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Article

Production of Squalene in *Bacillus subtilis* by Squalene Synthase Screening and Metabolic Engineering

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ABSTRACT: Squalene synthase (SQS) catalyzes the conversion of two farnesyl pyrophosphates to squalene, an important intermediate in between isoprene and valuable triterpenoids. In this study, we have constructed a novel biosynthesis pathway for squalene in *Bacillus subtilis* and performed metabolic engineering aiming at facilitating further exploitation and production of squalene-derived triterpenoids. Therefore, systematic studies and analysis were performed including selection of multiple SQS candidates from various organisms, comparison of expression vectors, optimization of cultivation temperatures, and examination of rate-limiting factors within the synthetic pathway. We were, for the first time, able to obtain squalene synthesis in *B. subtilis*. Furthermore, we achieved a 29-fold increase of squalene yield (0.26−7.5 mg/L) by expressing SQS from *Bacillus megaterium* and eliminating bottlenecks within the upstream methylerythritol-phosphate pathway. Moreover, our findings showed that also *ispA* could positively affect the production of squalene.

KEYWORDS: *Bacillus subtilis, MEP pathway, squalene, squalene synthase*

INTRODUCTION

Bacillus subtilis, Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA), has long been investigated and widely used in various fields of industry ranging from food, feed additive, pharmaceuticals, and fine chemicals.[1−3] In addition, *B. subtilis* has been reported to be a high isoprene producer,[4] which indicates its potential to become a cell factory for high-value terpenoids.[5,6] Terpenoids, also referred to as isoprenoids, constitute a large class of natural products with a great diversity in both structural and biochemical properties. Moreover, many of them have health-enhancing properties and therapeutic potential, such as ginsenosides and artemisinin.[7] Recently, biosynthesis of these terpenoids in *B. subtilis* has attracted ample attention because of the numerous advantages of this microbial cell factory.[6,8] In *B. subtilis*, isoprenoid precursors are synthesized through the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway,[9,10] where the common building blocks of terpenoids, the two five-carbon precursors isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) are produced.[11] The two isoprene units condense to form geranyl pyrophosphate (GPP, C_{10}) and farnesyl pyrophosphate (FPP, C_{15}) by addition of another IPP, and geranylgeranyl pyrophosphate (GGPP, C_{20}) by condensation of another two IPPs. Then, they are cyclized, glycosylated, and modified to produce various terpenoids, where GPP produce monoterpenoids, FPP yield sesquiterpenoids and triterpenoids, and GGPP produce diterpenoids and tetraterpenoids.

Squalene, an acyclic isoprenoid, is a crucial intermediate for the synthesis of many bioactive triterpenoids, such as hopanoids and sterols, which play vital cellular functions in organisms.[12] In addition, squalene itself shows very promising physiological activities such as antioxidant effect, decreasing cancer risks and enhancing the immune system, which promotes its wide applications as additive, supplement, or nutraceutical in the food and personal care industry.[13] Squalene synthase (SQS) (EC 2.5.1.21) catalyzes the head-to-head condensation of two molecules of FPP to form linear C_{30} squalene in a two-step reaction, which is the first committed step toward many triterpenoids (Figure 1).[14,15] In the first step, two molecules of FPP sequentially enter into the catalytic center of SQS to form presqualene pyrophosphate (PSPP), which is a stable cyclopropylcarbinyl pyrophosphate intermediate.[15,16] In the following step, further carbon-skeleton rearrangement including heterolysis and isomerization occur to form squalene, with the NADPH-dependent reduction happening at the same time.[17,18] In the past several decades, SQSs from different eukaryotic species have been extensively characterized, and the catalytic mechanism has been clarified supported by 3D-structure elucidation, truncation, and site-directed mutagenesis.[15,19−22]

Because most studies focus their attention on eukaryotic SQSs, prokaryotic SQSs have rarely been explored.[19,20,23] In *B. subtilis*, no squalene producing capacity has been reported, and the *yisP* gene, which was initially annotated as SQS, has now been characterized as a phosphatase with no squalene catalytic activity.[24,25] Because the aim of this work is researching *B.*

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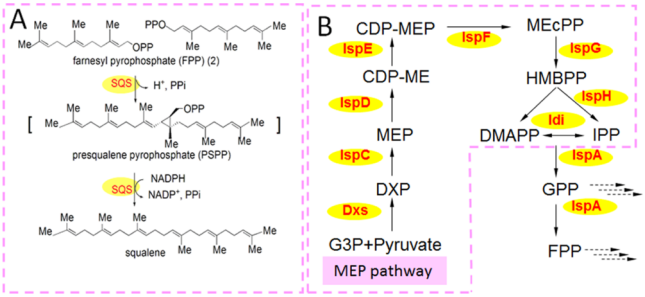

Figure 1. SQS reaction and 2C-methyl-D-erythritol-4-phosphate pathway. (A) Scheme of SQS reaction steps. (B) MEP pathway in *B. subtilis*. Dxs, 1-deoxy-D-xylulose-5-phosphate synthase; IspC, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; IspD, 4-pyrophosphocytidyl-2-C-methyl-D-erythritol synthase; IspE, 4-pyrophosphocytidyl-2-C-methyl-D-erythritol kinase; IspF, 2C-methyl-D-erythritol 2,4-cyclopyrophosphate synthase; IspG, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-pyrophosphate synthase; IspH, 1-hydroxy-2-methyl-butenyl 4-pyrophosphate reductase; Idi, IPP isomerase; and IspA, FPP synthase; metabolite abbreviations: G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5′-pyrophospho)-2-C-methyl-D-erythritol; CDP-MEP, 2-phospho-4-(cytidine 5′-pyrophospho)-2-C-methyl-D-erythritol; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate; HMBPP; 1-hydroxy-2-methyl-2-butenyl 4-pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; and PSPP: presqualene pyrophosphate.

subtilis for the production of the most important committed triterpenoid intermediate (squalene), which has not yet been synthesized in *B. subtilis*, this study explored the synthesis of squalene directed by SQSs from different species and engineered the host strain to improve squalene production. Four representative SQS candidates from bacteria species, fungi, and plants were selected and expressed in *B. subtilis* to detect their squalene production. Different plasmids were employed to carry the SQSs, and the upstream MEP pathway genes were also combined and overexpressed to explore their effect on squalene production. In addition, the fermentation temperature was optimized to boost the production level of squalene. This paves the way for future metabolic engineering work in *Bacillus* to improve the production levels of other triterpenoids.

MATERIALS AND METHODS

Strains and Culture Conditions. The plasmids and strains used in this study are listed in Tables S1 and S2. Single colonies of *B. subtilis* were picked up and inoculated into LB media with appropriate antibiotics and incubated at 37 °C overnight. Then, the overnight culture mixture was inoculated at a ratio of 1:100 (v/v) into 10 mL 2SR media (5% yeast extract, 3% tryptone and 0.3% K_2HPO_4) in 50 mL CELLSTAR cellreactor tube with a filter screw cap (Greiner Bio-One, Germany) for fermentation, in triplicates per strain. When the OD_{600} of the bacteria reached around 0.5−0.7, expression was induced by adding D-xylose to a final concentration of 1% (m/v) or isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM when necessary. Then, bacterial cells were harvested after 48 h of fermentation at 37 °C (unless indicated), 230 rpm. Antibiotics were added where appropriate (ampicillin at 100 μg/mL for *Escherichia coli*, spectinomycin at 100 μg/mL, chloramphenicol at 5 μg/mL, and erythromycin at 10 μg/mL for *B. subtilis*).

Plasmid Construction and Transformation. Candidate SQS genes were obtained from *Bacillus acidocaldarius* (BaSQS) (Genbank: WP_012811689.1), *Bacillus megaterium* (BmSQS) (GenBank: ADF40697.1), *Panax ginseng* (PgSQS) (GenBank: AJV26445.1), and *Saccharomyces cerevisiae* (ScSQS) (GenBank: AAB68360.1). They were synthesized and codon optimized to *B. subtilis 168* (Eurofins, Netherlands). Eukaryotic SQSs normally contain their transmembrane regions (TMRs) at the C-terminus of the proteins. Therefore, 25 and 24 amino acid residues at the C-terminus of PgSQS and ScSQS, which according to the literature reports and prediction results of TMHMM Server (v. 2.0) were supposed to be the TMRs, were truncated respectively (Figure S1).[19,26,27]

The plasmids were constructed by the prolonged overlap extension polymerase chain reaction (POE-PCR) method, as described before.[28] The ribosome binding site (RBS) and the spacer between RBS and start codon (AAAGGGGG) were added at the N-terminus of the SQS candidates. The 6×His-tag (CATCATCATCATCATCAT) were placed at the C-terminus of the SQS candidates upstream of the stop codon. The POE-PCR product was transformed directly to *E. coli*. The MEP pathway genes (*dxs*, *ispD*, *ispF*, and *ispH*) were cut from previously constructed plasmid pHB201-SDFH, as reported by Xue et al.,[5] with restriction enzymes XbaI and SpeI and ligated with linearized pBS0E (digested with the same enzymes XbaI and SpeI) by T4 Ligase (Thermo Fisher Scientific, USA), leading to the construction of pBS0E-SDFH. *ispC*, *ispE*, *ispG*, and *ispA* amplified from pHCMC04G-CEGA were individually cloned into pBS0E-SDFH and placed downstream of *ispH* by the POE-PCR method, generating plasmids, pBS0E-SDFHC, pBS0E-SDFHE, pBS0E-SDFHG, and pBS0E-SDFHA, respectively. Samples with positive colony-PCR results were further confirmed by sequencing the fragments (Macrogen, Netherlands). Plasmids were transformed to *B. subtilis* under the standard methods described by Kunst and Rapoport.[29] Primers used in this study are listed in Supporting Information (Table S3).

Sample Preparation for Gas Chromatography Detection. Bacterial cells were harvested by centrifugation (11,000*g*) at 4 °C. Prechilled (−20 °C) 50% methanol (methanol: Milli-Q water, 1:1, v/v) was added to the pellets to quench the cells. After centrifugation, the quenched cell pellets were quickly washed by 4 °C Milli-Q water. To lyse the cells, 1 mL of 50% cold methanol was added to the washed pellets and repeated the freeze−thaw process five times by using liquid nitrogen.[30] The supernatants were collected in a new tube. In the following extraction procedure, acetone was used two times and ethyl acetate was used five times to extract the desired components. All the extracts of one sample were collected in the same tube. Then, samples were dried under nitrogen and dissolved in 250 μL of isopropanol−acetonitrile (7:3, v/v). Prior to use, all the samples were filtered through a 0.22 μm membrane.

Squalene Detection and Quantification. Sample analysis was performed on a Shimadzu GCMS-QP2010SE system equipped with a GC-2010 Plus gas chromatograph (GC) and AOC-20i autoinjector. Samples (4 μL) were injected in a split mode onto the HP-5MS (5% phenyl)-methylpolysiloxane GC column (Agilent J&W 0.25 mm inner diameter, 0.25 μm thickness, 30 m length), with helium as the carrier gas. The injector temperature was 280 °C, and the column oven initial temperature was 210 °C with an increase of 15 °C per minute up to 260 °C and then 5 °C per minute till 280 °C. Subsequently, the temperature was raised to 310 °C with an increase of 25 °C per minute and held for 8 min. The solvent cutoff was set at 8 min. The selected ion mode was applied for acquisition, monitoring *m*/*z* ion 136 and 384 for squalene and internal standard cholesterol, respectively. The integration tools in GCMSsolution 1.20 software (Shimadzu, Den Bosch, The Netherlands) was used to determine the chromatographic peak areas for squalene and cholesterol. To quantify the amount of squalene in the different samples, a calibration curve of standard squalene (Sigma-Aldrich S3626, purity ≥98%) was generated with the concentration range from 10 to 500 μg/mL and cholesterol (Sigma-Aldrich C8667, purity ≥99%) as the internal standard at a concentration of 80 μg/mL.

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RESULTS

Candidate Selection and Analysis. To investigate the evolutionary relations of SQSs across multiple kingdoms of life, an unrooted phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0. Apart from SQSs from *B. acidocaldarius* and *B. megaterium*, the other SQSs among the 28 candidates have been investigated and validated to maintain the capacity to convert FPP to squalene *in vitro* or *in vivo*. Results (Figure 2A) showed that these SQSs can be divided into four categories, including plants, bacteria, mammals, and fungi.

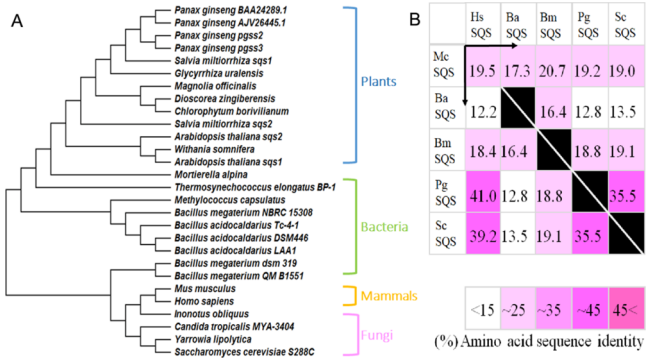

Figure 2. Phylogenetic tree analysis of SQSs and sequence alignment. (A) Phylogenetic tree analysis of SQSs from different species constructed by MEGA 7.0. (B) SQS sequence alignment. McSQS, HsSQS, BaSQS, BmSQS, PgSQS, and ScSQS represent SQSs originating from *M. capsulatus*, *Homo sapiens*, *B. acidocaldarius*, *B. megaterium*, *P. ginseng*, and *S. cerevisiae*, respectively.

SQSs exist both in prokaryotic and eukaryotic organisms. The candidates selected were either reported to be functional SQSs in their native hosts or have been annotated as SQSs (Table 1). After sequence alignment and literature comparison, candidates with a high sequence identity with reported functional SQSs or one with an available crystal structure were chosen. Four candidates were selected for experimental analysis, that is, BaSQS, BmSQS, PgSQS, and ScSQS, which originate from *B. acidocaldarius*, *B. megaterium*, *P. ginseng*, and *S. cerevisiae*, respectively. BaSQS was annotated as SQS with its crystal structure available (4HD1),[31] which would facilitate further exploration if a high squalene synthesis capacity is being detected. The discovery of squalene cyclase in *B. megaterium* implied the existence of SQS in this bacterium.[32] Hence, the annotated squalene/phytoene synthase in *B. megaterium* was selected as a candidate. *P. ginseng* is famous for producing ginsenosides, the bioactive triterpenoids derived from squalene.[27] SQSs from this plant were thought to possess high catalytic efficiency. The ScSQS in the candidate list was selected because of its high squalene synthesis capacity in both yeast and *E. coli*.[19,33]

Then, the four candidates BaSQS, BmSQS, PgSQS, and ScSQS were selected to align with the well-studied SQS HsSQS and McSQS, originating from eukaryotes (*Homo sapiens*) and prokaryotes (*Methylococcus capsulatus*), to compare their sequences (Figure 2B).[15,23] Results demonstrate that prokaryotic SQSs from different bacteria can show quite low amino acid identities among each other even within the same genus (up to 16.4%). SQS identities between prokaryotes and eukaryotes are even lower, with the percentages ranging from only 12.2 to 19.1%. Only PgSQS and ScSQS shared higher identities (41 and 39%) with HsSQS, which is consistent with the previous observation that SQSs from eukaryotic species are more conserved. The figures implied that SQSs from different species can be significantly distinctive, at least at the primary sequence level. This inspired us to investigate the squalene-synthesis capacities of different SQSs in *B. subtilis*.

Level of Production of Squalene by Different SQSs in *B. subtilis* 168. *E. coli*−*Bacillus* shuttle vector pHCMC04G was introduced to express SQS candidates (BaSQS, BmSQS, PgSQS, and ScSQS) by placing SQS downstream of an engineered *B. subtilis mntA* RBS.[5] The xylose-inducible promoter facilitated their precise expression. The constructs are shown in Table S1. All these constructs were transformed to *B. subtilis*, generating BA, BM, PG, and SC (Table S2). The negative control strain BC was *B. subtilis* containing plasmid pHCMC04G without SQS. Because the eukaryotic SQS candidates (PgSQS and ScSQS) possess the TMRs, which will anchor the protein to membranes,[22,27] these fragments were truncated (Figure S1).

B. subtilis strains were cultured in the 2SR medium at 37 °C. After 48 h incubation, metabolites were extracted and squalene production was quantified by GC−mass spectroscopy (MS). The squalene production levels produced by different SQSs were compared (Figure 3). In the negative control, non-SQS

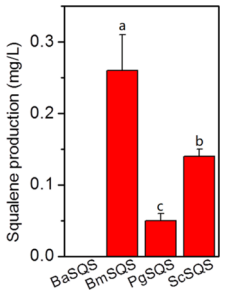

Figure 3. Production levels of squalene in *B. subtilis* strains expressing different SQSs. Error bars represent standard deviations of biological triplicates. Different letters indicate significant statistical differences (Scott Knott 5%). BaSQS, BmSQS, PgSQS, and ScSQS are SQSs originating from *B. acidocaldarius*, *B. megaterium*, *P. ginseng*, and *S. cerevisiae*, respectively.

Table 1. Information of SQS Candidates

SQS candidates	original species	characterization	crystal structure	amino acid length	references
BaSQS	*B. acidocaldarius*	crystal structure available	yes	291	PDB: 4HD1[31]
BmSQS	*B. megaterium*	annotated as squalene/phytoene synthase	No	272	48
PgSQS	*P. ginseng*	validated in *E. coli* and restores SQS function in plant	No	415	26,27
ScSQS	*S. cerevisiae*	validated in *E. coli*	No	444	19

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containing strain BC, no squalene could be detected. Surprisingly, strain BA that is carrying the SQS candidate from *B. acidocaldarius* also showed no detectable squalene. For the other two eukaryotic SQSs, PgSQS and ScSQS originating from a plant and yeast, the conversion of precursor FPP into squalene could be measured in *B. subtilis* after the predicted TMR regions were truncated. SC produced a higher level of squalene than PG. BM that is containing the SQS from *B. megaterium* produced the highest level of squalene among the tested candidates, reaching 0.26 mg/L.

Effect of the Vector System on Squalene Production. BmSQS produced the highest level of squalene among the tested candidates when expressed in pHCMC04G. Subsequently, we explored multiple plasmids of maintaining the BmSQS genes in *Bacillus*. The first construct uses a rolling-circle replicating plasmid pHY300PLK (strain HBM).[34] A second construct is pDR-BmSQS, in which the original integrative plasmid pDR111 contains the strong IPTG inducible promoter $P_{hyperspank}$ which is responsible for BmSQS expression upon insertion into the *amyE* locus of the *B. subtilis* genome (strain DBM) (Figure 4A).[8] In the same way, pHY-

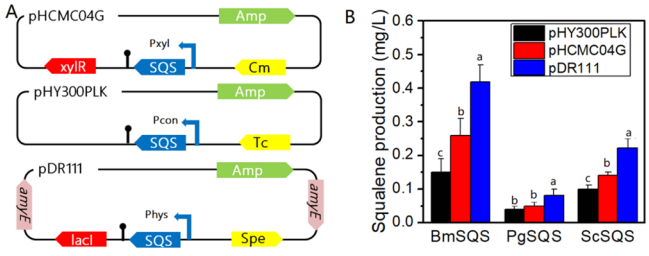

Figure 4. Effect of the vector system on squalene production in *B. subtilis*. (A) Plasmids used for SQS expression. Pxyl: xylose inducible promoter P_{xylA}; Pcon: constitutive promoter; and Phys: IPTG inducible promoter $P_{hyperspank}$. (B) Squalene production levels when expressed in different plasmids in *B. subtilis*. Error bars represent standard deviations of biological triplicates. Different letters indicate significant statistical differences (Scott Knott 5%). BmSQS, PgSQS, and ScSQS are SQSs originating from *B. megaterium*, *P. ginseng*, and *S. cerevisiae*, respectively.

PgSQS, pHY-ScSQS, pDR111-PgSQS, and pDR111-ScSQS were constructed and transformed to *B. subtilis* 168 generating HPG, HSC, DPG, and DSC, respectively (Tables S1 and S2). As shown in Figure 4, pDR-BmSQS produced the highest level of squalene at 0.4 mg/L, whereas pHY-BmSQS showed much lower squalene production with only 0.1 mg/L. Consistently, similar results displayed that DPG and DSC result in higher squalene production than using plasmids pHY300PLK and pHCMC04G (Figure 4B). Subsequently, SQSs integrated into the *B. subtilis* genome have been used for further experiments.

Upregulation of MEP Pathway Genes Increased the Metabolic Flux toward Squalene. Previous literature indicates that *B. subtilis* is a high isoprene producer, and downstream terpenoids production could be enormously improved when the upstream pathway was boosted.[5] Hence, we tried to combine the upregulation of the MEP pathway with the expression of SQSs and evaluated the downstream squalene production. In *B. subtilis*, the MEP pathway consists of *dxs*, *ispD*, *ispF*, *ispH*, *ispC*, *ispE*, and *ispG*. In addition, *ispA*, encoding prenyltransferase, is responsible for subsequent elongation of isoprene units (Figure 1B).[6] A part or all of these genes were combined as an entire operon in pHCMC04G. Four genes (*dxs*, *ispD*, *ispF*, and *ispH*) were used to form pHCMC04G-SDFH. Additionally, another four genes *ispC*, *ispE*, *ispG*, and *ispA* were assembled to form pHCMC04G-SDFHCEGA, as described before.[8] Plasmids pHCMC04G-SDFH and pHCMC04G-SDFHCEGA were transformed to DBM, DPG, and DSC, respectively. The resulting strains were tested using the same fermentation protocol. The results are displayed in Figure 5. Overall, all the

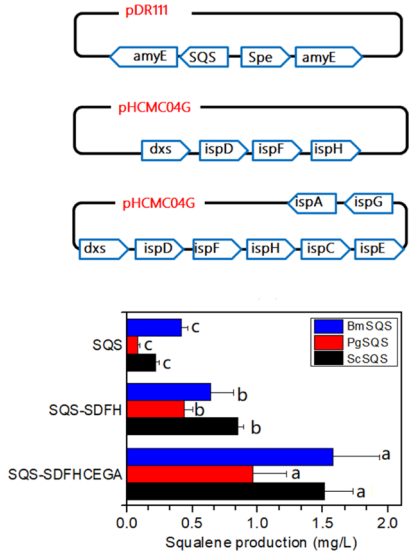

Figure 5. Effect of overexpressing MEP pathway genes on squalene production in *B. subtilis*. *B. subtilis* strains were cultured at 37 °C for 48 h. SQSs were expressed in pDR111 and subsequently integrated into genome of *B. subtilis*; MEP pathway-related genes were expressed in pHCMC04G. S, C, D, E, F, G, H, and A represent *dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*, and *ispA*, respectively. The second and third plasmids are pHCMC04G-SDFH and pHCMC04G-SDFHCEGA, respectively. Error bars represent standard deviations of biological triplicates. Different letters indicate significant statistical differences (Scott Knott 5%). BmSQS, PgSQS, and ScSQS are SQSs originating from *B. megaterium*, *P. ginseng*, and *S. cerevisiae*, respectively.

three SQSs showed improved squalene production when coexpressed with pHCMC04G-SDFH. PgSQS showed the strongest increase possibly because of its low basal production and reached 0.44 mg/L. The BmSQS-containing strain reached 0.6 mg/L, and the ScSQS-containing strain reached 0.85 mg/L squalene. When all eight MEP pathway-related genes were overexpressed, all the three different SQS strains had an around 4- to 10-fold increase of squalene production. DBM-MEP8 (containing pHCMC04G-SDFHCEGA) produced the highest level of squalene, reaching around 1.6 mg/L.

Improved Production Level of Squalene by Temperature Optimization. Given that the SQS candidates originate from organisms living at different temperatures, it is useful to determine the optimal temperature for their expression and activity. To determine this, the influence of temperature on squalene production in *B. subtilis* was explored. Strains expressing SQSs, without and with MEP pathways genes, were fermented at 30, 25, and 20 °C for 48 h. Results (Figure 6) showed that the squalene production increased when the culture temperature was decreased to 30 or 25 °C and performed best at 25 °C. The maximum squalene production was around 4 mg/L produced by DBM-MEP8, which is around 2.5-fold more than the yield found at 37 °C. When the

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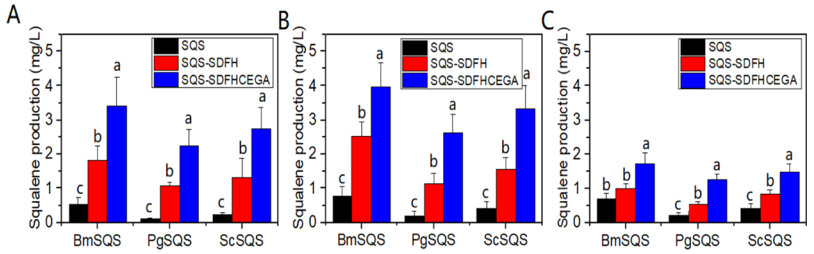

Figure 6. Effect of fermentation temperature on squalene production in *B. subtilis*. (A) Production of squalene in *B. subtilis* cultured 48 h at 30 °C; (B) production of squalene in *B. subtilis* cultured 48 h at 25 °C. (C) Production of squalene in *B. subtilis* cultured 48 h at 20 °C. SQSs were expressed in pDR111 and subsequently integrated into genome of *B. subtilis*; MEP pathway-related genes were expressed in low-copy-number plasmid pHCMC04G. S, C, D, E, F, G, H, and A represent *dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*, and *ispA*, respectively. Error bars represent standard deviations of biological triplicates. Different letters indicate significant statistical differences (Scott Knott 5%). BmSQS, PgSQS, and ScSQS are SQSs originating from *B. megaterium*, *P. ginseng*, and *S. cerevisiae*, respectively.

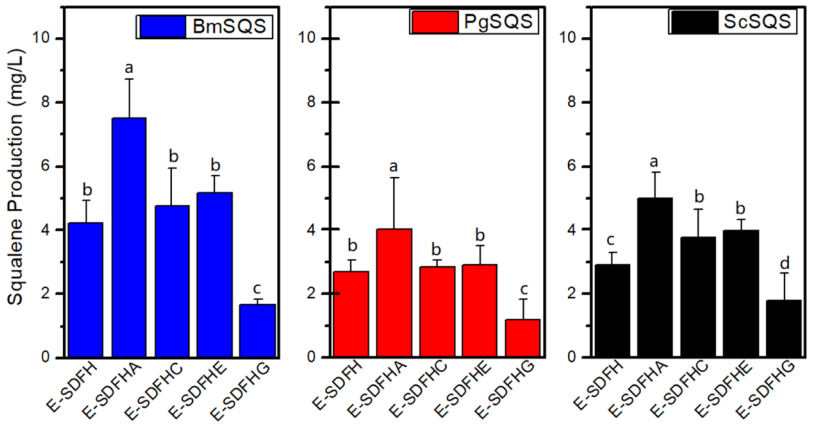

Figure 7. Effect of different combinations of MEP pathway genes in pBS0E plasmid on squalene production in *B. subtilis* cultured 48 h at 30 °C. Error bars represent standard deviations of biological triplicates. Different letters indicate significant statistical differences (Scott Knott 5%). SQSs were expressed in pDR111 and subsequently integrated into genome of *B. subtilis*. MEP pathway-related genes were overexpressed in high-copy-number plasmid pBS0E. S, C, D, E, F, G, H, and A represent *dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*, and *ispA*, respectively. BmSQS, PgSQS, and ScSQS are SQSs originating from *B. megaterium*, *P. ginseng*, and *S. cerevisiae*, respectively.

temperature decreased to 20 °C, all of the strains showed similar squalene production as when being cultured at 37 °C. Considering that comparable squalene production was observed when strains were cultured at 30 and 25 °C, both of these temperatures were chosen for further experiments.

Improved Production Level of Squalene by Releasing the Rate-Limiting Factor in the MEP Pathway. In this step, investigation of whether further improvement of a precursor level could improve squalene production was performed. Compared to pHCMC04G (5−6 units per chromosome), pBS0E has relatively a high copy number (15−25 units per chromosome).[35−37] Hence, the vector pBS0E was employed to express MEP pathway genes and compare it to pHCMC04G. Four MEP pathway genes were first expressed in pBS0E (*dxs*, *ispD*, *ispF*, and *ispH*, as pBS0E-SDFH) with SQSs. Results showed that overexpressing four MEP genes in high-copy-number plasmid pBS0E (pBS0E-SDFH) can lead to similar squalene production to the strain with eight genes overexpressed in low-copy-number plasmid pHCMC04G (pHCMC04-SDFHCEGA) (Figures 6 and 7). To further evaluate contributions of *ispC*, *ispE*, *ispG*, and *ispA* to terpenoids production, which have not been extensively investigated and discussed, each of them was combined individually with pBS0E-SDFH, respectively generating pBS0E-SDFHC, pBS0E-SDFHE, pBS0E-SDFHG, and pBS0E-SDFHA, respectively. In this case, effects of each individual enzyme could be investigated, with potential bottlenecks identified and released. Subsequently, these constructs were coexpressed with SQSs in *B. subtilis* and fermented for 48 h at both 30 and 25 °C to test squalene production. Higher squalene productions were observed when strains were cultured at 30 °C (Figure 7). Results display that compared with pBS0E-SDFH containing strains, no enormous changes on squalene production were observed when pBS0E-SDFHC or pBS0E-SDFHE were overexpressed. Overexpression of pBS0E-SDFHA leads to 1.5- to 1.8-fold increase in squalene production, indicating that FPP concentration limited the synthesis of squalene. The maximum squalene production reached 7.5 mg/L acquired by BmSQS coexpressed with pBS0E-SDFHA. However, pBS0E-SDFHG overexpression decreases squalene production to 0.38- and 0.62-fold compared to pBS0E-SDFH strains.

DISCUSSION

Squalene is a pivot precursor for the biosynthesis of many triterpenoids, and its synthesis is catalyzed by SQS. For decades, most researchers mainly focused on SQSs from eukaryotes such as human, yeast, rats, and plants, where they were studied by recombinant expression, crystallization, and site-specific mutations to explore their catalytic sites. Limited efforts have been given to study prokaryotic SQSs, and only those from *M. capsulatus*, *Thermosynechococcus elongates*, and

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Bradyrhizobium japonicum have been cloned and expressed.[23,38,39] SQSs from multiple other microbial species have yet to be comprehensively and systematically investigated. Therefore, four SQS candidates from both prokaryotic and eukaryotic organisms were selected to compare and explore their capacity to synthesize squalene in *B. subtilis*.

In general, a lack of sequence homology among tested SQSs was observed after sequence alignment analysis (Figure 2). Canonical SQSs contain two conserved aspartate-rich motifs (DxxxD) associated with catalytic active sites,[23] and these two motifs could be observed in Ba (Figure S2). However, no squalene was detected in the extract from *B. subtilis* containing pHCMC04G-BaSQS or pDR111-BaSQS, even with the MEP pathway genes overexpressed. This result attracted our attention to "SQS like" protein. Recently, it was discovered that FPP can be converted to squalene by three steps/three enzymes from the hopanoid biosynthesis pathway in the bacteria *Zymomonas mobilis* and *Rhodopseudomonas palustris*.[40] In this pathway, HpnD combines two molecules of FPP to form PSPP; then, HpnC converts PSPP to hydroxysqualene (HSQ), and HpnE subsequently reduces HSQ to squalene (Figure S3). BaSQS is also annotated as SQS HpnC according to the KEGG database and Uniprot, and sequence alignment of BaSQS with HpnC from *Z. mobilis* and *R. palustris* showed higher identities than when aligned with typical SQSs from *H. sapiens* and *M. capsulatus* (Table S3). Therefore, further exploring hydroxySQS provides us new insights into understanding functions and characterizations of BaSQS. BmSQS is annotated as squalene/phytoene synthase, and it is the first SQS originating from a *Bacillus* species that has been validated to synthesize squalene. Interestingly, among the tested candidates expressed in *B. subtilis*, the highest squalene production was achieved by BmSQS. Considering that the first amino acid of the second aspartate-rich motif was not the conserved aspartate (Figure S2), mutation of this residue to aspartic acid provides a promising strategy to further improve the catalytic efficiency of BmSQS.

SQSs from eukaryotes (PgSQS and ScSQS) contain a TMR domain at C-terminus, which will target the protein to organelle membranes.[22,27] Thus, TMR regions of PgSQS and ScSQS were removed to permit their functional folding in the cytoplasm of bacteria. As expected, squalene could be readily detected in *B. subtilis* metabolites upon expression of truncated PgSQS or ScSQS. However, the squalene accumulations were not as high as in BmSQS strains. Identifying more nonessential domains of eukaryotic SQSs and truncating them proved to be vital for accumulating more squalene by improving enzyme expression, solubility, and activity.[20,41] This method has been demonstrated to be successful on human SQS, where 30 N-terminal amino acids and 47 C-terminal amino acids have been deleted in *E. coli* leading to higher productions.[20,42] In future, truncating both the N-terminal and C-terminal unnecessary residues could also be explored and tested on PgSQS and ScSQS in *B. subtilis*.

Compared to growth at 30 °C, the truncated human SQS obtained higher squalene production in *E. coli* when cultured at 37 °C.[40] In contrast, with the three tested SQSs expressed in *B. subtilis*, the highest squalene accumulations were observed at 25 °C or at 30 °C instead of culturing at 37 °C, and a similar observation was made on taxadiene synthase when expressed in *B. subtilis*.[8] *In vitro* experiments further demonstrated that both BmSQS and ScSQS showed highest catalytic activity at 30 °C instead of 37 °C (Figure S4). Taken together, properly decreasing the cultivation temperature can serve as a candidate strategy to improve terpene synthases performance when expressed in *B. subtilis*.

Different levels of squalene production were observed, when three different types of plasmids were employed to express SQSs. It was reported that high level terpenoid production could be guaranteed with ample strength of terpene synthesis pathway and minimized plasmid-borne metabolic burden at the same time.[41,42] In our study, the high copy number plasmid pHY300PLK might burden the growth of host cells, and its rolling circle replication made it unstable during long time cultivation, thus leading to the lowest level of squalene. The genome integrative plasmid pDR111 performed best among the three tested plasmids. The stability of genome-integrated expression cassette and the strong IPTG inducible promoter $P_{hyperspank}$ in pDR111 gave SQSs the advantages to reach higher squalene titers in *B. subtilis*. In addition, *in vitro* assay showed that higher SQS activities were also measured in crude extracts from strains with higher squalene titers (Figure S5). Previous reports showed that overexpression of the MEP pathway genes could dramatically increase terpenoid production in *B. subtilis*, including isoprene, carotenoids, amorphadiene, and taxadiene.[5,8,43,44] Similar consistent trends were observed with SQSs, regardless of fermentation temperature (37, 30, and 25 °C). The squalene production increased 3.4-, 5.7-, and 3.8-fold, when four MEP pathway genes (*dxs*, *ispD*, *ispF*, and *ispH*) were coexpressed with BmSQS, PgSQS, and ScSQS and fermented at 25 °C, respectively. Also, these fold changes further increased to 5.3-, 13.2-, and 8.3-fold when eight MEP pathway-related genes were overexpressed. These results indicate that enough supply of precursor is indispensable for high production of squalene. However, in *B. subtilis*, linearized C_{30} terpenoids (carotenoids) production could reach around 10 mg/g after four MEP pathway genes were overexpressed, and 20 mg/L amorphadiene could be achieved when additional *dxs* and *idi* were overexpressed. Therefore, it is deduced that the rate-limiting factor did not come from the precursor supply; instead it might exist within other metabolic factors or the step catalyzed by SQS, for instance, the insufficient supply or regeneration of NADPH in *B. subtilis*.[45] This hypothesis was confirmed by the *in vitro* assay result (data not shown) demonstrating that squalene cannot be detected without additional NADPH added to the reaction samples, indicating that the NADPH concentration in the cell extract is insufficient to run the conversion.

Further experiments provided insights into effects of MEP pathway-related genes on squalene production. First, IspA made significant contribution to precursor supply for squalene synthesis (Figures 7 and S6). IspA catalyzes isoprenoid chain elongation reactions, that is, the formation of GPP and FPP. Previous results indicated that additional overexpression of *ispA* could increase C_{30} -terpenoid production, and it was demonstrated that this strategy also applies to squalene production improvement.[6] Second, more IspG overexpression led to negative effects on squalene production (Figure 7). IspG converts MEcPP to HMBPP, and subsequently, HMBPP will form the basic isoprene precursor IPP and DMAPP catalyzed by IspH. The tremendous HMBPP accumulation could decrease terpenoid production in bacteria.[46] Li et al. reported that increased *ispG* gene expression led to decreased *β*-carotene production in *E. coli* because of toxicity of HMBPP, and this negative effect could be further eliminated by an optimal expression level of downstream gene *ispH* to consume

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HMBPP.[46] Next, *ispC* overexpression level should be screened and optimized to guarantee improved terpenoid production. IspC uses DXP as the substrate to form MEP.[6] This study showed no sharp increase on squalene production when additional *ispC* was overexpressed. In contrast, previous results have been presented on effects of this enzyme. Xue et al. demonstrated IspC to be a rate-limiting factor in the MEP pathway as a 5.5-fold increase of carotenoids was obtained when *ispC* was overexpressed in *B. subtilis 168*.[5] However, the production level of isoprene remained unchanged with *ispC* overexpression in *B. subtilis DSM 10*.[43] It is theorized that different conclusions might be caused by varied expression levels of *ispC* in host strains, as effects of *ispC* overexpression could either increase or decrease lycopene production according to its overexpression levels in *E. coli*.[41] Overall, to further increase squalene production in *B. subtilis*, modulation of MEP pathway enzymes and fine-tuning of their expression levels, improving NADPH supply and regeneration, or protein engineering of SQS should be promising strategies in the future.[47]

A significant increase of squalene production (approximately 29-fold) has been achieved in this study. However, this is still far behind the level produced by selected *S. cerevisiae* strains, which yield around 2 g/L of squalene using 144 h fed-batch fermentation and optimized extraction.[33] Considering this as the first time that squalene being synthesized in *B. subtilis*, there are still many strategies that could be explored to further increase squalene production, such as metabolic engineering to release biosynthesis bottlenecks, squalene extraction methods, and optimization of fermentation conditions. In conclusion, this is the first time that squalene was synthesized in *B. subtilis*, and different SQSs derived from plant and microbial sources were expressed and analyzed. Among the tested SQSs, the one from *B. megaterium* produced the highest amount of squalene in *B. subtilis*. Also, when MEP pathway genes were overexpressed, the highest squalene production reached 7.5 mg/L after 48 h of fermentation. IspA and IspG were shown to be critical factors that positively and negatively affect squalene production, respectively. This information provides important suggestions for further fine-tuning of the MEP pathway to increase production of squalene and its triterpenoid derivatives.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c00375.

Plasmids used in this study, strains used in this study, oligonucleotides used in this study, sequence alignment result of SQS/HpnC from different species, analysis of the secondary structures of SQSs, sequence alignment of the SQS candidates from different species, biosynthesis pathway of squalene, effect of incubation temperature on the activity of crude SQS extracts, *in vitro* relative activity of crude SQS extracts, and effect of different combinations of MEP pathway genes in pBS0E plasmid on squalene production in *B. subtilis* cultured 48 h at 25 °C (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SQS, squalene synthase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-pyrophosphate; HSQ, hydroxysqualene

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