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REVIEW

Production of squalene by microbes: an update

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Abstract Squalene, a naturally occurring linear triterpene formed via MVA or MEP biosynthetic pathway, is widely distributed in microorganisms, plants and animals. At present, squalene is used extensively in the food, cosmetic and medicine industries because of its antioxidant, antistatic and anti-carcinogenic properties. Increased consumer demand has led to the development of microbial bioprocesses for the commercial production of squalene, in addition to the traditional methods of isolating squalene from the liver oils of deep-sea sharks and plant seed oils. As knowledge of the biosynthetic enzymes and of regulatory mechanisms modulating squalene production increases, opportunities arise for the genetic engineering of squalene production in hosts. In this review, we present the various strategies used up to date to improve and/or engineer squalene production in microbes and analyze yields.

Keywords Biosynthesis · Fermentation · Microbial production · Metabolic engineering · Squalene

Introduction

Squalene, a dehydro-triterpenic hydrocarbon, is a basic intermediate in the biosynthesis of sterols, hopanoids and triterpenes (Kohno et al. 1995). It was not until around a hundred years after it was first described (Tsujimoto 1916) to be isolated from the sea shark's (*Squalus* spp) liver oil (Gershbein and Singh 1969) that it was formally named. The amount of squalene varies in plant seeds (Berger et al. 2003; Martirosyan et al. 2007; Maguire et al. 2009), oils (Singhal and Kulkarni 1990; De Leonardis et al. 1998; Huang et al. 2009; Xiao et al. 2016) and freshwater fish (Ackman et al. 1968; KopiCoVá and VaVreiNoVá 2007). In humans, it is the principal hydrocarbon in surface lipids, making up approximately 10 % of the total surface fat (Liu et al. 1976). Currently, squalene is used widely in the food, cosmetics and medicine industries due to its multiple functions. Up until now, the liver oil of deep sea sharks (Hernández-Pérez et al. 1997) and plant oils (He et al. 2002) have been the major commercial source of squalene. However, the continuous supply and future availability of these sources are uncertain due to the endangerment of the shark species (Lack and Sant 2009) and the unpredictable effect of region and seasonal variation on crop production (Salvador et al. 2003). Therefore, in recent years, many studies have tried to develop microbial strains for squalene production using fermentation processes (Ghimire et al. 2016). This review focuses on these current methods of microbial fermentation and explores the impact that our improving knowledge of squalene biosynthesis may have on future production.

Structure and functions of squalene

Squalene (2, 6, 10, 15, 19, 23-hexamethyltetracosa-2, 6, 10, 14, 18, 22-hexaene,), an unsaponifiable lipid, is a dehydro-triterpenic hydrocarbon ($C_{30}H_{50}$) with six double bonds (Fig. 1). Squalene acts as an natural antioxidant that protects cells from free radicals and reactive oxygen species (Kohno et al. 1995; Obulesu et al. 2015). It has been proven to have radioprotective (Storm et al. 1993) and cardioprotective (Aguilera et al. 2005) functions. Squalene also

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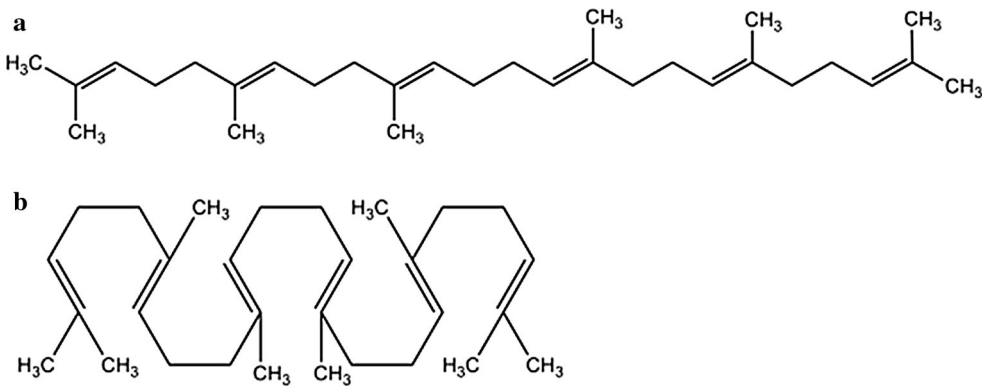

Fig. 1 **a** Squalene in linear type, **b** squalene in coiled type

participates in reducing serum cholesterol levels (Miettinen and Vanhanen 1994; Chan et al. 1996) and improving cellular and non-specific immune functions (Ohkuma et al. 1983; Kelly 1999). Various studies have demonstrated that it is an effective inhibitor of chemically induced tumorigenesis in the colon, lung and skin (Budiyanto et al. 2000; Ronco and De Stéfani 2013; Fang 2014). Since 1997, over 22 million squalene-containing flu vaccines have been administered for their potential capability of increasing immune responses (Kaya et al. 2011).

Recently, it has been found that squalene can modulate fatty acid metabolism (Ravi Kumar et al. 2016) and improve DSS-induced acute colitis by down-regulating signaling pathways (Sánchez-Fidalgo et al. 2015).

Squalene biosynthesis in natural microbial producers

Due to their fast and massive growth, microorganisms are considered a promising alternative to other sources for squalene production. Squalene isolation has been reported in *Saccharomyces cerevisiae* (Kamimura et al. 1994; Socaciu et al. 1995; Mantzouridou and Tsimidou 2010; Naziri et al. 2011; Garaiová et al. 2014; Hull et al. 2014), *Torulaspora delbrueckii* (Bhattacharjee et al. 2001), *Synechocystis* (Englund et al. 2014), *Pseudomonas* (Uragami and Koga 1986), *Candida* (Tsujiwaki et al. 1995; Lee et al. 2014), the microalgae *Traustochytrium* (Dessi et al. 2002), *Chlamydomonas reinhardtii* (Kajikawa et al. 2015) and *Schizochytrium mangrovei* (Jiang et al. 2004; Yue and Jiang 2009; Hoang et al. 2016), *Rhodopseudomonas palustris* (Welander et al. 2009; Xu et al. 2016), and *Botryococcus braunii* (Banerjee et al. 2002). In these microorganisms, each molecule of squalene is formed by fusing two molecules of farnesyl diphosphate (FPP), an isoprenoid compound (Spanova and Daum 2011). Herein, the squalene biosynthetic pathway is re-cast into two modules: the isoprenoid pathway and the fusion pathway.

Isoprenoid pathway

All isoprenoids are synthesized from common isopentenyl units including isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Lange et al. 2000) via either the classical mevalonate (MVA) pathway or 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway using non-homologous enzymes (Lange et al. 2000). The MEP pathway most commonly occurs in prokaryotes such as *E.coli* and the plastids of photosynthetic organisms (Rohmer et al. 1993; Orihara et al. 1998; Kuzuyama 2002; Dellas et al. 2013), although some parasitic prokaryotic microorganisms such as *Rickettsia* and *Mycoplasma* appear to simply obtain their isoprenoids from the host cells (Fraser et al. 1995; Andersson et al. 1998).

Isoprenoid biosynthesis begins with the formation of 1-deoxy-D-xylulose- 5-phosphate (DXP) catalyzed by DXP synthase (DXS), a rate-limiting enzyme of the MEP pathway (Kim et al. 2006; Lee et al. 2007). DXP then undergoes rearrangement and forms reduced MEP by DXP reductoismerase DXR or DRL encoded by the *dxr* or *drl* genes respectively (Sangari et al. 2010). A series of other enzymes are used in the following sequential reactions to convert MEP into the building blocks of IPP and DMAPP which are further isomerized via the enzyme isopentanyl diphosphate isomerase (IDI). Farnesyl diphosphate synthase (FPS) catalyzes the sequential 1′-4 coupling of IPP with DMAPP and GPP, resulting in the formation of FPP (Fig. 2).

Eukaryotic microorganisms such as fungi and yeasts lack the MEP pathway and rely almost entirely on the MVA pathway. The biosynthesis of IPP using the MVA pathway begins with the conversions of three molecules of acetyl-CoQ to MVA sequentially through acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), catalyzed by acetoacetyl-CoA synthsae (AAS) and HMG-CoA synthase (HMGS) (Maury et al. 2005). MVA is then converted to MVA 5-diphosphate by two phosphorylations that are mediated by MVA kinase and phospho-MVA kinase, respectively. MVA 5-diphosphate undergoes dehydration-decarboxylation in the presence of adenosine triphosphate

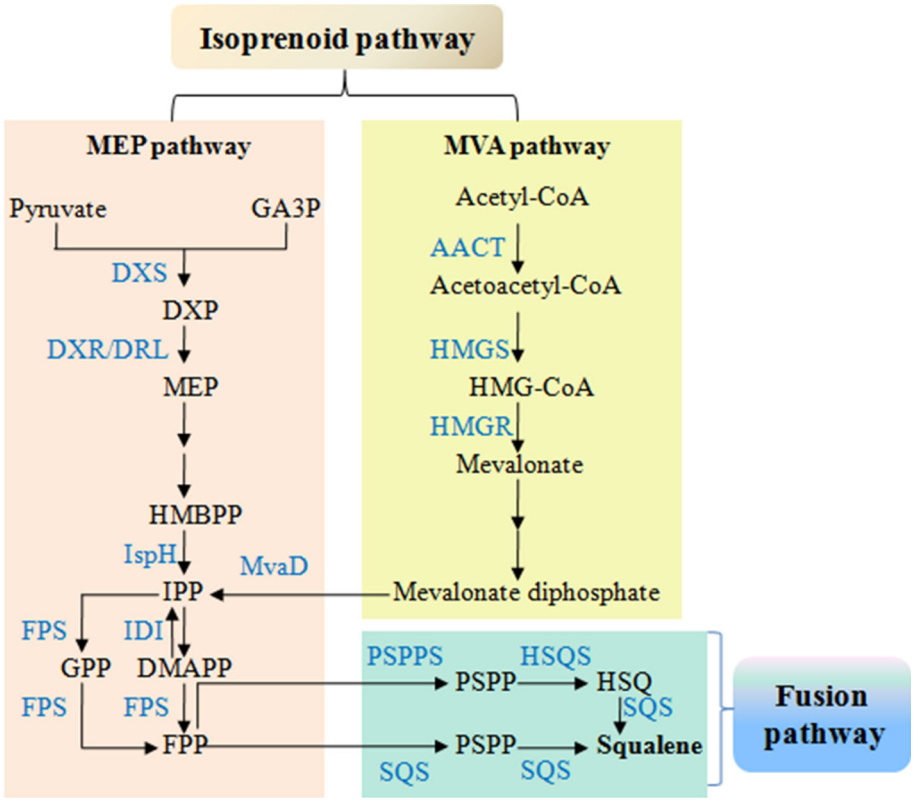

Fig. 2 Metabolic pathway involved in the synthesis of squalene. *AACT* acetoacetyl-CoA thiolase, *DMAPP* dimethylallyl diphosphate, *DXS* 1-deoxy-xylulose-5-phosphate synthase, *DXP* 1-deoxy-xylulose -5-phosphate, *DXR* DXP reductoisomerase, *DRL* DXR-like protein, *FPS* farnesyl diphosphate (FPP) synthase, *GA3P* D-glyceraldehyde-3-phosphate, *GPP* geranyl diphosphate, *HMGS* 3-hydroxy-3- methylglutaryl-CoA (HMG-CoA) synthase, HMGR HMG-CoA reductase, HMGR *HSQS* (R)-12-hydroxysqualene (HSQ) synthase, *IDI* isopentenyl diphosphate isomerase, *IPP* isopentenyl diphosphate, *IspH* IPP synthase, *PSPPS* presqualene diphosphate (PSPP) synthase, *SQS* squalene synthase

(ATP) to form IPP. An IPP isomerase (IDI) catalyzes the isomerization of IPP to DMAPP. Finally, GPP, formed by the condensation of IPP with DMAPP, is condensed with another molecule of IPP to create FPP (Fig. 2).

Fusion pathway

The studies in animals (Shechter et al. 1992; Thompson et al. 1998), plants (Nakashima et al. 1995), fungi (Jennings et al. 1991) and a few bacteria (Lee and Poulter 2008; Ohtake et al. 2014) show that squalene synthase (SQS), catalyzes the fusion of two FPP molecules into one molecule of squalene in two consecutive steps—coupling of two molecules of FPP into presqualene diphosphate (PSPP) and NADOH-dependent reductive rearrangement of PSPP to squalene (Epstein and Rilling 1970). Recently, a three-step pathway using a set of three enzymes including PSPP synthase (PSPPS), HSQ synthase (HSQS) and squalene synthase (SQS) has been discovered in bacteria (Pan et al. 2015) (Fig. 2).

Strategies for increasing squalene production in natural producers

Microorganisms have a great potential to become the sources for squalene production. Current investigations are focused on increasing the content of squalene in microorganisms. Multiple strategies, including seeking alternative sources, optimizing fermentation and using metabolic engineering as a tool, have been applied to improve the squalene production.

Exploration of squalene producing sources

Natural producers with high content of squalene are of importance for squalene production. Therefore, much effort has been directed towards exploring these new sources. Marine microorganisms such as *Pseudozyma* sp. JCC207 (Chang et al. 2008), *Schizochytrium* sp. CCTCCM209059 (Ren et al. 2010), *Schizochytrium mangrovei* (Jiang et al. 2004), *Rubritalea sabuli* sp. nov. (Yoon et al. 2008) and *Rubritalea squalenifaciens* sp. nov (Kasai et al. 2007) have been characterized as suitable squalene producers. Thraustochytrids *Aurantiochytrium* strains have a high potential for commercial production (Aasen et al. 2016). Kaya et al. successfully isolated the 18W-13a strain of *Aurantiochytrium* sp. from a mangrove area. It was found that the strain produced 198 mg/g dry cell weight (DCW) and 1.29 ± 0.13 g/L of squalene after 4 days of culture (Kaya et al. 2011).

Recently, Nakazawa et al. reported that 176 strains of thraustochytrids isolated from various regions in Asia were screened using thin layer chromatography (TLC) for squalene contents. A total of 38 strains were rated as "+"

(high), 29 as "±" (medium), and 109 as "−" (low). The strain with the highest squalene content (317.74 mg/g DCW) was *Aurantiochytrium* sp. Yonez 5-1 (Nakazawa et al. 2014).

Optimization of fermentation conditions

Mantzouridou et al. examined the growth dynamics of two wild-type strains of *S. cerevisiae* EGY48 and BY4741 for sterols production through investigation of the impact of oxygen supply, inoculums, and fermentation period on yield. The highest squalene yield were 3.13 mg/L for EGY48 and 2.97 mg/L for BY4741 respectively (Mantzouridou et al. 2009). The production peak was achieved by treating the strain EGY48 with terbinafine (0.05–0.55 mM) and methyl jasmonate (0–1.0 mM). Maximum squalene content (10.02 mg/g DCW) and yield (20.70 mg/L) were reached at 0.442 mM terbinafine plus 0.044 mM methyl jasmonate after 28 h of culture (Naziri et al. 2011). Bhattacharjee et al. used both *S. cerevisiae* and *Torulaspora delbrueckii* for fermentative squalene production under anaerobic conditions. It was demonstrated that the production of squalene from *Torulaspora delbrueckii* (237.25 μg/g DCW) was much higher than that from *S. cerevisiae* (41.16 μg/g DCW), suggesting *Torulaspora delbrueckii* could be a potential suitable alternative source (Bhattacharjee et al. 2001).

Fan et al. increased the squalene content and yield in the thraustochytrid, *Aurantiochytrium mangrovei* FB3, through medium optimization and terbinafine treatment to inhibit squalene monooxygenase in the sterol biosynthetic pathway. The highest biomass concentration of 21.2 g/L was found in media with a glucose concentration at 60 g/L. Although addition of terbinafine led to a slight inhibition of cell growth, an increase in squalene content was observed. Compared to the control, the squalene content increased 36 and 40 % in the presence of 10 and 100 mg/L of terbinafine respectively (Fan et al. 2010). For squalene production by microalga *Aurantiochytrium* sp.BR-MP4-A1 in heterotrophic cultures, Li et al. optimized the nitrogen sources. Monosodium glutamate, yeast extract and tryptone were founded to be able to enhance cell growth and squalene production during screenings. By using the optimal concentrations of the three nitrogen sources, the squalene content and squalene yield reached 0.72 mg/g DCW and 5.90 mg/L, respectively (Li et al. 2009). Nakazawa et al. optimized the culture conditions for *Aurantiochytrium* sp. 18W-13a, including media ingredients and temperature. With 25–50 % seawater, 2–6 % glucose and 25 °C, the highest squalene content and yield reached 171 mg/g DCW and 900 mg/L, indicating that 18W-13a strain is a potential source for commercial squalene (Nakazawa et al. 2012).

Recently, Englund et al. (2014) used the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 as a host for the production of squalene. Investigation of the impacts of light intensity and growth stage on squalene accumulation in the mutant strain found that normal light conditions (50 μmol photons m^{-2} s^{-2}) were better for squalene accumulation, compared to low and high light conditions (Englund et al. 2014). Drozdíková et al. (2015) tested the squalene production in *Kluyveromyces lactis*. By partial inhibition of squalene epoxidase with terbinafine and additional nutrients such as commercially available glucose diary wastes containing lactose, the level of produced squalene was high. In addition, this study shows a promising future for squalene production in *K. lactis* (Drozdíková et al. 2015).

Metabolic engineering

With better understanding of biosynthetic enzymes and regulatory mechanisms during squalene production, metabolic engineering in microbes becomes practical. Kamimura et al. (1994) was the first group that successfully increased squalene production in *S. cerevisiae*, by disrupting a gene involved in the conversion of squalene to ergosterol. The yield of squalene reached 5 mg/g DCW when culturing the mutant strains in aerobic condition. When grass juice was added as the feedstock together with 0.025 μg/mL of doxycycline to treat the recombinant *S. cerevisiae* YUG37-EGR1, the level of squalene reached 18.0 ± 4.18 mg/L (Kamimura et al. 1994). The ERG1 gene encodes an enzyme (squalene epoxidase) that catalyzes the rate-limiting step in the squalene biosynthetic pathway. It has been proven that targeted genetic manipulation of ERG1 gene is a promising tool for increasing squalene production in yeast. In the study from Garaiová et al. (2014), the highest squalene level (over 1000 μg/10^9 cells) was induced by specific point mutations in the ERG1 gene that were capable of reducing the activity of squalene epoxidase and inducing terbinafine hypersensitivity, without disturbing their cell growth. Treatment with squalene epoxidase inhibitor terbinafine revealed a limit of 700 μg/10^9 cells in squalene accumulation, which was attributed to pronounced growth defects (Garaiová et al. 2014). Mantzouridou and Tsimidou (2010) studied the capacity of squalene accumulation in *S. cerevisiae* via constructing the strains of AM63 and AM64. AM63 had an extra copy of the HMG2 gene with a K6R stabilizing mutation in Hmg2p under the control of the inducible galactose promoter, while AM64 was derived from AM63 with an additional deletion of the ERG6 gene. In AM63, the squalene content was 20-fold higher than that in the wild-type EGY48 parental strain. However, in AM64, the combined Hmg2p stabilization and ERG6 deletion did not

Table 1 Squalene production levels among different microorganisms

Source	Squalene level	Reference
Aurantiochytrium sp. BR-MP4-A	0.57 mg/g DCW[a]	(Li et al. 2009)
Aurantiochytrium sp. 18W-13a	900 mg/L	(Kaya et al. 2011)
Aurantiochytrium sp.	6940 mg/L	(Chen et al. 2010)
Aurantiochytrium sp. Yonez 5-1	1100 mg/L	(Nakazawa et al. 2014)
Aurantiochytrium mangrovei FB3	21.2 g/L	(Fan et al. 2010)
Chlamydomonas reinhardtii	1.1 mg/g DCW	(Kajikawa et al. 2015)
E. coli	230.00 mg/L	(Katabami et al. 2015)
E. coli	11.80 mg/L	(Ghimire et al. 2009)
Kluyveromyce lactis	600.00 μg/10^9 cells	(Drozdíková et al. 2015)
Rubritalea squalenifaciens sp. nov.	15.00 mg/g DCW	(Kasai et al. 2007)
Rhodopseudomonas palustris TIE-1	15.8 mg/g DCW	(Xu et al. 2016)
Pseudozyma sp. JCC207	340.52 mg/L	(Chang et al. 2008)
Schizochytrium mangrovei	1.30 mg/L	(Jiang et al. 2004)
S. cerevisiae EGY48	20.7 mg/L	(Mantzouridou et al. 2009)
S. cerevisiae AM63	5.20 mg/g DCW	(Mantzouridou and Tsimidou 2010)
S. cerevisiae YUG37-ERG1	18.0 mg/L	(Kamimura et al. 1994)
Synechocystis sp. PCC 6803	3.16 mg/g DCW	(Englund et al. 2014)
Torulasporadelbrueckii	237.25 μg/g DCW	(Bhattacharjee et al. 2001)

[a] *DCW* dry cell weight

further enhance squalene accumulation. It was believed that the lack of ergosterol feedback inhibition led to an elevated transfer of surplus squalene into C27 sterols (Mantzouridou and Tsimidou 2010).

Recently, Englund et al. (2014) engineered *Synechocystis* sp. PCC 6803 by deleting the *slr2089* gene encoding squalene hopene cyclase (Shc), an enzyme converting squalene into hopene. The accumulated squalene in the recombinant strain was over 70-times higher than that in wild-type cells, reaching 3.16 mg/g DCW (Englund et al. 2014). Similarly, our study (2016) on *Rhodopseudomonas palustris* showed that the deletion of the *shc* gene resulted in a squalene production of 3.8 mg/g DCW, which was 27-times higher than that in the wild-type strain. Moreover, it was found that the fusion expression of the two genes, *crt*E and *hpn*D, was better than the co-expression method for squalene production. Finally, the titer of squalene reached 15.8 mg/g DCW by fusing the two genes and co-expressing the *dxs* gene in the *shc*-deficient strain, corresponding to a 112-fold increase compared to that in the wild-type strain (Xu et al. 2016). Kajikawa et al. (2015) investigated the accumulation of squalene in microalgal *Chlamydomonas reinhardtii* on the basis of the characterization of squalene synthease (CrSQS) and squalene epoxidase (CrSQE). It was found that the overexpression of CrSQS increased the rate of conversion of C14-labeled farnesyl pyrophosphate into squalene, but not squalene content. However, in the CrSQE-deficient strain, the titer of squalene was increased significantly (0.9-1.1 μg/mg DCW), indicating that partially knocking down CrSQE is a promising approach for increasing squalene production in *C. reinhardtii cells* (Kajikawa et al. 2015). The squalene production levels among different microorganisms are presented in Table 1.

Recombinant engineering in host *Escherichia Coli*

Many efforts on recombinant techniques are focused on *E. coli* because this organism is suitable for genetic modification and large-scale fermentation. For instance, Ghimire et al. (2009) used *E. coli* for heterologous production of squalene up to 4.1 mg/L via expression of the *Streptomyces peucetius*-originated genes (*hopA*, *hopB* and *hopD*). Moreover, *E. coli* endogenous *dxs* and *idi* genes encoding 1-deoxy-D-xylulose 5-phosphate synthase and isopentenyl diphosphate isomerase were co-expressed, leading to the production up to 11.8 mg/L of squalene (Ghimire et al. 2009). Recently, *E. coli* was engineered successfully by introducing a three-step pathway for squalene synthesis using a set of three enzymes viz. PSPPS, HSQS and SQS (Pan et al. 2015). To establish an efficient *E. coli*-based system for squalene production, Katabami et al. (2015) tested two different squalene synthases and their mutants in combination with precursor pathways. By co-expressing a chimeric mevalonate pathway with human or *Thermosynechococcus* squalene synthase, *E. coli* was able to produce squalene up to 230 mg/L or 55 mg/g DCW in flask culture (Katabami et al. 2015). These studies suggest that

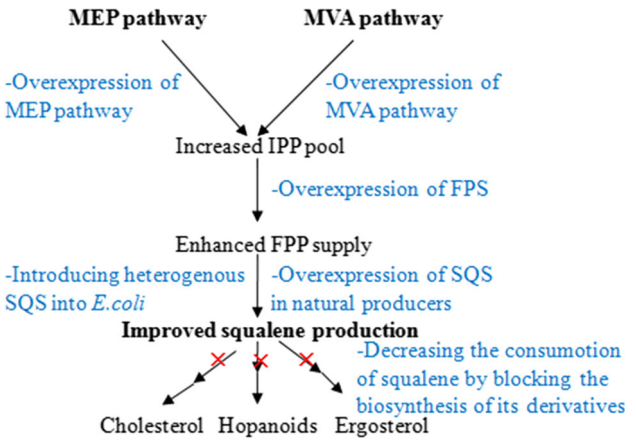

Fig. 3 Schematic representation of metabolic engineering approaches to improve squalene production. *FPS* farnesyl diphosphate (FPP) synthase, *IPP* isopentenyl diphosphate, *SQS* squalene synthase

E. coli is a promising host for squalene production. Metabolic engineering approaches to enhance squalene production are represented in Fig. 3.

Conclusion and future prospects

The biosynthesis pathway and biotechnological production of squalene have been of interest to many researchers because of its benefit for human health, as well as its applications in the pharmaceutical and cosmetics industries. So far, the isolation from natural squalene producers with optimized media has shown to be the most successful approach to increase squalene yield. Strategies need to be developed to explore alternative squalene producers with high squalene levels.

Using microorganisms such as *S. cerevisiae* and *E. coli* as hosts enables specific genes to be targetted and drives squalene production through specific biochemical pathways. Despite the promising results currently obtained, the yields are not sufficient for commercial production. Increasing the production of precursors by introducing foreign pathways (Zahiri et al. 2006) or blocking branch pathways (Xu et al. 2014) and enhancing the yield of target-soluble proteins via stress-minimization techniques (Wyre and Overton 2014) may be potential approaches to increase metabolic engineering efficiency for squalene production. Important questions that are still outstanding are whether the yields are limited by the carbon flux through the pathways or if there are some additional, yet unidentified, physiological factors that may limit squalene accumulation in the engineered strains. Further analysis of strains naturally producing high levels of squalene might be of help.

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