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SHORT COMMUNICATION

Metabolic engineering of *Rhodopseudomonas palustris* for squalene production

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Abstract Squalene is a strong antioxidant used extensively in the food, cosmetic and medicine industries. *Rhodopseudomonas palustris* TIE-1 was used as the host because of its ability to grow photosynthetically using solar energy and carbon dioxide from atmosphere. The deletion of the *shc* gene resulted in a squalene production of 3.8 mg/g DCW, which was 27-times higher than that in the wild type strain. For constructing a substrate channel to elevate the conversion efficiency, we tried to fuse *crt*E gene with *hpn*D gene. By fusing the two genes, squalene content was increased to 12.6 mg/g DCW, which was 27.4 % higher than that resulted from the co-expression method. At last, the titer of squalene reached 15.8 mg/g DCW by co-expressing the *dxs* gene, corresponding to 112-fold increase relative to that for wild-type strain. This study provided novel strategies for improving squalene yield and demonstrated the potential of producing squalene by *Rhodopseudomonas palustris*.

Keywords Squalene · *Rhodopseudomonas palustris* · Squalene-hopene cyclase · Fusion expression

Introduction

Squalene is an basic intermediate in the biosynthesis of sterols, hopanoids and triterpenes [1]. This terpenoid hydrocarbon can effectively inhibit chemically induced colon, lung and skin tumorigenesis in rodents [2–4]. Squalene intake becomes effective through the everyday diet or after intravenous injection, which appears to be critical in reducing incidence of coronary heart disease and cancers [5, 6]. Microorganisms have been studied to explore the potential for squalene production because of their advantages of renew ability and low cost. For instance, *Saccharomyces cerevisiae* (1.6, 10 and 18.5 mg/g DCW), *Aurantiochytrium* sp (0.72 mg/g DCW), *Aurantiochytrium mangrovei* (0.53–0.57 mg/g DCW), *Schizochytrium mangrovei* (1.17 mg/g DCW), *Pseudozyma* sp (70 mg/g DCW), *Thraustochytrid* (1.5 mg/g DCW) and cyanobacterium *Synechocystis* (0.67 mg/OD_{750}/l) have been well-investigated for producing squalene [7–16]. However, the levels of squalene in these microorganisms are still not high enough, compared to those in the *Amaranthus* seed oil (80 mg/g) and olive oil (30 %, w/w) [17, 18]. Therefore, novel microorganisms and strategies should be explored for higher microbial production of squalene.

Rhodopseudomonas palustris (*R. palustris*) is a typical purple non-sulfur bacteria (PNSB) which can obtain energy for growth from natural light anaerobically, whose fermentation processes have been well researched over the years [19–21]. *R. palustris* TIE-1 is a strain whose genome sequence is known, which is suitable for being developed through genetic engineering to be the squalene producer [22]. Previous studies have demonstrated that the disruption or deletion of the genes involved in the conversion of squalene to ergosterol or hopanoids improved squalene production significantly [16, 23]. Given that *R. palustris* TIE-1 contains more than 30 mg/g DCW of hopanoids under photoheterotrophic condition [24], we hypothesized that the blocking of hopanoids pathway through *shc* gene deletion could make an increase in squalene content.

For enhancing the yields of target products, one of the common strategies is elevating the expression levels of rate-limiting enzymes to enhance the pathway flux.

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For instance, the over-expression of DXS directed more precursors into the MEP pathway (2-C-methyl-D-erythritol 4-phosphate pathway) and increased the contents of downstream products [25]. However, sometimes, the branched pathways could compete with the target pathway for the intermediates. For instance, the squalene pathway shares farnesyl diphosphate (FPP) with the pathways of ubiquinone, carotenoids and polyterpenoids in *R. palustris* (Fig. 1). An alternative strategy proposed recently is expressing the fused rate-limiting enzymes to construct a substrate channel and prevent the intermediates from diffusing, thus increasing the utility of the intermediate [26, 27]. As shown in Fig. 1, the biosynthesis of squalene from FPP is catalyzed by three enzymes (HpnD, HpnC and HpnE) [28]. The fusion expression of FPP synthase (CrtE) and HpnD was expected to enhance the conversion efficiency, thus improving the content of squalene.

In this study, multiple strategies were employed for improving the squalene production, including blocking of hopanoids pathway, fused expression of *crtE* and *hpn*D genes and over-expression of *dxs* gene.

Materials and methods

General materials and methods

General molecular manipulations were performed according to standard protocols [29]. PCRs were performed using PrimeSTAR HS DNA polymerase (TaKaRa, Dalian City, China). Squalene standard was purchased from Sigma (Shanghai, China). Electroporator (Eppendorf, Humburg, Germany) was utilized for transforming the constructed plasmids into the *R. palustris* strains (12.5 kv/cm, 200 Ω, 25 μF). High-performance liquid chromatography system (Hitachi, Tokyo, Japan) was used for the analysis of squalene.

Strains, plasmids and culture conditions

R. palustris TIE-1 was adopted for genetic engineering. The *E. coli* strain DH5α was utilized for the propagation of the plasmids and *E. coli* S17-1 was used for gene deletion [30]. The plasmid pMG103 with *lacZ* promoter was used for the over-expression of genes while the plasmids of pRK2013 and pk18mobsacB were employed for gene deletion [31, 32].

The bacteria seed cultivation was performed at 30 °C in YP medium (0.3 % peptone, 0.3 % yeast extract pH 6.5) for 48 h under aerobic condition. The engineered *R. palustris* strains for squalene overproduction were cultured at 30 °C in a designed medium (0.2 % sodium succinate, 1 % glucose, 0.3 % peptone, 0.3 % yeast extract, 0.2 % KH_2PO_4, 0.2 % K_2HPO_4, 0.2 % $MgSO_4 \cdot 7H_2O$, 0.5 ‰ $FeSO_4 \cdot 7H_2O$, 0.5 % mineral solution, pH 6.5) under anaerobic condition. The mineral solution contained (per l): 1 g of $NaMoO_4 \cdot 2H_2O$, 2 g of $CuSO_4 \cdot 5H_2O$, 1 g of $ZnSO_4$, 1 g of H_3BO_3, 1 g of $MnCl_2$. Anaerobic cultivation was achieved via static culture in the presence of nitrogen with white fluorescent light (3200 lx). For chemoheterotrophic cultivation, the *R. palustris* strains were cultured at 30 °C in 250-ml shake flasks supplemented with 50-ml of the designed medium on a rotary shaker at 200 rpm. 350 mg/l kanamycin was added to the culture media to retain the constructed plasmids with corresponding antibiotic selection markers. All the cell cultures were grown for 120 h to be in the stationary phase, before the cells were collected for squalene analysis.

Deletion of chromosomal *shc* gene in *R. palustris* TIE-1

A 0.5-kb of *shc* upstream region amplified by primers Δ*shc*-L1 and Δ*shc*-L2 was inserted into the suicide vector pk18mobsacB between *Eco*RI and *Kpn*I to make pKSD1. Similarly, a 0.5-kb of of *shc* downstream region amplified by primers *shc*-R1 and Δ*shc*-R2 was inserted into pKSD-1between *Kpn*I and *Xba*I, generating the deletion plasmid pKSD2. Subsequently, *E. coli* S17-1 harboring pKSD2, *E. coli* HB101 harboring pRK2013 and *R. palustris* TIE-1 were co-cultured on the YP agar for 3 days, in order to transferred pKSD2 into *R. palustris* TIE-1 and integrated it onto the chromosome through homologous recombination [30]. Selection of single recombinants on plates containing 350 mg/l kanamycin, followed by selection

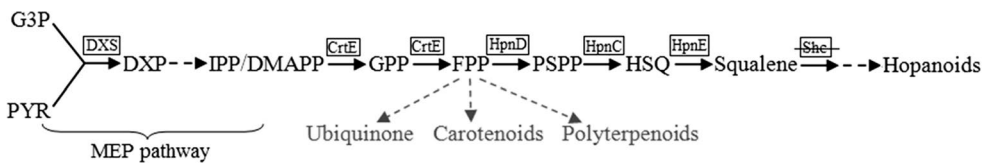

Fig. 1 Squalene synthesis and branched pathways. Relevant abbreviations: *G3P* D-glyceraldehyde-3-phosphate, *PYR* pyruvate, *DXS* 1-deoxy-xylulose-5-phosphate synthase, *DXP* 1-deoxy-xylulose-5-phosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *CrtE* geranylgeranyl pyrophosphate synthase, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate, *PSPP* presqualene diphosphate, *HSQ* (R)-12-hydroxysqualene, *Shc* squalene hopene cyclase

Table 1 Primers and plasmids used in this study

Primer	Sequence (5′-3′)
Δ*shc*-L1	G**GAATTC**TGTGAGGGCGGCGCGGACGTGA
Δ*shc*-L2	GG**GGTACC**CGGGCATTCGAAGGCGTCGACG
Δ*shc*-R1	GG**GGTACC**GCCCGATGAGGCCACGCTCGAT
Δ*shc*-R2	GC**TCTAGA**TTCGACATCACACGATCGCGTA
check-1	TCGAACAGATGGTCGCGCAGTC
check-2	TGCCGTTGTCGATGACAAGCC
scrtE-L	G**GAATTC**GTGAATGGACGTGACCAATCGGATC
scrtE-R	GC**TCTAGA**TCAGGCCGCAGTCGCGGCCAG
shpnD-L	G**GAATTC**GTGAATGACCGTTCACGCCACGCCA
shpnD-R	GC**TCTAGA**TCACACGATCGCGTATTGCAG
chpnD-L	GC**TCTAGA***AGGAA*ACAGCTATGACCGTTCACGCCACGCCA
chpnD-R	CCC**AAGCTT**TCACACGATCGCGTATTGCAG
fcrtE-R	TCAGGCCGCAGTCGCGGCCAG
fhpnD-L	CTGGCCGCGACTGCGGCCTGAGGCGGAGGCGGGAGTGGAGGTGGAGG CAGCGGTGGGGGCGGAAGTATGACCGTTCACGCCACGCCA
dxs-L	GC**TCTAGA***AGGAA*GTGACCGAATTTAGTAAAACAC
dxs-R	CCC**AAGCTT**TCAGGCCAGCTTCGAGGTCT

Bold: restrict enzyme sites, Italic: ribosome binding sites, Underlined: $(GGGGS)_3$ linker

of double recombinants on sucrose (10 %) plates, were conducted as previously described [32]. The *shc* deleted strain was verified by PCR with primers check-1 and check-2, resulting in a 0.8 kb PCR product. All primer sequences used for the construction of the mutant are listed in Table 1.

Construction of plasmids

For the expression of *crt*E (Gene ID: 6409364), the gene was inserted into the vector pMG103 between *Eco*RI and *Xba*I to generate pMG-SE, using the primers scrtE-L and scrtE-R for amplification. Similarly, *hpn*D (Gene ID: 6411947) amplified with the primers shpnD-L and shpnD-R was inserted into pMG103, generating pMG-SD. For constructing the co-expression plasmid, *hpn*D gene was amplified using the primers chpnD-L containing a ribosome bind site (RBS) and chpnD-R, followed by ligation into pMG-SE between *Xba*I and *Hind*III to generate pMG-CED. For constructing the fused expression plasmid, firstly, *crt*E was amplified using the primers scrtE-L and fcrtE-R while *hpn*D was amplified with shpnD-R and fhpnD-L containing the peptide linker $(GGGGS)_3$ encoding sequence. Secondly, the two genes were fused by fusion PCR using the primers scrtE-L and shpnD-R, followed by ligation into pMG103 between *Eco*RI and *Xba*I to generate pMG-FED. In order to co-express *dxs* (Gene ID: 6408677), the primers dxs-R and dxs-L containing a RBS were utilized for amplification, before inserted the gene into pMG-FED between *Xba*I and *Hind*III for generating pMG-FEDS. The plasmids constructed here were sequenced to ensure their accuracy.

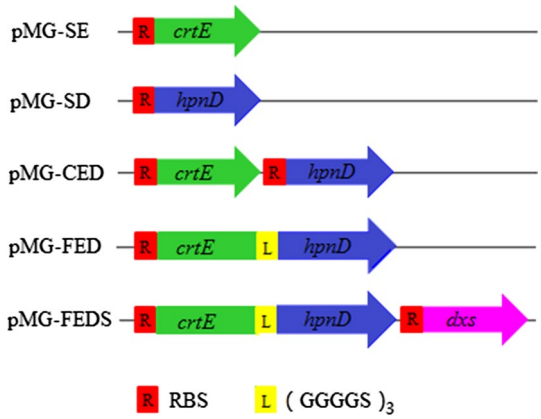

Fig. 2 Structures of the recombinant plasmids constructed in this study. *RBS* Ribosome binding site, $(GGGGS)_3$ peptide linker of GGGGSGGGGSGGGGS, *G* glycine, *S* Serine

The primer sequences are listed in Table 1 and the structures of the plasmids are shown in Fig. 2.

Determination of dry cell weight and squalene

Cell growth was measured using a spectrophotometer at 600 nm and converted to dry cell weight (DCW) upon a prepared standard curve of DCW versus OD600 ≤Vol. For squalene analysis, cells cultured for 120 h were harvested by centrifugation and freeze-dried. The extractions of total lipids in cells were carried out according to the procedure described previously [33]. Then, squalene was analyzed by HPLC based on the procedure reported previously [34].

Results and discussion

Effects of *shc* deletion on cell growth and squalene content

In previous study, it was demonstrated that the mutant *R. palustris* deficient in *shc* gene was unable to produce any hopanoids, with an increase in squalene content [24]. However, the level of squalene was ill-defined. In this study, *shc* was deleted to study the effects of blocking the hopanoids pathway on squalene content. After confirming the deletion of *shc* by PCR and sequencing, the effects of *shc* deletion on cell growth and squalene content were studied. Considering that there was a significant difference in doubling time between the WT and Δ*shc* strains [24], the cell cultures were grown for 120 h to make sure both of the two strains were in stationary phase. As shown in Fig. 3a, there were no differences in biomass between the wild-type strain and *R. palustris* (*Ωshc*) cultured under chemoheterotrophic and photoheterotrophic conditions. Moreover, based on the deletion of *shc*, 22-fold and 28-fold increases in squalene content were realized with the chemoheterotrophic and photoheterotrophic culture conditions respectively (Fig. 3b).

Results of Welander et al. and our study suggested that the accumulation of squalene was improved by reducing the consumption of squalene for hopanoids synthesis. For example, it has been reported that an over 70-fold increase in squalene content was realized by the interruption of *shc* gene in *Synechocystis sp*. PCC 6803, which was higher than that in this study [16]. A possible explanation might be that the *Synechocystis sp*. PCC 6803 contains more hopanoids or less original squalene than those in *R. palustris* TIE-1, leading to a more significant increase by contrasting the levels of squalene before and after blocking the hopanoids pathway. However, the biomass (less than 0.5 g/l) and squalene content (about 3.15 mg/g DCW) of the mutant *Synechocystis sp*. PCC 6803 were less than those for *R. palustris* (*Ωshc*) obtained in this study. Anyhow, blocking the biosynthesis pathway of hopanoids was an efficient strategy for improving the squalene content.

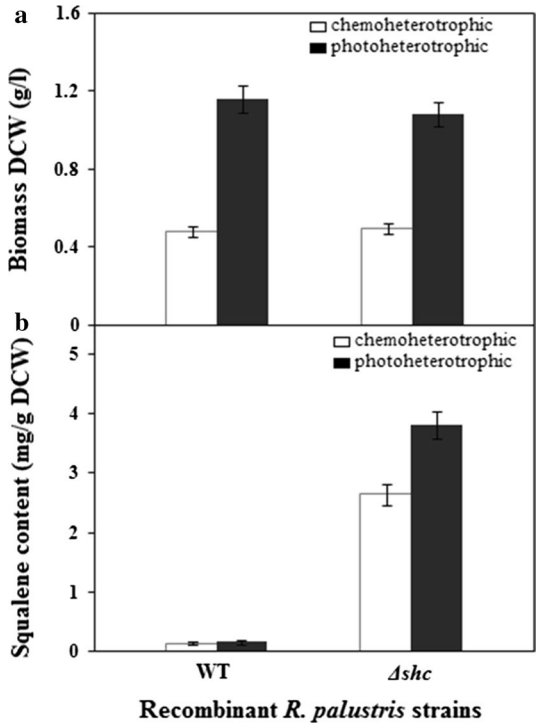

Fig. 3 Effects of *shc* deletion on the biomass and squalene content under different culture conditions. *WT* wild-type strain, Δ*shc* *shc* deleted strain, *DCW* dry cell weight. The results represent the mean value ± SD of duplicate samples in three independent experiments

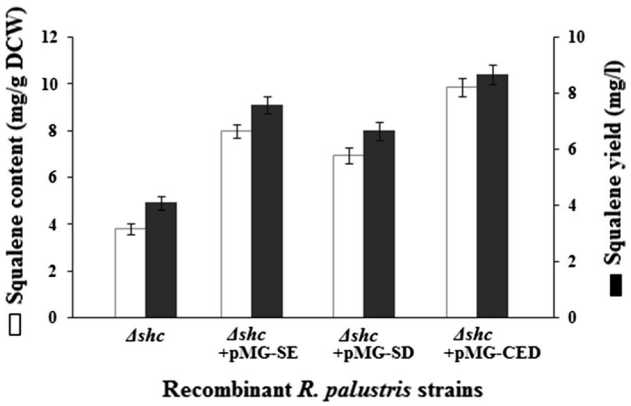

Fig. 4 Squalene production upon expression of genes. Δ*shc* shc deleted strain, Δ*shc*+*pMG-SE* *R. palustris* (Δ*shc*)/pMG-SE, Δ*shc*+*pMG-SD* *R. palustris* (Δ*shc*)/pMG-SD, Δ*shc*+*pMG-CED* *R. palustris* (Δ*shc*)/pMG-CED, *DCW* dry cell weight. The results represent the mean value ± SD of duplicate samples in three independent experiments

Squalene production improvement by the co-expression of *crt*E and *hpn*D

Farnesyl diphosphate (FPP) is the precursor of squalene biosynthetic pathway [35]. For pulling more upstream intermediates towards the synthesis of FPP, *crt*E gene was over-expressed to elevate the level of farnesyl diphosphate synthase. The result showed that a 2.1-fold increase in squalene content was realized (Fig. 4). This indicated that the accumulation of squalene could benefit from the enhancement of FPP pool. Moreover, it was found that the over-expression of *hpn*D enhanced the titer of squalene by 83 % (Fig. 4), indicating that more FPP had been pulled into the squalene pathway.

To study the effects of the union between the over-expressions of the two genes on squalene production, *crt*E and *hpn*D were co-expressed. The two genes each had their own ribosome binding site in the upstream region (Fig. 2). Upon the co-expression, the titer of squalene was increased to 9.9 mg/g DCW, which was 1.6 times higher than that in the *shc* deleted strain. Results demonstrated that the co-expression of the two genes directed more FPP into the squalene pathway by forming a push–pull model, suggesting a synergistic effect on squalene content.

Elevated conversion efficiency by fusion expression of proteins improved squalene production

In *R. palustris*, FPP is the common intermediate at the branch point of several pathways (Fig. 1). Thus, the squalene pathway shares FPP pool with other pathways for their biosynthesis. Although the expression of *hpn*D increased the competitiveness of squalene pathway, FPP could still be used by other pathways. With the purpose of constructing a substrate channel to eliminate the diffusion of intermediates and elevate the conversion efficiency, we fused CrtE and HpnD by a $(GGGGS)_3$ linker, which was reported to help the functional folding of the fused protein [27, 36]. The construct was transformed into *R. palustris* (*Ωshc*), resulting in the recombinant strain *R. palustris* (*Ωshc*)/pMG-FED.

Upon expressing the fused protein, the production of squalene was significantly improved in *R. palustris* (*Ωshc*)/pMG-FED. The content of squalene reached 12.6 mg/g DCW, corresponding to 3.3-fold and 1.27-fold increases relative to those for *R. palustris* (Δ*shc*) and *R. palustris* (Δ*shc*)/pMG-CED (Fig. 5). The results above suggested that the fusion expression resulted in higher efficiency of the conversion from IPP/DMAPP to PSPP, compared to the co-expression method. A possible explanation might be that the synthesized FPP was utilized preferentially by HpnD adjacent to CrtE, before it was diffused into the cell to be used by the branched pathways. In conclusion, expressing the fused protein was an efficient strategy to improve the squalene production.

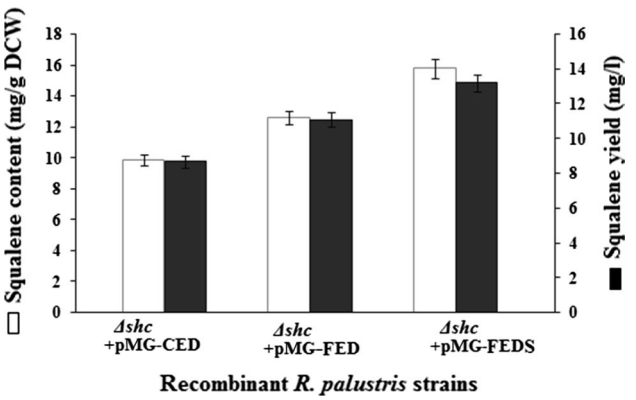

Fig. 5 Effects of fusion expression and *dxs* expression on squalene production. Δ*shc*+*pMG-FED R. palustris* (Δ*shc*)/pMG-FED, Δ*shc*+*pMG-FEDS R. palustris* (Δ*shc*)/pMG-FEDS, *DCW* dry cell weight. The results represent the mean value ± of duplicate samples in three independent experiments

Enhancing the expression level of rate-limiting enzyme of MEP pathway improved squalene content

Despite the successful elevation of the conversion efficiency by expression of the fused protein, the restricted IPP pool could limit the downstream metabolic flux to PSPP. DXP synthase encoded by *dxs* is the first rate-limiting enzyme of MEP pathway, which has been shown to play an important regulatory role in the MEP pathway for the synthesis of IPP/DMAPP [37]. With the purpose of expanding the IPP pool, the recombinant plasmid pMG-FEDS was constructed with an independent RBS for *dxs* expression (Fig. 2). Based on the expression of *dxs*, the content of squalene was increased to 15.8 mg/g DCW in *R. palustris* (*Ωshc*)/pMG-FEDS, which was 25.4 % higher than that in *R. palustris* (*Ωshc*)/pMG-FED. The results suggested that the expression of *dxs* enhanced the metabolic flux of MEP pathway, and thus accelerated the accumulation of IPP. Then the substrate channel constructed by fusion expression could benefit from the expanded IPP pool for squalene production.

Squalene is a useful compound with wide application in medicine chemistry, and as food additives. For large scale generation of squalene, purple non-sulfur bacteria including *R. palustris* would be preferable to be used as the hosts because of their ability to grow photosynthetically using solar energy and carbon dioxide from atmosphere for generating the desired product. *R. palustris* TIE-1 is one of the purple non-sulfur bacteria whose genome sequence is known, which is suitable for developing through genetic engineering.

In this study, a 28-fold increase in squalene content was realized by blocking the hopanoids pathway, without any growth impairment in the recombinant strain. Moreover, a substrate channel was constructed by fusion expression of CrtE and HpnD to elevate the conversion efficiency, resulting in a significant increase in squalene content. Importantly, it was demonstrated that the fusion expression of proteins was better than the co-expression method for squalene production. Finally, the metabolic flux of MEP pathway was enhanced by *dxs* expression, benefiting the synthesis of squalene. Based on the combination of the strategies above, the squalene content in the recombinant strain reached 15.8 mg/g DCW, corresponding to 112-fold increase relative to that for wild-type strain. In future, more

strategies such as branched pathways blocking and fed-batch fermentation will be employed for further enhancement of squalene production in *R. palustris*. The results represented here will enhance our ability to design the system with metabolic engineering techniques for higher squalene production.

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References

1. Kohno Y, Egawa Y, Itoh S, S-i Nagaoka, Takahashi M, Mukai K (1995) Kinetic study of quenching reaction of singlet oxygen and scavenging reaction of free radical by squalene in n-butanol. Biochimica et biophysica acta (BBA)-lipids and lipid. Metabolism 1256:52–56
2. Rao CV, Newmark HL, Reddy BS (1998) Chemopreventive effect of squalene on colon cancer. Carcinogenesis 19:287–290
3. Budiyanto A, Ahmed NU, Wu A, Bito T, Nikaido O, Osawa T, Ueda M, Ichihashi M (2000) Protective effect of topically applied olive oil against photocarcinogenesis following UVB exposure of mice. Carcinogenesis 21:2085–2090
4. Smith TJ (2000) Squalene: potential chemopreventive agent. Expert Opin Investig Drugs 9:1841–1848
5. Newmark HL (1997) Squalene, olive oil, and cancer risk: a review and hypothesis. Cancer Epidemiol Biomark Prev 6:1101–1103
6. Reddy LH, Couvreur P (2009) Squalene: a natural triterpene for use in disease management and therapy. Adv Drug Deliv Rev 61:1412–1426
7. Bhattacharjee P, Shukla V, Singhal R, Kulkarni P (2001) Studies on fermentative production of squalene. World J Microbiol Biotechnol 17:811–816
8. Lewis TE, Nichols PD, McMeekin TA (2001) Sterol and squalene content of a docosahexaenoic-acid-producing *thraustochytrid*: influence of culture age, temperature, and dissolved oxygen. Mar Biotechnol 3:439–447
9. Li Q, Chen G-Q, Fan K-W, Lu F-P, Aki T, Jiang Y (2009) Screening and characterization of squalene-producing *thraustochytrids* from Hong Kong mangroves. J Agric Food Chem 57:4267–4272
10. Mantzouridou F, Naziri E, Tsimidou MZ (2009) Squalene versus ergosterol formation using *Saccharomyces cerevisiae*: combined effect of oxygen supply, inoculum size, and fermentation time on yield and selectivity of the bioprocess. J Agric Food Chem 57:6189–6198
11. Yue C-J, Jiang Y (2009) Impact of methyl jasmonate on squalene biosynthesis in microalga *Schizochytrium mangrovei*. Process Biochem 44:923–927
12. Fan KW, Aki T, Chen F, Jiang Y (2010) Enhanced production of squalene in the *thraustochytrid Aurantiochytrium mangrovei* by medium optimization and treatment with terbinafine. World J Microbiol Biotechnol 26:1303–1309
13. Chen G, Fan K-W, Lu F-P, Li Q, Aki T, Chen F, Jiang Y (2010) Optimization of nitrogen source for enhanced production of squalene from *thraustochytrid Aurantiochytrium sp*. New Biotechnol 27:382–389
14. Mantzouridou F, Tsimidou MZ (2010) Observations on squalene accumulation in *Saccharomyces cerevisiae* due to the manipulation of HMG2 and ERG6. FEMS Yeast Res 10:699–707
15. Naziri E, Mantzouridou F, Tsimidou MZ (2011) Enhanced squalene production by wild-type *Saccharomyces cerevisiae* strains using safe chemical means. J Agric Food Chem 59:9980–9989
16. Englund E, Pattanaik B, Ubhayasekera SJK, Stensjö K, Bergquist J, Lindberg P (2014) Production of squalene in *Synechocystis sp.* PCC 6803. PLoS One 9:e90270
17. Bondioli P, Mariani C, Lanzani A, Fedeli E, Muller A (1993) Squalene recovery from olive oil deodorizer distillates. J Am Oil Chem Soc 70:763–766
18. He H-P, Corke H (2003) Oil and squalene in amaranthus grain and leaf. J Agric Food Chem 51:7913–7920
19. Gest H, Kamen MD (1960) The photosynthetic bacteria, in die CO2-assimilation/the assimilation of carbon dioxide. Springer, Berlin, pp 1582–1626
20. Carlozzi P, Sacchi A (2001) Biomass production and studies on *Rhodopseudomonas palustris* grown in an outdoor, temperature controlled, underwater tubular photobioreactor. J Biotechnol 88:239–249
21. Carlozzi P, Pushparaj B, Degl'Innocenti A, Capperucci A (2006) Growth characteristics of *Rhodopseudomonas palustris* cultured outdoors, in an underwater tubular photobioreactor, and investigation on photosynthetic efficiency. Appl Microbiol Biotechnol 73:789–795
22. Jiao Y, Kappler A, Croal LR, Newman DK (2005) Isolation and characterization of a genetically tractable photoautotrophic Fe(II)-oxidizing bacterium, *Rhodopseudomonas palustris* strain TIE-1. Appl Environ Microbiol 71:4487–4496
23. Lorenz RT, Casey WM, Parks L (1989) Structural discrimination in the sparking function of sterols in the yeast *Saccharomyces cerevisiae*. J Bacteriol 171:6169–6173
24. Welander PV, Hunter RC, Zhang L, Sessions AL, Summons RE, Newman DK (2009) Hopanoids play a role in membrane integrity and pH homeostasis in *Rhodopseudomonas palustris* TIE-1. J Bacteriol 191:6145–6156
25. Xu W, Yang S, Zhao J, Su T, Zhao L, Liu J (2014) Improving coenzyme Q(8) production in *E. coli* employing multiple strategies. J Ind Microbiol Biotechnol 41:1297–1303
26. Conrado RJ, Wu GC, Boock JT, Xu H, Chen SY, Lebar T, Turnšek J, Tomšič N, Avbelj M, Koprivnjak T (2012) DNA-guided assembly of biosynthetic pathways promotes improved catalytic efficiency. Nucleic Acids Res 40:1879–1889
27. Wang C, Yoon S-H, Jang H-J, Chung Y-R, Kim J-Y, Choi E-S, Kim S-W (2011) Metabolic engineering of *E. coli* for α-farnesene production. Metab Eng 13:648–655
28. Pan J-J, Solbiati JO, Ramamoorthy G, Hillerich BS, Seidel RD, Cronan JE, Almo SC, Poulter CD (2015) Biosynthesis of squalene from farnesyl diphosphate in bacteria: three steps catalyzed by three enzymes. ACS Cent Sci 1:77–82
29. Sambrook J, Russell DW (1989) Molecular cloning: a laboratory manual, vol 3. Cold Spring Harbor Laboratory Press, New York
30. Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Nat Biotechnol 1:784–791
31. Inui M, Roh JH, Zahn K, Yukawa H (2000) Sequence analysis of the cryptic plasmid pMG101 from *Rhodopseudomonas palustris* and construction of stable cloning vectors. Appl Environ Microbiol 66:54–63
32. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *E. coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69–73
33. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

34. Lu H-T, Jiang Y, Chen F (2004) Determination of squalene using high-performance liquid chromatography with diode array detection. Chromatographia 59:367–371
35. Ohto C, Muramatsu M, Obata S, Sakuradani E, Shimizu S (2009) Overexpression of the gene encoding HMG-CoA reductase in *Saccharomyces cerevisiae* for production of prenyl alcohols. Appl Microbiol Biotechnol 82:837–845
36. Trinh R, Gurbaxani B, Morrison SL, Seyfzadeh M (2004) Optimization of codon pair use within the $(GGGGS)_3$ linker sequence results in enhanced protein expression. Mol Immunol 40:717–722
37. Kim SW, Keasling J (2001) Metabolic engineering of the non-mevalonate isopentenyl diphosphate synthesis pathway in *E. coli* enhances lycopene production. Biotechnol Bioeng 72:408–415