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Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenosides

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ABSTRACT

Ginsenosides are the primary bioactive components of ginseng, which is a popular medicinal herb and exhibits diverse pharmacological activities. Protopanaxadiol is the aglycon of several dammarane-type ginsenosides, which also has anticancer activity. For microbial production of protopanaxadiol, dammarenediol-II synthase and protopanaxadiol synthase genes of *Panax ginseng*, together with a NADPH-cytochrome P450 reductase gene of *Arabidopsis thaliana*, were introduced into *Saccharomyces cerevisiae*, resulting in production of 0.05 mg/g DCW protopanaxadiol. Increasing squalene and 2,3-oxidosqualene supplies through overexpressing truncated 3-hydroxyl-3-methylglutaryl-CoA reductase, farnesyl diphosphate synthase, squalene synthase and 2,3-oxidosqualene synthase genes, together with increasing protopanaxadiol synthase activity through codon optimization, led to 262-fold increase of protopanaxadiol production. Finally, using two-phase extractive fermentation resulted in production of 8.40 mg/g DCW protopanaxadiol (1189 mg/L), together with 10.94 mg/g DCW dammarenediol-II (1548 mg/L). The yeast strains engineered in this work can serve as the basis for creating an alternative way for production of ginsenosides in place of extraction from plant sources.

1. Introduction

Natural products have many important medicinal properties, such as anticancer, anti-inflammatory, antiparasitic and anti-oxidation (Ajikumar et al., 2008). The majority of new drugs had been generated from natural products and their derived compounds (Li and Vederas, 2009). Two methods had been usually used for production of these valuable chemicals. One was direct extraction from plants. Due to low abundance of these secondary metabolites in plant tissues, extraction yield was usually very low. The second method was through chemical synthesis (Li and Vederas, 2009). However, complexity and chirality of these compounds had hampered development of this producing method (Chang and Keasling, 2006).

Metabolic engineering of microorganisms is an alternative and attractive route for production of these valuable natural products (Ajikumar et al., 2008, 2010; Chang and Keasling, 2006; Farhi et al., 2011; Kumar et al., 2012; Leonard et al., 2010; Olano et al., 2008; Ozaydin et al., 2013; Ro et al., 2006; Thykaer et al., 2010; Yang et al., 2011). This strategy has been widely used to engineer either *Escherichia coli* or *Saccharomyces cerevisiae* for successful production of many natural products and important precursors in their biosynthesis pathways, such as artemisinin (Ro et al., 2006; Westfall et al., 2012), taxol (Ajikumar et al., 2010; Engels et al., 2008), ginkgolides (Leonard et al., 2010), (S)-reticuline (Hawkins and Smolke, 2008; Nakagawa et al., 2011), tanshinones (Dai et al., 2012; Zhou et al., 2012) and so on.

Ginsenosides are a group of active triterpenoids found in Asian ginseng (*Panax ginseng* C.A. Meer) and American ginseng (*Panax quinquefolius* L.), which exhibit diverse pharmacological effects on the central nervous, cardiovascular, endocrine and immune systems (Christensen, 2008; Qi et al., 2011; Radad et al., 2011). Seven dammarene-type tetracyclic triterpenoids (ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1) are the major ginsenoside constituents, while Ro is the only oleanane-type pentacyclic ginsenoside, which is found minimally in *P. ginseng* (Han et al., 2011).

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These dammarane-type ginsenosides are divided into two groups according to the aglycon structure: the protopanaxadiol (Rb1, Rb2, Rc and Rd) and protopanaxatriol groups (Re, Rf, and Rg1) (Han et al., 2011). Protopanaxadiol had also been reported to have anticancer activity (Musende et al., 2012), and is now in clinical trials as an anticancer drug candidate (Saklani and Kutty, 2008). Protopanaxadiol exhibited apoptotic effects on cancer cells through various signaling pathways and was cytotoxic to multi drug-resistant tumors (Du et al., 2011; Musende et al., 2010, 2012; Zhu et al., 2011). It had also been used in adjuvant treatment for lung cancer and other solid tumors (Han et al., 2011).

Triterpenoids are derived from two common building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesized through either mevalonic acid (MVA) pathway (Fig. 1) in all eukaryotic cells and cytoplasm and mitochondria of plants or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in bacteria, other prokaryotes and plastids in plants (Ajikumar et al., 2010; Ro et al., 2006). In yeast, most of the IPP and DMAPP precursors enter the ergosterol synthetic pathway (Kennedy and Bard, 2001; Leber et al., 2001; Ma et al., 2011). In *P. ginseng*, cyclization of 2,3-oxidosqualene to dammarenediol-II by dammarenediol synthase (*PgDDS*) is the first reaction towards biosynthesis of dammarane-type ginsenosides (Han et al., 2012; Tansakul et al., 2006). Dammarenediol-II is further converted to protopanaxadiol through protopanaxadiol synthase (*PgPPDS*), which is a cytochrome P450 enzyme and catalyzes the hydroxylation of dammarenediol-II at the C-12 position (Han et al., 2011). Protopanaxadiol is further converted to ginsenosides compounds through uridin diphosphate glycosyltransferases (UGTs) (Augustin et al., 2011; Chen et al., 2011; Fig. 1).

Currently, ginsenosides are mainly produced through extraction from ginseng roots, which contain at least 4% ginsenosides by dry weight (Shibata, 2001). Wild ginseng roots are scarce, while most ginseng roots on the market are collected from farms cultivating ginseng in fields (Paek et al., 2009). However, cultivating ginseng is time-consuming, which takes 5–7 years from seeding to the final harvest. A 6-year-old root yields only about 90 g (fresh weight) (Choi et al., 2003). It is also a labor-intensive process since ginseng growth is influenced by many conditions

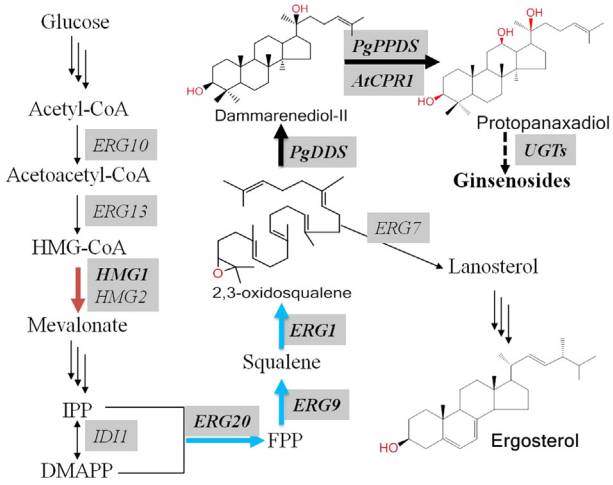

Fig. 1. Biosynthetic pathway for protopanaxadiol production in *S. cerevisiae*. 2,3-oxidosqualene is an essential precursor for protopanaxadiol synthesis. 2,3-oxidosqualene is converted to dammarenediol-II by dammarenediol synthase (*PgDDS*), while dammarenediol-II is further converted to protopanaxadiol through protopanaxadiol synthase (*PgPPDS*). The native yeast ergosterol synthetic pathway competes with protopanaxadiol synthetic pathway for the common precursor 2,3-oxidosqualene. Single arrows represent the one-step conversions, while triple arrows represent multiple steps. Dashed arrow represents unknown step. Abbreviation: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate.

such as soil, climate, pathogens and pests (Paek et al., 2009). Although many attentions had been transferred to ginseng cell cultures for ginsenosides production, this did not lead to a significant improvement due to low growth rate and low ginsenosides content (Thanh et al., 2006; Wu and Zhong, 1999). In this work, *S. cerevisiae* was metabolically engineered for efficient production of protopanaxadiol, which can serve as the basis for microbial production of ginsenosides.

2. Materials and methods

2.1. Strains and medium

S. cerevisiae BY4742, a derivative of S288C (Brachmann et al., 1998), was obtained from EUROSCARF and used as the parent strain for all yeast strains. Engineered yeast strains were grown either in SD medium (Burke et al., 2000; Dai et al., 2012) lacking leucine, uracil and histidine where appropriate, or in YPD medium (Burke et al., 2000). *E. coli* Trans-10 (TransGen Biotech, Beijing, China) was used for transformation and plasmid DNA extraction. Strains were cultivated at 37 °C in LB medium with 100 mg/L ampicillin.

2.2. Plasmids construction

The yeast expression plasmids pRS313 (Sikorski and Hieter, 1989) and pRS425 (Christianson et al., 1992) were kindly provided by Dr. Qinhong Wang at Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences. Plasmid pδ-UB (Lee and Da Silva, 1997) was kindly provided by Dr. Da Silva NA. Primers used during plasmids construction are summarized in Table S1.

P. ginseng cell and *Arabidopsis thaliana* were induced with 0.1 mg/L MeJA for 24 h. Total RNA was isolated by the Trizol method (Dai et al., 2011), and the cDNA was obtained by PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The *PgDDS* and *PgPPDS* genes were PCR amplified from cDNA of *P. ginseng* (using primer sets 2-Pac1-PqDDS/PqDDS-Asc1 and 2-Pac1-CYP716A47/CYP716A47-Asc1). The *AtCPR1* gene was amplified from cDNA of *A. thaliana* (using primer sets ZD-SexA1-AtCPR1/ZD-AtCPR1–Asc1). The amplified DNA fragments were cloned into pEASY-Blunt (TransGen Biotech, Beijing, China), resulting in p-PgDDS, p-PgPPDS and p-AtCPR1, respectively. The protopanaxadiol synthase gene with restriction sites SexAI and AscI were synthesized by GenScript (Nanjing, China) with codon optimization for improved expression in *S. cerevisiae*. The synthesized DNA fragment was cloned into pUC57 (GenScript, Nanjing, China), resulting in p-SynPgPPDS.

The *ERG20*, *ERG9* and *ERG1* genes were amplified from the genomic DNA of *S. cerevisiae* BY4742 (using primer sets SexA–ERG20/Asc1–FPS, SexA–ERG9/ERG9–Asc1 and Pac–ERG1/ERG1–ASC) and cloned into pEASY-Blunt, resulting in p-ERG20, p-ERG9 and p-ERG1, respectively.

Construction of pM3–ERG9, pM11–ERG1, pM14–PgDDS, pM13–PgPPDS, pM3–SynPgPPDS, pM11–AtCPR1, pδ–tHMG1, pEHIS–ERG20, prDNA–LEU, pTrp–HIS, pδ–AtCPR1 and pM3–tHMG1 are described in the Supplementary Data.

2.3. Strain construction

Transformation of *S. cerevisiae* strains was performed by standard lithium acetate method (Burke et al., 2000). For integration of *PgDDS*, *PgPPDS* and *AtCPR1* genes into rDNA sites of strain BY4742, strain ZD-PPD-000 was constructed using the DNA assembler method (Fig. S2) as described previously (Shao and Zhao, 2009). Five DNA fragments were amplified from pM14–PgDDS (using

primer set *1-M-pEASY–PGK1-F/3G-1-M–ADHt–TDH3-R*), pM13–PgPPDS (using primer set *3G-2-M-TPI1t–TEF1–F/M-CYC1t–pEASY-R*), pM11–AtCPR1 (using primer set *3G-3-M-ADHt–TDH3-F/3G-3-M-TPI1t–TEF1-R*) and prDNA–LEU (using primer sets *X1-M-pEASY-r-t-F/X1-r-t-R-rDNA* and *X2-r-t-F-rDNA/X2-M-pEASY-r-t-R*), and transformed into strain BY4742, followed by selection on SD–LEU plate. Ten colonies were randomly picked from the plate, and verified by PCR amplification using primer sets Sac11–pGK1/PqDDS–Asc1, Pac1–TDH3/ZD–AtCPR1–Asc1 and Pac1–TEF1/CYP716A47–Asc1. All right colonies were selected for flask fermentation, and the colony having the highest protopanaxadiol yield was designated ZD-PPD-000 and used for further engineering.

Strain ZD-PPD-010 was constructed by integrating *tHMG1* gene into *δDNA* site of ZD-PPD-000. Plasmid pδ-tHMG1 (Table 1) containing P_{PGK1}–*tHMG1*–T_{ADH1} cassette was digested with *Xho*I and transformed into strain ZD-PPD-000, followed by selection on SD–LEU–URA plates. Ten colonies were randomly picked from the plate, and verified by PCR amplification using primer set Sac11–pGK1/Asc1–HMG1. All right colonies were selected for flask fermentation, and the colony having the highest protopanaxadiol yield was designated ZD-PPD-010 and used for further engineering.

Strain ZD-PPD-013 was constructed by integrating *ERG20* and *ERG9* genes into *Trp1* site of ZD-PPD-010. Four DNA fragments were amplified from pEHIS–ERG20 (using primer set 1-M-pEASY–PGK1-F/1-M-ADHt–TEF1-R), pM3–ERG9 (using primer set 2-M-ADHt–TEF1-F/M-CYC1t–pEASY-R) and pTrp–HIS (using primer sets X1-M-pEASY-r-t-F/X1-r-t-R-Trp1 and X2-r-t-F-Trp1/X2-M-pEASY-r-t-R), and transformed into strain ZD-PPD-010, followed by selection on SD–LEU–URA–HIS plates. PCR verification was performed using primer sets Sac11–pGK1/Asc1–FPS and Pac1–TEF1/ERG9–Asc1 (Table 2).

Strain ZD-PPD-016 was constructed by integrating *ERG20*, *ERG9* and *ERG1* genes into *Trp1* site of ZD-PPD-010. Five DNA fragments were amplified from pEHIS–ERG20 (using primer set *1-M-pEASY–PGK1-F/3G-1-M-ADHt–TDH3-R*), pM3–ERG9 (using primer set *3G-2-M-TPI1t–TEF1-F/M-CYC1t–pEASY-R*), pM11–ERG1 (using primer set *3G-3-M-ADHt–TDH3-F/3G-3-M-TPI1t–TEF1-R*) and pTrp–HIS (using primer sets X1-M-pEASY-r-t-F/X1-r-t-R-Trp1 and X2-r-t-F-Trp1/X2-M-pEASY-r-t-R), and transformed into strain ZD-PPD-010, followed by selection on SD–LEU–URA–HIS plates. PCR verification was performed using primer sets Sac11–pGK1/Asc1–FPS, Pac1–TEF1/ERG9–Asc1 and Pac1–TDH3/ERG1–ASC. Removing URA3 marker from strain ZD-PPD-016 was performed as described previously (Ro et al., 2006), resulting in strain ZD-PPD-016 (URA3$^-$), and used for further engineering.

Strain ZD-PPD-018 was constructed by integrating codon optimized *PgPPDS*, *tHMG1* and *AtCPR1* genes into *δDNA* site of ZD-PPD-016 (URA3$^-$). Four DNA fragments were amplified from

Table 1
Plasmids used in this study.

Name	Description	Source
pRS313	CEN6/ARSH4, *HIS3*	Sikorski and Hieter (1989)
pRS425	2MICRON, *LEU2*	Christianson et al. (1992)
pδ-UB	δDNA site, *URA3*	Lee and Da Silva (1997)
pEASY-Blunt	Cloning vector with multiple cloning sites, *Amp*, *Km*	TransGen Biotech
pEASY-Blunt Simple	Cloning vector for blunt ligation, *Amp*, *Km*	TransGen Biotech
pUC57	Cloning vector with multiple cloning sites, *Amp*	GenScript
p-PgDDS	Cloning *PgDDS* gene into pEASY-Blunt	This study
p-PgPPDS	Cloning *PgPPDS* gene into pEASY-Blunt	This study
p-SynPgPPDS	Cloning *SynPgPPDS* gene into pUC57	This study
p-AtCPR1	Cloning *AtCPR1* gene into pEASY-Blunt	This study
pδ–tHMG1	Cloning P_{PGK1}–*tHMG1*–T_{ADH1} cassette into pδ-UB	This study
pM14–PgDDS	Cloning P_{PGK1}–*PgDDS*–-T_{ADH1} cassette into pEASY-Blunt simple	This study
pM13–PgPPDS	Cloning P_{TEF1}–*PgPPDS*–T_{CYC1} cassette into pEASY-Blunt simple	This study
pM3–SynPgPPDS	Cloning P_{TEF1}–*SynPgPPDS*–T_{CYC1} cassette into pEASY-Blunt simple	This study
pM11–AtCPR1	Cloning P_{TDH3}–*AtCPR1*–T_{TPI1} cassette into pEASY-Blunt simple	This study
pEHIS–ERG20	Cloning P_{PGK1}–*ERG20*–T_{ADH1} cassette and *HIS3* marker into pEASY-Blunt	This study
pM3–ERG9	Cloning P_{TEF1}–*ERG9*–T_{CYC1} cassette into pEASY-Blunt simple	This study
pM11–ERG1	Cloning P_{TDH3}–*ERG1*–T_{TPI1} cassette into pEASY-Blunt simple	This study
pTrp–HIS	Cloning *Trp1* and *HIS3* marker into pEASY-Blunt	This study
prDNA–LEU	Cloning *rDNA* and *LEU2* marker into pEASY-Blunt	This study
pM3–tHMG1	Cloning P_{TEF1}–*tHMG1*–T_{CYC1} cassette into pEASY-Blunt simple	This study
pδ–AtCPR1	Cloning P_{PGK1}–*AtCPR1*–T_{ADH1} cassette into pδ-UB	This study

Table 2
Strains used in this study.

Name	Description	Source
BY4742	*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *MET15*, *ura3Δ0*	Brachmann et al. (1998)
ZD-PPD-000	P_{PGK1}–*PgDDS*–T_{ADH1}, P_{TDH3}–*AtCPR1*–T_{TPI1} and P_{TEF1}–*PgPPDS*–T_{CYC1} cassettes and LEU2 marker gene were integrated into *rDNA* site of BY4742	This study
ZD-PPD-010	P_{PGK1}–*tHMG1*–T_{ADH1} cassette and URA3 marker gene were integrated into *δDNA* site of ZD-PPD-000	This study
ZD-PPD-013	P_{PGK1}–*ERG20*–T_{ADH1}, P_{TEF1}–*ERG9*–T_{CYC1} cassettes and HIS3 marker gene were integrated into *Trp1* site of ZD-PPD-010	This study
ZD-PPD-016	P_{PGK1}–*ERG20*–T_{ADH1}, P_{TDH3}–*ERG1*–T_{TPI1}, P_{TEF1}–*ERG9*–T_{CYC1} cassettes and HIS3 marker gene were integrated into *Trp1* site of ZD-PPD-010	This study
ZD-PPD-016 (URA3$^-$)	Removing *URA3* marker from strain ZD-PPD-016	This study
ZD-PPD-016H	P_{PGK1}–*tHMG1*–T_{ADH1} cassette and URA3 marker gene were integrated into *δDNA* site of ZD-PPD-016 (URA3$^-$)	This study
ZD-PPD-016HA	P_{PGK1}–*AtCPR1*–T_{ADH1}, P_{TEF1}–*tHMG1*–T_{CYC1} cassettes and URA3 marker gene were integrated into *δDNA* site of ZD-PPD-016 (URA3$^-$)	This study
ZD-PPD-018	P_{PGK1}–*tHMG1*–T_{ADH1}, P_{TDH3}–*AtCPR1*–T_{TPI1}, P_{TEF1}–*SynPgPPDS*–T_{CYC1} cassettes and URA3 marker gene were integrated into *δDNA* site of ZD-PPD-016 (URA3$^-$)	This study

pδ-tHMG1 (using primer sets *1-M-HisG–PGK1-F/3G-1-M-ADHt–TDH3-R* and *3-M-CYC1t–δ2-F/3-δ2-R*), pM3 -SynPgPPDS (using primer set *3G-2-M-TPI1t–TEF1-F/2-M-CYC1t-δ2-R*), pM11–AtCPR1 (using primer set *3G-3-M-ADHt–TDH3-F/3G-3-M-TPI1t–TEF1-R*). Another DNA fragment was obtained by *BamH*I and *Xho*I digestion of plasmid pδ-UB. These five DNA fragments were transformed into strain ZD-PPD-016(URA3$^-$), followed by selection on SD–LEU–HIS–URA plates. Ten colonies were randomly picked from the plate, and verified by PCR amplification using primer set Pac1–TEF1/SPPD-R. All right colonies were selected for flask fermentation, and the colony having the highest protopanaxadiol yield was designated ZD-PPD-018 and used for further fed-batch fermentation.

Strain ZD-PPD-016H was constructed by integrating *tHMG1* gene into *δDNA* site of ZD-PPD-016 (URA3$^-$). Plasmid pδ-tHMG1 containing P_{PGK1}–*tHMG1*–T_{ADH1} cassette was digested with *Xho*I and transformed into strain ZD-PPD-016 (URA3$^-$), followed by selection on SD–LEU–HIS–URA plates. Ten colonies were randomly picked from the plate, and verified by PCR amplification using primer set URA3-F/URA3-R. All right colonies were selected for flask fermentation, and the colony having the highest protopanaxadiol yield was designated ZD-PPD-016H.

Strain ZD-PPD-016HA was constructed by integrating *tHMG1* and *AtCPR1* genes into *δDNA* site of ZD-PPD-016 (URA3$^-$). Three DNA fragments were amplified from pδ-AtCPR1 (using primer sets *1-M-HisG–PGK1-F/1-M-ADHt–TEF1-R* and *3-M-CYC1t–δ2-F/3–δ2-R*) and pM3–tHMG1 (using primer set *2-M-ADHt–TEF1-F/2-M-CYC1t-δ2-R*). Another DNA fragment was obtained by *BamH*I and *Xho*I digestion of plasmid pδ-UB. These four DNA fragments were transformed into strain ZD-PPD-016 (URA3$^-$), followed by selection on SD–LEU–HIS–URA plates. Ten colonies were randomly picked from the plate, and verified by PCR amplification using primer sets Sac11–pGK1/ZD–AtCPR1–Asc1 and Pac1–TEF1/Asc1–HMG1. All right colonies were selected for flask fermentation, and the colony having the highest protopanaxadiol yield was designated ZD-PPD-016HA.

2.4. Yeast cultivation

YPD medium was used for cultivating the engineered yeast strains. All strains were firstly inoculated into 15 mL culture tubes containing 2 mL medium, and grown at 30 °C and 250 rpm to OD_{600} about 1.0. Flasks (100 mL) containing 10 mL medium were then inoculated to an OD_{600} 0.05 with these seed cultures. Strains were grown at 30 °C and 250 rpm for 6 days. All the flask fermentation results represented the means ± S.D. of three independent experiments.

2.5. Fed-batch and two-phase extractive fermentation of strain ZD-PPD-018

Strain ZD-PPD-018 was used for production of protopanaxadiol through fed-batch fermentation as described previously (Lenihan et al., 2008; Westfall et al., 2012). Medium used in this work were based on the medium described previously (Lenihan et al., 2008), and 5 g/L lysine was added for both seed preparation and fermentation. Seed culture was prepared by inoculating several colonies into a 250 mL flask containing 50 mL culture medium, and incubating at 30 °C and 250 rpm for 24 h. The seed was then transferred to a 7.5 L Infors-HT fermenter (Infors AG, Switzerland) containing 3 L medium with an initial OD_{600} 0.5. Fermentation was carried out at 30 °C with an agitation speed of 1000 rpm and air flow rate of 5 L/min L. The pH was controlled at 5.5 by automatic addition of 5 M ammonia hydroxide. A fed solution, containing 578 g/L glucose, 9 g/L KH_2PO_4, 5.12 g/L $MgSO_4 \cdot 7H_2O$, 3.5 g/L K_2SO_4, 0.28 g/L Na_2SO_4 and 5 g/L lysine, was fed to the fermentor by controlling ethanol concentration less than 0.5 g/L.

Two-phase extractive fermentation was performed to enhance protopanaxadiol production. Either methyl oleate or dodecane was used as the extractive solvent. Fed-batch fermentation was performed as described above, and 300 mL extractive solvent was added to the fermentor at 48 h.

2.6. Analysis

All optical densities at 600 nm (OD_{600}) were measured using a Shimadzu UV-2550 spectrophotometer. Dry cell weight (DCW) was calculated from the optical density at 600 nm (1 OD_{600} = 0.513 g DCW L^{-1}).

Cells were collected by centrifugation at 10,000*g* for 5 min. Cells were then crushed by BeadBeater (BioSpec, USA), followed by ultrasonic extraction with 1.5 mL acetone for 3 times.

Products secreted to the medium were extracted by ethyl acetate. 2 mL ethyl acetate was added to 8 mL culture medium, and the mixture was agitated by vortex for 20 s. After centrifugation at 12,000*g* for 10 min, the ethyl acetate phase was collected. This extraction process was repeated once.

Acetone or ethyl acetate extracts (1 μL) were analyzed by GC/MS using a Agilent technologies 5975C insert xl MSD with triple-Axis Detector equipped with a HP-5ms (30 m × 0.25 mm × 0.5 μm) GC column. Compound separation was achieved with an injector temperature of 300 °C, and a 30 min temperature gradient program for GC-separation starting at 80 °C for 1 min followed by heating the column to 300 °C at 20 °C min^{-1} and a final constant hold at 300 °C for 18 min. Mass detection was achieved with electric ionization using a SIM scan mode with diagnostic ion monitored: *m/z* 69, *m/z* 109, *m/z* 135, *m/z* 363 and *m/z* 411. A crystallized dammarenediol-II sample was used as the standard for quantification (purchased from BioBioPha, China). Squalene, lanosterol and ergosterol standards (purchased from Sigma Aldrich) were also used for quantification.

For determination of protopanaxadiol, acetone extracts (20 μL) were analyzed by LC/MS using an Agilent 1200 HPLC system coupled to a Bruker-micrOTOF-II with an electrospray ionization (ESI) interface. Data acquisition and processing were done with MicrOTOF control version 3.0/Data Analysis Version 4.0 software. For chromatographic separation, a Waters Symmetry C18® column (250 mm×4.6mm, 5 μm) was used. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), using a gradient program of 50–90% B in 0–8 min, 90% B in 8–15 min, 90–50% B in 15–20 min, and 50% B in 20-25 min. The solvent flow rate was 1.0 mL/min and the column temperature was set at 30 °C. Optimized MS operating conditions were as follows: all spectra were obtained in positive mode over an *m/z* range of 100–1200; dry gas flow, 6.0 L/min; dry temperature, 180 °C; nebulizer pressure, 1 bar; probe voltage +4.5 kV. Protopanaxadiol purchased from Sigma Aldrich was used as standard for analysis.

For quantitative analysis of protopanaxadiol, acetone or ethyl acetate extracts (20 μL) were injected on an Agilent 1200 HPLC with UV detection at 203 nm. For chromatographic separation, a Waters Symmetry® C18 column (250 mm×4.6 mm, 5 μm) was used. The mobile phase consisted of 1% water, 9% methanol and 90% acetonitrile. The solvent flow rate was 1.0 mL/min and the column was held at 30 °C during the separation. Protopanaxadiol was found to elute at 10.14 min. Protopanaxadiol purchased from Sigma Aldrich was used for quantification.

2.7. Analysis of the components of black solids accumulated in the fed-batch fermentation

The black solids (10 mg) were crushed by BeadBeater (BioSpec, USA), followed by ultrasonic extraction with 1.5 mL acetone for 3 times. 20 μL acetone extracts were used for quantitative analysis

of protopanaxadiol by HPLC as described above. 1 μL acetone extracts were used for quantitative analysis of dammarenediol-II, squalene, lanosterol and ergosterol by GC–MS as described above.

The black solids (10 mg) was crushed by BeadBeater (BioSpec, USA), followed by ultrasonic extraction with 1.5 mL water for 3 times. 20 μL water extracts were used for quantitative analysis of glucose by HPLC as described previously (Underwood et al., 2002). Phenol-sulfuric acid method was used to measure polysaccharide as described previously (Cuesta et al., 2003).

For protein analysis, the black solids (10 mg) were mixed with 50 μL 2% SDS-loading buffer, boiled for 10 min, followed by analysis using 10% SDS-PAGE gel.

For analysis of fatty acids and other compounds, the black solids (10 mg) was mixed with 2 mL 1% (v/v) H_2SO_4–MeOH solution, followed by incubation at 70 °C for 60 min. The mixed solution was then extracted with 2 mL hexane twice. 1 μL hexane extracts were analyzed by GC/MS using a Agilent technologies 5975C insert xl MSD with triple-Axis Detector equipped with a HP-5ms (30 m × 0.25 mm × 0.5 μm) GC column. Compound separation was achieved with an injector temperature of 300 °C, and a temperature gradient program for GC-separation starting at 80 °C for 1 min followed by heating the column to 280 °C at 14 °C min^{-1} and a final constant hold at 280 °C for 40 min. Mass detection was achieved with electric ionization using a TIC mode. Methyl oleate standard (purchased from Sigma Aldrich) was used for quantification analysis of fatty acids. Other compounds were identified by the data library of NIST08.

3. Results and discussion

3.1. Construction of protopanaxadiol synthetic pathway in S. cerevisiae

In order to construct protopanaxadiol synthetic pathway in *S. cerevisiae*, the *PgDDS* and *PgPPDS* genes of *P. ginseng*, together

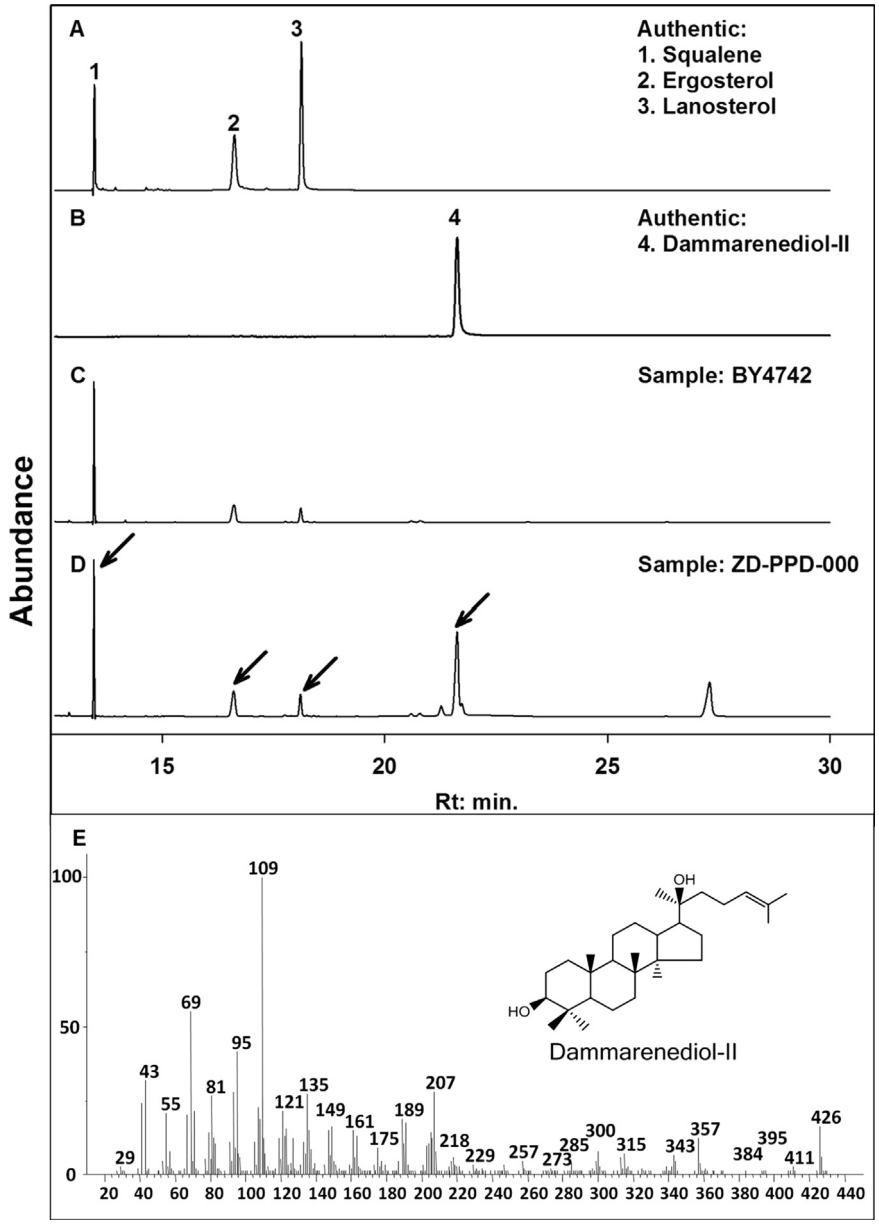

Fig. 2. Identification of fermentation products of strain ZD-PPD-000. This strain was cultivated in YPD medium with 2% glucose for 6 days. (A) GC–MS analysis of squalene (1), ergosterol (2) and lanosterol (3) standards; (B) GC–MS analysis of dammarenediol-II (4) standard; (C) GC–MS analysis of cell extraction of the parent strain BY4742; (D) GC–MS analysis of cell extraction of strain ZD-PPD-000; (E) mass spectra of dammarenediol-II.

with a cytochrome P450 reductase gene of *A. thaliana* (*AtCPR1*) (Urban et al., 1997) were firstly integrated into chromosome of *S. cerevisiae*. The *PgPPDS*, *AtCPR1* and *PgDDS* genes were controlled by *TEF1*, *TDH3* and *PGK1* promoters, respectively. These 3 strong promoters are constitutive and compatible with yeast fermentation using glucose as the carbon source (Engels et al., 2008; Sun et al., 2012). After integrating these 3 genes into chromosome of *S. cerevisiae* BY4742 at *rDNA* site, 10 colonies were randomly picked from the plate and verified by PCR amplification. All right colonies were cultivated in YPD medium with 2% glucose for 6 days. GC/MS and LC/MS analysis of cell extraction confirmed production of both dammarenediol-II (Fig. 2) and protopanaxadiol (Fig. 3).

Protopanaxadiol yields varied from 0.02 to 0.05 mg/g DCW, suggesting that different gene copies were integrated in these 10 colonies (Fig. S3A). The second colony, which had the highest protopanaxadiol yield, were named ZD-PPD-000 and used for further engineering. It was suggested that multiple copies of *PgPPDS*, *AtCPR1* and *PgDDS* genes were integrated in strain ZD-PPD-000. This strain produced 0.05 mg/g DCW dammarenediol-II and 0.05 mg/g DCW protopanaxadiol (Fig. 4). It also produced 0.27 mg/g DCW squalene, 0.42 mg/g DCW lanosterol and 5.09 mg/g DCW ergosterol (Fig. 4).

3.2. Overexpressing tHMG1 gene to improve squalene supply for increasing protopanaxadiol production

The low quantity of protopanaxadiol produced by strain ZD-PPD-000 was assumed to be caused by deficient supply of triterpenoid precursors. Squalene is the universal triterpenoid precursor (Augustin et al., 2011). In order to increase squalene supply in *S. cerevisiae*, several genes responsible for squalene synthesis were overexpressed.

HMG-CoA reductase is a key rate-limiting enzyme in the MVA pathway, and overexpression of a truncated HMG-CoA reductase gene (*tHMG1*) was commonly used to remove the feedback regulation, thus increasing carbon flux through the MVA pathway (Donald et al., 1997). This strategy had been used to successfully increase production of many terpenoids, such as artemisinic acid (Ro et al., 2006), taxadiene (Engels et al., 2008), geranylgeraniol (Tokuhiro et al., 2009), cubebol (Asadollahi et al., 2010), patchoulol (Albertsen et al., 2011), beta-carotene (Verwaal et al., 2007) and so on.

After integrating *tHMG1* gene (controlled by *PGK1* promoter) into chromosome of ZD-PPD-000 at *δDNA* site, 10 colonies were randomly picked from the plate, and verified by PCR amplification. All right colonies were cultivated in YPD medium with 2% glucose

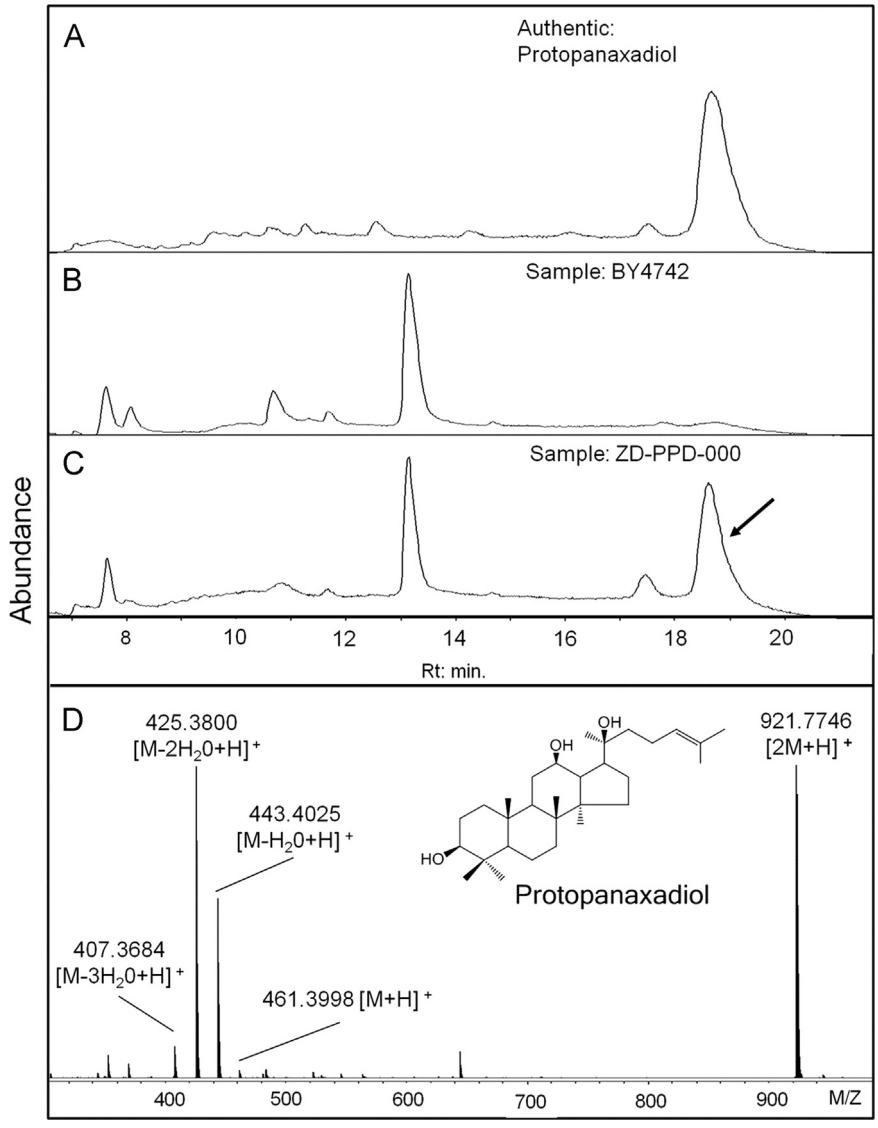

Fig. 3. Identification of fermentation products of strain ZD-PPD-000. This strain was cultivated in YPD medium with 2% glucose for 6 days. (A) LC–MS analysis of protopanaxadiol standard; (B) LC–MS analysis of the parent strain BY4742; (C) LC–MS analysis of strain ZD-PPD-000; (D) mass spectra of protopanaxadiol.

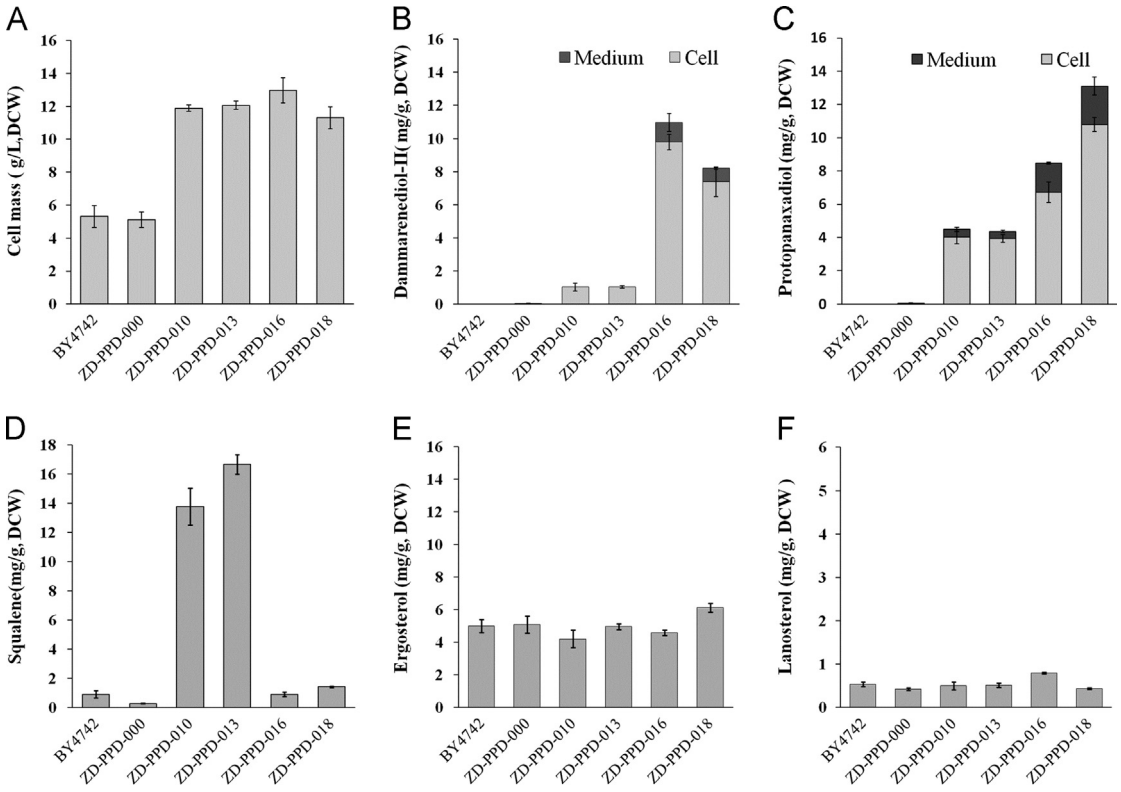

Fig. 4. Production of protopanaxadiol, dammarenediol-II, squalene, ergosterol and lanosterol by engineered *S. cerevisiae* strains. All strains were cultivated in YPD medium with 2% glucose for 6 days. (A) cell growth; (B) production of dammarenediol-II; (C) production of protopanaxadiol; (D) production of squalene; (E) production of ergosterol; (F) production of lanosterol. Dammarenediol-II was detected in the culture medium of strains ZD-PPD-016 and ZD-PPD-018. Protopanaxadiol was detected in the culture medium of strains ZD-PPD-010, ZD-PPD-013, ZD-PPD-016 and ZD-PPD-018. Three repeats were performed for each strain, and the error bars represented standard deviation.

for 6 days. Protopanaxadiol yields varied from 0.50 to 4.49 mg/g DCW, suggesting that different gene copies were integrated in these 10 colonies (Fig. S3B). The second colony, which had the highest protopanaxadiol yield, were named ZD-PPD-010 and used for further engineering. It was suggested that multiple copies of *tHMG1* gene were integrated in strain ZD-PPD-010.

To our surprise, some amounts of protopanaxadiol were detected in the culture medium, while dammarenediol-II, squalene, lanosterol and ergosterol were not detected (Fig. 4). The total amounts of protopanaxadiol (accumulated inside the cell and secreted to the medium) produced by strain ZD-PPD-010 were 4.49 mg/g DCW, which was 89.8-fold higher than parent strain ZD-PPD-000 (Fig. 4). Dammarenediol-II yield increased 21-fold (from 0.05 to 1.05 mg/g DCW). In addition, squalene yield increased 51-fold (from 0.27 to 13.78 mg/g DCW) (Fig. 4). Our results demonstrated that enough squalene supply was essential for efficient protopanaxadiol production.

Squalene is a common precursor for both protopanaxadiol and ergosterol synthesis (Fig. 1). However, improvement of lanosterol and ergosterol production was relatively smaller. Lanosterol yield increased 1.2-fold (from 0.42 to 0.50 mg/g DCW), and ergosterol yield decreased from 5.09 to 4.21 mg/g DCW (Fig. 4). It was suggested that some enzyme of the ergosterol synthetic pathway, such as cytochrome P450 lanosterol 14a-demethylase (encoded by *ERG11*), might be rate-limiting, thus limiting carbon flux towards ergosterol production (Veen et al., 2003).

On the other hand, there was a 2.3-fold increase of cell mass after integrating *tHMG1* gene in strain ZD-PPD-000 (Fig. 4A). It was thought that although auxotrophic strain ZD-PPD-000 could grow in YPD medium without uracil supply, uracil content in the medium might be limiting. Integrating *tHMG1* gene with *URA3* marker recovered uracil auxotrophy, thus providing enough uracil for cell growth. In order to investigate the effect of adding uracil on cell mass and protopanaxadiol production, 0.2 g/L uracil was added in the YPD medium when cultivating strains ZD-PPD-000 and ZD-PPD-010. Addition of uracil led to 2.1-fold increase of cell mass of strain ZD-PPD-000, while it had nearly no effect on cell mass of strain ZD-PPD-010 (Table 3). On the other hand, addition of uracil had nearly no effect on protopanaxadiol yields of both strains (Table 3). It was thus suggested that increase of cell mass after overexpressing *tHMG1* gene was mainly due to simultaneous integration of *URA3* gene.

Table 3
Effects of adding uracil on cell mass and protopanaxadiol production of strains ZD-PPD-000 and ZD-PPD-010.

Strains[a]	Media	Dry cell weight (g/L)	Protopanaxadiol yield (mg/g DCW)[b]
ZD-PPD-000	YPD	5.11 ± 0.36	0.05 ± 0.01
ZD-PPD-000	YPD + 0.2 g/L uracil	10.84 ± 0.87	0.04 ± 0.02
ZD-PPD-010	YPD	11.89 ± 0.27	4.49 ± 0.55
ZD-PPD-010	YPD + 0.2 g/L uracil	11.15 ± 0.32	4.76 ± 0.61

[a] Three repeats were performed for each strain, and the error bars represented standard deviation.
[b] Protopanaxadiol yields represented the total amounts accumulated inside the cell and secreted to the media.

3.3. Overexpressing ERG20 and ERG9 genes to further improve squalene supply

Farnesyl diphosphate synthase (encoded by *ERG20*) and squalene synthase (encoded by *ERG9*) are responsible for conversion of the common building blocks of isoprenoids (IPP and DMAPP) to

squalene (Fig. 1). Overexpression of *ERG20* gene either individually or together with GGPP synthase gene had been used to increase production of sesquiterpenoids and diterpenoids compounds, such as amorpha-4,11-diene (Wu et al., 2006) and geranylgeraniol (Ohto et al., 2010; Tokuhiro et al., 2009). Squalene synthase is the first enzyme dedicated to synthesis of sterols in yeast. Either deleting *ERG9* gene or down-regulating its expression by replacing the native promoter with a *MET3* promoter had been widely used to reduce flux toward ergosterol synthesis, thus enhancing production of sesquiterpenoids or diterpenoids compounds (Albertsen et al., 2011; Asadollahi et al., 2010; Kirby et al., 2008; Ro et al., 2006). On the other hand, overexpressing *ERG9* gene had been used to enhance production of phytosterols and triterpenoids in plants (Kim et al., 2011; Lee et al., 2004; Seo et al., 2005) and production of ergosterol and β-amyrin in engineered yeast (Kennedy and Bard, 2001; Madsen et al., 2011).

In order to further increase squalene supply for protopanaxadiol production, both *ERG20 and ERG9* genes were integrated into the chromosome of ZD-PPD-010 at *Trp1* site, resulting in strain ZD-PPD-013. Squalene production increased 21% (from 13.78 to 16.68 mg/g DCW), and ergosterol production increased 18% (from 4.21 to 4.96 mg/g DCW). However, production of dammarenediol-II and protopanaxadiol was almost the same as parent strain.

3.4. Overexpressing squalene epoxidase gene was essential for increasing protopanaxadiol production

Squalene epoxidase (encoded by *ERG1*) catalyzes the first oxygenation step in ergosterol biosynthesis and is suggested to represent one of the rate-limiting enzymes in this pathway (Leber et al., 2001). It had been demonstrated that squalene epoxidase activity is reduced in the presence of excess sterols through a mechanism like transcriptional repression (Leber et al., 2001), and overexpressing *ERG1* gene can increase sterol production (Leber et al., 2001; Veen et al., 2003).

The *ERG1* gene, together with *ERG20* and *ERG9* genes, were integrated into chromosome of ZD-PPD-010 at *Trp1* site, resulting in strain ZD-PPD-016. Some amounts of dammarenediol-II were also detected in the culture medium (Fig. 4). Squalene production decreased significantly from 16.68 to 0.91 mg/g DCW, while production of dammarenediol-II and protopanaxadiol increased significantly (Fig. 4). ZD-PPD-016 produced 10.97 dammarenediol-II and 8.48 mg/g DCW protopanaxadiol, which were 10-fold and 1.9-fold higher than strain ZD-PPD-013, respectively. Lanosterol production increased 57% (from 0.51 to 0.8 mg/g DCW), while ergosterol production was almost the same as strain ZD-PPD-013. These results demonstrated that squalene epoxidase was a rate-limiting step towards dammarenediol-II and protopanaxadiol production.

3.5. Utilization of codon-optimized PPDS gene for protopanaxadiol production

After overexpressing *ERG1* gene, the ratio of protopanaxadiol to dammarenediol-II changed from 4.1:1 to 1:1.3. It was suggested that protopanaxadiol synthase activity was limiting for protopanaxadiol production in strain ZD-PPD-016. The protopanaxadiol synthase of *P. ginseng* might not express well in *S. cerevisiae*. Codon optimized *PPDS* gene (*SynPgPPDS*), together with *tHMG1* and *AtCPR1* genes, were integrated into chromosome of ZD-PPD-016 (URA3$^-$) at *δDNA* site. Ten colonies were randomly picked from the plate, and four colonies were verified to be correct by PCR amplification. All right colonies were selected for flask fermentation. Protopanaxadiol yields varied from 10.32 to 13.11 mg/g DCW (Fig. S3C). The fourth colony, which had the highest protopanaxadiol yield, was named ZD-PPD-018. Protopanaxadiol yield increased 55% (from 8.48 to 13.11 mg/g DCW), while dammarenediol-II yield decreased 25% (from 10.97 to 8.20 mg/g DCW) compared to parent strain ZD-PPD-016 (Fig. 4).

In order to investigate the effect of additional *tHMG1* over-expression alone, this gene was integrated into chromosome of ZD-PPD-016 (URA3$^-$) at *δDNA* site. The highest protopanaxadiol-producing colony among 10 selected colonies was designated ZD-PPD-016H. Production of dammarenediol-II and protopanaxadiol by strain ZD-PPD-016H was almost the same as parent strain ZD-PPD-016 (Table 4), suggesting that HMG-CoA reductase was not limiting in strain ZD-PPD-016 for protopanaxadiol synthesis.

In order to investigate the effects of additional *tHMG1* and *AtCPR1* over-expression, these two genes were integrated into chromosome of ZD-PPD-016 (URA3$^-$) at *δDNA* site. The highest protopanaxadiol-producing colony among 10 selected colonies was designated ZD-PPD-016HA. Protopanaxadiol production of strain ZD-PPD-016HA increased from 8.48 to 9.81 mg/g DCW, while dammarenediol-II production increased from 10.97 to 14.66 mg/g DCW compared to parent strain ZD-PPD-016 (Table 4). However, the ratio of protopanaxadiol to dammarenediol-II in strain ZD-PPD-016HA was 1:1.5, which was almost the same as parent strain ZD-PPD-016 (1:1.3). It was suggested that increasing AtCPR1 activity was useful for improving both dammarenediol-II and protopanaxadiol synthesis. Redox partner is very important for protopanaxadiol synthase, which is a P450 enzyme. Since the NADPH-cytochrome P450 reductase (CPR) gene of *P. ginseng* was not identified yet, a CPR gene of *A. thaliana* (*AtCPR1*) was used in this study. More CPRs from other organisms need to be further investigated to identify a better one for protopanaxadiol production.

After additional over-expression of codon optimized *PgPPDS* gene, the total amount of dammarenediol-II and protopanaxadiol produced by strain ZD-PPD-018 was almost the same as strain ZD-PPD-016HA. However, the ratio of protopanaxadiol to dammarenediol-II increased from 1:1.5 to 1.6:1, suggesting that additional over-expression of codon optimized *PgPPDS* gene was essential for converting more dammarenediol-II to protopanaxadiol.

In order to evaluate the stability of strain ZD-PPD-018 for production of protopanaxadiol, this strain was serially transferred for 10 times in both mineral and YPD medium, leading to strains ZD-PPD-018-M10 and ZD-PPD-018-Y10 respectively. Strain ZD-PPD-018-M10 produced 7 mg/g protopanaxadiol in mineral medium, which was almost the same as the parent strain (Table S3). Strain ZD-PPD-018-Y10 produced 9.98 mg/g protopanaxadiol in YPD medium, which was 76% of the parent strain (Table S3). It was suggested that strain ZD-PPD-018 was stable when cultivated in mineral medium. This was reasonable since there was no uracil, histidine and leucine in mineral medium, and the *URA3*, *HIS3* and *LEU2* markers, together with genes for protopanaxadiol synthesis, needed to be kept in the strain to satisfy nutrition requirement.

Table 4
Effects of additional *tHMG1* over-expression and additional *tHMG1* and *AtCPR* over-expression on protopanaxadiol production.

Strains[a]	Dry cell weight (g/L)	Dammarenediol-II yield (mg/g DCW)[b]	Protopanaxadiol yield (mg/g DCW)[b]	Ratio[c]
ZD-PPD-016	12.97 ± 0.77	10.97 ± 1.01	8.48 ± 0.68	1:1.3
ZD-PPD-016H	11.31 ± 0.84	11.42 ± 0.75	8.10 ± 0.86	1:1.4
ZD-PPD-016HA	11.24 ± 0.37	14.66 ± 0.57	9.81 ± 0.45	1:1.5
ZD-PPD-018	11.30 ± 0.66	8.20 ± 0.95	13.11 ± 0.96	1.6:1

[a] Three repeats were performed for each strain, and the error bars represented standard deviation.
[b] Dammarenediol-II and protopanaxadiol yields represented the total amounts accumulated inside the cell and secreted to the media.
[c] The ratio of protopanaxadiol to dammarenediol-II.

3.6. Production of protopanaxadiol through fed-batch fermentation

In order to obtain higher protopanaxadiol production, fed-batch fermentation of strain ZD-PPD-018 was performed in a 7.5 L fermentor with pH controlled at 5.5. Cell growth reached stationary stage after 48 h, and continued to increase until 144 h, with the maximum OD_{600} 324. Protopanaxadiol and dammarenediol-II titers continued to increase until 72 h, but had big decreases at 96 h (Fig. 5). After that, protopanaxadiol titer continued to increase until 144 h (up to 232 mg/L), while it decreased to 221 mg/L at 192 h (Fig. 5). Dammarenediol-II titer continued to increase until 192 h (up to 304 mg/L) (Fig. 5). Ergosterol titer continued to increase until 120 h (up to 263 mg/L), while it decreased to 180 mg/L at 192 h. In addition, 16 mg/L squalene and 34 mg/L lanosterol were also produced.

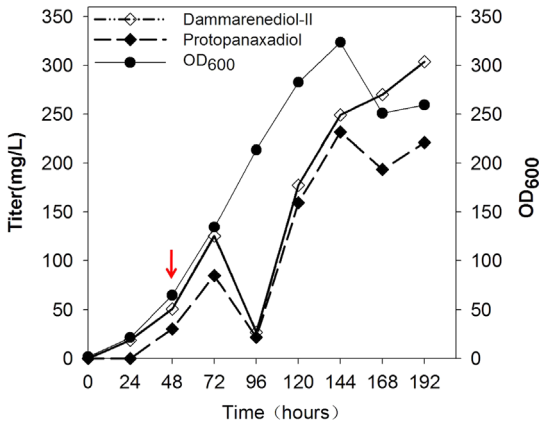

Fig. 5. Production of protopanaxadiol by strain ZD-PPD-018 through fed-batch fermentation. Black solids began to accumulate in the upper surface of the culture medium after 48 h.

Surprisingly, after 48 h, some black solids began to accumulate in the upper surface of the culture medium (Fig. 6). At 96 h, the amounts of these black solids increased significantly, while protopanaxadiol and dammarenediol-II contents within the cells had big decreases (Fig. 5; Fig. 6). It was thus thought that protopanaxadiol and dammarenediol-II accumulated in these black solids. After fermentation for 192 h, 5 L culture medium was washed through a gauze, and 25 g black solids were collected for analysis of its components. Protopanaxadiol, dammarenediol-II, glucose, water, fatty acid, ergosterol, squalene and lanosterol accounted for 11.81%, 9.45%, 2.14%, 15%, 0.62%, 0.1%, 0.01% and 0.01% (wt/wt) of the weight of these black solids (Fig. 6). Protein and polysaccharide were not detected. Many compounds having high similarities with triterpenoids were also detected in the black solids, which might be the main components of the remaining 60.86% weight of the black solids (Fig. S4; Table S4). The total protopanaxadiol titer (in both cells and black solids) was 812 mg/L, while the total dammarenediol-II titer was 776 mg/L.

Saponins have the ability to cause membrane perturbation (Augustin et al., 2011). The hydrophobic part of saponins (sapogenin) can form complexes with sterols of the membranes. Subsequently, these saponin–sterol complexes can accumulate into matrices or plaques, which might be driven by interaction of their extra-membranous orientated saccharide residues, and cause membrane curvature (Armah et al., 1999; Augustin et al., 2011). This curvature can result in the formation of pores, which would cause an increase in membrane permeability enabling ions and macromolecules up to proteins to pass the membrane bilayer (Armah et al., 1999; Augustin et al., 2011). It was assumed that high amounts of protopanaxadiol produced by the engineered yeast strains might form complexes with sterols and cause pore formation in the membrane. Protopanaxadiol, dammarenediol-II

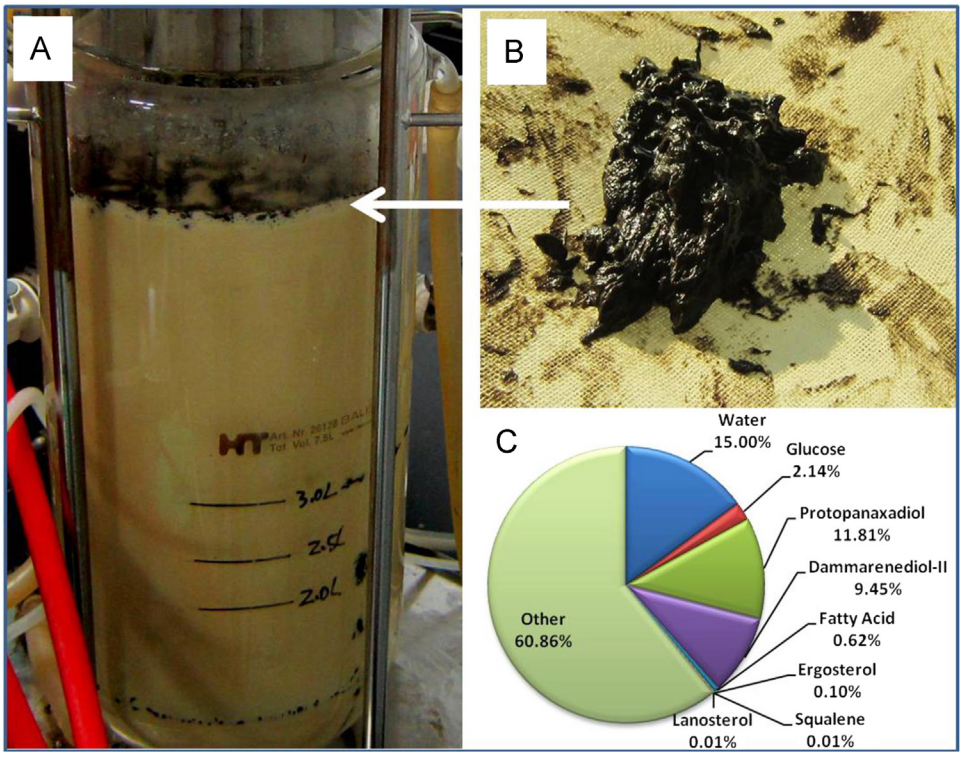

Fig. 6. Analysis of the components of black solids during fed-batch fermentation. (A) Accumulation of black solids in the upper surface of culture medium during fed-batch fermentation; (B) collection of black solids by washing the culture medium through a gauze; (C) components of the black solids.

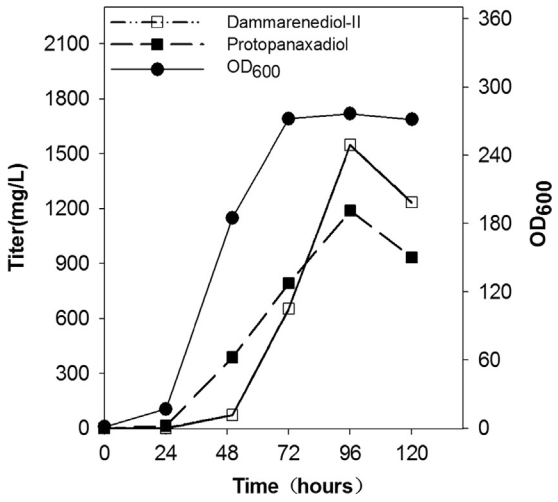

Fig. 7. Production of protopanaxadiol by strain ZD-PPD-018 through two-phase extractive fermentation. Protopanaxadiol and dammarenediol-II titers were calculated by adding products accumulated inside the cell and extracted in solvent.

and other compounds thus passed the membrane, and black solids might be formed due to polymerization of these compounds.

3.7. *Production of protopanaxadiol through two-phase extractive fermentation*

In order to avoid accumulation of black solids, a two-phase extractive fermentation process was performed to remove terpenoids in situ during fermentation using an extractive solvent. This strategy had been successfully used to enhance production and recovery of monoterpenes (Brennan et al., 2012) and sesquiterpenes such as amorpha-4,11-diene (Newman et al., 2006). Two solvents, methyl oleate and dodecane, were tested in this work. Adding dodecane to the fermentor resulted in lots of foams immediumtely, and was excluded from the remaining experiment. Black solids were not formed when using methyl oleate as the extractive solvent, while protopanaxadiol and dammarenediol-II production increased significantly. After 96 h, 1.8 L fed solution was added to the fermentor, and the final volume of the fermentation broth was 4.8 L. There were 1493 mg protopanaxadiol and 2256 mg dammarenediol-II accumulated inside the cell, while 4214 mg protopanaxadiol and 5174 mg dammarenediol-II were extracted in 300 mL methyl oleate. The total protopanaxadiol titer (in both cells and solvent) was 1189 mg/L (8.40 mg/g DCW), and the total dammarenediol-II titer was 1548 mg/L (10.94 mg/g DCW) (Fig. 7). Our results demonstrated that two-phase extractive fermentation process is also an effective method for enhancing triterpenoids production.

4. Conclusion

Through introducing *P. ginseng* dammarenediol-II synthase, protopanaxadiol synthase and *A. thaliana* CPR genes into *S. cerevisiae*, we have constructed cell factories for efficient protopanaxadiol production. Increasing supplies of squalene and 2,3-oxidosqualene through overexpressing *tHMG1*, *ERG20*, *ERG9* and *ERG1* genes, together with increasing protopanaxadiol synthase activity through codon optimization and using two-phase extractive fermentation process, resulted in production of 1189 mg/L protopanaxadiol (8.40 mg/g DCW). The yeast strains engineered in this work can serve as the basis for creating an alternative way for production of ginsenosides in place of extraction from plant sources.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.10.004.

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