Squalene Biosynthesis in Engineered *Escherichia Coli*

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Pharmaceut Fronts 2023;5:e321–e329.

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Abstract

Squalene is a natural triterpenoid that is important in medicine, and daily-use chemical and nutraceutical industries. The demand for squalene remains high and constant. Shark liver oil is the source of the compound; however, the source is unsustainable due to the protection of the gradually extinct animal. This study aimed to construct an engineered *Escherichia coli* strain to produce squalene in a green and sustainable manner. In this study, the activity of several squalene synthases from different species was screened. Gas chromatography-mass spectrometry and high-performance liquid chromatography were used for chemical structure identification. The mevalonate pathway (MVA) was constructed into BL21(DE3) to enhance the supply of farnesyl diphosphate to obtain squalene at a concentration of 69.3 mg/L. *IspH* was overexpressed in BL21(DE3) to decrease squalene production due to the accumulation of dimethylallyl diphosphate. By balancing the overexpression of *ispH* and *ispG*, the production of squalene increased to 298.3 mg/L. The genes of the MVA pathway were further separated upstream and downstream, and constructed into two plasmids with different origins to balance the metabolic flux. By optimizing the expression strength of genes in MVA and 2-C-methyl-d-erythritol-4-phosphate pathway, an engineered strain was finally obtained with squalene production of 974.3 mg/L. By replacing chassis cell BL21(DE3) with XL1-Blue, squalene production was further increased to 1,274 mg/L.

Keywords
- squalene
- MVA
- MEP
- biosynthesis
- *Escherichia coli*

Introduction

Squalene is a natural triterpenoid that acts as an important precursor in the biosynthesis of sterols, cholesterol, and terpenoids,[1] and plays a broad role in reducing cancer risk,[2,3] medication usage,[3,4] anti-infection,[4,5] and moisture retention,[5] suggesting its wide application in daily-use chemical, medical, and nutraceutical sectors. Squalene is abundant in the liver of deep-sea sharks,[1,6] but access to this source is limited due to animal protection.[7] Squalene can also be produced by genetically modified plants and microorganisms,[8] but these methods are still not widely used due to low yield and high cost.[6,7,9]

In nature, squalene is synthesized from the two pathways: 2-C-methyl-d-erythritol-4-phosphate pathway (MEP pathway, mainly presents in eubacteria and plant plastids) and mevalonate pathway (MVA pathway, mainly presents in plant and animals) (►**Fig. 1**).[10,11] In *Escherichia coli*, farnesyl diphosphate (FPP), a precursor substance, already exists, and squalene can be synthesized from two molecules of FPP head-to-head by the addition of squalene synthase (SQS). *Escherichia coli* does not consume squalene, yet produce and store squalene reliably. Thus, *E. coli* may be an excellent genetically engineered host for squalene production.[2,5]

In the past, Furubayashi et al proved that the expression of human SQSs is a predominant strategy for the production of squalene in *E. coli*,[12] Sun et al introduced the entire MVA pathway to enhance the production of squalene in *E. coli*,[13] and Katabami et al took the same measure and introduced the MVA pathway to increase the production of squalene to 230 mg/L,[9] Xu et al increased the production of squalene in *E. coli* by enhancing NADPH supply, and achieved net

received
April 13, 2023
accepted
September 30, 2023
article published online
December 1, 2023

DOI https://doi.org/10.1055/s-0043-1776291.
ISSN 2628-5088.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

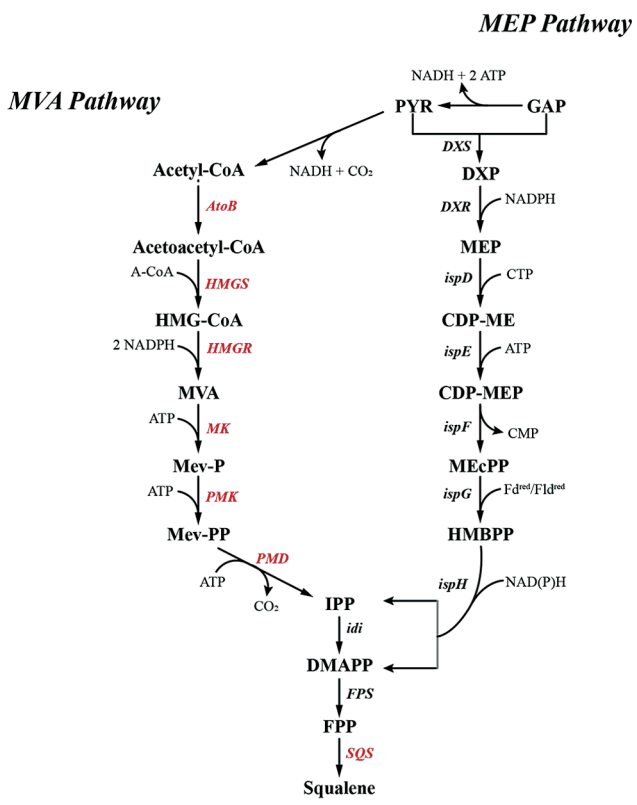

Fig. 1 The MEP and MVA pathways for squalene biosynthesis (enzymes *in red* were introduced into *E. coli* in this study). MEP, 2-C-methyl-d-erythritol-4-phosphate pathway; MVA, mevalonate pathway.

increment of 21-folds compared to the origin strain.[14] However, whether co-expressing the MEP and MVA pathways and increasing metabolic flow[15] would further improve the production of squalene remained largely unknown.

It is also favorable to increase the expression of rate-limiting enzymes and rate-limiting substrates,[5,16] and replace the chassis cell[17,18] to enhance the production of squalene. In this study, the activity of several SQSs from different species was screened. The MVA pathway was constructed into BL21(DE3) to enhance the supply of FPP, isopentenyl pyrophosphate (IPP), and dimethylallyl diphosphate (DMAPP). In addition, we tried to overexpress different combinations of *idi*, *ispA*, *dxs*, *ispG*, and *ispH* to further enhance FPP supply. Genes of the MVA pathway were all on plasmid p35151, and separated into two plasmid backbones with different copy numbers to balance the metabolic flux. Chassis cells were also changed to improve the production of squalene. The study constructed an engineered *E. coli* to achieve a biosynthetic pathway to obtain squalene efficiently.

Material and Methods

Strains, Plasmids, Reagents, and Shake Flask Cultivation

The bacterial strains and plasmids used in this study are listed in ►**Table 1**. DE3 (*E. coli*) and XL1-Blue (*E. coli*) were used as the host for metabolic engineering. DH5α (*E. coli*) was used in plasmids amplification. Plasmid p35151, pETDuet-1, pUC19m, and pBBR1MCS-2 were kept in our laboratory and used as the backbone for other plasmids' construction.

The standard of squalene was purchased from Sigma-Aldrich (Shanghai, China). Restriction enzymes, PrimeSTAR Max DNA Polymerase, and In-Fusion HD Cloning Plus were purchased from Takara (Dalian, China). Gel extraction kit, PCR purification kit, and plasmid purification kit were purchased from Generay (Shanghai, China).

Strains for plasmid amplification were cultured at 37°C in LB medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl) for 12 hours on a rotary shaker (230 rpm). Engineered strains for squalene production were cultured at 37°C in TB medium (12 g/L peptone, 24 g/L yeast extract, 4 ml/L glycerol, 0.17 mol/L KH_2PO_4, 0.73 mol/L K_2HPO_4) for approximately 3 hours on a rotary shaker (230 rpm). When the OD_{600} of cultures reached 0.6, isopropyl-1-thio-β-*D*-galactopyranoside (IPTG; 0.1 mmol/L) was added to induce the expression of genes under the control of IPTG-inducible promoters. Engineered strains were grown for a further 48 hours at 37°C on a rotary shaker (180 rpm) before harvesting and analyzing for squalene content. According to different plasmids containing various engineered strains, ampicillin (100 μg/mL), kanamycin (50 μg/mL), and chloramphenicol (25 μg/mL) were selected and added to the medium LB and TB.

Plasmid Construction

The primers used in this study are listed in ►**Table 2**. All primers were synthesized by GENEWIZ Inc. (Suzhou China). General molecular manipulations were performed according to standard protocols.[19]

pET-KN, pET-NN, pET-thN, and pET-YN

Plasmids pET-KN, pET-NN, pET-thN, and pET-YN were constructed from pETDuet-1, which contains ColE1 origin, ampicillin resistance gene, and T7 promoter.

The sequence of *dehydrosqualene desaturase* (*CrtN*) (UniProt ID: Q7A3E2) was synthesized and constructed into the second MCS of pETDuet-1 by GENEWIZ Inc. (Suzhou China) after codon optimization, yielding plasmid pET-CrtN.

The sequence of *KSS* (a SQS from *Bacillus subtilis*, UniProt ID: O06728), *YSS* (a SQS from *Yarrowia lipolytica*, UniProt ID: Q9Y753), *NSS* (a SQS from *Saccharomyces cerevisiae*, UniProt ID: P29704), and *thSQS* (a SQS from Human, UniProt ID: P37268, 31–370 residues) was synthesized and constructed separately into pET-CrtN by GENEWIZ Inc. (Suzhou China) after codon optimization, yielding plasmids pET-KN, pET-YN, pET-NN, and pET-thN (►**Fig. 2** and ►**Table 1**).

pET-YSS, pET-IAY, and pET-HIAY

YSS was amplified from plasmid pET-YN with primers pET-YSS-F and pET-YSS-R, and infusion cloned into backbone pETDuet-1 linearized by *Bam*H I and *Not* I, yielding plasmid pET-YSS (►**Fig. 3A** and ►**Table 1**).

Idi was amplified from plasmid p35151 with primers pET-IAY-1-F and pET-IAY-1-R. *IspA* was amplified from plasmid p35151 with primers pET-IAY-2-F and pET-IAY-2-R. *YSS* was

Table 1 The plasmids and strains used in this study

Name	Relevant characteristics	Source
Plasmids		
p35151	P15A origin; CmR; PlacUV5::atoB-*hmgs*-t*hmgr*-*mk*-*pmk*-*pmd*-idi-ispA	Our laboratory
pETDuet-1	ColE1 origin; AmpR; PT7	Our laboratory
pUC19m	ColE1 origin; AmpR; Plac	Our laboratory
pBBR1MCS-1	P15A origin; CmR; Plac	Our laboratory
pBBR1MCS-2	pBBR1 origin; KanR; Plac	Our laboratory
pET-CrtN	ColE1 origin; AmpR; PT7::; pT7::CrtN	This study
pET-KN	ColE1 origin; AmpR; PT7::KSS; pT7::CrtN	This study
pET-NN	ColE1 origin; AmpR; PT7::NSS; pT7::CrtN	This study
pET-thN	ColE1 origin; AmpR; PT7::thSQS; pT7:: CrtN	This study
pET-YN	ColE1 origin; AmpR; PT7::YSS; pT7:: CrtN	This study
pET-YSS	ColE1 origin; AmpR; PT7::YSS	This study
pET-IAY	ColE1 origin; AmpR; PT7::idi-ispA-YSS	This study
pET-HIAY	ColE1 origin; AmpR; PT7::ispH-idi-ispA-YSS	This study
pMEP-DG	pBBR1 origin; KanR; plac::dxs-ispG	This study
pMVA1	P15A origin; CmR; Plac::atoB-*hmgs*-t*hmgr*	This study
pMVA2	pBBR1 origin; KanR; plac:: *mk*-*pmk*-*pmd*-idi	This study
pUC-IAY	ColE1 origin; AmpR; Plac::idi-ispA-YSS	This study
pUC-HIAY	ColE1 origin; AmpR; Plac::ispH-idi-ispA-YSS	This study
Strains		
E. coli DH5α	F-ϕ80 lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk +) phoA, supE44 thi-1 gyrA96 relA1 λ-	Our laboratory
BL21 (DE3)	F-ompThsdSB (rB-mB-) gal dcm (DE3)	Our laboratory
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' roABlaclqZ? M15 Tn10 (Tetr)]	Our laboratory
N1	BL21(DE3)/pET-KN	This study
N2	BL21(DE3)/pET-NN	This study
N3	BL21(DE3)/pET-thN	This study
N4	BL21(DE3)/pET-YN	This study
H1	BL21(DE3)/p35151/pET-YSS	This study
H2	BL21(DE3)/p35151/pET-IAY	This study
H3	BL21(DE3)/p35151/pET-HIAY	This study
H4	BL21(DE3)/p35151/pET-IAY/pMEP-DG	This study
H5	BL21(DE3)/p35151/pET-HIAY/pMEP-DG	This study
H6	BL21(DE3)/pMVA1/pMVA2/ pET-YSS	This study
H7	BL21(DE3)/pMVA1/pMVA2/ pET-IAY	This study
XH3	XL1-Blue/p35151/pMEP-DG/pUC-IAY	This study
XH4	XL1-Blue/p35151/pMEP-DG/pUC-HIAY	This study
XH5	XL1-Blue/pMVA1/pMVA2/pUC-IAY	This study

amplified from plasmid pET-YN with primers pET-IAY-3-F and pET-IAY-3-R. These three genes were infusion cloned into backbone pETDuet-1 linearized by *Bam*H I and *Not* I, yielding plasmid pET-IAY (►**Fig. 3A** and ►**Table 1**).

The sequence of *ispH* (UniProt ID: P62623) was synthesized and constructed into plasmid pET-IAY by GENEWIZ Inc. (Suzhou China) after codon optimization, yielding plasmid pET-HIAY (►**Fig. 3A** and ►**Table 1**).

pMEP-DG

Sequences of *dxs* (UniProt ID: P77488) and *ispG* (UniProt ID: P62620) were synthesized and constructed into plasmid

Table 2 The primers used in this study

Primer	Sequence (5′ → 3′)
pET-YSS-F	agccaggatccgatgggaaaactcatc
pET-YSS-R	gccgcaagcttctaatctctcag
pET-IAY-1-F	tcaccacagccaggatccaggaggtaatgataatgcaaac
pET-IAY-1-R	ttctttatcctcctagatccttatttaagctgggt
pET-IAY-2-F	ggatctaggaggataaagaaatggactttccgcag
pET-IAY-2-R	tttgtaatcctcctagatccttatttattacgctg
pET-IAY-3-F	ggatctaggaggattacaaaatgggaaaactcatc
pET-IAY-3-R	ttatgcggccgcctaatctctcagagg
pMVA1-F	cggtatcgataagcttatgaagaactgtgtgattgtttctgcg
pMVA1-R	tagaactagtggatcctcagcttttaatgcacgtgacgct
pMVA2-F	cggtatcgataagcttatgtctctgccattcctgacg
pMVA2-R	tagaactagtggatccttatttaagctgggtaaatgcaga
pUC-IAY-F	tgattacgccaagcttatgataatgcaaacggaacacgtca
pUC-IAY-R	gacggccagtgaattcctaatctctcagaggaaacatcttagagtcgaaaatct
pUC-HIAY-F	tgattacgccaagcttatgcagatcctgttggccaac
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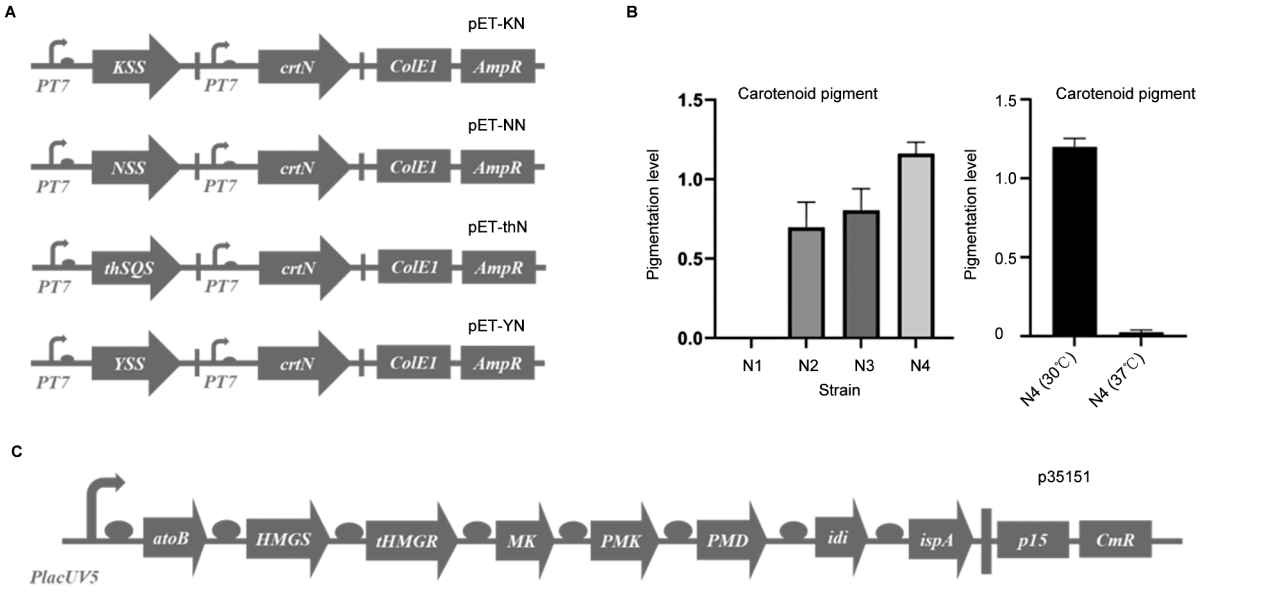

Fig. 2 Screening for SQS activity by colorimetric detection. (**A**) The SQS genes from different species and the crtN gene are cloned into plasmid pETDuet-1. (**B**) The colorimetric detection of squalene produced in the strain. (**C**) Plasmid p35151. SQS, squalene synthase.

pBBR1MCS-2 by GENEWIZ Inc. (Suzhou China) after codon optimization, yielding plasmid pMEP-DG (►**Fig. 3A** and ►**Table 1**).

pMVA1 and pMVA2

AtoB-hmgs-thmgr was amplified from plasmid p35151 with primers pMVA1-F and pMVA1-R, and infusion cloned into backbone pBBR1MCS-1 (with p15A origin) linearized by *Hin*d III and *Bam*H I, yielding plasmid pMVA1 (►**Fig. 3A** and ►**Table 1**).

Mk-pmk-pmd-idi was amplified from plasmid p35151 with primers pMVA2-F and pMVA2-R, and infusion cloned into backbone pBBR1MCS-2 linearized by *Hin*d III and *Bam*H I, yielding plasmid pMVA2 (►**Fig. 3A** and ►**Table 1**).

pUC-IAY and pUC-HIAY

Idi-ispA-YSS was amplified from plasmid pET-IAY with primers pUC-IAY-F and pUC-IAY-R, and infusion cloned into backbone pUC19m linearized by *Hin*d III and *Eco*R I, yielding plasmid pUC-IAY (►**Fig. 3A** and ►**Table 1**).

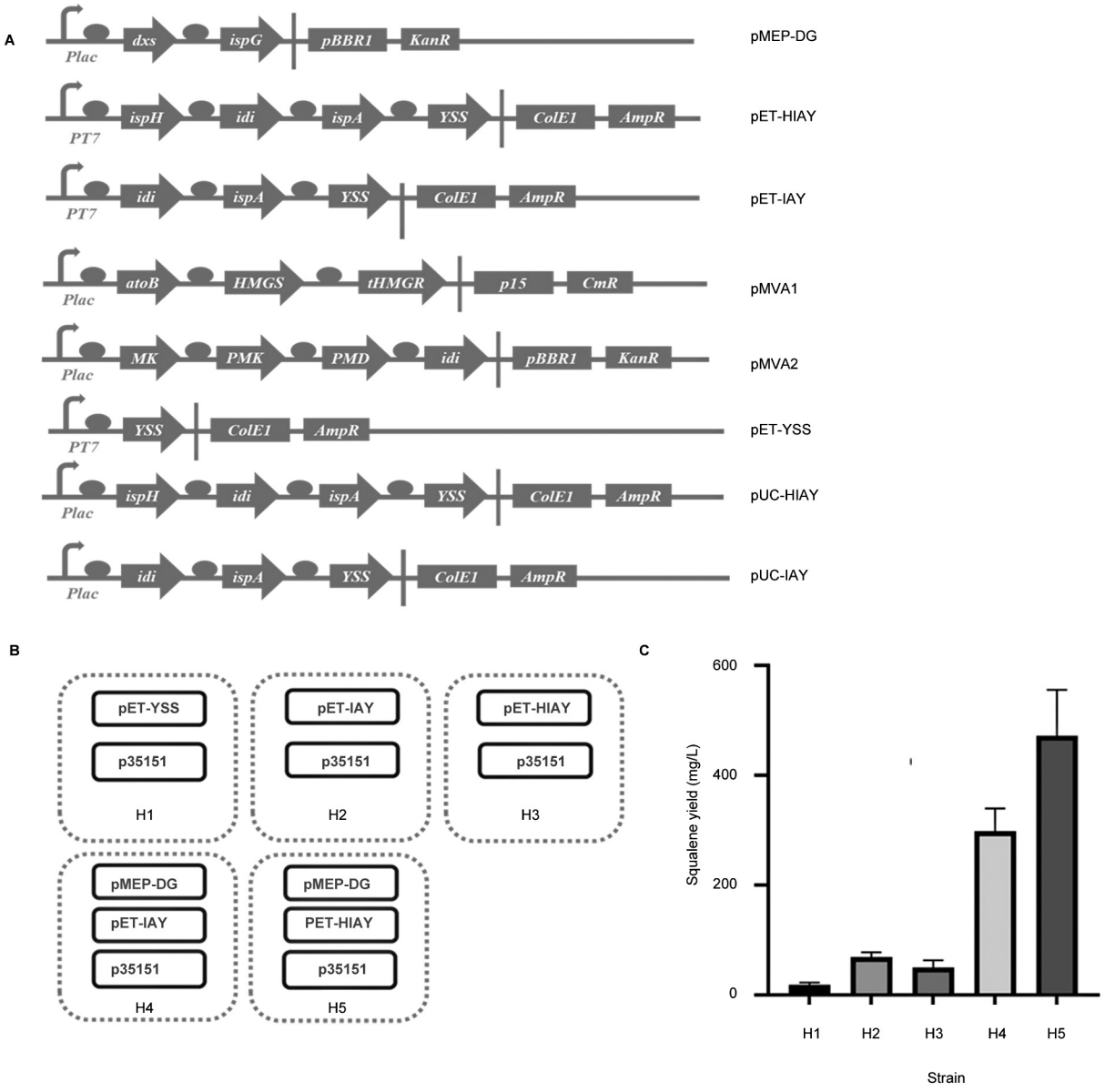

Fig. 3 Optimization of the MEP pathway. (**A**) Plasmids used in BL21 (DE3). (**B**) Plasmids in the strain of H1–H5. (**C**) Average yield of squalene in strain H1–H5. MEP, 2-C-methyl-d-erythritol-4-phosphate pathway.

IspH-*idi-ispA-YSS* was amplified from plasmid pET-HIAY with primers pUC-HIAY-F and pUC-HIAY-R, and infusion cloned into backbone pUC19m linearized by *Hin*d III and *Eco*R I, yielding plasmid pUC-HIAY (►**Fig. 3A** and ►**Table 1**).

Carotenoid Pigment Analysis

Carotenoid pigment analysis was performed according to a reported study.[20] Each strain was harvested from a 10 mL culture medium into a 50 mL centrifugal cube. After centrifuging at 8,000 rpm for 15 minutes and discarding the supernatants, bacterial pellets were obtained. Then, 0.5 to 1 mL of acetone was added to each tube, and immediately vortexed the tubes for 1 minute to extract the carotenoids. The mixture was centrifuged at 10,000 rpm for 30 minutes to obtain acetone extracts. The absorbance at 470 nm was analyzed by using Ultrospec 2100 pro (GE, Boston, United States). The pigmentation level of each culture was determined from the absorbance of the resulting extract by using the molar adsorption coefficients of carotenoids (470 nm, 147,000 M^{-1} cm^{-1}).

Identification and Quantification of Squalene

The identification and quantification of squalene was conducted according to a reported study.[21] Each strain was harvested from a 20 mL culture medium and added into a 50 mL centrifugal cube. The suspension was centrifuged at 8,000 rpm for 5 minutes. Discard the supernatants and obtain the bacterial pellets. To each tube, add 2 mL NaCl (1% w/w) and invert five times to mix gently. Add acetone (5 mL), invert five times, and disrupt the mixtures by ultrasonication for 30 minutes. Add hexane (2 mL) and invert five times. The resulting mixtures stood for 5 minutes

at room temperature, then centrifuged at 8,000 rpm for 5 minutes to obtain the hexane extracts, which were evaporated under reduced pressure. The residue was dissolved in acetonitrile (300 µL) and filtered through a 0.25 µm filter for gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) analysis. Squalene purchased from Aladdin; China was used as standard.

For squalene quantitative analysis, acetonitrile extract (20 µL) was loaded onto an HPLC system (Agilent 1260 infinity, California, United States) with UV detection at 210 nm. For chromatographic separation, an XB-C18 column (250 mm × 4.6 mm, 5 µm) was used. The mobile phase consisted of 2% water and 98% acetonitrile. The flow rate was 1.0 mL/min and the column was held at 40°C during the separation. The peak area was converted into squalene concentration according to a standard curve plotted with a set of known concentrations of squalene.

Table 3 The squalene production of engineered strains in this study

Strains	Plasmids transformed	Squalene yield (mg/L)
H1	p35151/pET-YSS	18.9
H2	p35151/pET-IAY	69.3
H3	p35151/pET-HIAY	50.4
H4	p35151/pMEP-DG/ pET-IAY	298.2
H5	p35151/pMEP-DG/pET-HIAY	472.3
H6	pMVA1/pMVA2/ pET-YSS	617.8
H7	pMVA1/pMVA2/pET-IAY	974.3
XH3	p35151/ pMEP-DG/ pUC-IAY	293.7
XH4	p35151/pMEP-DG/ pUC-HIAY	504.0
XH5	pMVA1/pMVA2/ pUC-IAY	1274

Results

Squalene production of engineered strains in this study is listed in ►**Table 3**.

Screening of Highly Active Squalene Synthase

In this study, *SQSs* from different species were employed for the heterologous production of squalene in *E. coli*.[9] *CrtN* was used to convert squalene into carotenoids with yellow pigment.[12]

Plasmid p35151, containing genes of the MVA pathway, was introduced into BL21(DE3). Plasmids pET-KN, pET-NN, pET-thN, and pET-YN were introduced separately into an engineered strain containing p35151, yielding strains N1, N2, N3, and N4 (►**Fig. 2**).

After a 48-hour culture, the carotenoid pigment of each strain showed that N4 had a higher carotenoid production, indicating that *YSS* had a higher activity.

Strain N4 was cultured at 30°C and 37°C, respectively, and the carotenoid production was compared. The result showed that *YSS* performed much better at 37°C (►**Fig. 2**).

SQS Functional Expression

Plasmids p35151 and pET-YSS were both introduced into BL21(DE3), yielding strain H1 (►**Fig. 3**). After cultured for 48 hours with 0.1 mmol/L IPTG induction, the production of squalene reached 18.9 mg/L. The result from GC-MS analysis showed that the structure of squalene produced by strain H1 was consistent with the standard (►**Fig. 4**).

Increase in the Supply of Precursor FPP

Plasmids p35151 and pET-IAY were both introduced into BL21(DE3), yielding strain H2 (►**Fig. 3**). Strain H2 overexpressed *idi* and *ispA* based on strain H1, and the production of squalene reached 69.3 mg/L.

Optimization of the MEP Pathway

Based on strain H2, *ispH* was overexpressed in strain H3 by introducing plasmids p35151 and pET-HIAY into BL21(DE3) (►**Fig. 3**). The production of squalene in strain H3 was 50.4 mg/L. Plasmids pMEP-DG was introduced into strains H2 and H3, yielding strain H4 and H5 (►**Fig. 3**). Plasmid pMEP-DG contained *dxs* and *ispG*, and by overexpressing these two genes, squalene production of strain H4 and H5 reached 298.2 and 472 mg/L, respectively.

MVA Pathway Optimization

Plasmid pMVA1 contained upstream genes of the MVA pathway (*atoB*, *hmgs*, *thmgr*), and plasmid pMVA2 contained downstream genes of the MVA pathway (*mk*, *pmk*, *pmd*, *idi*). Plasmids pMVA1, pMVA2, and pET-YSS were all introduced into BL21(DE3), yielding strain H6 (►**Fig. 5**), and squalene production reached 617.8 mg/L. Based on strain H6, strain H7 overexpressed *idi* and *atoB* by replacing plasmid pET-YSS with pET-IAY (►**Fig. 5**), increasing the production of squalene to 974.3 mg/L.

Replacement of Chassis Cells

Plasmids pUC-IAY and pET-IAY shared common genes, but the backbone pUC19m could perform in XL1-Blue while backbone pET-IAY could not. pET-HIAY was also replaced with pUC-HIAY. Plasmids p35153, pMEP-DG, and pUC-IAY were introduced into XL1-Blue, yielding strain XH3 (►**Fig. 6**), and pUC-IAY was replaced with pUC-HIAY to obtain strain XH4. Squalene production of strain XH3 and XH4 was 293.7 and 504.0 mg/L, respectively. Strain XH5 contained plasmids pMVA1, pMVA2, and pUC-IAY, and squalene production reached 1,274 mg/L (►**Fig. 6**).

Discussion

Genes *idi* and *ispA* were the key enzymes to produce FPP, and overexpressing the two genes could increase squalene production in strain H1 (18.9 mg/L) and H2 (69.3 mg/L). In strain H3, *ispH* was overexpressed and contributed to a DMAPP accumulation in cells, which has been proven to be harmful to cell culture and can decrease terpenoid production in

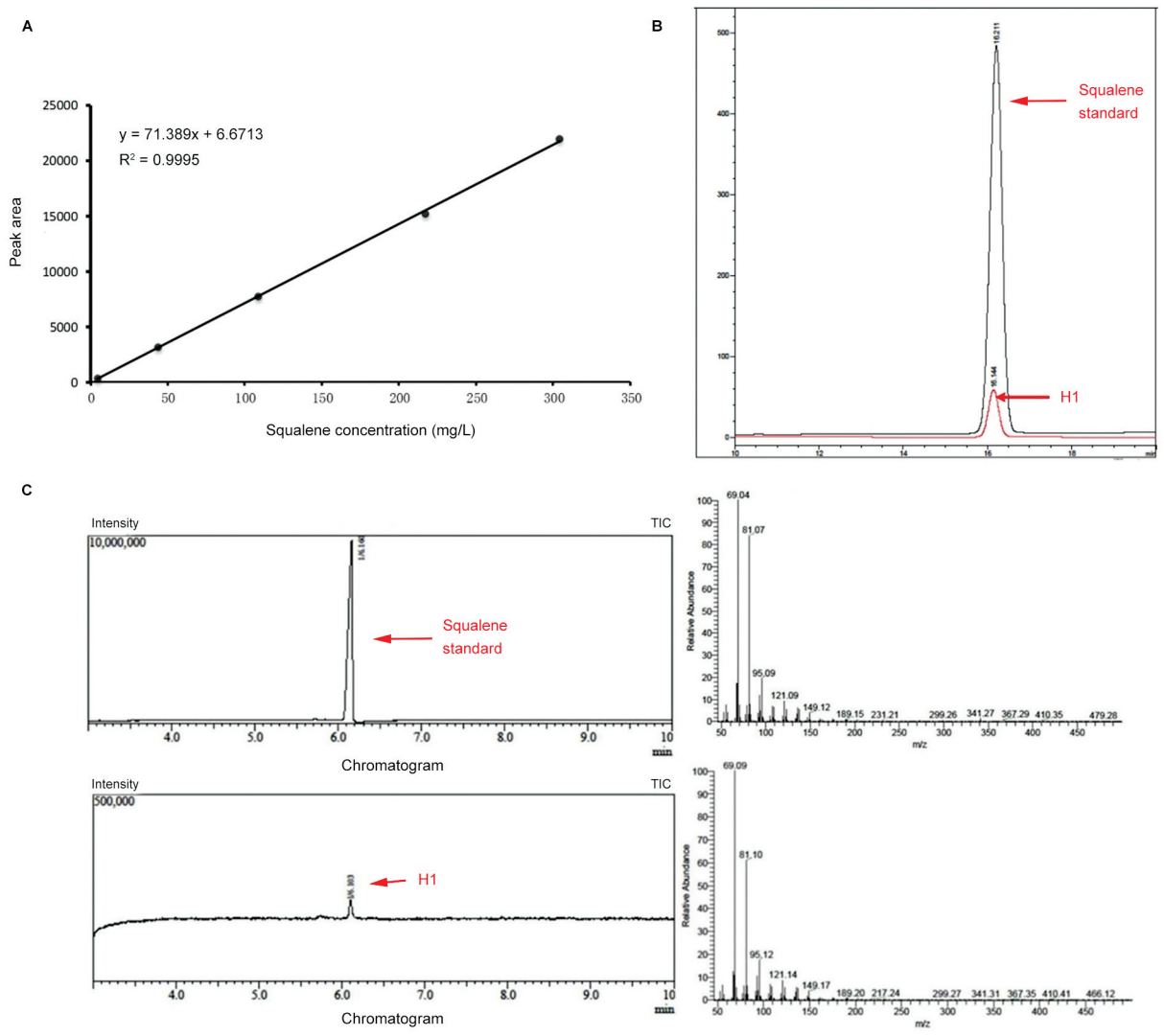

Fig. 4 HPLC and GC-MS analysis for squalene produced by strain H1. **(A)** Standard curve plotting to quantify squalene yield. **(B)** HPLC of squalene produced by strain H1. The peak of the sample overlaps with the standard. **(C)** GC-MS analysis of squalene produced by strain H1. GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography.

previous studies.[12,22,23] In strain H3, squalene production was indeed decreased to 50.4 mg/L in comparison to strain H2 (69.3 mg/L), and this may be due to the accumulation of DMAPP.

The MEP pathway is naturally present in *E. coli*, and yields IPP 1.48-fold higher than the MVA pathway.[24,25] Improving the metabolic flux of the MEP pathway may be helpful in squalene production. *dxs* was the key enzyme in the MEP pathway. By overexpressing *dxs*, the production of lycopene increased by 3.5-fold in engineered strain.[17] Evidence suggests that Gene *ispG* favors the production of lycopene and isopentenol.[26,27] Thus, in this study, *dxs* and *ispG* were both overexpressed in *E. coli*, as a result, squalene production in strain H4 (298.3 mg/L) was significantly increased in comparison to strain H2 (69.3 mg/L).

Overexpression of *ispG* leads to the accumulation of intermediate hydroxymethylbutenyl diphosphate, which seriously interferes with the synthesis machinery of nucleotide and protein in *E. coli*.[16] Li et al found that balanced activation of *ispG* and *ispH* could push the carbon flux away from methylerythritol cyclodiphosphate and increase β-carotene and lycopene titer by 73 and 77%, respectively.[16] Thus, in this study, *ispG* was constructed in plasmid pMEP-DG (low copy number, medium-strength promoter lac) and *ispH* was constructed into plasmid pET-HIAY (high copy number, strong promoter T7) to balance their expression; as a result, squalene production in strain H5 (472.3 mg/L) was elevated obviously in comparison to strain H4 (298.2 mg/L).

The MVA pathway could be divided into an upstream portion (MVU, contains *atoB*, *hmgs*, *hmgr*) and a downstream portion (MVD, contains *mk*, *pmk*, *pmd*, *idi*). Li et al showed that increasing the expression strength of MVD and decreasing the expression strength of MVU could contribute to the production of IPP and DMAPP.[28] In this study, genes of MVU were constructed into the backbone of pBBR1MCS-1 with P15A origin (~10 copy in *E. coli*), generating plasmid pMVA1, while genes of MVD were constructed into the backbone of pBBR1MCS-2 with pBBR1 origin (~19 copy in *E. coli*),

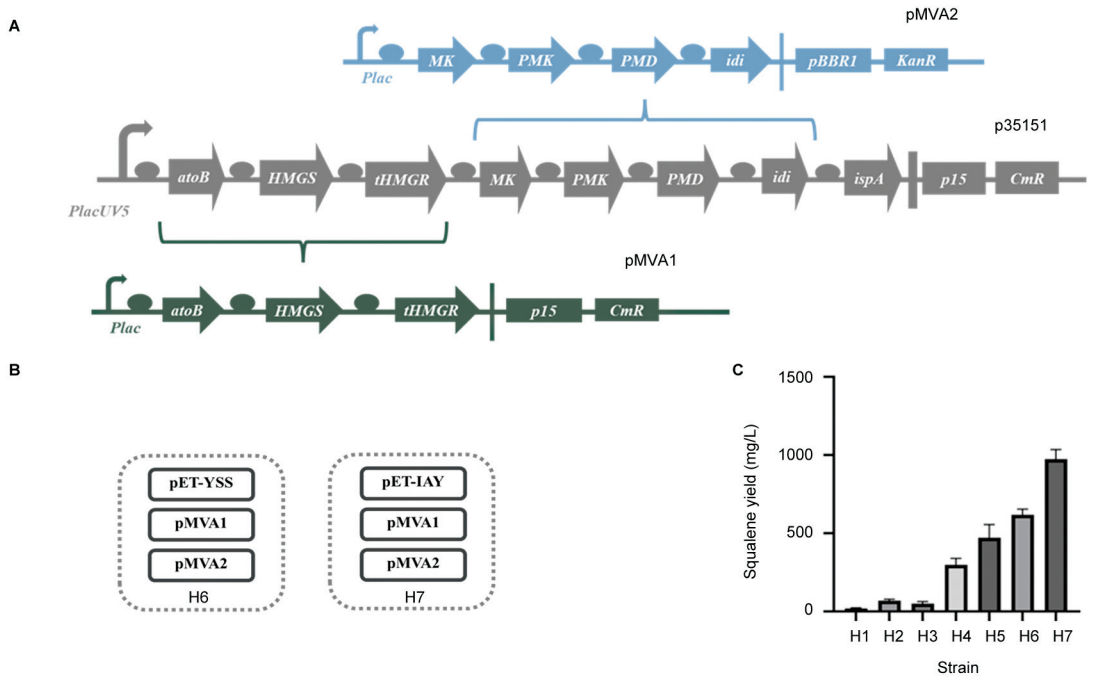

Fig. 5 Optimization of MVA pathway. (**A**) The MVA pathway genes included in plasmid p35151 were separated into two parts and cloned in two plasmids. (**B**) Plasmids in strains H6 and H7. (**C**) Average yield of squalene in strains H1–H7. MVA, mevalonate pathway.

generating plasmid pMVA2. By replacing plasmids p35151 with pMVA1 and pMVA2, squalene production in strain H6 increased to 617.8 mg/L in comparison to strain H1 (18.9 mg/L), and in strain H7 increased to 974.3 mg/L in comparison to strain H2 (69.3 mg/L).

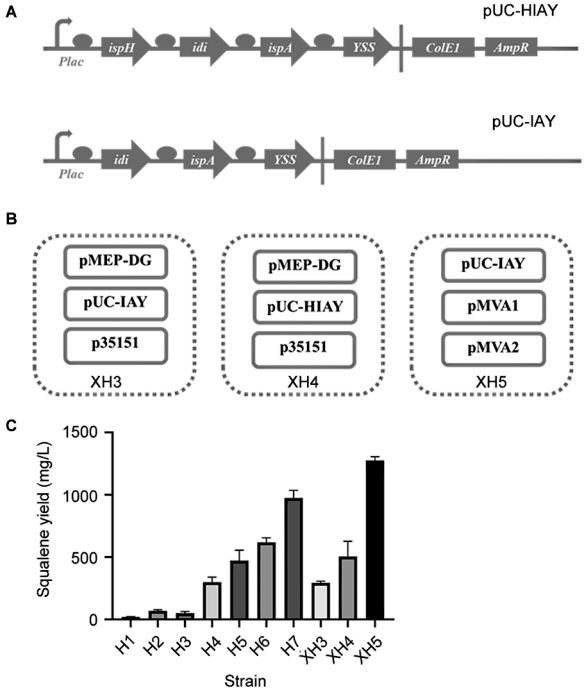

Fig. 6 Effect of chassis cell on squalene production. (**A**) Plasmids used in XL1-Blue. (**B**) Plasmids in strains XH3, XH4, and XH5. (**C**) Average yield of squalene in strains constructed in this study.

Katabami et al obtained 230 mg/L of squalene in XL1-Blue, which was higher than other engineered *E. coli* strains.[9,29] Thus, we replaced the chassis cell from BL21 (DE3) to XL1-Blue, and encouragingly, the production of squalene reached 1,274 mg/L.

The other strategies that may increase the production of squalene were further discussed. Squalene is a nonpolar lipid and is stored in the cell membrane of *E. coli*. Therefore, enlarging the cell membrane area may be beneficial in the accumulation of squalene in *E. coli*.[30] In addition, screening the key enzymes of MEP and MVA pathways of different species helps optimize the two pathways, and in turn, influences the subsequent production of squalene. Also, rational-design engineering could improve the biochemical properties of enzymes, including kinetic behaviors and substrate specificity,[31] and fusing upstream and downstream enzymes may make their activities more efficient.[32] Besides, glycerol was a potential carbon source for the production of terpenoids in *E. coli*.[33] Thus, constructing a glycerol-utilization pathway in *E. coli* by introducing genes like aldehyde reductase (*alrd*) and aldehyde dehydrogenase (*aldH*) may make it possible to improve the production of squalene and cut the cost.[34]

Conclusion

This study demonstrated that the production of squalene could be improved by the co-expression of MVA and MEP pathways and the introduction of a highly active SQS. The production of squalene was also facilitated through the use of promotors of different strengths and the adjustment of the genes' copy number of key enzymes in the MEP and MVA pathways. In this study, based on XL1-Blue, a strain

with squalene production of 1274 mg/L was constructed. This study also presented several promising strategies to produce squalene in *E. coli*, helping squalene biosynthesis through a potential low-cost and highly effective production source.

Conflict of Interest
None declared.

Acknowledgments
We thank the Instrumental Analysis and Research Center, China State Institute of Pharmaceutical Industry for GC-MS support.

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