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REVIEW OPEN ACCESS

Microbial Squalene: A Sustainable Alternative for the Cosmetics and Pharmaceutical Industry – A Review

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Received: 24 December 2023 | **Accepted:** 4 August 2024

Funding: This study was supported by the Science and Engineering Research Board (SERB), DST, Government of India for SERB International Research Experience (SIRE) (SIR/2022/000277) and the Science and Engineering Research Board (SERB), Department of Science and Technology, Government of India for Research Grant (CRG/2022/002239).

Keywords: metabolic engineering | squalene | synthetic biology | triterpene | yeast

ABSTRACT

Squalene is a natural triterpenoid and a biosynthetic precursor of steroids and hopanoids in microorganisms, plants, humans, and other animals. Squalene has exceptional properties, such as its antioxidant activity, a high penetrability of the skin, and the ability to trigger the immune system, promoting its application in the cosmetic, sustenance, and pharmaceutical industries. Because sharks are the primary source of squalene, there is a need to identify low-cost, environment friendly, and sustainable alternatives for producing squalene commercially. This shift has prompted scientists to apply biotechnological advances to research microorganisms for synthesizing squalene. This review summarizes recent metabolic and bioprocess engineering strategies in various microorganisms for the biotechnological production of this valuable molecule.

1 | Introduction

Squalene ($C_{30}H_{50}$, 2, 6, 10, 15, 19, 23-hexamethyltetracosa-2, 6, 10, 14, 18, 22-hexaene) is a linear, unsaturated triterpene hydrocarbon that is a biosynthetic precursor of steroids and hopanoids in eukaryotes [1, 2]. Squalene has a refractive index of 1.4, a molecular mass of 410.7 g/mol, a melting point of 75°C, and a boiling point of 285°C. The unsaturated nature of squalene renders it susceptible to oxidation. Through chain reactions, unsaturated carbons combine with ions to produce saturated forms of the molecule. Although squalene is resistant to peroxidation, it protects against oxidative damage. Thus, as an antioxidant, squalene has various potential applications in the pharmaceutical, nutraceutical, and personal care industries [3].

Mitsumaru Tsujimoto, a Japanese researcher and expert in oils and fats at the Tokyo Industrial Testing Station, discovered squalene in 1916. The term *squalene* originated from the family name of sharks, Squalidae [4]. Squalene also exists in humans and is secreted by sebaceous glands to protect the skin. It is a component of skin surface lipids (300–500 μg/g) and is present in such organs as the small intestine and liver (75 μg/g) [5].

[Correction added on 30 August 2024, after first online publication: The surname of the author Panam Kunnel Raveendranathan Karthikanath was corrected in this version.]

The growing trend in the use of natural ingredients in personal care products and cosmetics is expected to be the leading market driver over the next several years. In a market analysis by Grand View Research (California, US), the global market for squalene was estimated to be 120 million USD in 2021 and is anticipated to increase at a compound annual growth rate of 11% from 2022 to 2030.

Since its discovery, squalene has been obtained primarily from shark liver oil. Squalene comprises a significant amount of unsaponifiable matter that occupies 50% to 80% of shark liver. The techniques for extracting squalene from shark liver oil are less expensive and more straightforward. Over 98% of the pure squalene in shark liver oil can be extracted with just a single distillation under a vacuum at 200°C–300°C.

The long reproductive cycle and slow growth rate of sharks have contributed to a substantial decrease in the number of wild sharks [6]. Three to 6 million deep-sea sharks are killed yearly to satisfy the rising demand for squalene, which, although benefits human health, jeopardizes marine ecosystems. Thus, there is increasing pressure on industries to switch from obtaining squalene from sharks to more environment friendly sources, such as plants and microorganisms. Microbial sources of squalene are a viable alternative to plant-based or animal-derived squalene that avoids current unsustainable practices. The squalene biosynthesis pathway can be genetically altered to transform microorganisms into "cellular factories" that produce copious amounts of squalene [7].

Over the past 5 decades, significant advances in genetic engineering, metabolic engineering, and synthetic biology approaches have made it possible to insert heterologous genes and edit an organism's genome, leading to the production of many molecules with industrial value [8–10]. Recently, a variety of microorganisms, such as bacteria (including cyanobacteria), fungi, and yeast, have undergone genetic engineering, in which squalene biosynthesis pathways have been inserted or existing biosynthetic pathways have been modified to produce enormous amounts of squalene [7, 11]. Table 1 highlights recent strategies for generating squalene in prokaryotes and eukaryotes.

This review discusses the broad applications and various natural sources of squalene, ranging from microbes and plants to sharks. We summarize the two biosynthesis pathways of squalene and the recent metabolic engineering strategies that have been developed to improve squalene production, including the inhibition of key enzymes, the application of recombinant technologies, and compartmentalization strategies. We also highlight bioprocess engineering methods that have been reported to increase squalene output during fermentation. This review emphasizes the potential and importance of microorganisms as an alternative source for the industrial production of squalene, concluding with our outlook on what steps must be taken for this endeavor to succeed.

2 | Applications of Squalene

Squalene has wide applications in the pharmaceutical, nutraceutical, and personal care industries. It can serve as an antioxidant, drug carrier, and vaccine adjuvant and is a crucial ingredient in personal and skin care products. Recently, squalene has been used as an active ingredient in cosmetic products, including moisturizers, face creams, nail polish, body powder, lipstick, eye makeup, lotion, and sunburn remedies, based on its anti-inflammatory, detoxifying, moisturizing, and antioxidant properties. Most likely, the use of squalene in the cosmetic industry will be replaced by squalane, a saturated and thus oxidation-stable derivative [4] that can be synthesized chemically using the sesquiterpene farnesene produced by yeast. Leading cosmetic companies, such as L'Oréal Paris and Garnier, incorporate squalane into everyday skin care items, including moisturizing serums and oils, as well as hair conditioners [12]. Both molecules penetrate the deep skin layers and help counteract fine lines and dry patches by retaining moisture, providing elasticity, and improving the flexibility of the skin [13].

Squalene has garnered significant interest for its biomedical potential. It is a promising vaccine adjuvant, enhancing innate and adaptive immune responses. Notably, squalene-based emulsion adjuvants, such as MF59, AS03, and AF03, have been integrated into seasonal influenza vaccines and are being considered for SARS-CoV-2 vaccines, based on their ability to improve antigen presentation and antibody responses. Squalene also has utility in drug delivery, forming self-assembling nanoparticles that have enhanced pharmaceutical properties, particularly when conjugated with polyethylene glycol. Also, freeze-dried squalene-adenosine nanoparticles have advantages with regard to storage. Further, squalene has preventive and therapeutic potential in cancer and radiation therapy, given its antioxidant and cytoprotective properties, which could mitigate tumor formation and radiation-induced damage. Although its precise detoxification mechanisms are undetermined, squalene shows promise in eliminating lipid-soluble toxins [6, 14, 15].

3 | Natural Sources of Squalene

A wide range of organisms, from unicellular microbes to multicellular fungi, plants, and deep-sea sharks, are potential sources of natural squalene [14]. According to estimates, approximately 3000 sharks must be killed to produce 1 ton of squalene. To obtain >98% pure squalene from liver oil, a single distillation under a vacuum at a temperature of 200°C–230°C is necessary [16]. The slow growth rate and long reproductive cycle of sharks and the unsustainable massive killing of sharks have increased the demand for alternative sources of squalene [17, 18]. The resulting declining population of sharks has thus increased awareness regarding animal welfare and protection, leading governments to impose regulatory frameworks to protect marine animals. NGOs, such as Project AWARE Foundation (US, UK, Australia), Shark Angels, and Shark Allies, have made it difficult for cosmetics companies to rely on shark oil-derived squalene. This animal source predominates, mainly because the price of shark squalene is lower than that of squalene that is obtained from plants and other sources.

Certain plant oils also contain low levels of squalene [19], accounting for approximately 20% of the global market. Most plant oils contain minute amounts of squalene, particularly olive and amaranth [15]. Other plant sources include rice bran, palm

TABLE 1 | Recent strategies for the production of squalene in microorganisms.

S no.	Organism	Metabolic engineering and process strategy	Yield/Titer	References
1.	*E. coli*	OE: Mevalonate pathway HE: *S. cerevisiae* squalene synthase	16 mg/L	[143]
		OE: Mevalonate pathway and *tsr* HE: *S. cerevisiae* squalene synthase	612 mg/L	[53]
		OE: *idi, dxs, udhA, zwf, pgl* and *fps* KO: *pgi, menA* HE: Squalene synthase from human	28 mg/g DCW 52 mg/L	[51]
2.	*Bacillus subtilis*	OE: *dxs, ispD, ispF, ispH*, and *ispA* HE: Squalene synthase from *Bacillus megaterium*	8 mg/L	[58]
3.	*Corynebacterium glutamicum*	OE: *dxs, idi* R: *idsA* HE: *S. cerevisiae* squalene synthase	5 mg/g DCW or 105 mg/L	[144]
4.	*Phormidium autumnale*	Cultivation using agro-industrial waste (No metabolic strategies used)	0.2 g/kg DCW	[106]
5.	*Rhodopseudomonas palustris*	OE: *dxs, crtE, hpnD* KO: *shc, crtB* PS: Two-phase extraction	23 mg/g DCW	[69]
6.	*Yarrowia lipolytica*	OE: *ylHMG1, ylACL2, ylMnDH2, SQS* PS: C/N ratio optimization, two-phase extraction	503 mg/L	[11]
7.	*Saccharomyces cerevisiae*	OE: *ADH2, ERG10, ERG13, tHMG1, ERG13, tHMG1*, ADA from *ERG12, ERG8, MVD1, IDI1, ERG20, ERG9* KO: *GAL80* HE: NADH-HMGR from *Dickeya zeae* PS: Carbon source-controlled three-stage fed-batch fermentation, two-phase extraction	10 g/L	[100]
		OE: *ERG10, ERG13, tHMG1, ERG12, ERG8, MVD1, IDI1, ERG20, ERG9* genes fused with and without a C-terminal ePTS1 tag HE: NADH-HMGR from *Silicibacter pomeroyi*, *ACL1/2* from *Yarrowia lipolytica* R: P_{HXT1}-*ERG1* PS: Two-stage fermentation, two-phase extraction	11 g/L	[11]

Abbreviations: HE, heterologous expression; KO, knock out; OE, overexpression; PE, promoter engineering; PS, process strategy; R, repression.

oil, Brazil nut [13], avocado, sunflower seed, borage, soybean [19, 20], peanut [21, 22], macadamia, pistachio, almond, and walnut [22–24]. Plant seeds that are known for squalene production include ginseng, soybean, sunflower seed, flaxseed, pumpkin seed, coriander seed, sesame seed, grape seed, cottonseed, Rosaceae, and apricot kernels [19–21, 23, 25–29]. Plants are not considered ideal sources of squalene, due to their seasonal growth and low squalene content, requiring costly purification. Because the production of olive and soybean oils is massive, squalene production from them could be ramped up easily but might result in prices above those of shark oil-derived squalene. Squalene concentrations also differ between geographical locations, because they depend on temperatures, humidity, rainfall, soil texture, and other variables [13], making it more difficult to use plants as reliable resources.

Microorganisms are a prominent natural source of squalene. Despite their low physiological levels, their rapid growth and amenability to genetic engineering render them an alternative source of squalene. There are many microorganisms that produce squalene, such as the archaea *Halobacterium cutirubrum; Methylomonas methanica; Methylococcus capsulatus* [30]; Euglena [31];

Saccharomyces cerevisiae (yeast); fungi, such as *Torulaspora delbrueckii* [32, 33], *Aspergillus nidulans* [30], and *Kluyveromyces lactis* [34]; and the industrial yeast *Saccharomyces uvarum* [35]. *Pseudozyma sp*. produces over 2 g/L squalene without having to undergo any strain engineering [36]. *Candida famata* [13], *Rhodopseudomonas palustris* [37], and *Rhodosporidium sp*. [38] are other microorganisms that synthesize squalene. Before summarizing the current progress in generating squalene from microorganisms, we briefly describe its two synthetic pathways.

4 | Biosynthetic Pathways of Squalene Synthesis

Squalene can be produced through the 2-C-methyl-D-erythritol 4-phosphate (MEP) or mevalonate (MVA) pathway. The MEP pathway is active in bacteria and archaea, whereas eukaryotic organisms, such as yeast, higher fungi, plants, humans, and other animals use the MVA pathway [1].

4.1 | Synthesis of Squalene in Prokaryotes

In prokaryotes, squalene synthesis occurs through the MEP pathway, also called the DXP (1-deoxy-D-xylulose-5- phosphate) pathway. DXP synthase (DXS) catalyzes the condensation of glyceraldehyde-3-phosphate (GA3P) and pyruvate to DXP, which is the first step in the MEP pathway. DXP reductoisomerase (DXR) or its isozyme, DRL (DXR-like), reduces DXP to yield MEP. A series of enzymes then convert MEP into IPP and DMAPP in subsequent reactions; these steps, involving the conversion of IPP to FPP, are the same as in the MVA pathway. Squalene accumulates intracellularly, and no transporter for it has been identified.

Squalene is not generally produced by organisms that express the MEP pathway, although there are exceptions, such as *Pseudomonas sp*., *Methylomonas sp*., and *Halobacterium sp*. Metabolic engineering and synthetic biology have made it possible to extend the MEP pathway by introducing a heterologous squalene synthase (SQS) gene for squalene production [13, 39–41]. Figure 1A shows an overview of squalene synthesis in prokaryotes.

4.2 | Synthesis of Squalene in Eukaryotes

Squalene is synthesized via the MVA pathway in eukaryotes. The MVA pathway begins with the condensation of 3 acetyl-CoA molecules to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This reaction is catalyzed by acetoacetyl CoA thiolase (AACT) and HMG-CoA synthase (HMGS). HMG-CoA is further reduced to MVA by HMG-CoA reductase (HMGR). MVA is then phosphorylated twice by MVA kinase and phospho-MVA kinase in the presence of the cofactor NADPH to form MVA-5-diphosphate. Decarboxylation of MVA-5-diphosphate generates isopentyl diphosphate (IPP) using ATP, which converts into dimethylallyl diphosphate (DMAPP) through a reaction that is catalyzed by IPP isomerase. The condensation of IPP and DMAPP yields geranyl diphosphate (GPP), after which a condensation reaction converts GPP to FPP (farnesyl pyrophosphate, a 15-carbon isopentyl block unit), catalyzed by farnesyl diphosphate synthase (FPS). Squalene is synthesized through the condensation of two molecules of FPP in an NADPH-mediated reaction that is catalyzed by squalene synthase, which is encoded by ERG9. The pathway then synthesizes sterols [41, 42] by modifying squalene in the endoplasmic reticulum.

The shuttling of triterpenes in eukaryotes, including squalene, involves the coordinated interplay of transport proteins and intracellular membranes. Supernatant protein factor (SPF) is believed to mediate the transport of linear triterpenoids, squalene, and 2,3-oxidosqualene. It possesses a core domain that resembles SEC14, a protein that is involved in lipid binding, and a jelly roll barrel at its C-terminus. When the C-terminal jelly roll barrel is removed, SPF can bind to squalene and 2,3-oxidosqualene more effectively [43]. Figure 1B shows an overview of squalene synthesis in eukaryotes.

5 | Metabolic Engineering of Bacteria to Enhance Squalene Production

The sustainable synthesis of squalene by microorganisms is potentiated by the controlled expression of heterologous genes using modern genetic engineering methods and technologies. By deleting or amplifying metabolic genes, one can reconstruct metabolic pathways and fluxes to generate the desired product. Figure 2 illustrates strain engineering strategies that are used to improve the production of squalene in bacteria.

5.1 | *Escherichia coli*

Several metabolic strategies are used to increase squalene production by *E. coli*, based on their efficient fermentation and well-established genetic characteristics. *E. coli* is a mainstay in the industrial production of many molecules, such as 1,3 propanediol and proteins, due to its rapid growth and fermentation at high density and large genetic toolbox [44].

Recombinant *E. coli* strains that express truncated human squalene synthase (hSQS) have been constructed to synthesize squalene [40, 44]. Notably, higher amounts of squalene have been produced in an ECHSQ1 recombinant *E. coli* strain that harbors pTHS with hSQS cDNA [44].

The synthesis of squalene can benefit from the expression of rate-limiting enzymes in the isoprenoid pathway, because they direct carbon flux toward FPP, which is crucial for this production [45]. Squalene content rises with the overexpression of the *dxs*, *idi*, and *fps* genes, which encode for rate-limiting enzymes in the isoprenoid biosynthesis pathway [46].

E. coli produces isoprenoid precursors through the non-mevalonate pathway. The overexpression of 1-deoxyxylulose-5-phosphate synthase (Dxs), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr), and isoprenyl diphosphate isomerase (Idi) has been shown to improve the squalene titer of the pathway, increasing the production of various isoprenoid compounds (Figure 2A(a)). The coupled overexpression of *idi* and *dxs* increases squalene production in *E. coli* three-fold [40, 42].

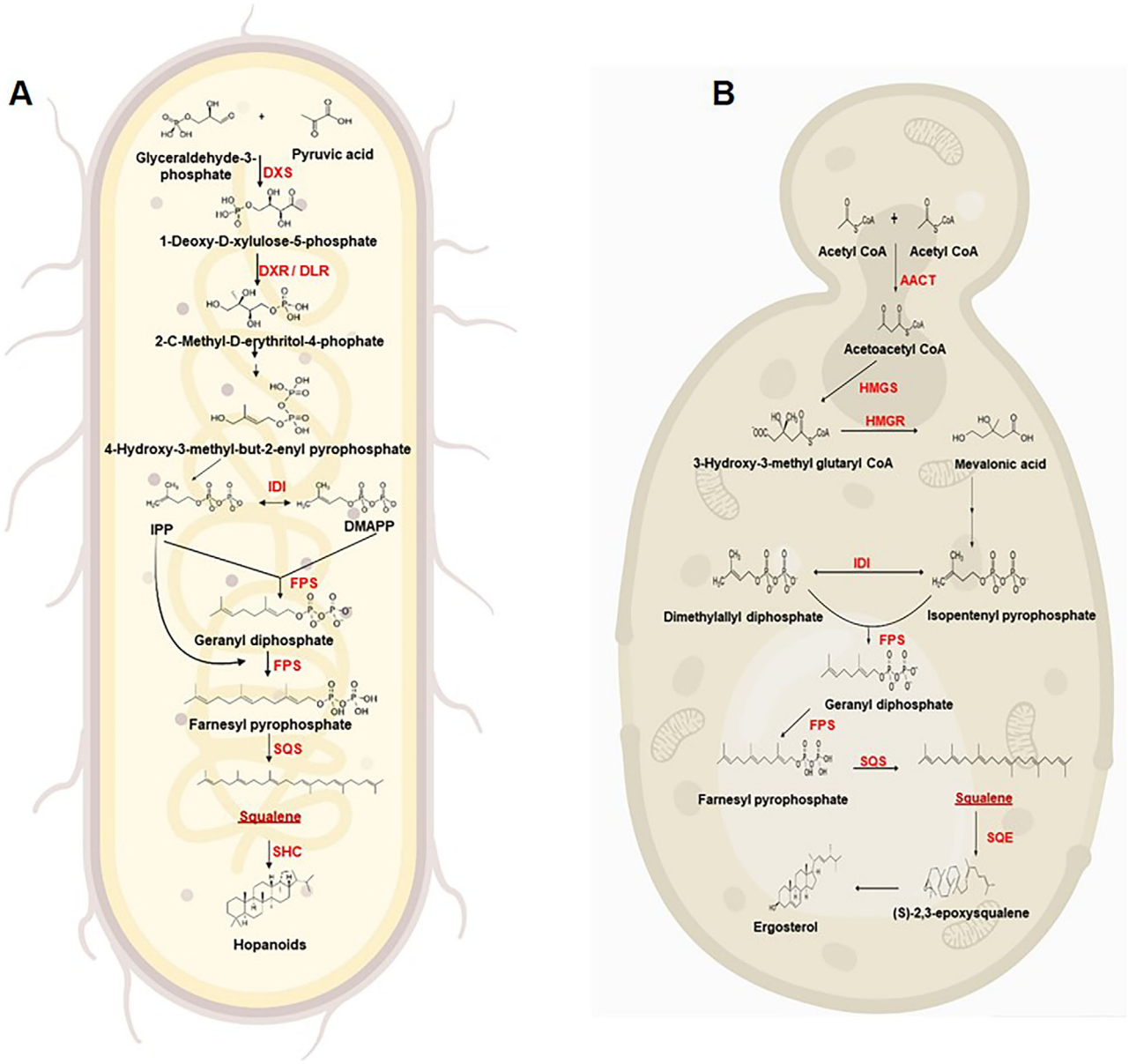

FIGURE 1 | Squalene biosynthesis pathway (A) MEP pathway (bacteria including cyanobacteria, archaea) (B) MVA pathway (eukaryotes). AACT – acetoacetyl-CoA thiolase; DLR – DXR-like enzyme; DMAPP – dimethylallyl diphosphate; DXR – DXP reductoisomerase; DXS – DXP synthase; FPS – farnesyl pyrophosphate synthase; HMGR – HMG-CoA reductase; HMGS – 3-hydroxy-3-methylglutaryl-CoA synthase; IDI – isopentenyl diphosphate isomerase; IPP – isopentenyl pyrophosphate; MEP – 2-C-methyl-d-erythritol 4-phosphate; MVA – mevalonate; SHC – squalene hopene cyclase; SQE – squalene epoxidase; SQS – squalene synthase.

In addition to improving titers, metabolic engineering is now focused on developing strategies to overcome the limitations in storage to account for the rise in squalene production. To this end, the primary approach is extension of the cell membrane [47]. The overexpression of the membrane protein Tsr, a chemoreceptor for serine, effects invagination of the inner membrane to form a multilayered structure on increased synthesis of squalene through the introduction of squalene synthase (Figure 2A(b)) [40, 47, 48]. Meng and colleagues [47] used the MVA route and *S. cerevisiae* squalene synthase to achieve *de novo* synthesis of squalene from glycerol in *E. coli*, resulting in a titer of 272 mg/L squalene in flask cultures. Additional Tsr expression increased this titer to 612 mg/L, which expanded the membrane volume for storage of squalene.

5.2 | *Bacillus subtilis*

B. subtilis can develop into a cell factory for industrial high-value terpenoid production due to its high production of squalene precursors [49, 50]. The overexpression of four genes in the MEP pathway (*dxs*, *ispD*, *ispF*, and *ispH*) can improve the production of squalene in *B. subtilis*, based on studies on terpenoid production (Figure 2B(a)) [51–53].

Novel metabolic engineering pathways have been constructed with SQS from *B. acidocaldarius, B. megaterium, P. ginseng*, and *S. cerevisiae* (BaSQS, BmSQS, PgSQS, and ScSQS, respectively) to establish squalene production in *B. subtilis* (Figure 2B(b)). All four metabolically engineered candidates have demonstrated

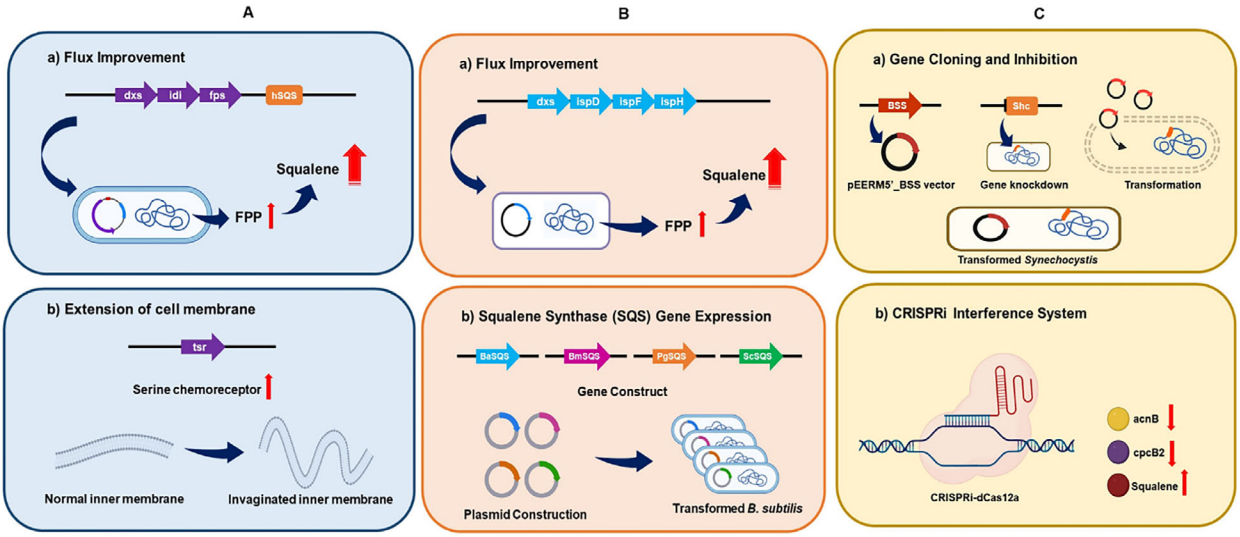

FIGURE 2 | Metabolic engineering and strain improvement strategies in bacteria for enhanced squalene production. (A) In *Escherichia coli* (a) Overexpression of rate-limiting enzymes in isoprenoid biosynthesis pathway. (b) overexpression of membrane protein. (B) In *Bacillus subtilis* (a) enhancing the precursor pool through chromosomal integration or episomal expression of genes, (b) gene addition from *B. acidocaldarius, B. megaterium, P. ginseng, and S. cerevisiae* (BaSQS, BmSQS, PgSQS, and ScSQS respectively). (C) In cyanobacteria (a) gene knockdown and vector transformation, (b) dCas12a-mediated CRISPRi interference system. acnB – aconitase; cpcB2 – phycocyanin β-subunit; dxs – 1-deoxy-D-xylulose-5-phosphate synthase; FPP – farnesyl pyrophosphate; fps – farnesyl pyrophosphate synthase; hSQS – human squalene synthase; idi – isopentenyl diphosphate isomerase; ispD – 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; ispF – 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ispH – 4-hydroxy-3-methyl but-2-enyl diphosphate reductase; Shc – squalene hopene cyclase; tsr – methyl-accepting chemotaxis protein I.

squalene production, wherein the highest production was observed in *B. subtilis* with truncated BmSQS [52].

5.3 | Cyanobacteria

Cyanobacteria can perform oxygenic photosynthesis using atmospheric CO_2 and light as its carbon and energy sources, respectively. Squalene is synthesized in cyanobacteria as an intermediate in the methylerythritol-phosphate (MEP) pathway to produce terpenoids and is then metabolized by the enzyme squalene hopene cyclase (Shc) to form hopanoids [54]. Englund and colleagues [55] demonstrated the accumulation of squalene in a *Synechocystis* strain that was constructed through the inactivation of Shc.

Botryocuccusbraunii, a type of green algae, shows properties of developing cyanobacteria as a sustainable source for squalene production, due to its accumulation of hydrocarbon oils. Triterpenes, such as squalene and botryococcene, constitute most of the oils that certain strains of *Botryococcus* produce [56]. The gene that encodes for *Botryococcus* squalene synthase (BSS) in *B. braunii* was optimized for expression in *Synechocystis* and cloned into the pEERM5' vector. The resulting pEERM5'_BSS vector was transferred into *Synechocystis*Δshc (Figure 2C(a)), improving squalene accumulation in the resulting engineered strain [57].

Another approach has been to optimize genetic and regulatory processes in *Synechococcus elongatus* PCC 7942. Two copies of SQS from *S. cerevisiae* and the *dxs*, *idi*, and *ispA* genes from *E. coli* were expressed, and the resulting strain was grown under optimized culture conditions in bioreactors with elevated levels of CO_2. Greater accumulation of squalene was reported [58].

Cellular metabolism was altered to improve squalene production by targeted repression of essential genes using the CRISPRi-dCas12a interference system in cyanobacteria to produce squalene from CO_2 photosynthetically. By dCas12a-mediated CRISPRi, the overexpression of crucial enzymes in the MEP pathway and the repression of *acnB*, which encodes for aconitase (essential for the citric acid cycle), and cpcB2, the gene for phycocyanin β-subunit, can maximize the production of squalene by cultures (Figure 2C(b)) and photosynthetic efficiency in *S. elongatus* PCC 7942 [59].

5.4 | *Rhodopseudomonas palustris*

A standard purple nonsulfur bacterium, *R. palustris*, has been studied extensively and genetically engineered to produce squalene [60, 61]. Welander et al. demonstrated that the deletion of Shc, encoding for squalene-hopene cyclase, increases the accumulation of squalene and reduces the levels of polycyclic triterpenoids [62].

In *R. palustris*, FPP is an intermediate in the production of ubiquinone, carotenoids, polyterpenoids, and hopanoids. The squalene pathway thus shares the pool of FPP with the synthesis of other compounds. The competitiveness of the squalene pathway, however, is enhanced with hpnD expression. The *crtE* gene has been overexpressed to increase farnesyl diphosphate synthase levels to shunt more upstream intermediates toward the synthesis of FPP. Studies in recombinant *R. palustris* have reported that squalene titers increase with the coexpression and fusion of *CrtE* and *HpnD*—1.6 and 3.3 times higher than in Shc-deleted strains, respectively; a practical method for improving squalene production is to express a fusion protein—in this case,

resulting in the preferential utilization of the synthesized FPP by HpnD, fused to CrtE, prior to it being absorbed by the cell and used by branching pathways [37]. Studies have reported that blocking the carotenoid pathway increases squalene production by 57%. Recombinant *R. palustris* strains with a deletion of the *crtB* and *shc* genes have higher squalene titers by combining the strategies of blocking branched pathways and expressing rate-limiting enzymes [63].

6 | Metabolic Engineering of Oleaginous Yeasts for Enhanced Squalene Production

Oleaginous yeasts can stockpile 90% of their cellular weight in lipids and are considered cellular factories of lipid-related products, chemicals, and fuels. Genetic engineering strategies for enhancing the production of squalene have been increasingly examined due to their high lipogenesis and utilization of various substrates [64, 65]. *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon*, and *Lipomyces* species are common oleaginous yeast strains [66], of which *Yarrowia lipolytica* is the most extensively studied [67–70]. The discovery of their genetic accessibility guided the creation of a more economically viable lipid synthesis technique [71–74].

6.1 | *Yarrowia lipolytica*

Many studies have reported that HMG-CoA reductase, encoded by HMG1 (3-hydroxy-3-methylglutaryl-CoA reductase gene), is the rate-limiting enzyme in the MVA pathway [75], as reported for baker's yeast. The overexpression of HMG1 improves squalene production in *Y. lipolytica* [11, 76, 77]. Some groups have studied the feasibility of engineering *Y. lipolytica* as a potential host for squalene production, reporting squalene levels of 180.3 and 188.2 mg/L from glucose or acetate minimal media, respectively [11].

Squalene accumulates when native HMGR is overexpressed. The recombinant *Y. lipolytica* SQ-1 strain, which co-overexpresses HMG1 and DGA1, encoding for diacylglycerol acyltransferase, an essential enzyme in lipid accumulation [77–81], enhances squalene synthesis [82]. Co-overexpression of HMG1 and DGA1 improves squalene production in *Y. lipolytica*, with squalene titers peaking at 731.18 mg/L. This study has provided the foundation for cost-efficient production of squalene.

Acetyl-CoA is a critical intracellular precursor in squalene biosynthesis [83]. The overexpression of endogenous HMG1 and ACL1 (the gene for ATP citrate lyase), coupled with ideal expression of acetyl CoA synthase (acs), boosts the acetyl-CoA supply and squalene production. This genome-scale metabolically engineered model directs carbon fluxes from intracellular precursors toward acetyl-CoA synthesis. Competing pathways are thus blocked from converting acetyl-CoA, and the flow of consumption is driven toward enhanced squalene production [84].

An innovative method for increasing the accumulation of squalene has been demonstrated in *Y. lipolytica* [85]. This lipid metabolic engineering strategy entailed the double deletion of PEX10, encoding peroxisomal membrane E3 ubiquitin ligase, and URE2, the gene for a dual-function protein that suppresses nitrogen catabolite formation and the response against oxidative stress; with this model, 115 times more squalene was produced (22.0 mg/g dry cell weight) than in the parental strain (0.2 mg/g dry cell weight).

7 | Metabolic Engineering of Non-Oleaginous Yeasts for Enhanced Squalene Production

Non-oleaginous yeasts have been studied extensively with regard to the development of efficient metabolic engineering strategies for lipid production and accumulation. These studies have demonstrated the viability of reprogramming cell metabolism pathways to produce lipids. Non-oleaginous yeasts have been modified to become oleaginous and accumulate lipids [86]. Figure 3 shows the strain engineering strategies that have been applied to oleaginous and non-oleaginous yeast to improve the production of squalene.

7.1 | *Saccharomyces cerevisiae*

S. cerevisiae is as a model organism for eukaryotic cells. One well-known metabolic engineering strategy for improving squalene production is the overexpression of truncated HMG1. Isoprenoid production can be enhanced by using a truncated variant of HMG-CoA reductase that lacks the N-terminal membrane targeting signal [11]. Several recombinant DNA techniques have been developed to produce strains that synthesize more squalene, including the overexpression of the genes *mvaE, tHMG1, ERG20, ispA*, and *GPPS2* in *S. cerevisiae* Y2805 [87]. NADPH is a cofactor in squalene synthesis reactions that involve HMG-CoA reductase (HMG1) and SQS (encoded by ERG9) [88]. POS5P, a protein that is encoded by *pos5*, functions as an NAD^+ and NADH kinase; thus, NADPH regeneration is higher in *pos5*-overexpressing strains [89]. This strategy has been used to improve squalene production [90].

According to some studies, overexpression of the gene for the cytosolic acetaldehyde dehydrogenase ALD6 increases acetate build-up in *S. cerevisiae* [91]. Excellent results have been obtained by overexpressing the genes that encode for acetyl-CoA synthase to improve the conversion of acetate to acetyl-CoA [92, 93]. An acetaldehyde dehydrogenase (ADA) from *Dickeyazeae* catalyzes the conversion of aldehydes to acetyl-CoA directly without ATP, which can lower the cost of generating ATP molecules and accelerate ethanol usage [83]. *S. cerevisiae* has been engineered by introducing HMG-CoA reductase (NADH-HMGR) from *Silicibacter pomeroyi*, which is highly selective for NADH, boosting the activity of HMG-CoA reductase and decreasing the dependence on NADPH. The capacity for using ethanol to synthesize squalene has been improved by overexpressing the native ethanol dehydrogenase ADH2 and ADA from *Dickeyazeae* [94]. *S. cerevisiae* that harbors genes that encode for trehalose synthesis and heat shock proteins has greater tolerance to ethanol and the highest squalene titer at 27.3 g/L [95].

The accumulation of squalene in the cellular membrane can influence membrane fluidity by promoting membrane softening [96]. The transcription of enzymes in cell wall synthesis is

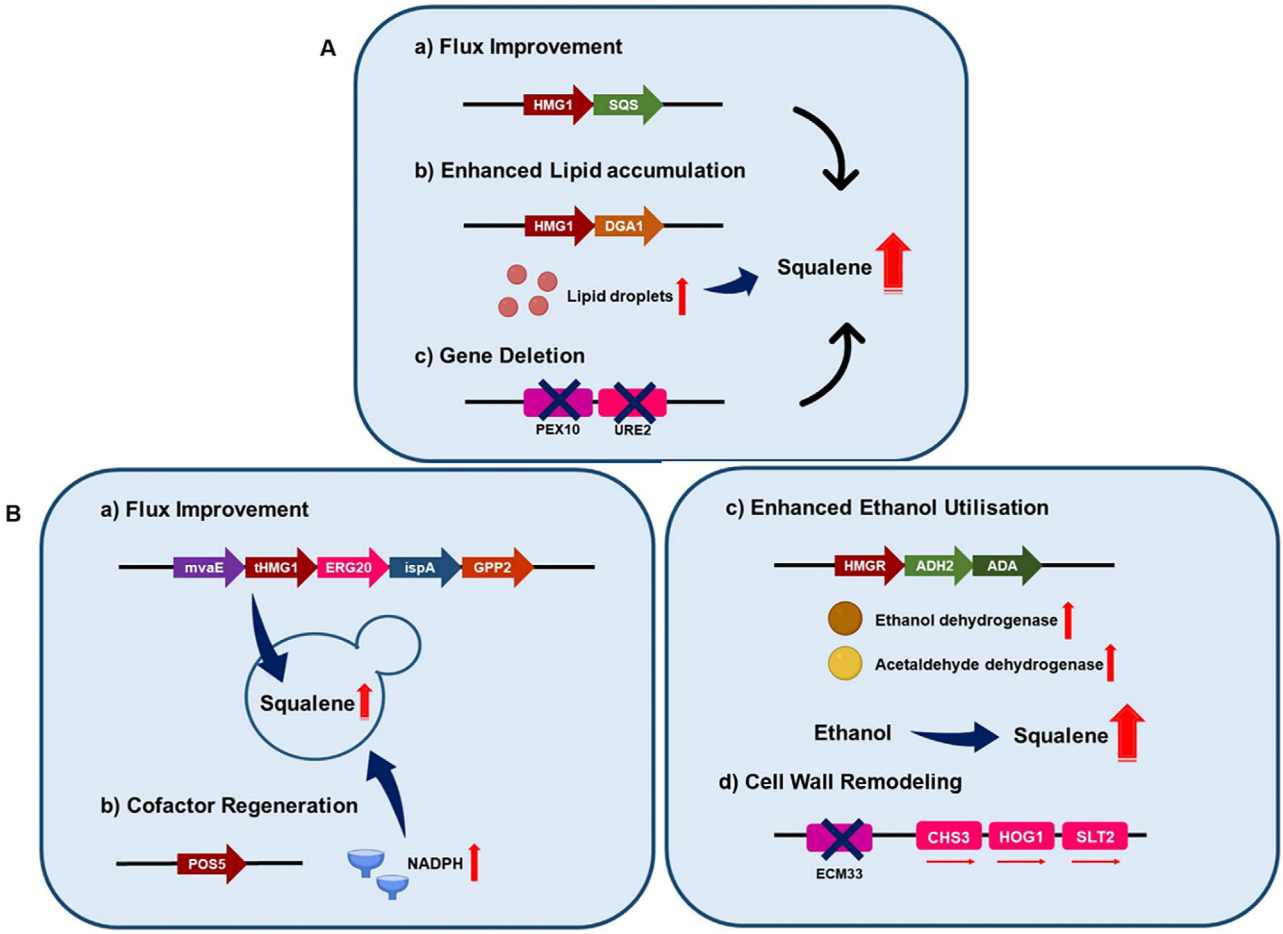

FIGURE 3 | Metabolic engineering and strain improvement strategies in yeast for enhanced squalene production. (A) In *Yarrowia lipolytica* (a) co-overexpression of rate-limiting enzymes in squalene biosynthesis pathway, (b) co-overexpression of genes for enhanced lipid accumulation, (c) dual gene knockout. (B) In *Saccharomyces cerevisiae* (a) enhancing the precursor pool through chromosomal integration and episomal expression of genes, (b) heterologous gene expression and cofactor regeneration, (c) overexpression of heterologous gene for the conversion of ethanol to squalene, (d) cell wall remodeling enhances stress tolerance and restore membrane integrity. ADA – acetaldehyde dehydrogenase; ADH2 – ethanol dehydrogenase; CHS3 – chitin synthase; DGA1 – diacylglycerol acyltransferase; ECM33 – glycosylphosphatidylinositol; ERG20 – farnesyl pyrophosphate synthetase; GPP2 – glycerol-1-phosphate phosphohydrolase 2; HMGR – HMG-CoA reductase; HOG1 – mitogen-activated protein kinase; ispA – farnesyl diphosphate synthase; mvaE – acetyl-CoA acetyltransferase; PEX10 – peroxisomal membrane E3 ubiquitin ligase; POS5 – NADH kinase pos5, mitochondrial; SLT2 – serine/threonine protein kinase; SQS – squalene synthase; tHMG1 – truncated HMG-CoA reductase; URE2 – ureidosuccinate transport 2.

triggered when the cell wall integrity (CWI) pathway is activated, often increasing the amount of chitin in the cell wall [97]. The ECM33 gene encodes for glycosylphosphatidylinositol (GPI)-anchored protein, a cell surface protein that controls the transcription of certain CWI pathway genes [98]. The CWI pathway has been engineered in a squalene-overproducing *S. cerevisiae* SQ03-INO2 strain through the deletion of ECM33 deletion, in the upregulation of CHS3 (a chitin synthase), HOG1 (a mitogen-activated protein kinase), and SLT2 (a serine/threonine protein kinase). This modification helps the cell wall remodel by restoring membrane rigidity and improving the tolerance to stress, increasing its production of squalene [99].

Coexpression of tHMG1 and DGA1 improves squalene production synergistically in *S. cerevisiae* through increased lipid content [80], demonstrating that this genetic modification is an effective means of hyperproducing hydrophobic molecules. In a promising cost-effective method of producing squalene, a novel industrial strain of *S. cerevisiae* was developed to enhanced its metabolic flux of the MVA pathway. A yeast strain was engineered with *Enterococcus faecalis* MvaE 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), combined with cofactor balance, β-alanine metabolism, and cytosolic acetyl-CoA synthesis [100].

7.2 | *Komagataella phaffii*

As a natural squalene producer, *K. phaffii* (*Pichia pastoris*) has been genetically modified to become an excellent host for production, significantly increasing its synthesis by decreasing the flux toward the ergosterol pathway [101]. The targeted expression of critical enzymes in the MVA pathway—HMG-CoA reductase (HMG1), squalene synthase (ERG9), farnesyl diphosphate synthase (ERG20), and squalene epoxidase (ERG1)—can also augment squalene levels [102].

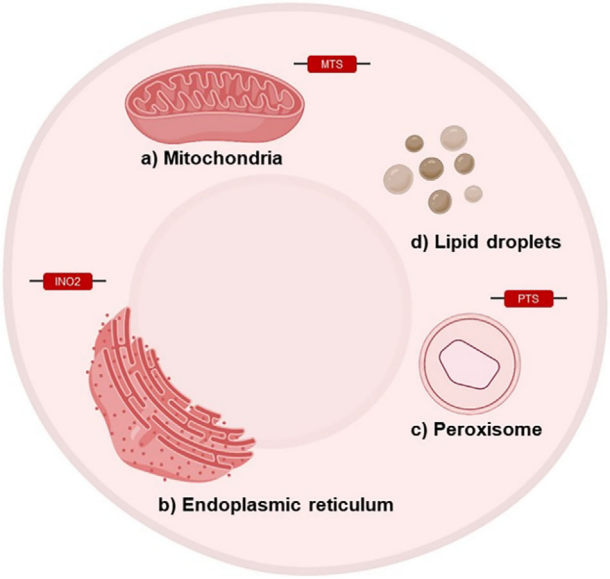

FIGURE 4 | Squalene synthesis pathway compartmentalization by peptide signaling sequence engineering. (a) Mitochondria (b) endoplasmic reticulum (c) peroxisome (d) lipid droplets. INO2 – inositol requiring transcription factor; MTS – mitochondrial targeting sequence; PTS – peroxisomal targeting sequence.

8 | Compartmentalization Strategies for Effective Squalene Production

Eukaryotes possess various organelles and membrane structures, including the endoplasmic reticulum (ER), Golgi complex, lipid droplets (LDs), peroxisomes, mitochondria, and the plasma membrane (PM), allowing them to produce and accumulate squalene [103]. Compartmentalization strategies can improve their catalytic efficiency and prevent toxicity [104, 105]. Figure 4 shows several compartmentalization strategies for generating squalene.

8.1 | Endoplasmic Reticulum

The ER has a significant function in the production of terpenoids—through the manufacture and processing of certain enzymes or the specific localization of some enzymes to the ER, including those in the MVA pathway [105]. The primary objective of engineering the ER has been to increase its ER [46]. Overexpression of the INO2 gene, the primary regulator of ER size, enlarges the ER and increases squalene production sharply [104, 106].

8.2 | Peroxisomes

Peroxisomes can house hydrophobic substances, because they are the sole locations of fatty acid oxidation during fatty acid catabolism [107–110]. Farnesyl diphosphate (FPP), a crucial metabolic precursor in the biosynthesis of squalene, can also be produced simultaneously by peroxisomes, supporting that peroxisomes are essential for terpenoid synthesis [104, 111].

Squalene has been observed to disperse in oil droplets when produced in excess in the cytoplasm of *S. cerevisiae*. Notably, these oil droplets are inflated peroxisomes that swell in tandem with squalene production, demonstrating that peroxisomes in *S. cerevisiae* are dynamic depots for squalene storage. This finding prompted the use of peroxisomes as subcellular compartments for squalene production [112].

Artificial metabolic pathways have been introduced and localized to peroxisomes with enhanced C-terminal peroxisome targeting signal type 1 (ePTS1) [113]. The entire squalene biosynthesis pathway, including *ERG10, ERG13, tHMG1, NADH-HMGR, ERG12, ERG8, MVD1, IDI1, ERG20*, and *ERG9*, was fused with C-terminal ePTS1 and targeted to yeast peroxisomes. The coordinated overexpression of Ant1, a peroxisomal adenine nucleotide transporter and a key component of the peroxisomal membrane that transports ATP to peroxisomes, peroxisomal Idp3, cytosolic Idp2, peroxisome-targeted Acs1, and cloned ACL (a *Y. lipolytica* gene) effected a significant rise in the pool of all cofactors in peroxisomes and thus a 138-fold increase (1312.82 mg/L) in squalene production [112, 114].

8.3 | Cytoplasm and Mitochondria

The potential for mitochondrial compartmentalization to aid in terpenoid synthesis is increased 20−30 fold by high acetyl-CoA content in mitochondria compared with the cytoplasm [115, 116]. In contrast, IPP and DMAPP are ATP analogs that are potent inhibitors of the mitochondrial respiratory chain and are produced from acetyl-CoA via the MVA pathway [117, 118]. Thus, incorporating the MVA pathway in mitochondria can cause cytotoxicity and poor cell growth. To lessen the metabolic burden of compartmentalization of the MVA pathway in mitochondria and enhance cell development, the integration of cytoplasmic and mitochondrial engineering has been proposed. The approach demonstrated that cytoplasmic and mitochondrial engineering has an additive impact (2.1-fold increase) on squalene production [119].

8.4 | Lipid Droplets

LDs are ER-derived, single-layer phospholipid membrane organelles that are used by cells as their primary lipid storage compartments. Triacylglycerols (TAGs) and sterol esters (SEs) are the main neutral lipids in the LD core [120, 121]. Observations of the coaccumulation and cooccurrence of terpenoids and neutral lipids in LDs have advanced our understanding of how bioactive defense compounds are isolated intracellularly, giving rise to metabolic engineering and synthetic biology methods for engineering high-yield terpene production and storage in LD-containing cells [104, 122–124]. Co-overexpression of tHMG1 and DGA1 increases the accumulation of squalene, given that lipid bodies are possible storage sites for squalene in *S. cerevisiae* and *Y. lipolytica* [80, 82].

9 | Squalene Production With Reduced Squalene Epoxidase Activity

Squalene epoxidase, a crucial oxygen-requiring enzyme that is encoded by the yeast ERG1 gene, converts squalene to squalene

epoxide during ergosterol biosynthesis [13]. Dysregulation of the ERG1 gene (even by point mutations) can boost squalene production without retarding growth [125, 126]. Many studies have shown that the targeting squalene epoxidase with inhibitors directs the ergosterol pathway to synthesize more squalene. Specific point mutations in the ERG1 gene decrease squalene epoxidase activity and increase its susceptibility to inhibitors [125]. Allylamine terbinafine (an antifungal agent) enhances squalene production by disrupting ergosterol biosynthesis in *S. cerevisiae* [27, 125] and *K. lactis* [34]. Methyl jasmonate, a signaling molecule in plants and another squalene epoxidase, induces the transcription of the genes that encode for 3-hydroxy-3-methylglutaryl coenzyme A reductase isoenzyme (Hmg1p), squalene synthase (Erg9p), and squalene epoxidase (Erg1p) [127] in *S. cerevisiae* [27].

Other studies [128] have shown the addition of terbinafine and the knockdown of CrSQE (encoding for squalene epoxidase) are effective strategies for accumulating squalene in *Chlamydomonas reinhardtii*. They sequenced squalene synthase (CrSQS) and squalene epoxidase (CrSQE). Overexpression of CrSQS increases the rate at which FPP is converted to squalene. The level of squalene was not substantially higher in cotransformed lines in which CrSQS was overexpressed and CrSQE was knocked down compared with knockdown of CrSQE alone. These findings suggest that squalene production in *C. reinhardtii* can be increased by partially knocking down CrSQE [13, 128]. Figure 4 shows the signal sequences that have been used for producing squalene in compartments.

10 | Optimization of Fermentation for Enhancing Squalene Production

There are many types of chemicals and bioactive compounds that are recognized as intermediates in various biosynthesis pathways and increases in these intermediates (with respect to titers) can be achieved only under optimized conditions [129–131]. Several commercially significant substances in the energy, pharmaceutical, chemical, and food industries are produced using fermentation technology on a large scale. Fermentation technology leverages the adaptability of natural pathways to synthesize a desired chemical from organisms using less expensive sources of substrates to improve the industrial-scale production of valuable products [132].

Squalene production in *S. cerevisiae* is strictly controlled and is negligible (0.041 mg/g of biomass) during its growth [13, 87]. Thus, unless fermentation conditions are improved, it is not a good candidate for squalene production [32]. Establishing fermentation conditions that support active growth and high squalene content in yeast cells is the goal in optimizing the industrial performance of *S. cerevisiae* with regard to squalene synthesis. Researchers have investigated how the intensification of bioprocesses for increasing squalene output and selectivity in two wild strains, *S. cerevisiae* BY4741 and EGY48, is affected by oxygen availability, inoculum size, and fermentation time [133]. Maximum squalene production was attained under anaerobic conditions (2.97 ± 0.12 and 3.13 ± 0.11 mg/L for BY4741 and EGY48, respectively), due to the inhibition of squalene epoxidase activity by low oxygen activity.

Suppression of carbon catabolites also limits squalene yields during growth on glucose. Physiological and metabolic characterizations of *S. cerevisiae* CEN.PK tHMG1 have demonstrated that the accumulation of squalene is influenced significantly by culture parameters, carbon source, composition of the media, and culture mode; further, glucose in the media can substantially reduce the availability of acetyl-CoA for the MVA pathway [134].

Creating anaerobic culture conditions is effective even for *Torulaspora delbrueckii* [32]. Comparative studies have evaluated the potential of *S. cerevisiae* and *T. delbrueckii* to produce squalene. Higher squalene production was reported in *T. delbrueckii* (237.25 μg/g dry cell weight), establishing its potential for commercial squalene production. Squalene yields of up to 11.12 mg/g can be obtained by supercritical fluid extraction (SFE) at a constant flow rate of 0.2 L/min of carbon dioxide (CO_2), a temperature of 60°C, and pressure of 250–255 bar. This extraction method typically benefits nonpolar compounds and low-molecular-weight compounds. By performing a lyophilization step before SFE, this yield can be improved, to 430.52 mg/g [32]. The composition of the media affects lipid accumulation, especially in *Y. lipolytica* [78, 135]. YPD-80, which contains an excess nitrogen, is more suited for enhancing lipid biosynthesis, further increasing the likelihood of squalene formation (731.18 mg/L) [84].

Optimizing the composition of media, including the concentration of glucose, peptone, yeast extract (optimizing carbon and nitrogen sources), and salinity, enhances squalene production six-fold in metabolically engineered *Aurantiochytrium* TWZ-97 (a heterotrophic unicellular marine thraustochytrid) that overexpresses squalene synthase [136]. Another group optimized the culture conditions for *Aurantiochytrium* 18W-13a, producing 171 mg/g DCW squalene content (and 0.9 g/L of squalene) using 2% glucose and 50% seawater in glucose-peptone-yeast media (GPY media), which was incubated at 25°C [137, 138]. Through the optimization of media, another prospective thraustochytrid strain, *Aurantiochytrium mangrovei* FB3, has been found to be an alternative squalene producer in the fermentation industry by adjusting glucose concentrations. Squalene concentrations rose to 2.21 mg/L in an experiment with 30 g/L glucose. Following treatment with 10 and 100 mg/L terbinafine, this yield increased by 36% and 40% compared with the control strain [139].

Squalene synthesis by *Schizochytrium mangrovei* has also been examined using 30-L and 150-L bioreactors. Squalene content of 33 mg/g DCW was recovered in both cases, and the yields were 0.99 and 1.01 g/L, respectively. Recently, this strain was subjected to a variety of fermentation processes, of which 48 h of incubation time in 15 L of media that contained 22% glucose generated the most squalene (98.07 mg/g of lipid) [140, 141].

Shakeri and colleagues [38] isolated *Rhodosporidium sp*. DR37, an oleaginous yeast, from a mangrove ecosystem, reporting it to be a potential source of squalene. On various carbon and nitrogen sources (glucose, sucrose, glycerol, olive oil, and starch), with varying quantities of saltwater, and at varying temperatures and pH levels, cell growth and lipid and squalene production were evaluated. After 72 h of incubation at 25°C and pH 7, maximum squalene synthesis of 619 mg/L was achieved in media that contained 20 g/L sucrose, 5 g/L peptone, and 20% v/v saltwater.

The novel yeast-like fungus strain *Pseudozyma* SD301 produced the highest titer of 2.44 g/L squalene by fed-batch fermentation, which involved being fed a 3:1 mixture of glucose: yeast extract at pH 6 and 25°C and supplemented with 15 g/L of sea salt [52]. A yield of 0.18 mg/g DCW was obtained by growing the microalgae *Phormidium autumnale* in a bubble column bioreactor with procured agroindustrial wastewater, a sustainable method for producing squalene [142].

11 | Conclusion and Outlook

Squalene is an interesting natural product with broad applications, especially in the cosmetics industry. To preserve marine life, new accessible natural sources of squalene and cost-effective methods of separating it are being sought after. Unfortunately, shark-based squalene remains the least expensive option. Clearly, this source must be replaced quickly with competitive alternatives with regard to cost and quality. Thus, techniques for obtaining non-animal squalene are being developed and scaled up to suit commercial needs.

Genetic modification of the natural squalene biosynthesis pathway can transform microorganisms into cell factories that produce squalene on an industrial scale, rendering microbial sources an option to replace plant-derived and animal-derived squalene. *S. cerevisiae, E. coli, C. glutamicum*, and *Y. lipolytica* are several well-known microbial platforms for squalene synthesis. Nevertheless, synthesis in these species and their fermentation can still be improved and optimized.

The use of microbial squalene in the cosmetics and pharmaceutical industries has many benefits. Squalene is a versatile compound with various applications, including skin care products, dietary supplements, and drug delivery systems. Its natural moisturizing properties, antioxidant effects, and compatibility with human skin render it a valuable ingredient in cosmetics. Further, squalene is a precursor in the synthesis of important molecules, such as sterols and hormones, adding to its pharmaceutical potential. The growing demand for sustainable and eco-friendly products provides excellent market opportunities for microbial squalene. The shift toward green alternatives in the cosmetics and pharmaceutical industries aligns with consumer preferences for environmentally conscious choices. The adoption of microbial squalene can reduce the ecological impact of these industries while meeting the increasing demand for high-quality and sustainable products.

Future research and development efforts should focus on improving the efficiency and scalability of microbial production of squalene, including optimizing fermentation conditions, exploring genetic engineering techniques to enhance squalene yields, and developing cost-effective production platforms. The challenges in positioning microbial squalene concern its yield, in situ extraction, and intracellular production, leading to high production costs. Process optimization and scale-up studies are crucial to ensure the commercial viability and competitiveness of microbial squalene as a sustainable alternative. In addition, continued exploration of its potential applications and benefits in the cosmetics and pharmaceutical industries is warranted. Such efforts should include examining its efficacy in various formulations, conducting clinical studies to validate its dermatological benefits, and developing novel delivery systems to enhance its bioavailability. Further, regulatory frameworks and certifications should be established to ensure the safety and quality of microbial squalene-based products. Standardization and certification processes will help build consumer trust and facilitate market acceptance.

Microbial squalene has significant promise as a sustainable green alternative for the cosmetics and pharmaceutical industries. With further advancements in production technology, increased awareness, and market acceptance, microbial squalene has the potential to revolutionize these industries, offering environmentally friendly and high-performance solutions.

Author Contributions

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Acknowledgments

The authors gratefully acknowledge the Science and Engineering Research Board (SERB), DST, Government of India for SERB International Research Experience (SIRE) fellowship to PAB (SIR/2022/000277). PAB acknowledge SERB-Core Research Grant from the Department of Science and Technology, Government of India (CRG/2022/002239). PAB acknowledges LMB and the Director of CSIR-NIIST for support for the lab facilities.

Open access funding enabled and organized by Projekt DEAL.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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