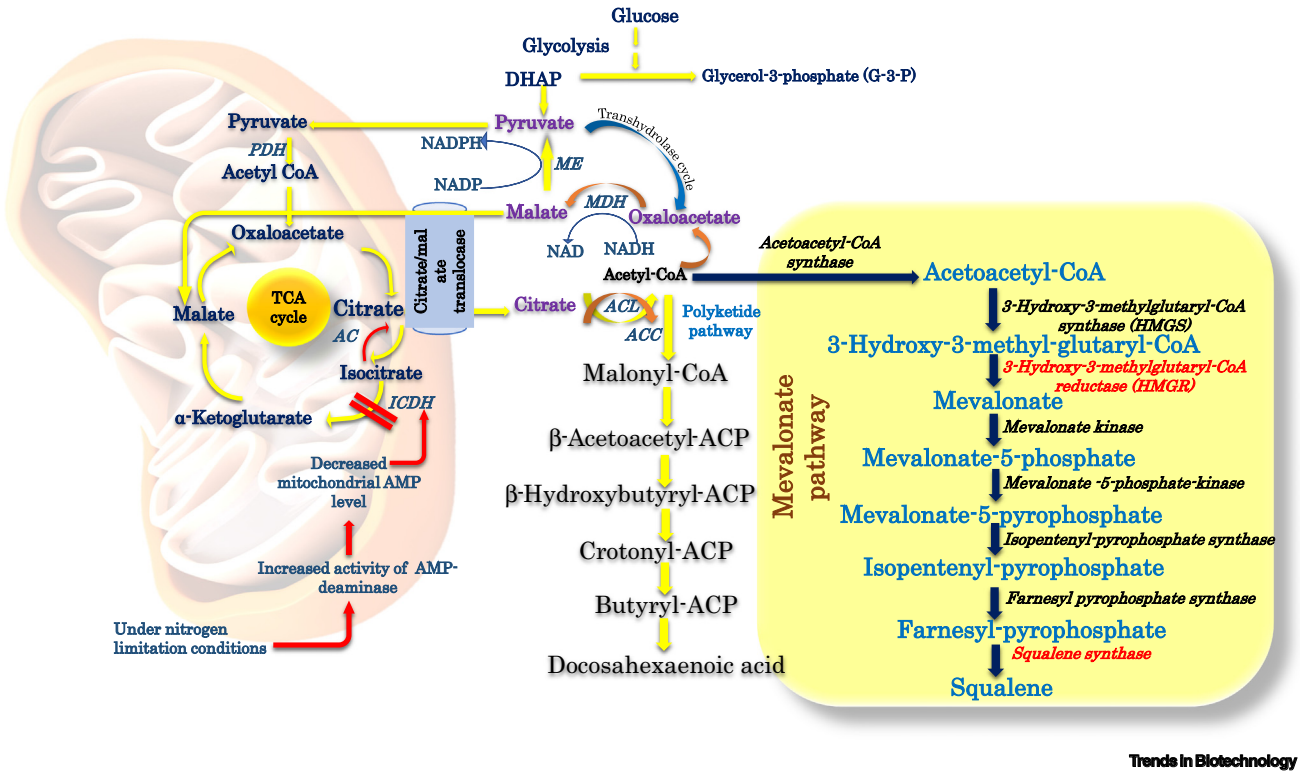

Figure 2. Possible targets of genetic engineering in the MVA pathway, whose modification improves squalene output in thraustochytrids. ACC, acetyl-CoA carboxylase; ACL, ATP:citrate lyase; ACP, acyl carrier protein; DHAP, dihydroacetone phosphate; ME, malic enzyme; MDH, malate dehydrogenase; PDH, pyruvate dehydroxylase; TCA cycle, tricarboxylic acid cycle.

regenerating gene *POS5* together with dual expression of *tHMG1* brought squalene content to 58.6 mg/g_{CDW}, a 27.5-fold increase compared to the original strain [45]. Overexpression of NADH/HMGR from *Silicibacter pomeroyi* in *S. cerevisiae* produced 1086.31 mg/l squalene, whereas coexpression of ethanol dehydrogenase *ADH2* and acetaldehyde dehydrogenase *ADA* from *Dickeya zeae* (a Gram-negative bacterial plant pathogen) improved ethanol utilization in fed-batch mode and yielded 9472 mg/l squalene (220.47 mg/g_{CDW}) [21]. In *Y. lipolytica*, overexpression of a series of rate-limiting enzymes, including acetoacetyl-CoA thiolase (*ERG10*) and *HMG* from the MVA pathway, as well as diacylglycerol acyltransferase (*DGA1*) from the lipid synthesis pathway, coupled to deletion of peroxisomal membrane E3 ubiquitin ligase (*PEX10*) and glutathione peroxidase (*URE2*, coding for a transcriptional regulator that governs nitrogen catabolite repression), enhanced squalene content by 115-fold to 22 mg/g_{CDW} [36].

Overexpression of squalene synthase

Squalene synthase (E.C. 2.5.1.21) is a membrane-bound enzyme that catalyzes the first step in the biosynthesis of sterols and other triterpenoids. Squalene synthase is thought to play an important role in regulating isoprenoid biosynthesis in eukaryotes. Upon overexpression, it speeds

up the conversion of FPP to squalene in some bacterial and yeast species. In the bacterium *Bacillus subtilis*, overexpression of squalene synthase from *Bacillus megaterium* enhanced squalene production 29-fold, from 0.26 mg/l to 7.5 mg/l [46]. Similarly, squalene titer was increased fivefold in *E. coli* upon overexpression of the squalene synthase gene, and then further enhanced by 59% following coexpression of pyridine nucleotide transhydrogenase (UdhA) [38] (Figure 3). Finally, squalene production was further improved to 28.5 mg/g_{CDW} (52.1 mg/l), corresponding to 104% of the original value, by overexpressing the *zwf* and *pgl* genes from the Entner–Doudoroff and pentose phosphate pathways [38].

Downregulation of squalene epoxidase

The steroid biosynthetic pathway is highly conserved and is key in eukaryote evolution. The flavoprotein squalene epoxidase catalyzes the first oxygenation reaction and rate-limiting step in this pathway. Downregulation or knockout of the genes responsible for the conversion of squalene to sterols and further to steryl esters has been shown to change the overall titer of squalene in yeast and the thraustochytrid *Aurantiochytrium limacinum* SR21 [47,48]. Steryl esters are synthesized by acyl-CoA:sterol acyltransferase (ASAT) through acylation of the hydroxyl group at the C3-position of sterols [48]. Disruption of ASAT is responsible for enhanced squalene synthesis in yeast cells [49]. Squalene titer in the bacterium *Rhodopseudomonas palustris* was enhanced by 57% after blocking the pathways involved in the carotenoid synthesis and by 50-fold after blocking hopanoid and carotenoid synthesis (Figure 4). Squalene content was 178-fold higher (23.3 mg/g_{CDW}) upon overexpressing the rate-limiting enzyme 1-deoxy-D-xylulose-5-phosphate synthase from the MEP pathway [50].

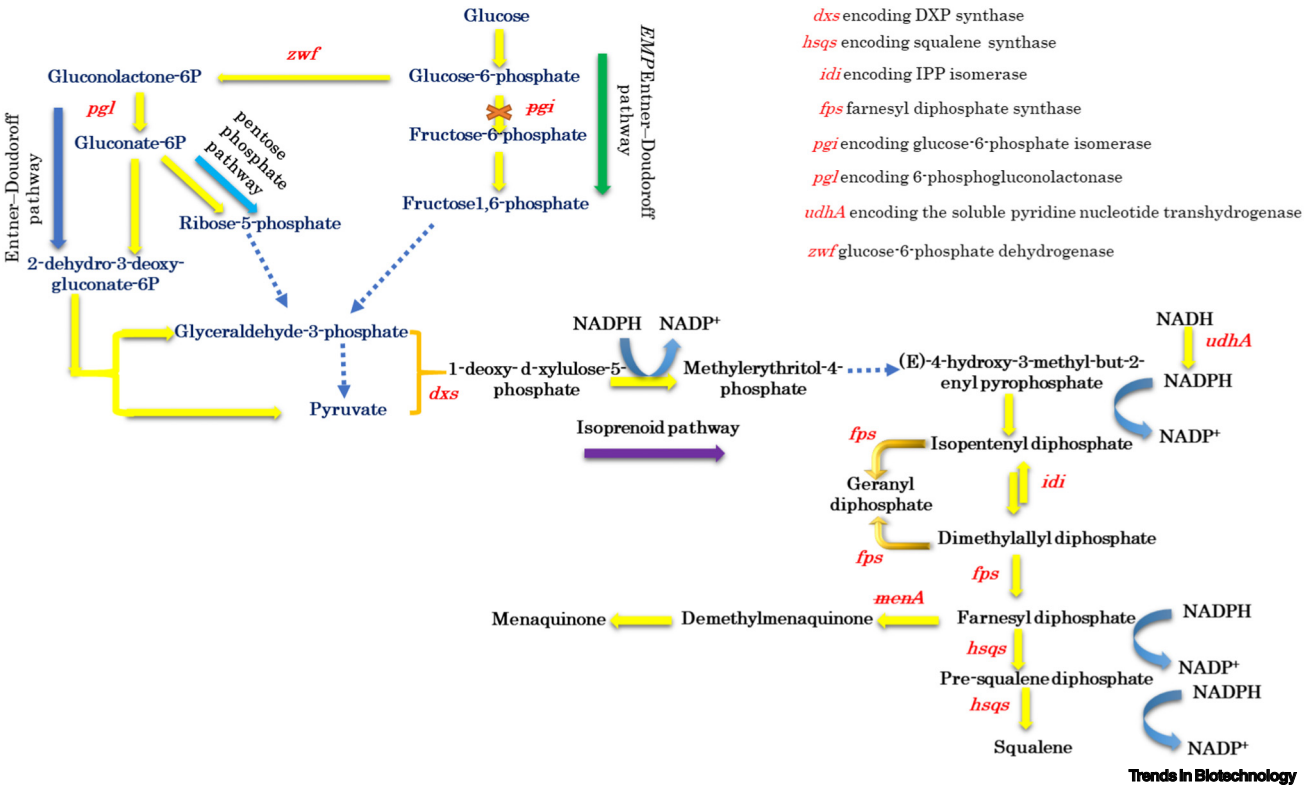

Figure 3. Biosynthesis of squalene through the isoprenoid pathway and the various modules feeding into it. Dashed arrows indicate multiple enzymatic steps. Abbreviations: DXP, 1-deoxy-d-xylulose 5-phosphate; IPP, isopentenyl pyrophosphate. The scheme of the pathway is reprinted from Xu and colleagues [38] under license CC BY-NC-ND 4.0.

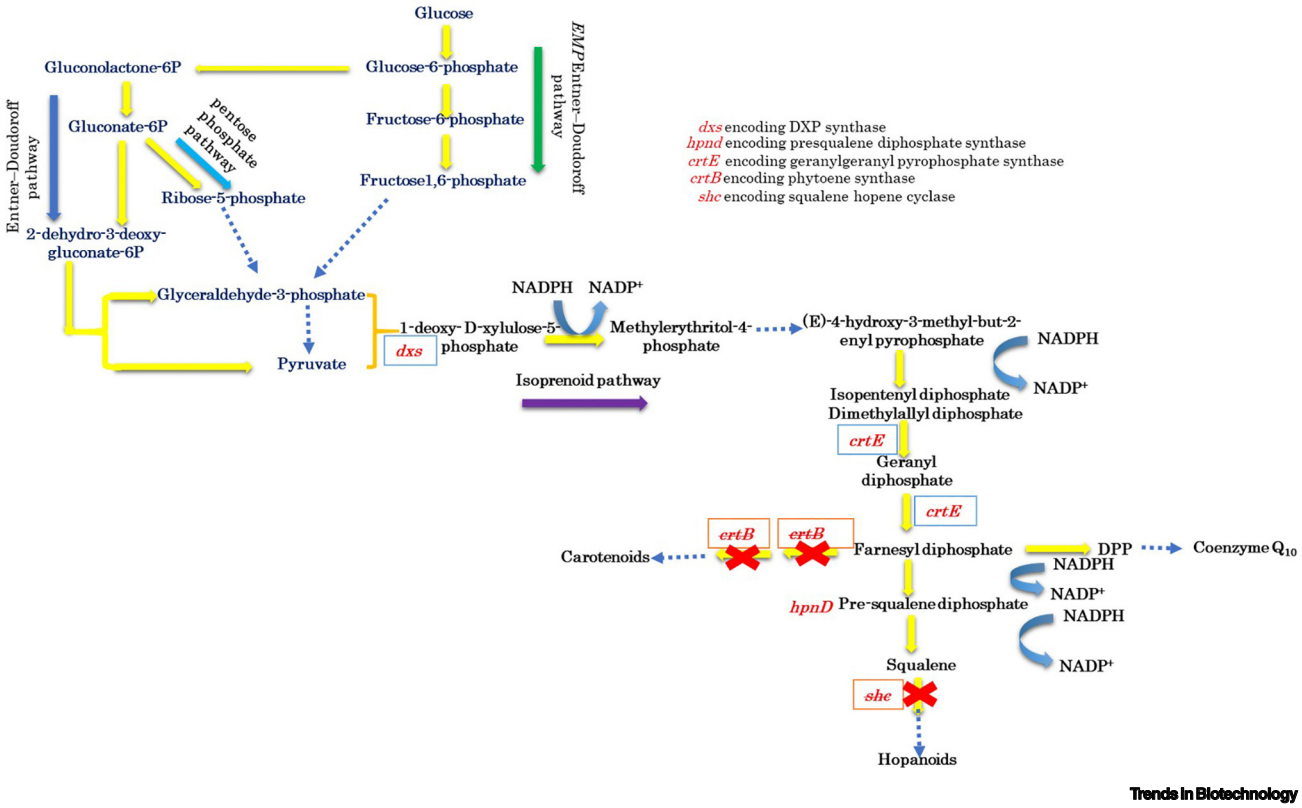

Figure 4. Biosynthesis of squalene and the branched pathways. Abbreviations: DPP, decaprenyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate. Figure reproduced from Xu and colleagues [50] with permission from John Wiley and Sons under license 5264161272333.

Garaiova *et al.* suggested that squalene biosynthesis could be increased by mutating the *ERG1* gene encoding squalene epoxidase that participates in ergosterol synthesis in *S. cerevisiae.* The two mutants *erg1L37P* and *erg1Q443UAG* showed decreased squalene epoxidase and consequent accumulation of over 1000 µg squalene per 10^9 cells [51]. Hull *et al.* suggested that complete deletion of *ERG1* hampered the growth of *S. cerevisiae* and that squalene production could also be enhanced by partial disruption of *ERG1* through transcriptional control via the doxycycline-repressible *tet07-CYC1* promoter. The resulting strain synthesized a high amount of squalene (7.89 ± 0.25 mg/g_{CDW}) when the cultivation medium was supplemented with water-soluble carbohydrates from grass juice and doxycycline [52]. An entirely different strategy, based on expanding the cell membrane, was employed to enhance the squalene titer in *E. coli*. Accordingly, expression of the pTispAErg9TC squalene synthesis module produced 272 mg/l squalene, which was further enhanced to 612 mg/l upon concomitant expression of the membrane elongation *Tsr* gene [53].

Overall, despite of various attempts to improve titers, squalene content (mg/g_{CDW}) in bacteria and yeast has nevertheless remained below that achieved by wild-type thraustochytrids (Table 1).

Genetic engineering to increase squalene content in cyanobacteria and microalgae

Botryococcus braunii is one of the most prominent microalgal species that can produce squalene by the native squalene biosynthesis pathway [54]. Several investigations have been performed to extract the hydrocarbon and oils from this species, but these efforts have been impeded by the

Table 1. Thraustochytrids and their squalene yields under different cultivation conditions

Thraustochytrids	Feedstock/cultivation conditions	Squalene concentration (mg/g_{CDW} unless otherwise indicated)	Refs
Schizochytrium sp. HX-308 (CCTCC M 209059)	At 24 h of cultivation	439.98	[64]
Aurantiochytrium sp. 9W-3a	Glucose (20 g/l)	236.04	[70]
Aurantiochytrium sp. AR-4a	Glucose (20 g/l)	316.64	
Aurantiochytrium sp. Yonez5-1	Glucose (20 g/l)	317.74	
Aurantiochytrium sp. 18W-13a	Glucose (20 g/l)	198	[58]
Aurantiochytrium sp. 18W-13a	Glucose (20 g/l)	173.7	[70]
Schizochytrium limacinum B4D1	Glucose (80 g/l) plus 3.2% of methanol	101	[62]
Schizochytrium limacinum CGMCC No: 14849	Glucose (60 g/l) with vitamin supplementation	55.23	[65]
Aurantiochytrium sp. T66 (ATCC PRA 276)	Glucose (30 g/l), 1.5% salinity	73	[59]
Aurantiochytrium sp. T66 (ATCC PRA 276)	Organosolv-pretreated birch hydrolysate, glucose (30 g/l)	69.31	[61]
Schizochytrium mangrovei FB1	Glucose (20 g/l)	0.162	[69]
Schizochytrium mangrovei FB2		~0.08	
Schizochytrium mangrovei FB3		~0.05	
Schizochytrium mangrovei	Glucose (20 g/l), methyl jasmonate (0.1 mM)	1.17	[68]
Schizochytrium mangrovei PQ6		33.00	[71]
Aurantiochytrium sp. BR-MP4-A1	Glucose (20 g/l)	0.72	[67]
Schizochytrium sp. CCTCC M209059	Glucose	33.7 mg/g_{lipids}	[72]

slow growth of the algae and the lack of genetic tools for this organism. However, the putative enzymes responsible for squalene synthesis can be utilized for heterologous expression in other strains for the overproduction of squalene. A few studies have also been performed to enhance squalene content by genetic engineering in other cyanobacterial species. For example, *Synechocystis* sp. PCC 6803, a cyanobacterium, was modified to produce squalene from CO_2 [55]. Squalene is synthesized through the MEP route for terpenoid biosynthesis in this organism and is finally utilized by the enzyme squalene hopene cyclase (Shc) for hopanoid production [55]. By inserting a gene encoding a squalene synthase from *Botryococcus braunii* under the control of an inducible promoter, the *Shc* gene in *Synechocystis* was inactivated (*Δshc*). Furthermore, the squalene level in this cyanobacterium was increased by overexpressing upstream genes in the MEP pathway, resulting in threefold more squalene per cell than the *Δshc:C* control strain [55]. Two enzymes that take part in squalene synthesis and metabolism, namely squalene synthase (CrSQS) and squalene epoxidase (CrSQE), were isolated and characterized in the model green microalga *Chlamydomonas reinhardtii* [56]. Transgenic experiments indicated that partial knockdown (KD) of CrSQE expression significantly increased the synthesis of squalene without inhibiting the growth of the microalga. On the other hand, overexpression of CrSQS did not affect the quantity of squalene in cells [56].

A biochemical engineering approach to boost squalene production in thraustochytrids

Marine thraustochytrids and yeasts are known terpenoid producers [57]. Most thraustochytrids synthesize squalene as a valuable terpenoid to protect ω-3 docosapentaenoic and docosahexaenoic

acids from oxidation [8]. Two thraustochytrid strains, *Aurantiochytrium* sp. 18W-13a and *Aurantiochytrium* sp. Yonez 5-1, are promising candidates for the production of squalene which has been shown to constitute up to 20% and 32% of their CDW, respectively [20,58]. *Aurantiochytrium* sp. 18W-13a cultivated on 20 g/l glucose, 10 g/l proteose-peptone, and 5 g/l yeast extract in 50% sea water synthesized 198 mg/g_{CDW} squalene on the fourth day of cultivation [58]. When cultivated on a similar medium, *Aurantiochytrium* sp. Yonez 5-1 and *Aurantiochytrium* sp. AR-4a accumulated 316.64 mg/g_{CDW} and 317.74 mg/g_{CDW} squalene, respectively [20], accounting for 31.63% and 31.47% of CDW and the highest squalene titer among microorganisms [20].

At 73 mg/g_{CDW}, squalene was the second most abundant lipid (17.8 ± 0.6% of biomass) when *Aurantiochytrium* sp. ATCC PRA276 was cultivated on 30 g/l glucose, 1.5% salinity, and 26°C, with its content declining slightly (16.1 ± 0.9%) at 3.5% salinity [59]. *Aurantiochytrium* sp. ATCC PRA276 cultivated on food-waste hydrolysate containing 30 g/l glucose and 9.89 g/l fructose produced 71.51 mg/g_{CDW} squalene [60]. When grown on an organosolv-pretreated non-edible lignocellulosic biomass from birch hydrolysate containing 60 g/l glucose, the same strain yielded 69.31 mg/g_{CDW} squalene [61]. The concentration of squalene in *Schizochytrium limacinum* B4D1, a less salt-tolerant derivative of *S. limacinum* SR21, was increased from 70 mg/g_{CDW} to 100 mg/g_{CDW} upon treatment with 3.2% methanol [62]. When the same strain (CGMCC No. 8313) was cultivated on 30 g/l glucose in the presence of butanol (6 g/l), squalene content was increased from 0.65 mg/g_{CDW} to 20.09 mg/$g_{CDW,}$ but decreased to 1.62 mg/g_{CDW} at 8 g/l butanol [63]. Ren and colleagues cultivated a *Schizochytrium* sp. (CCTCC M 209059) on 50 g/l glucose and artificial sea water, with 15 g/l glucose as intermittent feeding. Squalene content was maximal (439.98 mg/g_{CDW}) during the first 24 h of cultivation, declined rapidly after 48 h, and dropped to only 0.88 mg/g_{CDW} at 168 h [64]. Patent CN108004149B claims that 11.6 g/l squalene (55.23 mg/g_{CDW}) can be obtained using a *S. limacinum* strain isolated from the mangrove forest area of Haikou city in Hainan, China. The thraustochytrid in the patent was cultivated on medium containing 60 g/l glucose, yeast extract, corn steep liquor, soybean meal hydrolysate, sea salt, KH_2PO_4, and vitamins (B1, B5, and B7) [65]. A further example found in patent application WO2012159979A1 shows that a squalene titer of up to 120 mg/g_{CDW} was attained by Thraustochytriales sp. after optimizing cultivation temperature (30°C) and adding vitamin B12 [66]. The same authors investigated also other commercial strains, such as *Schizochytrium* sp. ATCC 20888 and *Aurantiochytrium* sp. ATCC PRA 276, which synthesized 61.66 mg/g_{CDW} and 23.61 mg/g_{CDW} squalene, respectively, whereas Thraustochytriales sp. attained 88.15 mg/g_{CDW} under the same culture conditions [66]. Squalene extracted from oleaginous microorganisms is free from contamination with heavy metals, polychlorobiphenyls, cholesterol, and other impurities, making extraction and purification much more convenient.

To facilitate the usage of squalene in the relevant industries, procedures for its separation from other lipids before further processing must be established. Few techniques for extracting squalene from total lipids have been published in the literature, such as fraction crystallization and the saponification. Yue *et al.* and Chen *et al.* reported squalene separation from freeze-dried cells after saponification followed by extraction with *n*-hexane [67,68], whereas Jiang and colleagues developed a fractional crystallization method in which the total isolated lipids were mixed with methanol/acetone (7:3 v/v) and kept at –20°C for 30 h, followed by filtration and washing with chloroform and methanol [69]. However, more research on the quality of squalene derived using these techniques is needed. Based on these findings, methodologies for extracting squalene from total extracted lipids and using the residual lipids for other purposes will be necessary for economic viability of the process.

Limitations of genetic modification in Thraustochytrids for squalene overproduction

Phylogeny and classification are initial challenges in the genetic manipulation of thraustochytrids. Thraustochytrids are referred in the literature under several names such as microalgae [73], heterokonts, chromists, protists, and marine heterotrophic fungi, causing uncertainty concerning their taxonomy [74]. Thraustochytrids were first classified as Phycomycetes or algal fungi, but more recently they have been placed in the Stramenopiles or Heterokonts [75]. In biotechnology, the value of taxonomy as a predictor of possible revolutionary inventions is sometimes underestimated. However, the close phylogenetic relationship between microorganisms can provide important insights into various trends in growth requirements, the production of compounds of interest, and ecological functions, all of which can lead to the discovery of a new group of functional microorganisms or functional bioactive compounds, or insight into the development of fermentation processes and methods. It is uncertain whether all thraustochytrid lineages belong to the Stramenopiles clade. The nomenclature and genera that make up this group are currently open to debate and are subject to modifications [4]. Moreover, there are significant differences in the phases of the thraustochytrid life cycle between various species. Vegetative cells, in general, form multinucleated cells that develop into sporangia, which contain zoospores [76]. After being released, zoospores colonize new places, settle, and repeat the cycle. The multinucleated structure of the cells makes genetic alteration and clone selection more difficult [76]. It is important to carefully identify mononucleated cells before attempting any genetic editing. Multinucleated cells have a low transformation efficiency, and rapid transgene loss, as well as loss of nuclei, in a single cell frequently leads to regeneration of the wild type.. As knowledge and skills are developed, these are becoming more extensively applied, with evident results. Furthermore, as will be seen later, the established thraustochytrid protocols appear to be limited to one or a few strains. This is a common problem for anyone working with non-model organisms because the transformation process must be tailored to the strain in question [29]. When a novel strain needs to be transformed, there is a major problem in identifying transformants when it is not known which antibiotic resistance indicators to use, which promoters and terminators to use, how to transfer the DNA, or how effective recombination is in that strain [23,29,30]. Genome-editing technologies are also necessary for identifying genes and determining genotype–phenotype relationships. One of the most used genome-editing techniques is based on a natural bacterial adaptive immune system known as CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) owing to its simple and efficient action. However, only one study on CRISPR/Cas9-based genetic alteration of *Aurantiochytrium* sp. (thraustochytrid) has been published [77]. The data on transformant selection in thraustochytrids using antibiotic resistance genes are very limited. Moreover, the antibiotic concentration required to select the transformants varies significantly between various strains; for example, to select transformants in *Thraustochytrium* sp. ONC-T18 the required concentration of zeocin was more than 100-fold higher than that used for *Schizochytrium* sp. HX-308 [78]. The difficulty in designing vectors and selection markers may be due to the differences in common antibiotic inhibition across thraustochytrids [79]. More research will be necessary to screen and determine optimal antibiotic selection protocols such that all thraustochytrids fall into the same inhibition level group for clone selection.

Disruption of acyl-CoA:sterol acyltransferase (ASAT) was associated with an increased titer of squalene in yeast [49]. By contrast, the thraustochytrid *Aurantiochytrium limacinum* SR21 showed a different behavior in which the expression of AlASATb and T66ASATb probably reduced the quantity of their sterol substrates and subsequently increased the amount of squalene [47], whereas inactivating both AlASATa and AlASATb reduced squalene levels. This suggests that the control of this pathway in thraustochytrids (SR21) differs from that in other species. These results suggest a more complex relationship between the

squalene and carotenoid biosynthetic pathways than merely competition for the same precursors [47].

Concluding remarks and future perspectives

Even though squalene has been extracted mainly from shark liver, it is also found in several non-animal sources such as olive oil, bacteria, yeasts, and thraustochytrids. Although shark-based squalene remains the cheapest option, its extraction is unsustainable and methods for obtaining non-animal squalene are being developed and scaled up to meet commercial needs. As more customers are becoming aware of the cruel techniques used to harvest shark liver oil, the industry has begun to move towards plant-based squalene. However, low productivity, the need for arable land, and the costs of separating and purifying squalene without altering the original qualities of the oils have limited plant-based squalene output, thus sustaining the practice of sourcing squalene from shark liver. Importantly, recent developments in synthetic biology and metabolic engineering have made microbial squalene a promising alternative (see Outstanding questions). An increase in the market share of microbial squalene will decrease the need for harvesting shark liver, and therefore contribute to two key United Nations **sustainable development goals (SDGs)**: 'Preserving and utilizing the seas and marine resources in a sustainable way for sustainable development' (SDG14) and 'Ensure sustainable consumption and production patterns' (SDG 12). Bioactive compounds from thraustochytrids such as *Schizochytrium* sp. (now *Aurantiochytrium* sp.) have been found to be safe for use in humans and animals. Squalene obtained from this source would thus contribute to 'Ensure healthy lives and promote the well-being of everyone of all ages' (SDG3) and constitute a viable alternative to its plant- and animal-based counterparts. This review has provided a comprehensive overview of recent advances in microbial squalene synthesis, highlighting their potential for process scale-up using biochemical and metabolic engineering. Of the squalene-producing microorganisms reported so far, the marine heterotrophic protists, thraustochytrids, seem to be advantageous because of their fast heterotrophic growth, high toxin-free squalene content, and suitability for commercial-scale fermentation. Although recent research has focused on bioprocess optimization for enhancing the squalene content in thraustochytrids, only yeast species have been explored for metabolic engineering to boost their content [21]. Therefore, better understanding of the biosynthetic genes and mechanisms of squalene production in thraustochytrids will be necessary to develop successful strategies for metabolic engineering. Most importantly, the elucidation of squalene biosynthetic pathways in thraustochytrids would allow dissection of the mechanisms underlying enhanced production upon culture optimization.

Acknowledgments

The authors thank the Swedish Research Council (FORMAS) and Kempestiftelserna, Sweden, for supporting this work as part of the projects 'Green and sustainable approach to valorize high saline and oily fish processing effluents for the production of nutraceuticals' (INVENTION; 2020-01028) and 'Boosting the squalene content in thraustochytrids by genetic engineering using the CRISPR–Cas9 system to replace shark-based squalene as an adjuvant for COVID 2019 vaccine' (JCK-2115).

Declaration of interests

The authors declare no conflicts of interest.

Resources

[i]www.alliedmarketresearch.com

[ii]www.marketsandmarkets.com/Market-Reports/squalene-market-542345.htm

Supplemental information

Supplemental information associated with this article can be found online at https://doi.org/10.1016/j.tibtech.2022.03.008

Outstanding questions

What is the best microbial source of squalene to replace shark liver oils?

Is vegan squalene in my skincare products? Who would choose cosmetics manufactured from endangered sharks when microbe-based alternatives are available?

Shark-based squalene is cheaper than non-animal squalene according to manufacturers; however, what methods of production of non-animal squalene can be developed and scaled up to meet commercial needs?

Is a large-scale process for isolating and purifying squalene from extracted oils available?

Can overexpression of squalene synthase in thraustochytrids further improve the squalene titer?

Is it possible to enhance and improve the level of squalene synthesis in thraustochytrids by genetic manipulation or the introduction of squalene-producing gene(s) into native squalene biosynthesis pathways by novel CRISPR/Cas9 genome-editing tools?

For many years, squalene-adjuvanted vaccines have been used to prevent diseases caused by different strains of influenza and coronaviruses (H1N1, H5N1, H7N9, H7N7, SARS-CoV, and MERS-CoV, influenza) and rabies. It is also currently being tested in COVID-19 vaccines. Does the COVID-19 outbreak bring an extra burden on shark livering for squalene?

MF59 is an adjuvant using squalene that has been developed for use in influenza vaccines. Was shark-based squalene the ideal source for the MF59 adjuvant?

What inexpensive raw materials can be used for the cultivation of microorganisms for squalene production to reduce the overall production cost?

References

1. Berghoff, S.A. *et al.* (2021) Microglia facilitate repair of demyelinated lesions via post-squalene sterol synthesis. *Nat. Neurosci.* 24, 47–60
2. Gohil, N. *et al.* (2019) Engineering strategies in microorganisms for the enhanced production of squalene: advances, challenges and opportunities. *Front. Bioeng. Biotechnol.* 7, 50
3. Chua, N.K. *et al.* (2020) Squalene monooxygenase: a journey to the heart of cholesterol synthesis. *Prog. Lipid Res.* 79, 101033
4. Morabito, C. *et al.* (2019) The lipid metabolism in thraustochytrids. *Prog. Lipid Res.* 76, 101007
5. Stonik, V.A. and Stonik, I.V. (2018) Sterol and sphingoid glycoconjugates from microalgae. *Mar. Drugs* 16, 514
6. Wang, W. *et al.* (2021) The mysterious route of sterols in oomycetes. *PLoS Pathog.* 17, e1009591
7. Reddy, L.H. and Couvreur, P. (2009) Squalene: a natural triterpene for use in disease management and therapy. *Adv. Drug Deliv. Rev.* 61, 1412–1426
8. Selvaraj, M. *et al.* (2019) Squalene biosynthesis and its role in production of bioactive compounds, a proper scientific challenge – a review. *J. Emerg. Technol. Innov. Res.* 6, 505–526
9. Garcia-Bermudez, J. *et al.* (2019) Squalene accumulation in cholesterol auxotrophic lymphomas prevents oxidative cell death. *Nature* 567, 118–122
10. Brown, A.J. *et al.* (2019) The shape of human squalene epoxidase expands the arsenal against cancer. *Nat. Commun.* 10, 888
11. Tateno, M. *et al.* (2020) Synthetic biology-derived triterpenes as efficacious immunomodulating adjuvants. *Sci. Rep.* 10, 17090
12. Martínez-Beamonte, R. *et al.* (2021) Dietary squalene modifies plasma lipoproteins and hepatic cholesterol metabolism in rabbits. *Food Funct.* 12, 8141–8153
13. Pollier, J. *et al.* (2019) A widespread alternative squalene epoxidase participates in eukaryote steroid biosynthesis. *Nat. Microbiol.* 4, 226–233
14. Hadinoto, S. *et al.* (2020) Characteristics squalene of smallfin gulper shark (*Centrophorus moluccensis*) livers from Aru Islands, Mollucas, Indonesia. *J. Phys. Conf. Ser.* 1463
15. Wejnerowska, G. *et al.* (2013) Separation of squalene and oil from *Amaranthus* seeds by supercritical carbon dioxide. *Sep. Purif. Technol.* 110, 39–43
16. Lozano-Grande, M.A. *et al.* (2018) Plant sources, extraction methods, and uses of squalene. *Int. J. Agron.* 2018, 1829160
17. Naziri, E. *et al.* (2011) Enhanced squalene production by wild-type *Saccharomyces cerevisiae* strains using safe chemical means. *J. Agric. Food Chem.* 59, 9980–9989
18. Bhattacharjee, P. and Singhal, R.S. (2003) Extraction of squalene from yeast by supercritical carbon dioxide. *World J. Microbiol. Biotechnol.* 19, 605–608
19. Blagovi, B. *et al.* (2001) Lipid composition of brewer's yeast. *Food Technol. Biotechnol.* 39, 175–181
20. Nakazawa, A. *et al.* (2014) TLC screening of thraustochytrid strains for squalene production. *J. Appl. Phycol.* 26, 29–41
21. Liu, H. *et al.* (2020) Genetic and bioprocess engineering to improve squalene production in *Yarrowia lipolytica*. *Bioresour. Technol.* 317, 123991
22. Wei, L.J. *et al.* (2018) Improved squalene production through increasing lipid contents in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 115, 1793–1800
23. Riley, L.A. and Guss, A.M. (2021) Approaches to genetic tool development for rapid domestication of non-model microorganisms. *Biotechnol. Biofuels* 14, 30
24. Morabito, C. *et al.* (2020) Illumina and PacBio DNA sequencing data, de novo assembly and annotation of the genome of *Aurantiochytrium limacinum* strain CCAP_4062/1. *Data Br.* 31, 105729
25. Liu, B. *et al.* (2016) Draft genome sequence of the docosahexaenoic acid producing thraustochytrid *Aurantiochytrium* sp. T66. *Genomics Data* 8, 115–116
26. Ji, X.-J. *et al.* (2015) Genome sequence of *Schizochytrium* sp. CCTCC M209059, an effective producer of docosahexaenoic acid-rich lipids. *Genome Announc.* 3, e00819-15
27. Liang, L. *et al.* (2020) Genome and transcriptome analyses provide insight into the omega-3 long-chain polyunsaturated fatty acids biosynthesis of *Schizochytrium limacinum* SR21. *Front. Microbiol.* 11, 687
28. Iwasaka, H. *et al.* (2018) A possible trifunctional β-carotene synthase gene identified in the draft genome of *Aurantiochytrium* sp. strain KH105. *Genes (Basel)* 9, 200
29. Faktorová, D. *et al.* (2020) Genetic tool development in marine protists: emerging model organisms for experimental cell biology. *Nat. Methods* 17, 481–494
30. Rau, E.M. and Ertesvåg, H. (2021) Method development progress in genetic engineering of thraustochytrids. *Mar. Drugs* 19, 515
31. Queiroz, N. *et al.* (2019) Global spatial risk assessment of sharks under the footprint of fisheries. *Nature* 572, 461–466
32. Pacoureau, N. *et al.* (2021) Half a century of global decline in oceanic sharks and rays. *Nature* 589, 567–571
33. Macdonald, C. and Soll, J. (2020) Shark conservation risks associated with the use of shark liver oil in SARS-CoV-2 vaccine development. *BioRxiv* Published online October 15, 2020. https://doi.org/10.1101/2020.10.14.338053
34. Mastralexi, A. and Tsimidou, M.Z. (2021) On the squalene content of cv chondrolia chalkidikis and chalkidiki (Greece) virgin olive oil. *Molecules* 26, 6007
35. Srivastava, S. *et al.* (2021) Effect of processing on squalene content of grain amaranth fractions. *J. Cereal Sci.* 100, 103218
36. Wei, L. *et al.* (2021) Increased accumulation of squalene in engineered *Yarrowia lipolytica* through deletion of PEX10 and URE2. *Appl. Environ. Microbiol.* 87, e0048121
37. Li, T. *et al.* (2020) Metabolic engineering of *Saccharomyces cerevisiae* to overproduce squalene. *J. Agric. Food Chem.* 68, 2132–2138
38. Xu, W. *et al.* (2019) Improving squalene production by enhancing the NADPH/$NADP^+$ ratio, modifying the isoprenoid-feeding module and blocking the menaquinone pathway in *Escherichia coli*. *Biotechnol. Biofuels* 12, 68
39. Thapa, H.R. *et al.* (2016) A squalene synthase-like enzyme initiates production of tetraterpenoid hydrocarbons in *Botryococcus braunii* Race L. *Nat. Commun.* 7, 11198
40. Kawamura, K. *et al.* (2021) Large-scale screening of natural genetic resource in the hydrocarbon-producing microalga *Botrycoccus braunii* identified novel fast-growing strains. *Sci. Rep.* 11, 7368
41. Cao, M. *et al.* (2019) Characterization of the squalene-rich *Botryococcus braunii* Abt02 strain. *J. Oceanol. Limnol.* 37, 675–684
42. Fagundes, M.B. *et al.* (2021) Green microsaponification-based method for gas chromatography determination of sterol and squalene in cyanobacterial biomass. *Talanta* 224, 121793
43. Huang, Y.Y. *et al.* (2018) Enhanced squalene biosynthesis in *Yarrowia lipolytica* based on metabolically engineered acetyl-CoA metabolism. *J. Biotechnol.* 281, 106–114
44. Tang, W.Y. *et al.* (2021) Metabolic engineering of *Yarrowia lipolytica* for improving squalene production. *Bioresour. Technol.* 323, 124652
45. Paramasivan, K. and Mutturi, S. (2017) Regeneration of NADPH coupled with HMG-CoA reductase activity increases squalene synthesis in *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* 65, 8162–8170
46. Song, Y. *et al.* (2020) Production of squalene in *Bacillus subtilis* by squalene synthase screening and metabolic engineering. *J. Agric. Food Chem.* 68, 4447–4455
47. Rau, E.M. *et al.* (2022) Overexpression of two new acyl-CoA: diacylglycerol acyltransferase 2-like acyl-CoA:sterol acyltransferases enhanced squalene accumulation in *Aurantiochytrium limacinum*. *Front. Microbiol.* 13, 822254
48. Korber, M. *et al.* (2017) Steryl ester synthesis, storage and hydrolysis: a contribution to sterol homeostasis. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 1862, 1534–1545
49. Zweytick, D. *et al.* (2000) Contribution of Are1p and Are2p to steryl ester synthesis in the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 267, 1075–1082
50. Xu, W. *et al.* (2021) Improving squalene production by blocking the competitive branched pathways and expressing rate-limiting enzymes in *Rhodopseudomonas palustris*. *Biotechnol. Appl. Biochem.* Published online July 18 2021. https://doi.org/10.1002/bab.2222
51. Garaiová, M. *et al.* (2014) Squalene epoxidase as a target for manipulation of squalene levels in the yeast *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 14, 310–323

52. Hull, C.M. *et al.* (2014) Co-production of ethanol and squalene using a *Saccharomyces cerevisiae* ERG1 (squalene epoxidase) mutant and agro-industrial feedstock. *Biotechnol. Biofuels* 7, 133
53. Meng, Y. *et al.* (2020) Extension of cell membrane boosting squalene production in the engineered *Escherichia coli*. *Biotechnol. Bioeng.* 117, 3499–3507
54. Cheng, P. *et al.* (2019) High-value chemicals from *Botryococcus braunii* and their current applications – a review. *Bioresour. Technol.* 291, 121911
55. Pattanaik, B. *et al.* (2020) Introduction of a green algal squalene synthase enhances squalene accumulation in a strain of *Synechocystis* sp. PCC 6803. *Metab. Eng. Commun.* 10, e00125
56. Kajikawa, M. *et al.* (2015) Accumulation of squalene in a microalga *Chlamydomonas reinhardtii* by genetic modification of squalene synthase and squalene epoxidase genes. *PLoS One* 10, e0120446
57. Xu, W. *et al.* (2016) Production of squalene by microbes: an update. *World J. Microbiol. Biotechnol.* 32, 195
58. Kaya, K. *et al.* (2011) Thraustochytrid *Aurantiochytrium* sp. 18W-13a accumulates high amounts of squalene. *Biosci. Biotechnol. Biochem.* 75, 2246–2248
59. Martins, G. *et al.* (2018) Simultaneous evaluation of the production of squalene and fatty acids by *Aurantiochytrium*. *J. Mar. Biol. Aquac.* 4, 14–20
60. Patel, A. *et al.* (2020) Mining of squalene as a value-added byproduct from DHA producing marine thraustochytrid cultivated on food waste hydrolysate. *Sci. Total Environ.* 736, 139691
61. Patel, A. *et al.* (2019) Simultaneous production of DHA and squalene from *Aurantiochytrium* sp. grown on forest biomass hydrolysates. *Biotechnol. Biofuels* 12, 255
62. Du, H. *et al.* (2019) Effects of methanol on carotenoids as well as biomass and fatty acid biosynthesis in *Schizochytrium limacinum* B4D1. *Appl. Environ. Microbiol.* 85, e01243-19
63. Zhang, K. *et al.* (2017) Effects of butanol on high value product production in *Schizochytrium limacinum* B4D1. *Enzym. Microb. Technol.* 102, 9–15
64. Ren, L.J. *et al.* (2014) Compositional shift in lipid fractions during lipid accumulation and turnover in *Schizochytrium* sp. *Bioresour. Technol.* 157, 107–113
65. Lu, Y. Marine protist and method for producing high value-added lipid product by fermenting marine protist, Qingdao Hongbang Biotechnology Co. Ltd., CN108004149B
66. Pora, B. *et al.* Method for the preparation and extraction of squalene from microalgae, Roquette Frères, WO2012159979A1
67. Chen, G. *et al.* (2010) Optimization of nitrogen source for enhanced production of squalene from thraustochytrid *Aurantiochytrium* sp. *New Biotechnol.* 27, 382–389
68. Yue, C.J. and Jiang, Y. (2009) Impact of methyl jasmonate on squalene biosynthesis in microalga *Schizochytrium mangrovei*. *Process Biochem.* 44, 923–927
69. Jiang, Y. *et al.* (2004) Fatty acid composition and squalene content of the marine microalga *Schizochytrium mangrovei*. *J. Agric. Food Chem.* 52, 1196–1200
70. Nakazawa, A. *et al.* (2012) Optimization of culture conditions of the thraustochytrid *Aurantiochytrium* sp. strain 18W-13a for squalene production. *Bioresour. Technol.* 109, 287–291
71. Hoang, M.H. *et al.* (2014) Extraction of squalene as value-added product from the residual biomass of *Schizochytrium mangrovei* PQ6 during biodiesel producing process. *J. Biosci. Bioeng.* 118, 632–639
72. Ren, L.J. *et al.* (2010) Development of a stepwise aeration control strategy for efficient docosahexaenoic acid production by *Schizochytrium* sp. *Appl. Microbiol. Biotechnol.* 87, 1649–1656
73. Leyland, B. *et al.* (2017) Are thraustochytrids algae? *Fungal Biol.* 121, 835–840
74. Fossier Marchan, L. *et al.* (2018) Taxonomy, ecology and biotechnological applications of thraustochytrids: a review. *Biotechnol. Adv.* 36, 26–46
75. Ganuza, E. *et al.* (2019) Genomics, biology and phylogeny *Aurantiochytrium acetophilum* sp. nov. (Thraustrochytriaceae), including first evidence of sexual reproduction. *Protist* 170, 209–232
76. Dellero, Y. *et al.* (2020) The zoospores of the thraustochytrid *Aurantiochytrium limacinum*: transcriptional reprogramming and lipid metabolism associated to their specific functions. *Environ. Microbiol.* 22, 1901–1916
77. Watanabe, K. *et al.* (2021) Improvement of fatty acid productivity of thraustochytrid, *Aurantiochytrium* sp. by genome editing. *J. Biosci. Bioeng.* 131, 373–380
78. Merkx-Jacques, A. *et al.* (2018) Engineering xylose metabolism in thraustochytrid T18. *Biotechnol. Biofuels* 11, 248
79. Sakaguchi, K. *et al.* (2012) Versatile transformation system that is applicable to both multiple transgene expression and gene targeting for thraustochytrids. *Appl. Environ. Microbiol.* 78, 3193–3202