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Review

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Advances in Biochemistry and Microbial Production of Squalene and Its Derivatives

Gopal Prasad Ghimire[1†], Nguyen Huy Thuan[2†], Niranjan Koirala[1†], and Jae Kyung Sohng[1*]

[1]*Department of BT-Convergent Pharmaceutical Engineering, Institute of Biomolecule Reconstruction, Sun Moon University, Asan 31460, Republic of Korea*

[2]*Center for Molecular Biology, Institute of Research and Development, Duy Tan University, Danang City, Vietnam*

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*Corresponding author
Phone: +82-41-530-2246;
Fax: +82-41-544-2919;
E-mail: sohng@sunmoon.ac.kr

†These authors contributed equally to this work.

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Squalene is a linear triterpene formed via the MVA or MEP biosynthetic pathway and is widely distributed in bacteria, fungi, algae, plants, and animals. Metabolically, squalene is used not only as a precursor in the synthesis of complex secondary metabolites such as sterols, hormones, and vitamins, but also as a carbon source in aerobic and anaerobic fermentation in microorganisms. Owing to the increasing roles of squalene as an antioxidant, anticancer, and anti-inflammatory agent, the demand for this chemical is highly urgent. As a result, with the exception of traditional methods of the isolation of squalene from animals (shark liver oil) and plants, biotechnological methods using microorganisms as producers have afforded increased yield and productivity, but a reduction in progress. In this paper, we first review the biosynthetic routes of squalene and its typical derivatives, particularly the squalene synthase route. Second, typical biotechnological methods for the enhanced production of squalene using microbial cell factories are summarized and classified. Finally, the outline and discussion of the novel trend in the production of squalene with several updated events to 2015 are presented.

Keywords: Squalene, biosynthesis, microbial cell factory, terpenes, squalene production

Introduction

Structurally, squalene is a unique 30-carbon, polyunsaturated hydrocarbon of the triterpene group, consisting of six isoprene units, and is consequently an isoprenoid compound (Fig. 1). Several antioxidants such as lycopene and beta-carotene are either isoprenoids or have an isoprenoid tail. The well-known compounds requiring a prenyl group for their synthesis include vitamins A, D, E, and K, carotenes, and lycopene, and all have antioxidant properties. In recent years, scientists have been interested in studying the biochemistry and production of squalene owing to its potential pharmaceutical applications. Historically, inhabitants of the Japanese island of Izu used to drink oil that was locally named "Samedawa," meaning "cure oil" [1]. Squalene was first described by Tsujimoto, a Japanese industrial engineer, and 10 years after his initial discovery, Samedawa was found to contain high proportions of a novel highly unsaturated hydrocarbon [67]. It received its name owing to the fact that it was first isolated from sea shark (*Squalus* spp.) liver oil, which was proven to bear a plentiful amount of squalene [17]. However, squalene is a naturally occurring polyprenyl compound found widely in differing amounts in nature, such as in olive oil [40], amaranth seeds [2, 46], wheat germ oil, palm oil, and rice bran oil [25]. It is also found in other plant materials, freshwater fish, and human tissue or sebum [34, 42] and has various beneficial effects, being useful as a nutrient and as a preventive and therapeutic medicine. The inhibition of cancer risk [50], enhancement of the antitumor action of chemotherapeutic agents [49], and efficient improvement of the immune system [23, 51, 52] are confirmed as dietary squalene properties. It is a critical agent to protect the skin from short wavelength radiant energy [66] and is also effective in lowering blood cholesterol [8]. From various studies, it was found that squalene can effectively inhibit induced

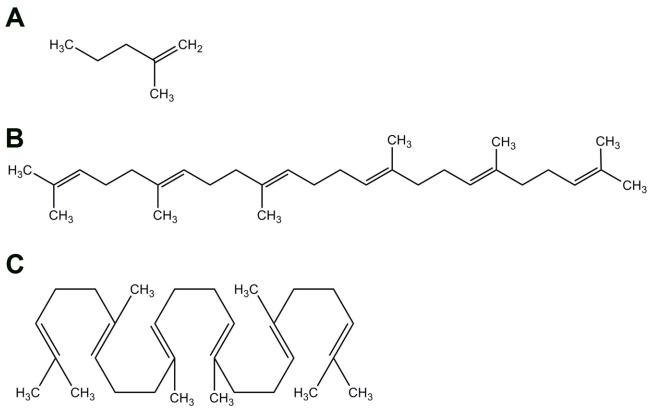

Fig. 1. (**A**) Structure of isopentenyl pyrophosphate, (**B**) squalene in linear form, and (**C**) squalene in coiled form.

tumor genesis of the lung, skin, and colon in rodents [14, 60], indicating that it is a highly potential pharmaceutical reagent.

Biochemistry of Squalene

General Biosynthetic Routes of Squalene and Its Derivatives

Isoprenoids are synthesized from the isopentenyl units formed by two different metabolic pathways, leading to isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) [36]. These distinctive pathways utilize non-homologous enzymes that have evolved independently, to generate the same universal C_5 precursors IPP and DMAPP. The classical mevalonate (MVA) pathway was discovered in the 1960s and is considered to be the only source of the precursors IPP and DMAPP [32, 43]. The MVA pathway is effective in plants, animals, and fungi, and functions in water-soluble components in the cytoplasm to generally supply the precursors for production of sesquiterpenes and triterpenes, such as squalene and its related compounds such as oxidosqualene and *bis*-oxidosqualene, which are considered to be the biosynthetic routes of nearly 200 triterpene skeletons [70], and the more recently discovered 1-deoxyxylulose-5-phosphate (DXP)/methylerythritol phosphate (MEP) pathway [59] primarily found in prokaryotes, including *Escherichia coli* and the plastids of photosynthetic organisms [11, 35]. This pathway, named after the first committed precursor 2-*C*-methyl-D-erythritol-4-phosphate (MEP; the pathway is also sometimes referred to as the DXP pathway), is used to obtain monoterpenes, diterpenes, and tetraterpenes [66].

Naturally, the synthesis of squalene via the MVA pathway starts with the condensation of one acetyl-CoA with another acetyl-CoA by acetoacetyl-CoA synthase. This enzyme is so-called acetyl-CoA transferase functioning in the formation of acetoacetyl-CoA. The reaction chain from acetoacetyl-CoA to mevalonate continues to form 3-hydroxy-3 methylglutaryl-CoA (HMG-CoA) via condensation in the presence of 3-hydroxy-3 methylglutaryl-CoA synthase [33]. In the presence of HMG-CoA reductase plus NADPH as a cofactor, HMG-CoA is later reduced to mevalonate [21], which is subsequently phosphorylated by mevalonate kinase in the presence of adenosine triphosphate (ATP) to form mevalonate 5-phosphate, and is further phosphorylated by phosphomevalonate kinase in the presence of ATP to form mevalonate 5-diphosphate and then decarboxylated by mevalonate 5-diphosphate decarboxylase to form IPP, which is used as the chemical base to build most known polyprenyl compounds. IPP is then isomerized to DMAPP by isopentanyl diphosphate isomerase (IDI). The condensation of IPP with DMAPP by farnesyl diphosphate synthase results in geranyl diphosphate, and the subsequent condensation with another IPP to form farnesyl diphosphate (FPP). Finally, squalene synthase has been identified as the enzyme that catalyzes the NADPH-mediated formation of squalene using FPP as the substrate [66] (Fig. 2A). Besides these, glyceraldehyde-3-phosphate and pyruvate are initial precursors for the formation of DXP by DXP synthase (DXS). This substrate is consequently reduced to MEP by a reaction using DXP reductoisomerase (DXR) as biocatalyst. Some bacteria lack DXR but have DRL (DXR-like) enzymes that perform the same reaction [62] (Fig. 2B).

Depending on the species, squalene is continuously utilized as a substrate for the synthesis of sterols, including cholesterol, and as a typical product or carbon source in eubacteria (*Corynebacterium*, *Pseudomonas*, or *Arthrobacter*) to form various metabolites [6, 64, 71]. Recently *Spterp25*, from *Streptomyces peucetius* ATCC 27952 was successfully heterologously characterized in *E. coli*. The experimental results showed that it is an enzyme functioning as a squalene-hopene cyclase [18] (Fig. 3).

Squalene Synthase and Its Application

Squalene synthase (SQS) is very important enzyme catalyzing the first step of sterol/hopanoid biosynthesis in various organisms. Many studies have reported on SQS in plants and fungi [3, 4, 22, 24, 28, 38, 39, 53, 54, 72, 74–77]. Detailed characteristics of squalene synthases are described in Table 1.

An SQS from *Poria cocos* was isolated and characterized

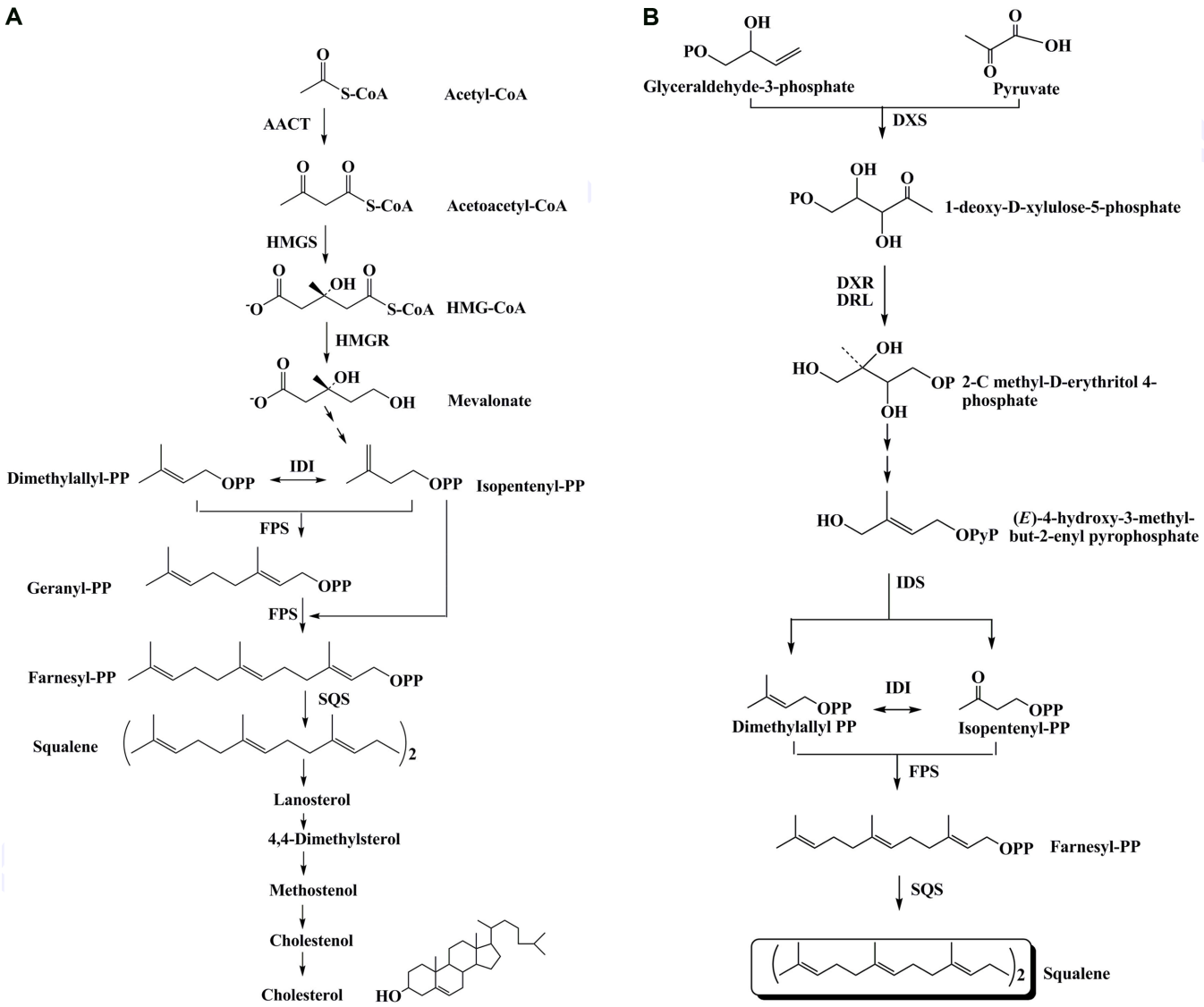

Fig. 2. Biosyntheses of squalene via the MVA and MEP pathways.

(**A**) Squalene synthesis via the mevalonate pathway in mammalian cells. AACT, acetoacetyl-CoA thiolase; FPS, farnesyl diphosphate synthase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, HMG-CoA reductase; IDI, isopentenyl diphosphate isomerase; SQS, squalene synthase. (**B**) Squalene synthesized via the MEP pathway in *E. coli*. DMAPP, dimethylallyl diphosphate; DXS, 1-deoxyxylulose-5-phosphate synthase; DXR, 1-deoxyxylulose-5-phosphate reductoisomerase; FPP, farnesyl diphosphate; FPS, FPP synthase; G-3-P, glycerol-3-; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; DRL, DXR-like protein found in *B. abortus*.

using degenerate and inverse PCR. The transcriptional level of this SQS gene was increased about 4-fold by treatment with *P. cocos* cells with 300 μM methyl jasmonate, enhancing the content of cellular squalene (128.62 μg/g) after 72 h induction [68], which helps in metabolic regulation, defense, and stress response.

Recently, directed evolution has become a powerful technique to screen and recruit an appropriate SQS for the production of dehydrosqualene. High-throughput screening of *Pantoea ananatis*–originated phytoene desaturase was accomplished to find out the appropriate candidate converting dehydrosqualene into yellow carotenoid pigments, resulting in the successful construction of various mutants able to convert SQS into a "dehydrosqualene synthase." In the similar manner, it is applicable for the production of diaponeurosporene and diapolycopene using human SQS

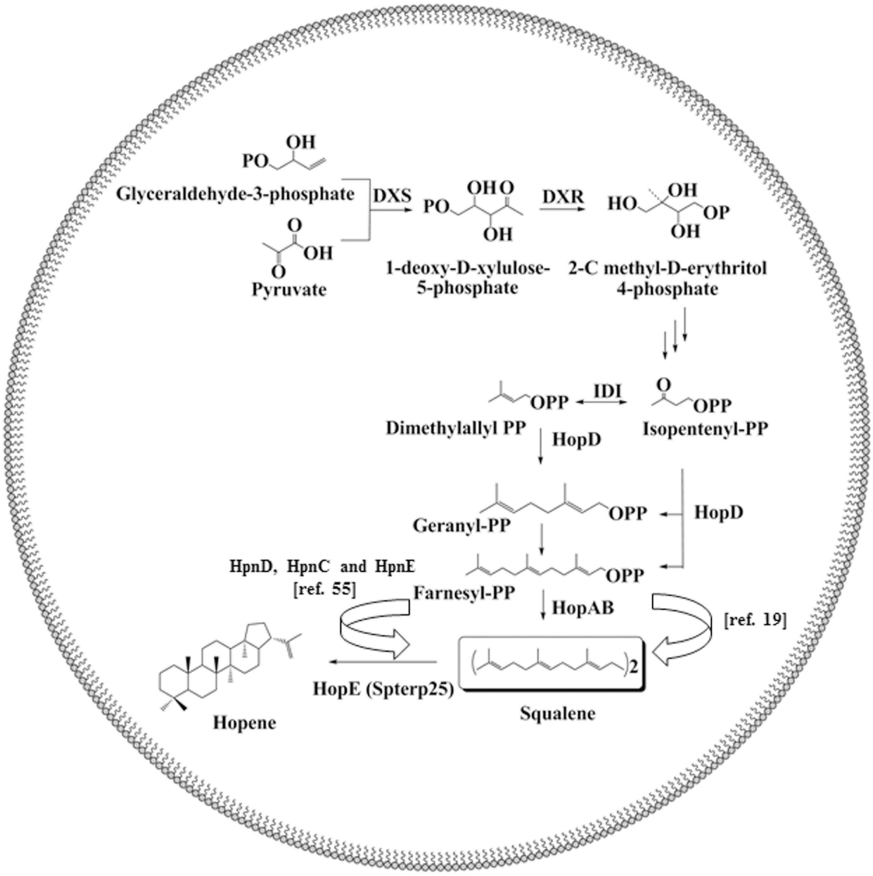

Fig. 3. Strategy for heterologous production of squalene and hopene in recombinant *E. coli* [18, 19].

and *Staphylococcus aureus* dehydrosqualene (C_{30} carotenoid) desaturase, respectively. Experimentally, the desaturation of squalene was proven as the main pathway to synthesize those carotenoids rather than the direct synthesis of dehydrosqualene [15].

RNAi-mediated disruption of squalene synthase genes would remarkably improve drought tolerance in rice via the change of various physiological properties, including more soil water conserved, delayed wilting, and improved recovery. Furthermore, the yield of transgenic positive rice was 14–39% higher than wild type; thereby, RNAi-mediated inactivation was proven as an efficient tool for improvement of the strain toward drought tolerance [44].

Squalene synthase from *Hepacivirus* genus of the *Flaviviridae* family is also significant for hepatitis C virus (HCV) production. Therefore, this enzyme was proposed as a potential anti-HCV target. For instance, Saito *et al*. [61] tested some chemicals, including Sandoz 58-035, zaragozic acid A, and YM-53601, as squalene synthase inhibitors. The experimental results showed that the compound YM-53601 repressed the biosynthesis of cholesterol and its derivatives (cholesteryl esters) in the same concentrations for indinavir, a protein inhibitor used as a component of highly active antiretroviral therapy. Furthermore, YM-53601 and zaragozic acid A were proven to cause positive effects, such as reduced viral protein, offspring production, and RNA in HCV-infected cells. The authors examined the impact of the cholesterol biosynthetic pathway for HCV production and indicated that SQS as a potential pharmaceutical target for antiviral strategy against HCV [61].

Chagas disease (or American trypanosomiasis) is caused by *Trypanosoma cruzi*, a type of tropical protozoan parasite that occurs mostly in Latin America. Biochemically, this parasite uses sterols and their derivatives rather than cholesterol to construct its cell membranes. Thus, inhibiting endogenous sterol biosynthesis is a critical therapeutic target. Shang *et al*. [65] reported five structures of the parasite's squalene synthases and a series of squalene synthase inhibitors (four classes, including the substrate-analog S-thiolo-farnesyl diphosphate, quinuclidines E5700 and ER119884, several lipophilic bisphosphonates, and the thiocyanate WC-9).

Table 1. Characterization of squalene synthases in various organisms using microbial expression hosts.

No.	Species	Expression host	Apperent molecular mass (kDa)	Enzymatic characteristics	References
1	*Botryococcus braunii*, race B	*E. coli*	52.50	*Botryococcus* squalene synthase gene expression is preferential during rapid growth	[54]
2	*Aurantiochytrium* sp. KRS101	*E. coli*	42.70	Conversion of farnesyl-diphosphate to squalene in the presence of NADPH and Mg^{2+}	[24]
3	*Thermosynechococcus elongatus* BP-1	*E. coli*	42.00	Bifunctional enzyme due to catalyzing the formation of squalene and presqualene-diphosphate	[39]
4	*Candida tropicalis*	*Pichia pastoris*	57.00	Specific reactivity of the purified recombinant squalene synthase with infected mice sera	[38]
5	*Leishmania donovani* (MHOM/IN/80/Dd8)	*E. coli*	Not mentioned	Catalyzing an unusual head-to-head reductive dimerization of two molecules of farnesyl-pyrophosphate in a two-step reaction to form squalene.	[3]
6	*Saccharomyces cerevisiae*	*E. coli*, *S. cerevisiae*	47.00	Catalyzing the two-step conversion of farnesyl diphosphate (FPP) to squalene	[74]
7	*Schizochytrium limacinum*	*E. coli*	47.42	Recombinant squalene synthase catalyzed FPP to squalene	[77]
8	*Fusarium fujikuroi* (*Gibberella fujikuroi* MP-C)	*E. coli*	53.40	Catalyzing the reaction from FPP to squalene	[75]
9	*Withania somnifera* (L.) Dunal	*E. coli*	47.06	Catalyzing dimerization of two FPP molecules intosqualene	[4]
10	*Glycyrrhiza uralensis* Fisch	*E. coli*	GuSQS1: 47.28 GuSQS2: 47.02	Catalyzing FPP into squalene	[14]
11	*Chlorophytum borivilianum* Sant. and Fernand	*E. coli*	46.00	Key enzyme of saponin biosynthesis pathway	[28]
12	*Methylococcus capsulatus*	*E. coli*	41.40	Water-soluble.^{58}DXX ^{61}E ^{62}D motif and the two basic residues (R55 and K212) were responsible for the binding of farnesyl diphosphate	[53]

Biotechnological Progress for Production of Squalene Using Microbial Factories

Owing to its clinical, cosmetic, and pharmaceutical importance, much effort has been directed towards exploring squalene-producing sources such as in marine microorganisms *Pseudozyma* sp. JCC207 [7], marine microalga *Schizochytrium mangrovei* [26], squalene-producing *Aurantiochytrium* from Hong Kong mangroves [41], *Rubritalea sabuli* sp. nov., *Rubritalea squalenifaciens* sp. nov. [30, 73], and especially *Saccharomyces cerevisiae* [29, 45, 51].

Herein, we categorize two types of basic methods used for the enhanced production of squalene.

Media and Fermentative Optimization

Fan *et al*. [13] reported the strain *Aurantiochytrium mangrovei* FB3 as a highly potential source for the enhanced production of squalene via optimized medium conditions and the use of terbinafine as an inhibitor of squalene monooxygenase enzyme. Their experiments recorded the highest biomass concentration of 21.2 g/l with the supplementation of a glucose concentration 60 g/l. Moreover, efficient concentration of terbinafine was used in the amount of 10 and 100 mg/l, because of 36% and 40% increased rates in squalene content, respectively, compared with the control [13]. In the similar manner, Naziri *et al*. [51] treated a wild-type laboratory *S. cerevisiae* strain with 14.57–160.27 mg/l terbinafine and 0–224.30 mg/l methyl jasmonate for the increased production of squalene. Maximum squalene content (10.02 ± 0.53 mg/g dry biomass) and yield (20.70 ± 1.00 mg/l) were obtained by using 128.81 mg/l of terbinafine and 9.86 mg/l methyl jasmonate after 28 h and 87.43 mg/l terbinafine after 30 h, respectively. In addition, the squalene content in the cellular lipid fraction was remarkably increased (12–13%), therefore promising a scale-up study

for the squalene production in this yeast [51]. In addition, the growth dynamics of two wild-type strains of *S. cerevisiae* EGY48 and BY4741 were tested for production of sterols under different aeration conditions. Specifically, they studied oxygen concentration, inoculum size, and fermentation time as followed by the central composite statistical design. The highest squalene yield and productivity were 2.9676 ± 0.1187 mg/l of culture medium and 0.104 ± 0.042 mg/l h for BY4741 and 3.129 ± 0.1095 mg/l of culture medium and 0.1559 ± 0.055 mg/l h for EGY48, respectively [47].

S. cerevisiae and *Torulaspora delbrueckii* were used for the fermentative production of squalene under anaerobic conditions. Experimental data revealed that the yield of squalene from *T. delbrueckii* (237.25 μg/g) was much higher than that from *S. cerevisiae* (41.16 μg/g) and could be used as a high potential alternative source [5]. Nakazawa *et al*. [48] optimized the culture conditions, including media ingredients (salinity, glucose concentration) and temperature, for *Aurantiochytrium* sp. 18W-13a as a squalene producer. The highest squalene titer of ~171 mg/g dry weight and 900 mg/l, respectively, were achieved at 25–50% seawater, 25ºC, and 2–6% glucose concentration. The experimental data indicate that strain 18W-13a has a high potential for exploring commercial squalene [48]. Chen *et al*. [9] investigated the effect of nitrogen sources (monosodium glutamate, tryptone, and yeast extract) for the optimized production of squalene using microalga *Aurantiochytrium* sp. in heterotrophic cultures. Central composite experimental design was used as an analytical tool to determine the critical parameters. The experimental data showed that the interaction of all these nitrogen sources is the most important property to obtain the highest squalene content and squalene yield. Specifically, optimal concentrations of monosodium glutamate, yeast extract, and tryptone were predicted to be 6.61, 6.13, and 4.50 mg/l for squalene content and 6.94 g/l, 6.22 mg/l, and 4.40 mg/l for squalene yield, respectively. By using those parameters the practical squalene content and squalene yield of 0.72 mg/g and 5.90 mg/l, respectively, were obtained [9].

Recently, Drozdíková *et al*. [12] used *Kluyveromyces lactis* as a host for the enhanced production of squalene. By using the commercially cheap glucose and lactose-containing diary industry wastes as the nutrient, they treated the bacterium with squalene epoxidase inhibitor, terbinafine. The data revealed that 7.5 mg/l terbinafine in the lactose medium resulted in a high production of squalene. Thereby, this study provided evidence of the use of lactose-containing diary industry wastes as a nutrient for the lactose-fermenting yeast like *K. lactis* for the production of a high-value liquid and also verified squalene epoxidase as a promising target for the overproduction of squalene [12].

Metabolic Engineering for Enhanced Production of Squalene

Recently, based on the advances of modern molecular biology (recombinant DNA and protein engineering), recombinant plasmids bearing squalene biosynthetic genes as well as relative genes were constructed and cloned in genetically engineered *S. cerevisiae* and *E. coli*. For example, Kamimura *et al*. [29] was the first group that successfully constructed squalene-accumulated *S. cerevisiae* via disruption of a gene involved in the conversion of this compound to ergosterol. Two mutants that required ergosterol for fast growth were aerobically cultured, affording the yield of squalene up to 5 mg/g dry cells. Furthermore, recombinant *S. cerevisiae* YUG37-ERG1 containing a doxycycline-repressible tet07-CYC1 promoter was treated with grass juice to investigate the production of ethanol and squalene. Experiments showed that using grass juice feedstock added with 0.025 μg/ml doxycycline, the yeast accumulated a high amount of squalene with a yield of 18.0 ± 4.18 mg/l. Moreover, grass juice was discovered as a rich source of water-soluble carbohydrates and can be used as excellent material for culture of the YUG37-ERG1 strain [29].

Recently, the accumulation of squalene in microalgal *Chlamydomonas reinhardtii* was investigated based on the characterization of squalene synthase (CrSQS) and squalene epoxidase (CrSQE). In particular, the overexpression of CrSQS increased the rate of conversion of ^{14}C-labeled farnesyl pyrophosphate into squalene but did not overaccumulate squalene. Alternatively, in the CrSQE-knockdown strain, the yield of squalene was enhanced remarkably (0.9–1.1 μg/mg cell dry weight). Therefore, they concluded that the partial knockdown of CrSQE is an important alternative for the enhanced production of squalene in *C. reinhardtii* cells [27].

Mantzouridou and Tsimidou [45] studied the mechanism for regulation of the ergosterol pathway by genetically constructing HMG2 with a K6R stabilizing mutation named AM63. Moreover, they also generated AM64, a derivative of AM63 with an additional deletion of the ERG6 gene, and used these strains as host for testing squalene accumulation in *S. cerevisiae*. Experimentally, strain AM63 produced 20-fold higher yield than the wild-type EGY48 strain. They suggested that in the case of AM64, because of lack of ergosterol feedback inhibition, this strain did not enhance the production of squalene [45]. Back in 1998, HMG-CoA reductase was reconstructed by deletion of its membrane-binding region (*i.e*., amino acids 1–552) and then overexpressed

Table 2. Production of squalene in various microorganisms.

No	Strains	Yield of squalene	Sources
1	*Pseudozyma* sp. JCC207	340.52 mg/l	[7]
2	*Schizochytrium mangrovei*	1.31 mg/l	[26]
3	*Aurantiochytrium* sp. BR-MP4-A1	0.57 mg/g dried cell	[41]
4	*Aurantiochytrium* sp.18W-13a	900.00 mg/l	[48]
5	*Aurantiochytrium* sp.	6,940.00 mg/l	[9]
6	*Aurantiochytrium mangrovei* FB3	21.2 g/l	[13]
7	*Kluyveromyce lactis*	600.00 μg/10^9 cells	[12]
8	*Rubritalea squalenifaciens* sp. nov.	15.00 mg/ g dried cell	[30]
9	*Saccharomyces cerevisiae* AM63	5.20 mg/g dried cell	[45]
10	*S. cerevisiae*YUG37-ERG1	18.0 ± 4.18 mg/l	[29]
11	*Torulaspora delbrueckii*	237.25 μg/g	[5]
12	*Chlamydomonas* sp	1.1 μg/mg cell dry weight	[27]
13	*E. coli*	230.00 mg/l	[31]
14	*E. coli*	11.80 mg/l	[19]

in the host, *S. cerevisiae*. The recombinant yeast resulted in remarkable yield of squalene rather than its derivatives [58].

Our results on *Streptomyces peucetius*-originated putative hopanoid genes, including *hopA*, *hopB* (squalene/phytoene synthases), and *hopD* (farnesyl diphosphate synthase), expressed in *E. coli* as host for heterologous production of squalene yielded 4.1 mg/l of squalene. By contrast, the production of squalene reached 11.8 mg/l together with the coexpression of *E. coli dxs* and *idi* genes encoding 1-deoxy-D-xylulose 5-phosphate synthase and isopentenyl diphosphate isomerase, respectively [19] (Fig. 3). Recently, an *E. coli*-based system was designed for high squalene production using two different types of squalene synthases and their mutants in combination with precursor pathways. The increased squalene concentration of 230 mg/l under the optimal conditions was experimentally found [31].

Squalene epoxidase (encoded by *ERG1*) is an enzyme that is the limiting step for the biosynthesis of squalene, and its biochemical role has been determined via site-directed mutagenesis. Specifically, point mutation carried out on the *ERG1* gene reduced the activity of deduced protein and caused hypersensitivity to terbinafine. The highest squalene level of 1,000 μg/10^9 cells was achieved, without disturbing the cell growth. Furthermore, terbinafine could be used as the squalene epoxidase inhibitor restricting squalene accumulation at 700 μg squalene/10^9 cells, associated with pronounced growth defects. Their data indicate that ERG1 is a critical objective for increased squalene production in yeast [16]. Detailed production of squalene in various microorganisms is shown in Table 2.

Suggested Strategies for Enhanced Production of Squalene Using Microbial Cell Factory

The fast development of genetic engineering, recombinant protein, bioinformatics as well as –omics technologies led to a novel concept for biosynthesis of natural and/or unnatural products named system metabolic engineering. This integration method totally reconstructs or modifies the organism for the optimized production of the desired target. As introduced by SY Lee's research group [10, 37, 57], system metabolic engineering (including synthetic biology, system biology, and evolutionary engineering) has become one of the most important methodologies for the biological production of chemicals and materials. Because *S. cerevisiae* contains all the genes necessary for the production of triterpenoid (ergosterol), it is an excellent genetically engineered host for the production of squalene. However, the endogenous ergosterol pathway is essential to this yeast's survival; hence, it is impossible to disrupt the whole corresponding genes and ergosterol pathway, which may eventually affect the heterologous expression of a novel gene and the actual yield and productivity of squalene and its derivatives [32, 47]. Although *E. coli* does not produce triterpenoid, it has a good chance to reconstruct an ideal host bearing recombinant plasmids such as for squalene. Herein, we proposed the following strategy: (i) directed evolution of important genes such as *hopAB*, *hopD*, *dxs*, *idi*, and *dxr* to screen the mutated genes possessing high capability of substrate conversion; those genes can be

clustered in a pET vector system containing the T7 promoter [18, 19]; (ii) increased production of precursors, including glyceraldehyde -3-phosphate and pyruvate, via the positive regulated genes and the removal of feedback inhibitions [20, 57]; (iii) restriction of the generation of byproducts via blocking branch biosynthesis pathways; and (iv) improve the fermentation techniques based on recent novel discoveries such as rocking-motion-type bioreactor [47], stress-minimization techniques to enhance the yield of target soluble protein [69], and feeding substrate as time-dependent concentration [63]. Pan *et al*. [55] have succeeded to demonstrate a three-step pathway for the synthesis of squalene using a set of three genes (*viz*., HpnC, HpnD, and HpnE) involved in the squalene biosynthesis from farnesyl diphosphate synthase using *E. coli* [55].

Conclusions and Perspectives

The results of the studies outlined in this review provide a current understanding on the biochemistry and biotechnological production of squalene. In addition to the traditional methods such as isolation, screening of potential strain, and media optimization, combinatorial biosynthesis of squalene using *S. cerevisiae* and *E. coli* as the hosts has just started but highly potential results have been obtained. The improvement of engineered microbial hosts, applications of directed evolution on key enzymes, development of strong promoter-bearing plasmid to construct DNA recombinants, regulation of carbon flux, and feedback with media optimization can be considered to be possible alternatives for the enhanced production of this compound in the near future.

The documented results suggest that squalene has a variety of beneficial properties, such as antiaging and anticancer agents. More preclinical studies are needed for establishing the efficacy and the safety issues of squalene with doses effective for cancer prevention and therapy in humans. Based on our current scientific understanding, squalene offers great hope for the prevention of chronic human disease.

Collectively, although squalene has been an important chemical in science as well as human life for years, the discovery of its novel and useful functions is still continuous, thus increasing the demand of squalene, as it is an attractive target for biotechnological production.

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