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REVIEW

Recent advances in the microbial production of squalene

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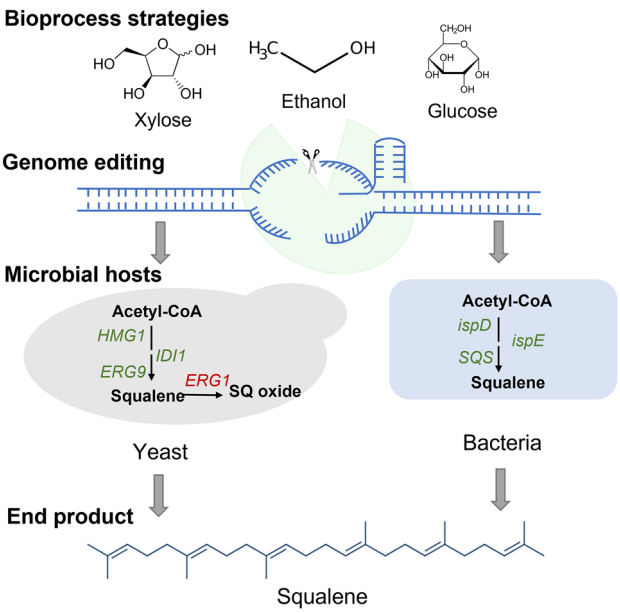
Abstract

Squalene is a triterpene hydrocarbon, a biochemical precursor for all steroids in plants and animals. It is a principal component of human surface lipids, in particular of sebum. Squalene has several applications in the food, pharmaceutical, and medical sectors. It is essentially used as a dietary supplement, vaccine adjuvant, moisturizer, cardio-protective agent, anti-tumor agent and natural antioxidant. With the increased demand for squalene along with regulations on shark-derived squalene, there is a need to find alternatives for squalene production which are low-cost as well as sustainable. Microbial platforms are being considered as a potential option to meet such challenges. Considerable progress has been made using both wild-type and engineered microbial strains for improved productivity and yields of squalene. Native strains for squalene production are usually limited by low growth rates and lesser titers. Metabolic engineering, which is a rational strain engineering tool, has enabled the development of microbial strains such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, to overproduce the squalene in high titers. This review focuses on key strain engineering strategies involving both *in-silico* and *in-vitro* techniques. Emphasis is made on gene manipulations for improved precursor pool, enzyme modifications, cofactor regeneration, up-regulation of limiting reactions, and downregulation of competing reactions during squalene production. Process strategies and challenges related to both upstream and downstream during mass cultivation are detailed.

Graphical abstract



Keywords *S. cerevisiae* · Squalene · Strain engineering · Terpene

Extended author information available on the last page of the article

Introduction

Squalene is a naturally occurring linear triterpene hydrocarbon ($C_{30}H_{50}$) comprising six double bonds (Fig. 1). Squalene was named after the shark family "Squalidae" (Tsujimoto 1916). The molecular weight of squalene is 410.7 g/mol, it has a melting point of − 75 °C, a boiling point of 285 °C, and a refractive index of 1.49 (Popa et al. 2015). Squalene has several applications in the food, chemical, and healthcare sectors. It is used as a dietary supplement (Naziri et al. 2011a), moisturizer, cardioprotective agent, anti-tumor agent, a natural antioxidant in addition to its antifungal (Smith 2000), antibacterial, and antivirulence activities (Bavisetty et al. 2015). It has been used as a vaccine adjuvant in seasonal influenza and COVID-19 vaccines (Tateno et al. 2020). Squalene intake in the Mediterranean region and the United States is in the 200–400 mg/day and 30 mg/day ranges, respectively (Newmark 1999). Squalane, a stable hydrogenated derivative of natural squalene is favored in the cosmetic industries as it penetrates human skin and imparts smoothness without greasy feels (Kim and Karadeniz 2012). With the increased demand for squalene along with regulations on shark-derived squalene, there is a need to find alternatives for squalene production which are low-cost as well as sustainable (Paramasivan 2019). In contrast to plant sterols

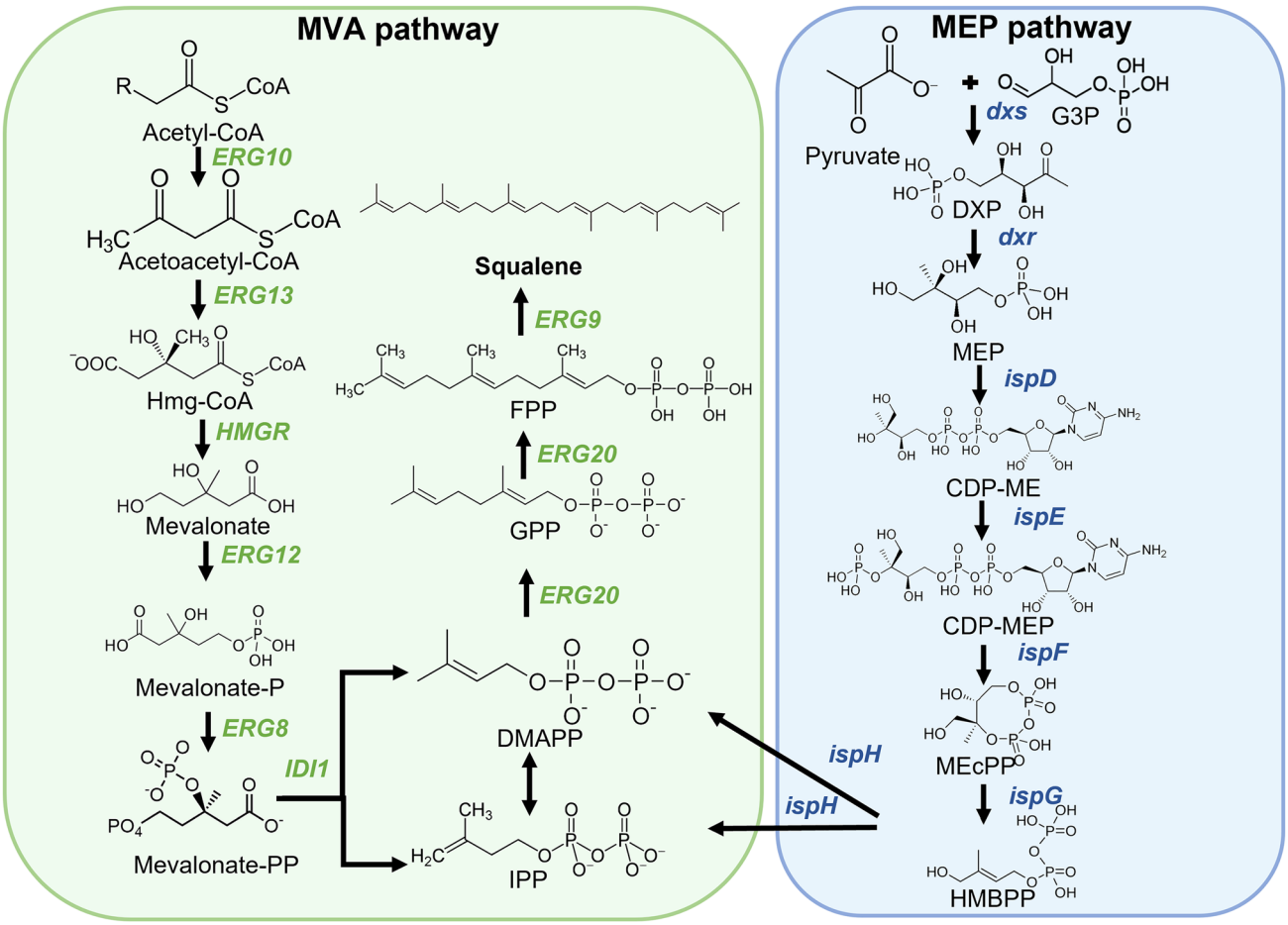


Fig. 1 Squalene biosynthesis pathway in yeast (the MVA pathway) and bacteria (the MEP pathway). CDP-ME, 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP, CDP-2-C-methyl-D-erythritol 2-phosphate; DMAPP, dimethylallyl pyrophosphate; DXP, 1-Deoxy-D-xylulose 5-phosphate; dxr-1-deoxy-D-xylulose-5-phosphate reductoisomerase; dxs, 1-deoxyxylulose-5-phosphate synthase; ERG8, Phosphomevalonate kinase; ERG10, Acetyl-CoA Cacetyltransferase; ERG13, Hydroxy methyl glutaryl-CoA synthase; ERG12, Mevalonate kinase; ERG19, Mevalonate pyrophosphate decarboxylase; ERG9, Squalene synthase; ERG20, farnesyl pyrophosphate synthase; FPP, Farnesyl Pyrophosphate; GPP, Geranyl Pyrophosphate; HMB-PP, 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; IDI1, isopentenyl pyrophosphate: dimethylallyl pyrophosphate isomerase; IPP, Isopentenyl Pyrophosphate; ispD-2-C-methyl-D-erythritol-4-phosphate cytidylyl transferase; ispE, 4-diphophocy-tidyl-2-C-methyl-D-erythritol kinase; ispF, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ispG, HMB-PP synthase; ispH, HMB-PP reductase; HMGR, Hydroxy methyl glutaryl CoA reductase; MEP, 2-C-methylerythritol 4-phosphate; MEcPP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate

Table 1 Strategies for production of squalene in *Saccharomyces cerevisiae*

Sl. No	Yield/Titer	Metabolic engineering and process strategy	Bioreactor type, volume and operation mode	Fold increase	References
1	5 mg/g DCW	KO: *ERG1*	NR	NR	Kamimura et al. (1994)
2	20 mg/l	OE: *tHMG1*	NR	NI—~tenfold	Donald et al. (1997)
3	1% (w/w)	OE: *tHMG1*	Flask cultivation 250-mL Batch mode	NI—~40-fold	Polakowski et al. (1998)
4	41.16 µg/g DCW	PS: Anaerobic fermentative condition	Flask cultivation 250-mL Batch mode	NR	Bhattacharjee et al. (2001)
5	3129 ± 109.5 µg/l	PS: Semianaerobic condition	Flask cultivation 500-mL Batch mode	PS—twofold	Mantzouridou et al. (2009)
6	191.9 mg/l	OE: *HMG1*	10-L Jar fermentor Fed-batch mode	NR	Tokuhiro et al. (2009)
7	5.2 mg/g DCW	SSM: Hmg2p (K6R) KO: *ERG6*	Flask cultivation 500-mL Batch mode	NI—~20-fold	Mantzouridou and Tsimidou (2010)
8	42 µg/OD_{600}	KO: *HEM1, DGA1, LRO1, ARE1, ARE2*	NR	KO-HEM1- 300-fold	Spanova et al. (2010)
9	10.02 ± 0.53 mg/g DCW or 20.70 ± 1.00 mg/L	CM: Terbinafine and Methyl jasmonate	Flask cultivation 500-mL Batch mode	NI—tenfold	Naziri et al. (2011a, b)
10	1000 µg per 10^9 cells	SSM: Erg1p (Q443UAG) CM: Terbinafine	Flask cultivation	SSM—30–40-fold	Garaiová et al. (2014)
11	7.89 ± 0.25 mg/g DCW or 18.0 ± 4.18 mg/l	R: Promoter replacement of *ERG1* with tet07-CYC1 PS: Grass juice as feedstock	Flask cultivation 50-mL Batch mode	NR	Hull et al. (2014)
12	9.91 mg/g DCW	OE: *tHMG1*	Flask cultivation 250-mL Batch mode	OE-30-fold	Thompson et al. (2014)
13	270 mg/l	ALE: Nystatin, Squalestatin for introducing SUE mutations HE: Squalene synthase from *Botryococcus braunii* OE: *tHMG1* KD: *ERG1* by insertional mutation	Flask cultivation Batch mode	*tHMG1*: 2.8-fold	Zhuang and Chappell (2015)

Table 1 (continued)

Sl. No	Yield/Titer	Metabolic engineering and process strategy	Bioreactor type, volume and operation mode	Fold increase	References
14	304.49 mg/l	OE: *HMG1, IDI1, ERG20,* and *ERG9* CM: Terbinafine KD: *ERG1, ERG7, ERG11, ERG24, ERG25, ERG26, ERG27, ERG6, ERG5, HEM1, PDC5, ADH1, ADH2, ADH4, ADH5, ADH7*	Flask cultivation 500-mL Batch mode	OE—tenfold CM- 35-fold	Rasool et al. (2016a)
15	304.16 mg/l	OE: *ERG10, ERG13, HMG1, ERG12, ERG8, ERG19, IDI1, ERG20, ERG9* (2X)	Flask cultivation 500-mL Batch mode	NI—89.5-fold	Rasool et al. (2016b)
16	58.6 mg/g DCW	OE: *tHMG1, ZWF1, POS5* (with and without MTS)	Flask cultivation 250-mL Batch mode	NI—27.5-fold	Paramasivan and Mutturi (2017a, b)
17	532 mg/l	OE: *tHMG1* and *ERG10* PS: Xylose feeding	Fed-batch mode	Xylose as carbon source -130%	Kwak et al. (2017)
18	59 mg/g DCW	OE: *tHMG1* PS: Carbon-limited exponential feeding, ethanol feeding, medium modification and nitrogen limitation, two-phase extraction	Mini-scale stirred-tank bioreactors 3-L Fed-batch mode, Continuous mode	OE- Ethanol feeding—eightfold Modified medium—13-fold	Ebert et al. (2018)
19	2011 ± 75 mg/l	HE: *ispA* OE: *tHMG1* CM: Terbinafine PS: Fermentation with terbinafine, extraction optimization, two-phase extraction using dodecane	5-L Fed-batch mode	HE-*ispA*—twofold CM—1.9-fold PS—2.5-fold	Han et al. (2018)
20	43 mg/g DCW	OE: *tHMG1* KO: *ERG6* KD: *ERG11* PS: Extraction standardization	Flask cultivation 250-mL Batch mode	KO-1.8-fold KD-3.4-fold PS—fivefold	Paramasivan et al. (2018)
21	445.6 mg/l	OE: *tHMG1, DGA1* PS: Nitrogen-limited fermentation	1.5-L Fed-batch mode	OE- *tHMG1*—20-fold OE- *tHMG1-DGA1*- 250-fold	Wei et al. (2018)
22	635 mg/l	OE: *tHMG1, ERG20* and *INO2*	Flask cultivation 250-mL Batch mode	NI—71-fold	Kim et al. (2019)
23	1.9 g/l	OE: *tHMG1* and *POS5* (with MTS) KO: *LYS1* and *ADK1* PS: Exponential feeding (openloop strategy)	5-L Fed-batch mode	KO—*LYS1-ADK1- tHMG1-POS5* -4.5-fold	Paramasivan et al. (2019)

Table 1 (continued)

Sl. No	Yield/Titer	Metabolic engineering and process strategy	Bioreactor type, volume and operation mode	Fold increase	References
24	255 mg/l	OE: *DGA1, HMG1, ERG20, IDI1* and *ERG9* PE: Promoters engineered with $TFBS_{TEF1}$ and $TFBS_{HHF2}$ R: AFT1p/CTR1p-*ERG1*	Flask cultivation 20-mL Batch mode	NI—73.49-fold	Manzoor et al. (2020)
25	9.5 g/l	OE: *ADH2, ERG10, ERG13, tHMG1, ERG13, tHMG1, Silicibacter pomeroyi,* 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	5-L Fed-batch mode	NR	Li et al. (2020)
26	11 g/l	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	5-L Fed-batch mode	ePTS1 tag fusion—138-fold	Liu et al. (2020a)
27	420 mg/l	ALE: Terbinafine SSM: ERG1p (F420I)	Flask cultivation 250-mL Batch mode	ALE—16.5-fold	Paramasivan et al. (2021)
28	3.53 g/l	OE: *ERG10, ERG13, HMG1, ERG13, HMG1, ERG12, ERG8, ERG19, IDI1, ERG20, ERG9* SSM: ERG1p (G30S) PE: Insertion of marO into P_{ERG1} and P_{ERG11} PS: Cane molasses as feedstock	Stirred-tank bioreactor 1-L Fed-batch mode	PE- 4.9-fold OE, PE—215-fold	Zhou et al. (2021)
29	21.1 g/l 437.1 mg/g DCW	OE: *ERG10, ERG13, tHMG1, ERG12, ERG8, ERG19, IDI1* genes fused with N-terminal MLS, *ERG20, ERG9, ACS1, tHMG1* R: P_{HXT1}-*ERG1* PS: Two stage fermentation, two-phase extraction	Tank fermenter 5-L Fed-batch mode	Fusion with MLS-48.5-fold *tHMG1*—2.4-fold	Zhu et al. (2021)

Table 1 (continued)

ADA Adenosine deaminase, *ACL1, ACL2* ATP citrate lyase, *ACS1* Acetyl-coenzyme A synthetase, *ADH1, ADH2, ADH4, ADH5, ADH7* alcohol dehydrogenase, *ADK1* Adenylate kinase, ALE Adaptive laboratory evolution, CM Chemical Means, CYC1 Cytochrome C1, DCW Dry cell weight, *DGA1* Diacylglycerol acyltransferase, *ERG1* Squalene epoxidase, *ERG5 C-22* sterol desaturase, *ERG6* Delta(24)-sterol C-methyltransferase, *ERG7* Lanosterol synthase, *ERG8* Phosphomevalonate kinase, *ERG9* Squalene synthase, *ERG10* Acetyl-CoA C-acetyltransferase, *ERG11* Lanosterol 14-alpha-demethylase, *ERG12* Mevalonate kinase, *ERG13* Hydroxy methyl glutaryl-CoA synthase, *ERG19* Mevalonate pyrophosphate decarboxylase, *ERG20* Farnesyl pyrophosphate synthetase, *ERG24* C-14 sterol reductase, *ERG25* C-4 methyl sterol oxidase, *ERG26* C-3 sterol dehydrogenase, *ERG27* 3-keto sterol reductase, *GAL80* Transcriptional regulator, HE Heterologous Expression, *HEM1* 5-aminolevulinate synthase, HHF2 Histone H Four, *HMG1, HMG2, HMGR* Hydroxy methyl glutaryl CoA reductase, *HXT1* glucose transporter, *IDI1* isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, *INO2* INOsitol requiring transcription factor, ispA Farnesyl diphosphate synthase, KD Knock Down, KO Knock Ou, *LYS1* Saccharopine dehydrogenase, marO mar operator, MLS mitochondrial localization signal, MTS1 Mitochondrial targeting signal, *MVD1* Diphosphomevalonate decarboxylase, NI Net increment, OE Overexpression, *PDC5* Pyruvate decarboxylase, *POS5* Mitochondrial NADH kinase, PE Promoter engineering, PS Process Strategy, PTS1 Peroxisomal targeting signal, R Repression, SSM Site-Specific Mutagenesis, TEF1 Elongation factor 1-alpha, tet07 Tetracycline operator, TFBS Transcription factor binding site, *ZWF1* Glucose-6-phosphate dehydrogenase

such as sitosterol and stigmasterol, and animal sterols such as cholesterol, ergosterol acts as a predominant sterol in the cell wall of the fungus. Squalene is a ergosterol pathway intermediate in *S. cerevisiae*. It is produced in yeast through the mevalonate (MVA) biosynthesis process and in bacteria through the methylerythritol 4-phosphate (MEP) biosynthesis process (Fig. 1). However, squalene, as an intermediary chemical, is produced at lower quantities in wild-type strains than other macromolecules and is usually below the detection level.

Squalene is a biomolecule of economic importance, and efforts are undertaken in recent years to synthesize it utilizing *S. cerevisiae* as a cell factory (Table 1) (Mantzouridou and Tsimidou 2010; Naziri et al. 2011b; Rasool et al. 2016a; Han et al. 2018; Wei et al. 2018; Paramasivan et al. 2019). In the last decade, only a few patents on the generation of squalene in modified yeast have been granted (Walker et al. 2009; Choi et al. 2012). Amyris, Inc., a biotech firm, has commercially synthesized squalene using a modified yeast strain (Fisher et al. 2013). Biotechnological synthesis of squalene has also been pursued in recent years in various other species such as *Escherichia coli, Synechocystis* sp, *Yarrowia lipolytica,* and *Kluyveromyces lactis* other than *S. cerevisiae* (Table 2) (Bergquist et al. 2014; Drozdíková et al. 2015; Huang et al. 2018). Squalene synthesis in microbes, plants, and mammals and their applications has been recently reviewed (Gohil et al. 2019). In the current review, metabolic engineering and process strategies for squalene enhancement in different microbes have been comprehensively discussed with emphasis on squalene synthesis in yeast. The discussed engineering strategies could also be effectively applied for the synthesis of different terpenes in yeast as well as in other microbes.

Genetic modifications to improve squalene synthesis

Precursor pool rebalancing

In *S. cerevisiae*, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase is translated as two different isomers, Hmg1p and Hmg2p. Hmg1p accounts for nearly 83 percent of overall enzyme activity in the wild-type strain (Basson et al. 1986). Further analysis revealed that these two variants have a membrane-binding region (1-552 amino acids) that covers the ER (endoplasmic reticulum) as well as the catalytic domain (553-1046 amino acids) (Basson et al. 1988). HMG-CoA reductase is a major regulatory enzyme in the ergosterol biosynthesis pathway and its overexpression has proven essential in most metabolic engineering efforts of *S. cerevisiae* towards terpene synthesis (Rodwell et al. 1976). Later, it was revealed that upregulation of the catalytic

Table 2 Strategies for production of squalene in other microbes

SI. No	Organism	Yield/Titer	Metabolic engineering and process strategy	Bioreactor type, volume and operation mode	Fold increase	References
1	*Bacillus subtilis*	7.5 mg/l	OE: *dxs, ispD, ispF, ispH*, and *ispA* HE: Squalene synthase from *Bacillus megaterium*	NR	NI—29-fold	Song et al. (2020)
2	*Chlamydomonas reinhardtii* (Cr)	0.9–1.1 µg/mg DCW	KO: Cr Squalene Epoxidase	NR	NR	Kajikawa et al. (2015)
3		1.6 µg/10^6 cells	M: UV mutagenesis CM: Terbinafine	NR	NI—50%	Potijun et al. (2021)
4	*Corynebacterium glutamicum*	5.4 ± 0.3 mg/g DCW or 105.3 ± 3.0 mg/l	OE: *dxs, idi* R: *idsA* HE: *S. cerevisiae* squalene synthase	Flask cultivation 250-mL Batch mode	NI—5.2-fold	Park et al. (2018)
5		1.5 g/l	PrE: truncated ScSQS and HsSQS KO: *pyc, idsA, crtE* PS: Dodecane based two-phase extraction	Jar fermentor 5-L Batch or fed-batch mode	HsSQS[1 − 370]-4.6 fold	Park et al. (2021)

Table 2 (continued)

SI. No	Organism	Yield/Titer	Metabolic engineering and process strategy	Bioreactor type, volume and operation mode	Fold increase	References
6	*E. coli*	11.8 mg/l	OE: *idi* and *dxs* HE: hopD and hopAB from *Streptomyces peucetius*	Flask cultivation Batch mode	NI—threefold	Ghimire et al. (2009)
7		2.7 mg/l	HE: *SQS* from human	Flask cultivation 200-mL Batch mode	NR	Furubayashi et al. (2014)
8		230 mg/l or 55 mg/g-DCW	OE: *atoB, idi,* and *ispA* HE: chimeric MVA pathway (*tHMG1, ERG13, ERG12, ERG8, MVD1*) from *S. cerevisiae* with SQS from human or *Thermosynechococcus elongatus*	Flask cultivation Batch mode	NI—55-fold	Katabami et al. (2015)
9		NR	HE: hpnC, hpnD, and hpnE from *Zymomonas mobilis* and *Rhodopseudomonas palustris*	NR	NR	Pan et al. (2015)
10		28.5 mg/g DCW 52.1 mg/l	OE: *idi, dxs, udhA, zwf, pgl* and *fps* KO: *pgi, menA* HE: Squalene synthase from human	Flask cultivation 250-mL Batch mode	NI—21-fold	Xu et al. (2019)
11		612 mg/l	OE: Mevalonate pathway and *tsr* HE: *S. cerevisiae* squalene synthase	Flask cultivation Batch mode	OE -*tsr*—2.25-fold	Meng et al. (2020)
12		16.0 ± 2.0 mg/l	OE: Mevalonate pathway HE: *S. cerevisiae* squalene synthase	NR	NR	Sun et al. (2020)
13	*Kluyveromyces lactis*	600 µg per 10^9 cells	CM: Terbinafine	NR	NR	Drozdıkova et al. (2015)
14	*Methylomonas sp. DH-1*	39.3 mg/g DCW or 31.3 mg/l	OE: sqs KO: pds-ald-crtN2 gene cluster CM: ferulenol PS: Nitrate supplementation	5-L Fed-batch mode	OE—sqs—6.7-fold PS- 1.7-fold NI—8.2-fold	Kang et al. (2021)
15	*Phormidium autumnale*	0.18 g/kg DCW	No strategies reported (Cultivation using agroindustrial waste)	Bubble column bioreactor Batch mode	NR	Fagundes et al. (2019)
16	*Torulaspora delbrueckii*	237.25 µg/g DCW	PS: Anaerobic cultivation	Flask cultivation 250-mL Batch mode	NR	Bhattacharjee et al. (2001)

Table 2 (continued)

SI. No	Organism	Yield/Titer	Metabolic engineering and process strategy	Bioreactor type, volume and operation mode	Fold increase	References
17	Thraustochytrid *Aurantiochytrium sp.*	0.72 mg/g DCW or 5.90 mg/l	PS: Nitrogen source optimization	Flask cultivation 250-mL Batch mode	NR	Chen et al. (2010)
18	Thraustochytrid *Aurantiochytrium mangrovei*	0.37 mg/g DCW	PS: Medium optimization CM: Terbinafine	Flask cultivation 500-mL Batch mode	CM- 40%	Fan et al. (2010)
19	Thraustochytrids *Aurantiochytrium sp.* 18 W-13a	171 mg/g DCW or 0.9 g/l	PS: Temperature, seawater and glucose concentration optimization	Flask cultivation 500-mL Batch mode	PS- > 100-fold	Nakazawa et al. (2012)
20		198 mg/g DCW or 1.29 g/l	No strategies reported (Strain isolated from mangrove area)	NR	NR	Kaya et al. (2011)
21	Thraustochytrids *Aurantiochytrium sp.* Yonez5–1	1 g/l	No strategies reported (Strain isolation and screening)	Flask cultivation 500-mL Batch mode	NR	Nakazawa et al. (2014)
22	Thraustochytrid *Aurantiochytrium sp.* S02-459	41.19 ± 1.86 mg/l or 10.85 ± 0.10 mg/g DCW	PS: Temperature, Shaking speed, glucose and nitrogen concentration	Flask cultivation 500-mL Batch mode	NR	Saengwong et al. (2018)
23	Thraustochytrid *Aurantiochytrium sp.* TWZ-97	188.6 mg/l	PS: Medium optimization, reactor cultivation	5-L Batch mode	PS—2.2-fold	Zhang et al. (2019)
24	Thraustochytrid *Aurantiochytrium sp.*	88.47 mg/g DCW or 1.0 g/L	PS: Batch cultivation	Batch mode	NR	Patel et al. (2019)
25	*Schizochytrium mangrovei*	1.17 ± 0.06 mg/g DCW	CM: Methyl jasmonate	Flask cultivation 250-mL Batch mode	CM—1.6-fold	Yue and Jiang (2009)
26	*Schizochytrium mangrovei PQ6*	33.04 ± 0.03 mg/g DCW or 1.019 g/l	PS: Reactor cultivation	Photobioreactor 30-L/150-L	NR	Hoang et al. (2014)
27	*Synechococcus elongatus*	11.98 ± 0.49 mg/l/OD_{730} or 79.2 mg/g DCW	OE: {CpcB1-SQS} fusion protein PS: Light optimization in photo bioreactor	Photobioreactor 6-L	OE-CpcB1-1.9-fold	Choi et al. (2017)
28		7.16 mg/l/OD_{730}	OE: *CpcB:SQS* HE: *dxs, Idi, IspA* from *E. coli, SQS* from *S. cerevisiae* PS: Two-phase extraction	Closed photobioreactor 100-L	NR	Choi et al. (2020)
29	*Synechocystis sp*	0.67 mg/L/OD_{730}	KO: *shc*	NR	KO-70-fold	Englund et al. (2014)
30	*Rhodopseudomonas palustris* TIE-1	15.8 mg/g DCW	OE: {*crt*E-*hpn*D}—fusion protein KO: *shc*	Flask cultivation 250-mL Batch mode	KO-27-fold NI -112-fold	Xu et al. (2016)

Table 2 (continued)

SI. No	Organism	Yield/Titer	Metabolic engineering and process strategy	Bioreactor type, volume and operation mode	Fold increase	References
31	*Rhodopseudomonas palustris*	23.3 mg/g DCW	OE: *dxs, crtE, hpnD* KO: *shc, crtB* PS: Two-phase extraction	Flask cultivation 250-mL Batch mode	KO-50-fold NI -178-fold	Xu et al. (2021)
32	*Rhodosporidium sp. DR37*	619 mg/l	PS: Medium optimization	Flask cultivation 250-mL Batch mode	NI—9.6-fold	Shakeri et al. (2021)
33	*Yarrowia lipolytica*	10 mg/g DCW	OE: *ylHMG1, ylACL1, acs**	Flask cultivation 250-mL Batch mode	OE-*ylHMG1*-3.2-fold NI—16.4-fold	Huang et al. (2018)
34		502.7 mg/l	OE: *ylHMG1, ylACL2, ylMnDH2, SQS* PS: C/N ratio optimization, two-phase extraction	Flask cultivation 250-mL Batch mode	NI—28-fold	Liu et al. (2020b)
35		731.18 mgl	OE: *ylHMG1* and *ylDGA1* PS: Fermentation optimization, two-phase extraction	Flask cultivation 250-mL Batch mode	*ylHMG1*- 20-fold *ylDGA1*—2.1-fold	Tang et al. (2021)
36		22.0 mg/g DCW	OE: *ERG9, HMG1, DGA1* KO: *PEX10, URE2*	Flask cultivation 250-mL Batch mode	NI—115-fold	Wei et al. (2021)
37	Yeast-like fungus *Pseudozyma sp.* JCC 207	340.52 mg/l	PS: C/N source and ratio optimization	Flask cultivation 250-mL Batch mode	NR	Chang et al. (2008)
38	Yeast-like fungus *Pseudozyma sp.* SD301	2.4 g/L	PS: C/N ratio, intermittent feeding, pH and temperature optimization	5-L Batch or fed-batch mode	NR	Song et al. (2015)

ACL1, ACL2 ATP citrate lyase, *acs* Acetyl-coenzyme A synthetase, *atoB* Acetyl-CoA acetyltransferase, C/N Carbon/Nitrogen, CM Chemical Means, *CpcB1* phycocyanin β-subunit protein, *crtB* Phytoene synthase, *crt*E GGPP synthase, DGA1 Diacylglycerol acyltransferase, *dxs* 1-deoxyxylulose-5-phosphate synthase, *ERG13* Hydroxy methyl glutaryl-CoA synthase, *ERG8* Phosphomevalonate kinase, *ERG12* Mevalonate kinase, *fps* Farnesyl pyrophosphate synthase, HE Heterologous Expression, *HMG1* Hydroxy–methyl-glutaryl-coenzyme A reductase, *hopAB squalene/phytoene synthases, hopD farnesyl diphosphate synthase,* hpnC Hydroxysqualene synthase, hpnD Presqualene diphosphate synthase, hpnE Hydroxysqualene dehydroxylase, *ispD* 2-C-methyl-D-erythritol 4-phosphate cytidylyl transferase, *idi* Isopentenyl-diphosphate Delta-isomerase, *idsA* geranylgeranyl pyrophosphate synthase, *ispA* Farnesyl diphosphate synthase, *ispE*-4-diphosphocytidyl-2-C-methylerythritol kinase, *ispF*-2-C-methyl-D-erythritol 2,4-cyclo diphosphate synthase, *ispH* 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, KD Knock Down, KO Knock Out, *menA* 1,4-dihydroxy-2-naphthoate octaprenyltransferase, *MnDH2* Mannitol dehydrogenase, *MVD1* diphosphomevalonate decarboxylase, NI Net increment, OE Overexpression, *pgi* Glucose-6-phosphate isomerase, *pgl* 6-phosphogluconolactonase, *PEX10* Peroxisomal membrane E3 ubiquitin ligase, PS Process strategy, R Repression, shc Squalene hopene cyclase, SSM Site-Specific Mutagenesis, *SQS* Squalene synthase, tsr serine chemoreceptor protein, *udhA* Pyridine Nucleotide Transhydrogenase, *URE2* Nitrogen catabolite repression transcriptional regulator, *zwf* Glucose-6-phosphate dehydrogenase

domain of HMG1, known as *tHMG1*, enhanced squalene levels in *S. cerevisiae* (Donald et al. 1997; Polakowski et al. 1998). Overexpression of membrane-bound Hmg1p results in the development of ER membrane stacks, also known as karmellae (Wright et al. 1988). To make a soluble and non-membrane bound, a truncated form of the HMG-CoA reductase enzyme (tHMG1) has been overexpressed (Polakowski et al. 1998). Moreover to avoid feed-back inihibition of ergosterol, it was expressed under a constitutive promoter in their studies. This theory established the basis for the upregulation of *tHMG1* in an array of studies targeting the synthesis of terpenes in *S. cerevisiae* (Donald et al. 1997; Polakowski et al. 1998; Thompson et al. 2014; Zhuang and Chappell 2015; Kwak et al. 2017; Han et al. 2018; Paramasivan et al. 2019; Liu et al. 2020a, b; Zhu et al. 2021) (Table 1). Overexpression of *tHMG1* encoding solely the catalytic domain of protein to circumvent regulation did not raise ergosterol content as predicted, but it did boost squalene production by nearly 30 times in *S. cerevisiae* (Thompson et al. 2014). Full-length *HMG1* overexpression has also been attempted and has been shown to increase squalene levels despite the feedback regulation (Tokuhiro et al. 2009). It might be attributable to the multi-copy integration of this gene in their studies; however, the exact theory is unclear. Hmg2p, a major isoform of this reductase in low oxygen conditions, has therefore turned into a target for upregulation during anaerobic growth to synthesize squalene (Mantzouridou and Tsimidou 2010).

Upregulation of the MVA pathway

Upregulation of structural genes, particularly those in the sterol pathway ahead of squalene, have been identified as key targets for squalene production in *S. cerevisiae* (Fig. 1) (Rasool et al. 2016a; Li et al. 2020; Liu et al. 2020a, b; Zhou et al. 2021; Zhu et al. 2021; Meng et al. 2020; Sun et al. 2020). The genes include *ERG10* coding for acetyl-CoA C-acetyltransferase, *ERG13* coding for HMG-CoA synthase, *HMGR, ERG12* coding for mevalonate kinase, *ERG8* coding for phosphomevalonate kinase, *IDI1* coding for isopentenyl pyrophosphate: dimethylallyl pyrophosphate isomerase, *ERG20* coding for farnesyl pyrophosphate synthase, and *ERG9* coding for squalene synthase. In a study by Rasool et al. (2016a), overexpression of the structural genes in the MVA pathway such as *HMG1, IDI1, ERG20,* and *ERG9* has been attempted during the synthesis of squalene and has enhanced the squalene up to 10-folds (Table 1; Figs. 1, 2a). Further, the presence of squalene monooxygenase inhibitor, terbinafine, together with overexpression of the MVA pathway genes has increased squalene titer to 304.49 mg/l (Rasool et al. 2016a). Overexpression of the MVA pathway genes combined with the carbon source controlled fed-batch fermentation has resulted in a strain with squalene synthesis ability of 9.5 g/l (Table 1) (Li et al. 2020).

Knockout of competing pathways

Deletion of the competing reactions is an effective method for constructing an efficient microbial chassis (Pickens et al. 2014) (Fig. 2b). This approach is referred to as either knockout which is typically applied by deleting a gene engaged in the competing reactions or knockdown which is reducing the levels of gene expression by introducing a weak promoter, and occasionally by allowing a system to regulate expression through the use of a programmable or customizable promoter (referred as repression). Through a random mutation in the *ERG1* gene, Kamimura et al. (1994) achieved a squalene production of 5 mg/g DCW (dry cell weight) by disrupting the ergosterol biosynthesis pathway in *S. cerevisiae*. Garaiová et al. (2014) investigated squalene increase in the mutant strains of *ERG1* grown in the medium supplemented with terbinafine, ergosterol biosynthesis inhibitor and obtained a squalene production of 1000 µg per 10^9 cells. Knockout of the *ERG6* gene coding for delta (24)-sterol C-methyltransferase along with a site-specific mutation in *HMG2* (K6R) has enhanced squalene to 20-fold (Mantzouridou and Tsimidou 2010). Likewise, knockout of *ERG6* gene along with knockdown of *ERG11* gene involved in ergosterol biosynthesis has enhanced squalene up to 43 mg/g DCW in *S. cerevisiae* (Paramasivan et al. 2018). In another study, knockdown of downstream genes involved in ergosterol synthesis and the alcohol dehydrogenase genes involved in ethanol biosynthesis combined with several other strategies has enhanced squalene to 304.49 mg/l (Rasool et al. 2016a). Also, knockout of the *SHC* gene coding for squalene hopene cyclase in the *Rhodopseudomonas palustris* and the cyanobacterial *Synechocystis* sp has enhanced squalene up to 70-fold and 27-fold, respectively (Englund et al. 2014; Xu et al. 2016) (Table 2). In *Yarrowia lipolytica,* knockout of *PEX10* encoding a peroxisomal membrane E3 ubiquitin ligase, and *URE2* encoding a transcriptional regulator involved in the repression of nitrogen catabolite has enhanced squalene yield (Wei et al. 2021). Rox1p is a repressor transcriptional factor protein involved in the regulation of ergosterol biosynthesis (Henry et al. 2002). *ROX1* gene deletion has enhanced the mevalonate and terpenoid synthesis in *Saccharomyces cerevisiae* (Özaydın et al. 2013). So far, this approach has not been demonstrated in yeast squalene synthesis.

Enzyme engineering towards squalene enhancement

Site-specific mutagenesis is a critical enzyme engineering technique to improve target compounds in the cells (Fig. 2c). Directed evolution improves enzyme characteristics such as stability, solubility, and specificity, resulting in the generation of customized enzymes (Kaur and Sharma 2006).

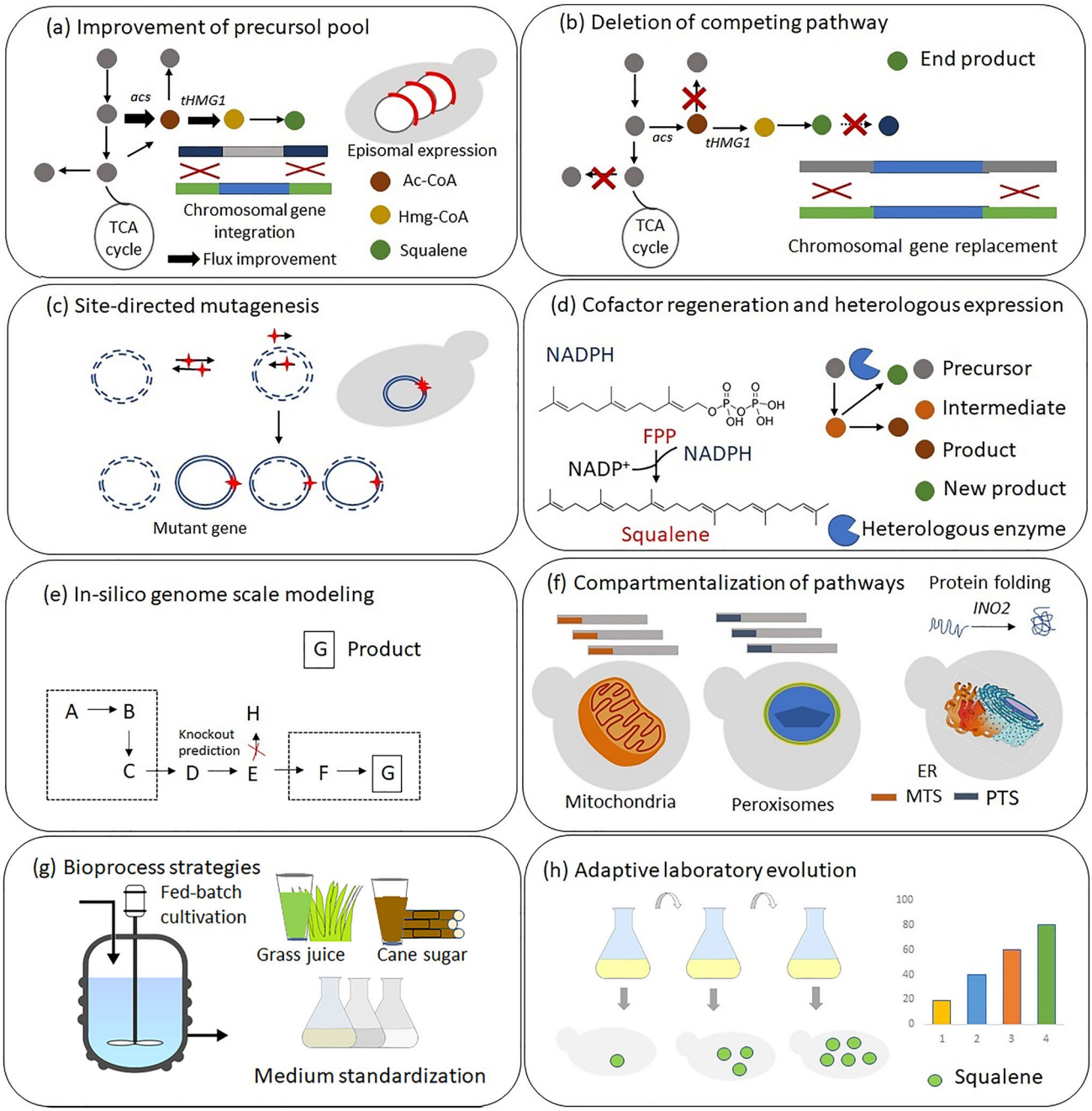


Fig. 2 Strain improvement strategies and tools applied towards squalene synthesis in microbes. (a) Improvement of precursor pool by episomal expression of genes and chromosomal integration of genes towards product flux improvement; (b) Deletion of competing reactions by chromosomal gene replacement; (c) Site-directed mutagenesis for expression of mutant genes; (d)Cofactor regeneration and heterologous gene expression; (e) In-silico genome scale modeling towards gene knockout predictions; (f) Compartmentalization of pathways by expression of genes with specific signaling sequences; (g) Bioprocess strategies by reactor cultivation and medium standardization; (h) Adaptive laboratory evolution. acs – Acetyl-CoA synthetase; Ac-CoA – Acetyl-CoA; FPP – Farnesyl pyrophosphate; HMG-CoA – Hydroxy Methyl Glutaryl-CoA; INO2 – Inositol requiring transcription factor; MTS – Mitochondrial targeting sequence; PTS – Peroxisomal targeting sequence; tHMG1-truncated HMG-CoA reductase

Site-directed mutagenesis aims to introduce deliberate mutations at a specific codon in the genes, resulting in a changed codon. This method has shown to be an invaluable tool in synthetic biology for improving protein characteristics, and it has been used to promote squalene synthesis in yeast and other microbes. As squalene epoxide synthesis reaction is catalyzed by the *ERG1* gene, attempts were made to modify the *ERG1* gene to reduce the flux towards the

downstream ergosterol biosynthesis pathway. In a study, glutamine Q at position 443 was mutagenized to UAG in Erg1p that in turn increased squalene up to 30–40-fold in the engineered *S. cerevisiae* (Garaiová et al. 2014) (Table 1). Several other point mutational studies have been made in the *ERG1* gene. In a study by Zhou et al. (2021), glycine G at position 30 was mutagenized to serine S in Erg1p to reduce the specific activity. The study resulted in a strain with squalene production ability up to 3.5 g/l (Table 1). Site-specific mutations have also been made in another critical gene, *HMG2*. Primarily, lysine K at positions 6, and 357 on Hmg2p were identified to be crucial for protein degradation, and so these lysines were substituted to evaluate the Hmgp2 stability (Gardner and Hampton 1999). In a study by Mantzouridou and Tsimidou (2010), lysine K at position 6 has been mutagenized to asparagine R in Hmg2p which resulted in a strain that produced 5.2 mg/g DCW of squalene (Table 1). Hmg2P (K6R) has also been successful in improving several other terpenes (Paramasivan and Mutturi 2017a, 2017b). During the synthesis of squalene, Garaiová et al. (2014) downregulated squalene epoxidase coded by *ERG1* and expressed multiple enzyme mutant variants of the *ERG1* gene. Based on reverse engineering approaches, a causal mutation for high levels of squalene accumulation in yeast cells has been identified in Paramasivan et al. (2021) when *S. cerevisiae* cells were adapted in the presence of terbinafine. In this study it was demonstrated that phenylalanine at position 420 of Erg1p was replaced with isoleucine (F420I), which resulted in improved squalene and terbinafine resistance. Under aerobic growth, Upc2p (uptake control protein) mutants are involved in sterol uptake (Vik Å and Rine 2001). Mutations in Upc2p such as G888A has been demonstrated to enhance external sterol (ergosterol) uptake, downregulation of internal ergosterol formation, and thereby realigning the allocation of resources for improvement in specific terpene synthesis. So far, this strategy has not been applied towards squalene synthesis studies in yeast.

Heterologous gene expression towards squalene synthesis

This section describes the heterologous expression-based metabolic engineering approaches that have been developed to augment squalene synthesis in *S. cerevisiae* and other microbes (Fig. 2d). Heterologous enzymes are sometimes more efficient in catalysis when compared to the native enzyme. In this regard, several attempts have been made to enhance the squalene synthesis in microbes by heterologous expression of non-native enzymes. In a study, as an alternative to *ERG20,* heterologous expression of IspA coding for farnesyl diphosphate synthase from *E. coli* was carried to obtain squalene levels of 1.24 g/l in *S. cerevisiae* (Choi et al. 2012) (Table 1). In another study by Zhuang and Chappell (2015), squalene synthase from *Botryococcus braunii* was expressed in *S. cerevisiae* which led to squalene synthesis of nearly 270 mg/l. Expression of squalene synthase from humans has also become another predominant strategy towards squalene improvement in host cells such as *E. coli.* (Furubayashi et al. 2014; Katabami et al. 2015; Xu et al. 2019). Human SQS has a high catalytic efficiency towards FPP (kcat/Km = 0.51 μM^{-1} s^{-1}). Similarly, squalene synthase from *T. elongatus* BP-1 has an even higher catalytic efficiency (kcat/Km = 1.8 μM^{-1} s^{-1}) and has been expressed in *E. coli* for increased squalene synthesis (Katabami et al. 2015). Also, the squalene synthase from *S. cerevisiae* (ScSQS) along with the entire MVA pathway genes has been expressed in *E. coli* towards squalene enhancement (Sun et al. 2020). In another study by Ghimire et al. (2009), hopD (farnesyl diphosphate synthase) and hopAB (squalene/phytoene synthases) genes from *Streptomyces peucetius* have been heterologously expressed to augment squalene synthesis in yeast cells leading to an increased titer of 11.8 mg/l in *E. coli*. A novel heterologous pathway of squalene biosynthesis from *Zymomonas mobilis* and *Rhodopseudomonas palustris* has been expressed and characterized in *E. coli.* The pathway consists of three genes, hpnD, hpnC, and hpnE expressing enzymes that convert FPP to PSPP (presqualene diphosphate), PSPP to HSQ (hydroxysqualene) and HSQ to squalene, respectively (Pan et al. 2015).

Manipulations in the central carbon metabolism

Since the biosynthesis of a metabolite includes multiple reactions within the cell, improvements in essential upstream molecules generated may affect enhancing downstream pathway components. Acetyl-CoA is an example of an early intermediate metabolite that contributes to the TCA cycle, sterol, fatty acids, and polyketides biosynthesis. In *S. cerevisiae*, cytoplasmic acetyl-CoA is formed via a PDH-bypass, which predominantly promotes sterol and fatty acid pathway (Shiba et al. 2007; Chen et al. 2013; Krivoruchko et al. 2015). Pulling the flux towards squalene biosynthesis exhausts acetyl-CoA supply in *S. cerevisiae* cells impairing its functions. To overcome this, the *ACS1* gene coding for acetyl-CoA synthetase has been overexpressed in a study targeting squalene improvement where *ACS1* combined with the *ERG10* gene expression has significantly enhanced squalene biosynthesis in yeast (Zhu et al. 2021). Cytoplasmic acetyl-CoA augmentation is one of the primary strategies applied to improve squalene synthesis. Erg10p is the foremost enzyme in the MVA pathway that pulls the flux towards squalene synthesis by converting acetyl-CoA to acetoacetyl-CoA. *ERG10* gene overexpression has been

attempted in several studies that aim to improve squalene synthesis (Rasool et al. 2016b; Kwak et al. 2017; Li et al. 2020; Liu et al. 2020a, b; Zhou et al. 2021; Zhu et al. 2021). Overexpression of *tHMG1* along with the *ERG10* gene in a xylose-fermenting strain has improved the squalene levels to 150 mg/l using xylose as a carbon source (Kwak et al. 2017). Likewise, endogenous gene *atoB* coding for acetyl-CoA acetyltransferase has been overexpressed in an attempt to enhance squalene synthesis in *E. coli* (Katabami et al. 2015). Yet another promising strategy to improve acetyl-CoA is by conversion of ethanol to acetyl-CoA. In an interesting study by Li et al. (2020), the *ADH2* gene that converts ethanol to acetyl-CoA has been overexpressed together with the heterologous expression of ADA from *Dickeya zeae* which converts acetaldehyde to acetyl-CoA in *S. cerevisiae*. As the ADA gene coding enzyme does not require ATP consumption, the metabolic burden on the cells during squalene synthesis is reduced. In addition, *ACL1* and *ACL2* genes coding for ATP citrate lyase from *Yarrowia lipolytica* that converts citrate to acetyl-CoA have been overexpressed in *S. cerevisiae* towards squalene synthesis. This strategy has proven to enhance the squalene content in the cells significantly (Liu et al. 2020a, b).

Co-factor regeneration

In yeast cells, NADPH is one of the ubiquitous cofactors (Agledal et al. 2010). Several enzymes in the sterol pathway of *S. cerevisiae* require NADPH as a cofactor. Overexpression of the MVA pathway genes such as *tHMG1, HMG2,* and *ERG9* would deplete cytosolic NADPH. Hence, the expression of NADPH regeneration-related genes has become a popular approach to stimulate terpenes and other metabolite syntheses (Brown et al. 2015) (Fig. 2d). In yeast cells, NADH kinases and $NADP^+$-dependent dehydrogenases are responsible for NADPH synthesis, and regeneration respectively (Shi et al 2005, 2011). Precisely, NADH kinase is involved in the phosphorylation of NADH to NADPH while the dehydrogenases are involved in the reduction of $NADP^+$ to NADPH. *POS5* gene codes for the enzyme that harbors both NAD kinase as well as NADH kinase activity (Strand et al. 2003) while another enzyme glucose-6-phosphate dehydrogenase coded by *ZWF1* has $NADP^+$-dependent dehydrogenase activity. In *S. cerevisiae, ZWF1* is a prime target for promoting cytosolic NADPH regeneration. *ZWF1* gene overexpression enhanced squalene synthesis to 2.3-fold, whereas *POS5* overexpression with mitochondrial targeting sequence (MTS) has enhanced squalene production to sixfold upon overexpression in yeast cells (Paramasivan & Mutturi, 2017a, b). In the same study, *ZWF1* and *POS5* (with MTS) gene expression combined with *tHMG1* expression has synergistically enhanced squalene to 22.5-fold and 27.5-fold respectively. In another study, *POS5* gene expression was combined with *tHMG1* expression and knockout of *ADK1* and *LYS1* genes identified by *in-silico* based genome-scale models towards squalene increase (Fig. 2e). The genetic modifications combined with fed-batch cultivation have enhanced squalene levels to 1.9 g/l. Similarly, zwf expression in *E. coli* combined with several other strategies has enhanced squalene synthesis to 52 mg/l with net increment up to 21-folds (Xu et al. 2019) (Table 1). In addition, to reduce the metabolic burden caused by the NADPH cofactor, an alternative NADH-based enzyme has been used as a target towards squalene synthesis (Li et al. 2020; Liu et al. 2020a, b). Since NADH is more readily available compared to NADPH, NADH-based HMGR from *Silicibacter pomeroyi* has been heterologously expressed in *S. cerevisiae* for squalene improvement. NADH-HMGR overexpression combined with several other strategies has resulted in a strain with squalene levels of 9.5 g/l (Li et al. 2020) and 11 g/l (Liu et al. 2020a, b) (Table 1).

Promoter based engineering strategies towards squalene synthesis

Promoters are critical for the control of gene expression thereby regulating the metabolic pathways in *S. cerevisiae*. Promoter-based engineering strategies are gaining an advantage in recent times as they could significantly affect gene expression levels. *ERG1* gene codes for squalene monooxygenase in the MVA pathway downstream of squalene in *S. cerevisiae*. Being an essential gene, disruption of *ERG1* leads to a lethal phenotype and therefore repression of *ERG1* gene by promoter replacement has become a prominent strategy in the development of cell factories towards squalene biosynthesis (Hull et al. 2014; Manzoor et al. 2020; Liu et al. 2020a, b; Zhu et al. 2021). Also, the usage of repressible promoters provides better economic feasibility when compared to the enzyme inhibitors at an industrial level. Initially, doxycycline repressible tet07-CYC1 promoter has been utilized to control the *ERG1* expression by substituting the native *ERG1* promoter at the chromosome. In a study by Hull et al. (2014), *ERG1* gene under repressible tet07-CYC1 promoter system along with grass juice as a carbon source has enhanced squalene up to 18 mg/l. Later, two iron and copper repressible promoters P_{AFT1} and P_{CTR1}, respectively are characterized and used to repress the *ERG1* gene in *S. cerevisiae* towards squalene bioproduction. *ERG1* repression with metal ion repressible P_{CTR1} promoter has enhanced squalene up to twofold when compared to P_{AFT1} promoter (Manzoor et al. 2020). In the same study, $TFBS_{TEF1}$ and $TFBS_{HHF2}$ were engineered into different constitutive promoters thereby enhancing the strength of the yeast constitutive promoters. The engineered promoters such as P_{HHF2}, P_{IRA1}, P_{RHO1}, and P_{PET9} with $TFBS_{TEF1}$ were utilized to drive the transcription of

HMG1, IDI1, ERG20, and *ERG9* genes respectively. This study resulted in a strain with squalene improvement to nearly 74-fold (Manzoor et al. 2020). Further, P_{HXT1} is a glucose inducible promoter and causes repression when the glucose levels in the medium become low or zero. The native promoter of *ERG1* when replaced with the HXT1 promoter combined with multiple other strategies has significantly enhanced squalene titer to 11 g/l (Liu et al. 2020a, b). Similarly, the P_{HXT1} has been used to repress the *ERG1* which in combination with the compartmentalization of genes in mitochondria leads to the highest production of squalene reported to date which is 21.1 g/l (Table 1; Zhu et al. 2021). Another noteworthy and most commonly used repressible promoter is the methionine repressible promoter (P_{MET3}) which has been extensively used to repress the *ERG9* gene towards other terpene production in yeast (Westfall et al. 2012). However tunable P_{MET3} has not been used towards squalene synthesis so far. *GAL80* negatively regulates gal regulon by binding to P_{GAL4} in the absence of galactose as well as in the presence of carbon sources such as glucose. Since galactose is an expensive carbon source compared to glucose, *GAL80* gene knockout is critical to express the genes under the strong galactose inducible promoters P_{GAL1} and P_{GAL10} (Westfall et al. 2012). Thus, Li et al. (2020) have overexpressed the mevalonate pathway gene *tHMG1* under the galactose inducible promoter in the *GAL80* deletion strain where the gene activation happens in response to glucose as the negative regulator is not present. This leads to a 33-fold improvement in squalene synthesis. In an interesting investigation by Zhou et al. (2021), the SRE (sterol response element) motif in *ERG1* and *ERG11* genes was replaced with the heterologous marO motif to downregulate the expression of both the genes thereby improving squalene and lanosterol accumulation, respectively. Replacement of the SRE motif reduces the feedback regulation by SREBP (sterol response element-binding proteins) at high sterol levels in the cells while marO insertion helps in controlling the metabolic flux. marO is a cis-element of mar operator bound by the transcriptional repressor protein, marR in *E. coli*. marO engineered near the TATA box of *ERG1* and *ERG11* promoter could efficiently repress the respective gene expression proving to be a promising alternative for gene repression studies in yeast *S. cerevisiae.*

Compartmentalization for squalene synthesis

Owing to the abundant availability of precursors and cofactors such as acetyl-CoA and redox equivalents, respectively, harnessing organelles such as mitochondria, ER, and peroxisomes are considered as a viable strategy towards squalene production (Fig. 2f). In a study by Zhu et al. (2021), to surpass the cell toxicity and the metabolic strain imposed by the phosphorylated intermediates of the MVA pathway, a partial pathway from acetyl-CoA to MVA has been incorporated into the mitochondria by merging the mitochondrial targeting signal sequence with overexpression genes. Upon combining the cytoplasmic and mitochondrial engineering with a two-stage fed-batch fermentation, the squalene titers have synergistically improved to 21.1 g/l with 437.1 mg/g DCW (Table 1). Similarly, introducing the MVA pathway in the peroxisomes by fusing the genes with peroxisomal targeting sequence has enhanced the squalene titers up to 11 g/l upon combining with P_{HXT1}-*ERG1* and a two-stage fermentation (Table 1) (Liu et al. 2020a, b). Another noteworthy ER-based metabolic engineering has enhanced squalene levels up to 71-fold where the overexpression of the *INO2* gene has expanded the ER functions. The key function of ER is protein folding and overexpression of genes causes metabolic burden in the ER. Therefore, the size regulatory factor gene *INO2* has been overexpressed in the yeast to restore the normal ER functions (Kim et al. 2019). Hence, organelle engineering could be considered as a novel and effective alternative to improve squalene production.

Storage of squalene in lipid droplets and cell membrane

Squalene is stored in the cell membrane, peroxisomes, and lipid droplets in yeast cells. A lipid droplet found in all eukaryotic cells is a lipid storage organelle that is made of sterol esters, triacylglycerols, and squalene. It consists of neutral lipids and serves as a depot for non-polar storage lipids such as squalene and fatty acids. Neutral lipid production and storage is a complex and dynamic process. Lipid droplets are ER-derived storage organelles whose quantity, size, and content vary depending on nutritional requirements and growth circumstances (Farese and Walther 2009; Fei et al. 2009). Triacylglycerides and steryl esters are inert and are found in the lipid bodies unless they are hydrolyzed while squalene is either sequestered in lipid particles or involved in membrane biogenesis (Spanova et al. 2010; Grillitsch et al. 2011). In a study by Wei et al. (2018), the *DGA1* gene coding for the diacylglycerol acyltransferase gene involved in fatty acid metabolism has been overexpressed in *S. cerevisiae* which enhanced squalene up to 250-fold upon the combination with the *tHMG1* gene. *DGA1* gene expression has proven to enhance lipid body formation in yeast cells thereby increasing the storage ability of the cells. Cell membrane engineering has been carried out to enhance the storage of squalene in *E. coli.* In this

regard, *tsr* gene coding for a serine chemoreceptor protein has been overexpressed which in turn enhanced the fatty acid composition in membrane lipid composition leading to the formation of a multi-layered structure. The engineered strain has synthesized squalene up to 612 mg/l which is the maximum known squalene synthesis to date in *E. coli* (Meng et al. 2020) (Table 2).

Process strategies for improving squalene synthesis

Bioprocess conditions play a key role in the high-level production of compounds in microbial fermentation (Fig. 2g). Under anaerobic fermentative conditions, Bhattacharjee et al. (2001) have generated squalene in a commercial yeast strain with a yield of up to 41 µg/g DCW. Besides, under optimized semi-anaerobic cultivation, inoculation levels, and growth conditions, Mantzouridou et al. (2009) reached a maximum squalene yield of 3.1 mg/l. Subsequently, overexpression of *tHMG1* and *DGA1* and growth under nitrogen-limited conditions have led to a squalene titer of 445.6 mg/l in galactose-based fed-batch cultivation (Wei et al. 2018). In another study by Han et al. (2018), *tHMG1* and bacterial *ispA* gene in tandem with terbinafine-based inhibition of squalene epoxidase have enhanced the squalene synthesis up to 2 g/l in glucose-based fed-batch cultivation. In some cases, the carbon sources to supplement the growth have been varied. For instance, grass juice and cane molasses have been used as a feedstock to increase squalene synthesis to 18 mg/l and 3.5 g/l respectively (Hull et al. 2014; Zhou et al. 2021). In an interesting study by Kwak et al (2017), xylose utilizing engineered strain was subjected to growth under xylose-based fed-batch cultivation. This study resulted in a strain with 532 mg/l of squalene titer. On the other hand, open loop-based exponential feeding of glucose in fed-batch cultivation has resulted in 1.9 g/l of squalene production using an engineered *S. cerevisiae* (Paramasivan et al. 2019). An optimized two-stage fed-batch cultivation using glucose and ethanol as a carbon source has been used to achieve a squalene titer of 11 g/l in an engineered strain (Liu et al. 2020a, b). C/N ratio, pH, and temperature optimization have led to a significantly high squalene production of 2.4 g/l in the yeast-like fungus *Pseudozyma* sp. SD301, a native producer of squalene (Song et al. 2015).

Adaptive laboratory evolution and downstream inhibition by terbinafine

Adaptive evolution is the use of selection pressure to leverage the innate capacity of an organism to undergo mutations toward the desired phenotype (Conrad et al. 2014) (Fig. 2h). It entails a process of continuous evolution with a selective gain for the variant exhibiting the desired trait (Sonderegger and Sauer 2003; Çakar et al. 2005). Adaptive laboratory evolution in combination with sequencing is a critical strategy in metabolic engineering for biomolecule synthesis (Conrad et al. 2014). Lowering the activity of the downstream enzyme, squalene epoxidase is a key technique for enhancing squalene levels and may be accomplished using a specialized non-competitive enzymatic inhibitor, terbinafine (Ta et al. 2012; Garaiová et al. 2014; Rasool et al. 2016a). Terbinafine has been extensively used in various studies and in different microbes to increase the squalene accumulation (Fan et al. 2010; Naziri et al. 2011a, b; Drozdıkova et al. 2015; Han et al. 2018). Naziri et al. (2011a, b) pioneered the cultivation of *S. cerevisiae* in terbinafine which led to at least ten times improvement in squalene. Following that, Garaiová et al. (2014) have produced 1000 µg of squalene per 10^9 cells in the presence of terbinafine with an engineered strain with Erg1p (Q443UAG). An engineered yeast strain has been subjected to grow in the presence of terbinafine as a selection pressure to enhance the squalene compound. This study resulted in a strain that produced 198 mg/l which was a 16.5-fold enhancement in comparison to the parent strain (Paramasivan et al. 2021). Further, terbinafine addition to the medium for the native squalene producer *Aurantiochytrium mangrovei* has increased squalene up to 40% (Fan et al. 2010). Besides, terbinafine has also been used to enhance squalene content in *Kluyveromyces lactis* (Drozdıkova et al. 2015). Han et al. (2018) have cultivated an engineered yeast strain in a fed-batch fermentation medium in the presence of terbinafine which has improved squalene synthesis up to twofold. However, large-scale cultivation of *S. cerevisiae* in the presence of terbinafine towards squalene synthesis could be an expensive strategy. Hence some studies reported the site-specific mutagenesis of squalene epoxidase and replacement of the promoter for *ERG1* gene to downregulate the activity of this enzyme (Garaiová et al. 2014; Hull et al. 2014). Such strategies resulted in improved squalene accumulation as discussed earlier.

Engineering towards squalene synthesis in other microbes

The genes involved in the MEP pathway are upregulated in combination with the heterologous expression of squalene synthase from *Bacillus megaterium* has enhanced the squalene synthesis up to 29-fold in *Bacillus subtilis* (Song et al. 2020) (Table 2). Overexpression of *dxs* and *idi* genes, repression of *idsA* gene, and heterologous expression of *ERG9* from *S. cerevisiae* has led to improved squalene levels (105 mg/l) in *Corynebacterium glutamicum* (Park et al. 2018) (Table 2). Further, when protein engineering by

expression of human squalene synthase was coupled with pathway engineering, fed-batch cultivation, and dodecane extraction, the *C. glutamicum* strain generated 1.5 g/l (Park et al. 2021) (Table 2). Squalene synthase from *S. cerevisiae* along with the overexpression of the MVA pathway genes and *tsr* gene has increased squalene in the engineered *E. coli* (Meng et al. 2020) (Table 2). Cyanobacterial species such as *Synechococcus elongatus* has been utilized for squalene synthesis by heterologous expression of *dxs, idi, ispA* from *E. coli,* and overexpression of the fusion protein CpcB1-ScSQS along with light optimization in photobioreactor which has increased the squalene content up to 79.2 mg/g DCW (Choi et al. 2017) (Table 2). Metabolic engineering of a novel species *Rhodopseudomonas palustris* has enhanced squalene up to 23.3 mg/g DCW with 178-fold net increment by the knockout of *shc* and *crtB* genes as well as the overexpression of dxs, crtE, and hpnD (Xu et al. 2021) (Table 2). Overexpression of *ylHMG1* and *ylDGA1* along with fermentation optimization enhanced squalene to 731 mg/l in *Yarrowia lipolytica.* This is the highest reported so far in that species (Tang et al. 2021) (Table 2). In a study by Kang et al (2021), overexpression of squalene synthase, inhibition of squalene-hopene cyclase by ferulenol, knockout of pds-crtN-ald gene cluster, and fed-batch cultivation with potassium nitrate supplementation has improved squalene levels to 31.3 mg/l in *Methylomonas* sp. DH-1 (Kang et al. 2021) (Table 2).

Conclusion and future prospects

Concerns about squalene production from shark liver oil, and lower yields from plant sources, microbial platforms for squalene production have been sought. However, there exists challenges with microbial production systems as well. Some of the significant limitations of existing microbial squalene manufacturing platforms is lower squalene TRY (titer, productivity and yields) meant for industrial production. Moreover, obtaining toxin-free squalene from the microbes that naturally produce squalene such as *Schizochytrium* sp. and *Aurantiochytrium* sp. is challenging. Besides, physiology, genetics, and process constraints needs to be well studied for such organisms. Some of the prominent microbial platforms for squalene production includes *S. cerevisiae*, *E. coli*, *C. glutamicum* and *Y. lipolytica*. Metabolic engineering strategies in *S. cerevisiae* such as co-factor engineering, improved precursor supply, alterations outside sterol pathway, and process strategies have been thoroughly investigated and highest squalene titer of 21.1 g/l has been reported so far. However, majority of studies in *S. cerevisiae* focused on episomal expression, which presents a process issue when grown in a bioreactor. Amyris Inc. commercial synthesis of squalene is implemented in two stages, (i) farnesene is synthesized in the first stage using modified *S. cerevisiae*, and (ii) the obtained farnesene is transformed to squalene in the second stage utilizing chemical catalysis. Such hybrid production platforms can be exploited for commercial viability. Several microbial strains with high squalene productivity are developed in recent times which could serve as potential chassis for commercial production of squalene and also to compete with other low cost non-sustainable alternatives such as shark liver oil and vegetable oils. The present review on strain engineering and bioprocess strategies might be highly useful in improving squalene synthesis, leading to the development of a feasible and robust microbial platform for industrial production squalene.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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