High production of triterpenoids in Yarrowia lipolytica through manipulation of lipid components

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Abstract

Background

Lupeol exhibits novel physiological and pharmacological activities, such as anti-cancer and immunity enhancement. However, cytotoxicity is still a challenge for triterpenoids overproduction in microbial cell factories. As lipophilic and relatively small-molecular compounds, triterpenes are generally secreted to the extracellular space. The effect of increasing triterpenes efflux on the synthesis capacity remains unknown.

Results

In this study, we developed a strategy to enhance the triterpenes efflux through manipulation of lipid components in Y. lipolytica by overexpressing the enzyme of Δ9-fatty acid desaturase (OLE1) and disturbing the phosphatidic acid phosphatase (PAH1) and diacylglycerol kinase (DGK1). As a result, we obtained a high-yield lupeol strain with the highest lupeol production of 411.72 mg/L in shake flasks reported to date, reaching a 33.2-fold improvement over the initial strain. The lipid manipulation led to a two-fold increase of unsaturated fatty acids (UFAs) content, up to 61%~73%, and an exceptionally elongated cell morphology, which might be caused by enhanced membrane phospholipids biosynthesis flux. Both of the phenotypes accelerated the export of toxic products to the extracellular space and ultimately stimulated the capacity of triterpenoids synthesis, which were proved by 5.11-fold higher ratio of extra-/intra-cellular lupeol concentrations, 2.7-fold higher biomass accumulation and 2.60-fold higher lupeol productivity per unit OD in modified strains. This strategy was also highly efficient for biosynthesis of other triterpenes and sesquiterpenes, including α-, β-arymin, longifolene, longipinene and longicyclene.

Conclusions

To conclude, we successfully created a high-yield lupeol strain via lipid manipulation. And we demonstrated that the enhancement of lupeol efflux and synthesis capacity was induced by the increased unsaturated fatty acids content and elongated cell morphology. Our study provides a novel strategy to promote the biosynthesis of valuable but toxic products in microbial cell factories.

Background
Triterpenoids are a class of antineoplastic natural products, which serve as significant resources of pharmaceuticals for the treatment of various diseases, such as anti-inflammatory, antibacterial, antimalarial, anti-oxidation, anti-HIV, and immunity enhancement [1]. To keep pace with the increasing commercial demands of triterpenoids, metabolic engineering of cell factories has emerged as a promising and attractive alternative in industrial production, which is an environmentally friendly and cost-efficient approach independent on plants [2]. Nevertheless, terpenoids, known for their in vivo anti-inflammatory activity, are generally highly toxic to microorganisms, inducing apoptosis and resulting in negative impact on terpenes production [3, 4]. To remedy the intractable deficiency, extensive efforts aimed at creating oleaginous subcellular organelles by increasing intracellular lipid content and lipid size, so that lipophilic terpenes can be accumulated in these storage compartments to decrease the cytotoxicity [5-8]. For instance, researchers successfully increased lycopene accumulation by creating supersized lipid droplets through manipulation of triacylglycerol (TAG) metabolism in Saccharomyces cerevisiae, which resulted in the highest yield of 73.3 mg/g cdw and 2.37 g/L lycopene in S. cerevisiae reported to date [6]. Similarly, knocking out POX1 to POX6 and GUT2 in Y. lipolytica increased the size of lipid bodies, which enabled Y. lipolytica to withstand higher lycopene concentrations [7]. And Gao et al. [8] have successfully applied lipid droplets as a storage sink to enhance β-carotene production in Y. lipolytica, resulting in the yield of β-carotene up to 4 g/L in fed-batch fermentation, the highest level so far. It is worth noting that the above strategies were biased to the biosynthesis of tetraterpenoids with large molecule, which can be accumulated in lipophilic compartments. However, the terpenoids with relatively small-molecule, such as triterpenes and flavonoids, are generally excreted to extracellular space [9, 10]. These products cross plasma membrane preferentially in the way of passive diffusion, since cell membrane is mainly composed by lipid bimolecular layer [11]. As such, two-phase fermentation is widely utilized to extract terpenes from cells into extracellular lipophilic solvent and thus reduced products cytotoxicity [3]. However, the relatively low permeability of cell membrane is still a barrier to the efflux of triterpenoids [11]. Manipulation of the lipid composition in membrane, such as the ratio of unsaturated fatty acids/saturated fatty acids (UFAs/SFAs) or the content of
membrane phospholipids, is an efficient approach to tune membrane permeability [11-16]. For example, up-regulation of SFAs content in E. coli resulted in a tightening membrane with enhanced tolerance to toxic n-hexane in culture [17]. And yeast cells can resist the damage of extracellular ethanol through altering membrane phospholipids properties [18]. However, these modifications are mostly adopted to reduce cell membrane permeability to block the entry of extracellular hazardous substances. Few applications are committed to promoting toxic terpenes secretion through lipid regulation in microbial cell factories.

As a “generally regarded as safety” (GRAS) host, *Y. lipolytica* has emerge as an attractive host preferentially employed in the production of numerous pharmaceuticals and nutraceuticals [19]. And naturally high flux of acetyl-CoA in *Y. lipolytica* provided abundant terpene synthesis precursors. Moreover, *Y. lipolytica* is a remarkable oleaginous yeast, in which lipid accumulation is over 40% of the dry cell weight (DCW). The tractability of lipid metabolism in *Y. lipolytica* make it a promising platform organism for lipophilic products biosynthesis [20]. Herein, we applied a strategy of engineering lipid components to achieve a high production of lupeol, a pentacyclic kind of triterpene, in *Y. lipolytica*. Specifically, we (i) established heterologous lupeol synthesis pathway in *Y. lipolytica* by chromosomally integration of genes encoding lupeol synthases from different sources, (ii) optimized the precursor supply through engineering structural genes in the MVA pathway, (iii) significantly elevated lupeol biosynthesis by disturbing essential genes in lipid metabolism, (iv) revealed accelerated export of lupeol caused by a ~2-fold increase of unsaturated fatty acids (UFAs) proportion and remarkably elongated cell morphology (Fig. 1). In doing so, the titer of lupeol reached up to 411.72 mg/L in shake flasks, representing the highest production reported to date. This strategy was also highly efficient to other triterpenes and sesquiterpenes biosynthesis, which provides a new insight into improving the production of valuable products in microbial cell factories.

**Results And Discussion**

**Establishment of lupeol synthesis in *Y. lipolytica***

Lupeol, a pentacyclic triterpene, is an important intermediate metabolite from 2,3-oxidosqualene to a series of lupane-type triterpenoids, which attracted increasing attention due to its anti-HIV, anti-
cancer and anti-inflammatory activity [24, 25]. To produce lupeol from acetyl-CoA through the mevalonate (MVA) pathway in *Y. lipolytica*, we assembled heterologous gene encoding lupeol synthases from different sources in strain ATCC 201249, that were *AtLus* from *Arabidopsis thaliana*, *GuLus* from *Glycyrrhiza uralensis*, *OeLus* from *Olea europaea*, *BgLus* from *Bruguiera gymnorrhiza*, *KdLus* from *Kalanchoe daigremontiana* and *RcLus* from *Ricinus communis*. All of these genes were codon-optimized (Additional file 2: Table S1) and integrated into the *Ku70* site in the genome of *Y. lipolytica* under the control of *Hp4d* promoter. Then we quantified their lupeol production after five days of cultivation. Of these variants, strain LU-6 containing *RcLus* achieved the highest lupeol titer of 12.4 mg/L (Additional file 1: Fig. S1A), subjected as the initial strain.

Subsequently, we sought to improve the expression of *RcLus* by testifying other promoters [26-28] (Additional file 1: Fig. S1B) and changed the subcellular locations of lupeol synthase by fusing different subcellular location signal peptides to *RcLus* (Additional file 2: Table S2), which were confirmed by laser scanning confocal microscopy analysis (Additional file 1: Fig. S1C). Collectively, the strain LU-9 containing the *RcLus* gene expressed by pTEFin promoter and localized in cytosol achieved the highest lupeol titer of 29.0 mg/L, a 2.3-fold increase than the initial strain LU-6 (Additional file 1: Fig. S1D). Thus, the optimized heterologous lupeol synthesis pathway was successfully established in *Y. lipolytica*. The strain LU-9 was selected to subsequent engineering, which was designated as control strain in this study.

**Engineering of MVA and lipid metabolism to improve lupeol production**

In order to improve lupeol production, we first up-regulated the mevalonate pathway by overexpressing the rate-limiting enzyme *HMG1* (3-hydroxy-3-methylglutaryl coenzyme A reductase) [29] in strain LU-9, generating strain LU-10. It has been reported that synchronous overexpression of *ERG1* (squalene synthase) and *ERG9* (squalene monooxygenase) [30] exhibited cooperativity towards enhancing terpenes synthesis [22]. Hence, we further performed this combination based on the *HMG1* overexpression to generate strain LU-11. In sum, *HMG1* and *ERG9* was expressed by pTEFin promoter, and *ERG1* was controlled by pEXP1 promoter, which were chromosomally integrated into the rDNA site of the *Y. lipolytica*, marked with red rectangles in Fig. 2A. Compared with the control strain LU-9,
the resulting strain LU-10 with single HMG1 overexpression, and LU-11 with HMG1, ERG9 and ERG1
overexpression only presented slight improvement of lupeol yield at 1.2- and 1.5-fold, respectively
(Fig. 2B). The achieved yield of lupeol in the present host was still at a low level.
In addition to modification of MVA pathway, lipid metabolism was also reported as an implicated
effector on terpenoid biosynthesis, which is mainly affected the accumulation and tolerance capacity
of lipophilic products [5, 6]. As such, we manipulated eight structural and regulatory genes related to
lipid metabolism in LU-9 (Fig. 2A), including β-oxidation disruption (knockout of PXA1, MFE1, PEX10 or
POT1) [31-33], and lipid synthesis regulation (overexpression of the limiting step gene ACC1, OLE1
and its activator MGA2G643R, and disturbing regulatory and structural genes RPD3, SNF1, LRO1, PAH1 and DGK1) [34, 35] (Fig. 2A. and Additional file 1: Fig. S2). The overexpression of each gene was
performed by homologous recombination and the knockout of each gene was achieved by
CRISPR/Cas9 system, generating a series of strains from LU-12 to LU-21 based on the LU-9. Among
them, LU-20 with overexpression of OLE1 and LU-21 with knockout of PAH1-DGK1 promoted the lupeol
production most significantly, with 3.16- and 3.30-fold improvement over strain LU-9, respectively.
Then, a LU-22 strain was generated by combination of overexpression of OLE1 and knockout of PAH1-
DGK1, which further improved lupeol production to 137.52 mg/L, 4.74-fold higher than the strain LU-9.
Subsequently, the manipulation of MVA pathway and lipid metabolism were combined, generating
strain LU-23 (with overexpression of RcLus, HMG1, ERG1, ERG9, OLE1 and knockout of PAH1-DGK1).
As shown in Fig. 2B, the production of lupeol in strain LU-23 was up to 280.46 mg/L in shake flasks.
Further improvement was achieved through optimization of carbon source in medium, and the final
lupeol production reached to 411.72 mg/L (Additional file 1: Fig. S3), 33.2-fold higher than initial
strain LU-6 and 9.67-fold higher than control strain LU-9.
In summary, the MVA pathway modifications on lupeol production only slightly enhanced the lupeol
biosynthesis. The lipid manipulation, however, presented remarkable effect on lupeol overproduction,
especially the overexpression of OLE1 and the disruption of PAH1-DGK1. Thus, these three modified
strains and the control strain LU-9 (marked with asterisks in Fig. 2B) were selected for further
investigations to reveal the mechanism of high production.
Effect of overexpression of OLE1 and disruption of PAH1-DGK1 on the UFAs proportion and cell morphology

To elucidate the underlying relationship between lupeol production and the lipid regulation, we measured the lipid content and the composition of unsaturated fatty acids (UFAs) and saturated fatty acids (SFAs) in high-producing strains (LU-20, LU-21, LU-22, marked with asterisks in Fig. 2B), and the control strain (LU-9). As shown in Fig. 3A, there was no obvious change of the total amount of fatty acids among different strains. However, the ratio of UFAs/SFAs was dramatically increased in all of the modified strains (Fig. 3B). The UFAs content in control strain (LU-9) was only 35%, but the UFAs ratios in all high-producing strains increased up to 61%~73%, approximately two-fold greater than that in the control strain. These findings suggested that overexpressing OLE1 and deleting PAH1-DGK1 mainly enhanced the proportion of unsaturated fatty acids instead of total fatty acids accumulation. The increased UFAs proportion caused by OLE1 overexpression and PAH1-DGK1 knockout was consistent with previous studies. OLE1 gene encodes a Δ9-fatty acid desaturase, which is mainly responsible for catalyzing the dehydrogenation of the 9-position in saturated fatty acids to form relative unsaturated fatty acids [36]. Meanwhile, knockout of PAH1 also could resulted in increased levels of UFAs in yeast even though it was responsible for phosphatidic acid (PA) transformation [37] (Additional file 1: Fig. S2).

Except for the change of lipid components, we also observed that the morphologic profiles of PAH1-DGK1 knockout strains changed significantly. As detected by optical microscope (Fig. 4A) and transmission electron microscopy (Fig. 4B), the PAH1-DGK1 knockout in strain LU-21 and LU-22 resulted in extraordinarily elongated morphology, while the strain LU-20 with OLE1 overexpression exhibited the same spherical cells as the control strain LU-9. PAH1 gene encodes a phosphatidate phosphatase. Single knockout of PAH1 caused shortened chronological life span and serious cell lethality, which can be rescued by knocking out DGK1 with PAH1 synchronously [38, 39]. The transformation from PA to DAG was blocked out with PAH1-DGK1 knockout, which could result in elevated level of PA [37], and consequently lead to higher biosynthesis of phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) through CDP-diacylglycerol (CDP-DAG)
pathway [17]. We performed an RNA-seq analysis for the four strains, and as shown in Fig. 5, the genes related to CDP-DAG pathway were obviously up-regulated more than 1.5-fold in PAH1-DGK1 deleted strain (LU-21 and LU-22) relative to the control strain (LU-9), including the genes of YALI0_C00209g (SCT1), YALI0_E18964g (SLC1), YALI0_D08514g (CHO1) and YALI0_E12441g (OPI3) (Additional file 2: Table S3). But in LU-20 strain, no similar up-regulation of these genes was observed. PS, PE and PC are the main components of membrane phospholipids. As such, the higher synthesis flux of PS, PE and PC might result in accelerated synthesis of membrane phospholipids, which was speculated as the main factor for the elongated cell morphology in PAH1-DGK1 disruption strains. In summary, OLE1 overexpression and PAH1-DGK1 disruption led to a remarkable change in lipid components. All the three modified strains obviously increased the proportion of unsaturated fatty acids instead of the lipid accumulation. And knockout of PAH1-DGK1 elongated cell morphology, which might be caused by the enhanced flux of membrane phospholipids synthesis.

**Effect of lipid modifications on the efflux of metabolites**

Various studies demonstrated that the increased ratio of UFAs/SFAs disrupts the order of the phospholipid bilayer, contributing to the enhanced membrane permeability of toxic substances across membrane [11-16]. Meanwhile, the resulting elongated cell increased the contact area between the cells and the external environment. Both of the results described above were supposed to accelerate the efflux of products. To validate this deduction, we first testified the extra- and intra-cellular lupeol titer and analyzed the ratio of extra-/intra- cellular lupeol content in two-phase extraction fermentations of LU-9, LU-20, LU-21 and LU-22 (Fig. 6A, 6B and Additional file 1: Fig. S4). Compared to LU-9, the total lupeol production and the extracellular lupeol titer in engineered strains increased significantly, while there was no obvious change of lupeol accumulation intracellularity (Additional file 1: Fig. S4). Similarly, as shown in Fig. 6B, the ratio of extra-/intra- cellular lupeol content also significantly increased to 13.71 in LU-20 with OLE1 overexpression and 9.71 in LU-21 with PAH1-DGK1 disruption, exhibiting 4.81- and 3.27-fold higher than LU-9, in which the ratio is 2.81. And optimal ratio of 14.38 was achieved in LU-22 with combinatorial engineering, 5.11-fold higher than that in LU-9. These results supported that lipid modifications indeed resulted in the improvement of products...
efflux. Then we determined the biomass growth and cell integrity. As shown in Fig. 6C, engineered cells grew much faster in logarithmic phase and the final bio-mass accumulation of strain LU-20, LU-21 and LU-22 exhibited 2.7-, 1.6- and 2.7-fold higher than strain LU-9, respectively. And cell integrity was also improved as PI up-take factor decreased in engineered strains (Additional file 1: Fig. S5). These results confirmed that the improvement of lupeol efflux was beneficial to higher cell growth and integrity, indicating that cytotoxicity was reduced indeed in engineered strains. We then calculated the lupeol productivity after 120h fermentation. As shown in Fig. 6D and Additional file 2: Table S4, notable improvements of lupeol specific productivity in the engineered strains were also observed. Compared to 0.24mg/L/OD/d in strain LU-9, lupeol productivity increased to 0.40mg/L/OD/d, 0.49mg/L/OD/d and 0.56mg/L/OD/d in LU-20, LU-21 and LU-22, respectively, indicating that the capacity of lupeol synthesis was also apparently increased in engineered strains. It is worth emphasizing specially that the improved lupeol productivity was induced by single-cell lupeol synthesis capacity, but not the improvement of biomass growth.

Collectively, these results validated that the elevated ratio of UFAs/SFAs and elongated cells significantly enhanced the export of lupeol, which was contributed to higher cell growth and integrity, and consequently enabled higher lupeol synthesis capacity.

**Effect of the lipid modifications on the production of other terpenoid classes**

To testify if our strategy can be widely applied to enhance the production of other terpenoids, we first established the synthesis of other two kinds of triterpenoids (α- and β-amyrin) by expressing α-amyrin synthetase from *Malus domestica* (αAS) and β-amyrin synthetase from *Arabidopsis thaliana* (βAS), respectively [40] (Fig. 7A). The positive effects of lipid metabolism modification on the α-amyrin and β-amyrin production were obtained, with 1.76-fold and 8.83-fold improved titer over the parent strain, respectively (Fig. 7B). Other than the triterpenoids, we further investigated the capacity of the lipid modifications for the production of sesquiterpenes. Longifolene class of sesquiterpenoids were employed, which serve as high-value therapeutic molecules [41] (Fig. 7C). The biosynthesis of longifolene, longipinene and longicyclene was realized by expressing terpene synthases (TPS) from *Pinus sylvestris* [42]. After five days of cultivation, obvious increased production was obtained in the
lipid modifications strain LO-3, with 5.68-fold higher sesquiterpenes production compared to the parent strain LO-1 (Fig. 7D).

Conclusions

In this study, we performed a strategy to create a high-yield lupeol strain which was facilitated via the regulation of lipid components. To conclude, we first constructed a host with optimized heterologous lupeol pathway, and achieved a lupeol titer at 29.0 mg/L. Compared with MVA pathway optimization that has little effect on lupeol yield, lipid manipulation exhibited obvious improvement by regulating a serious of genes involved in β-oxidation and lipid synthesis. Among them, the combinatorial manipulation of OLE1 overexpression and PAH1-DGK1 disruption with MVA modification (HMG1, ERG9 and ERG1 overexpression) resulted in a marvelous production of lupeol as high as 280.46 mg/L. With optimization of culture, final lupeol production reached up to 411.72 mg/L in shake flasks, a 33.2-fold improvement over the initial strain, and to the best of our knowledge, it is the highest lupeol yield reported to date.

Lipid manipulation were also employed to improve other terpenoids production, such as lycopene and β-carotene [5, 6]. They made efforts to improve lipids contents to create larger size of lipid-drops, which enhanced the storage of the lipophilic products and thus improved terpenoids production. In this study, we also successfully increased the capacity of terpenes synthesis via lipid engineering. Notably, we accelerated the efflux of small-molecule terpenes through altering lipid components, resulting in remarkable increase of UFAs proportion and elongated cell morphology. Our strategy was also valid for other triterpenes and sesquiterpenes overproduction. Accordingly, this work provided a novel and general strategy to improve the biosynthesis of valuable but toxic terpene products in microbial cell factories.

Methods

Strains, media and culture conditions

The Y. lipolytica strain ATCC 201249 (MATA, ura3-302, leu2-270, lys8-11, PEX17-HA) was chosen as the background strain [21] for all constructs and the genotypes of all derivatives constructed in the present study are listed in Additional file 2: Table S5. The above Y. lipolytica strains were cultivated in
yeast medium at 28 °C with 250 rpm shaking speed. The rich YPD medium containing 50 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract was used for cultivation and fermentation purposes of Y. lipolytica strains. SC medium used for screening Y. lipolytica transformants contained 20 g/L glucose H2O, 5 g/L (NH4)2SO4, 1.7 g/L yeast nitrogen base without amino acids, and 2 g/L complete supplement mixture (CSM) lacking uracil (SC-Ura) or leucine (SC-Leu), supplemented with uracil or leucine depending on the selection marker requirements. If necessary, add appropriate amount of hygromycin B (final concentration 100 mg/L) to 50-60 °C for screening of recombinant strains containing hygromycin resistance gene (Hph). DH5α E. coli was used for routine plasmid propagation and construction of recombinant vectors, and grown in Luria-Bertani broth at 37 °C and 250 rpm shaking speed. Suitable antibiotics were added when necessary at the following final concentrations: ampicillin with 100 mg/L or kanamycin with 50 mg/L. 20 g/L agar was added for solid plate preparation. For terpene shake-flask fermentation, freshly streaked single colony of strains was first cultivated in 25 ml polypropylene tube with 5 ml YPD medium and cultured at 28 °C and 250 rpm shaking speed overnight. After pre-culturing, the seed cultures were inoculated into 50 ml fresh YPD medium with initial OD600 at 0.2 and fermented in a 250 ml shake flask for 120 h. 10 ml of isopropyl myristate (IPM) was added for two-phase extraction fermentation to reduce the toxicity inhibition of steroid products on cell growth. All the flask fermentations were performed in triplicates.

**Strain and plasmids construction and yeast transformation**

All the plasmids and primers applied in this work are listed in Additional file 2: Table S6 and S7, respectively. All the enzymes involved in this study were obtained from New England Biolabs. TIANprep Mini Plasmid Kit and TIANgal Midi Purification Kit were used to plasmid extraction and DNA fragment purification, respectively, and Zymogen Frozen EZ Yeast Transformation Kit II (Zymo Research Corporation) was employed to Y. lipolytica transformation. The relevant processes were carried out in accordance with instructions.

Six endogenous lupeol synthase-coding genes (AtLus from *Arabidopsis thaliana*, GuLus from *Glycyrrhiza uralensis*, OeLus from *Olea europaea*, BgLus from *Bruguiera gymnorrhiza*, KdLus from *
*Kalanchoe daigremontiana* and *RcLus* from *Ricinus communis*) were codon-optimized and synthesized by GenScript (Nanjing, China), (codon-optimized sequences in Additional file 2: Table S1). Three native genes in the mevalonate pathway, that was *HMG1* (3-hydroxy-3-methylglutaryl coenzyme A reductase), *ERG9* (squalene synthase), *ERG1* (squalene monoxygenase), and three native genes in lipid metabolism pathway, that was *ACC1* (acetyl-CoA carboxylase from *Y. lipolytica*), *OLE1* (Δ9-fatty acid desaturase) and its activator *MGA2* substitution *MGA2*^{G643R} were amplified from genomic DNA in *Y. lipolytica* by a normal PCR method using the primers given in Additional file 2: Table S7. All of these genes were cloned into the expression plasmids at the corresponding sites with different promoters and LEU2, URA3 or Hph marker. The resulting recombinant plasmids were digested with *NotI* and purified on a gel. Then, approximately 2 μg of linearized DNA was used in the transformation reaction and the transformants were centrifugated at 6,000 ×g for 2 min, plated on SC agar plates without the auxotrophic compound supplemented by corresponding markers, and cultured at 28 °C for 2~3 days. Positive transformants were confirmed by colony polymerase chain reaction with KOD FX DNA polymerase (Toyobo Co., Ltd.; Shanghai, China). The removal of the Ura3 selection marker was carried out by shaking the transformants in YPD liquid medium for 2~3 days and then incubating the transformants on YPD solid medium containing 1.2 mg/mL 5-fluoroorotic acid for 2 days. Obtained colonies were then streaked onto SC and SC-Ura plates, and incubated at 28 °C for 2~3 days.

With regard of disruption of target genes in *Y. lipolytica*, it was achieved by the CRISPR system [19], which is widely used in various studies. The plasmid pMCS-Cen1 was used to construct CRISPR-Cas9 system. The synthesized gRNA was incorporated into pMCS-Cen1-URA, and then the resulting plasmids were digested with restriction enzyme *BamHI* and *HindIII* followed by ligated with the segment of Cas9, thereby forming the corresponding plasmids. For CRISPR plasmid transformations, *Y. lipolytica* were transformed with corresponding plasmids and cultivated in SC-Ura liquid medium for 4 days. Then the cells were plated onto SC-Ura plates and cultured for 2 days at 28 °C and confirmed via sequencing analysis.

**Extraction and analysis of triterpenoids** (*lupeol, α-amyrin, and β-amyrin*) and **sesquiterpenes** (*longifolene, longicyclene and longipinene*)
For the extraction of triterpenes (lupeol, α-arymin, β-arymin) and sesquiterpene products (longifolene, longifolene, longifolene), it is mainly divided into organic phases and cell phases extraction, respectively. For the organic phase sample, the two-phase fermentation broth was centrifuged at 5000 rpm for 5 min, and then the supernatant was filtered by 0.22-µm polypropylene organic filter for extracellular terpenoids detection. The precipitate was prepared for the extraction of cell phase samples. After being centrifugated and washed 3 times with distilled water to ensure adequate removal of residual organic phase, the precipitate was mixed with 0.5 mL of ethyl acetate and 0.1 mL of quartz sand, and then vortexed for 20 min. After being centrifuged at 15000 rpm for 15 min, the organic mixture was filtered by 0.22µm organic filter membrane. Appropriate concentrations of lupeol (Aladdin Industrial Corporation, US), α-amyris, β-amyris (Sigma-Aldrich Corporation, US) and longicyclene (Shanghai Yuanye Bio-technology Corporation, China) were dissolved in IPM as external standard to ensure the production of terpenoids. All of the final samples in two phases were stored at -80 °C.

The methods of the analysis of terpenoids by GC-MS were modified based on that reported in previous study [22]. The combination of GC-MS system equipped with DB-5MS gas chromatography column (30 m×0.25 mm×0.5 μm) and Masslynx software (Version 4.1, Waters Corp., USA) was applied for the qualitative and quantitative analysis of triterpenoids. The details of analysis process are as follows. For the GC system, 1 μL of the obtained sample was injected by an Agilent 7683 autosampler under the split ratio of 10, and the temperatures of injector and GC interface were 250 °C. The carrier gas was high-purity helium with a constant flow of 1.2 mL/min. After 2 min at 80 °C, the column temperature was increased with the speed of 20 °C/min to 300 °C, holding for 17 min. For the MC system, the mass ionization method was electro-impact ionization (EI +) in positive ion mode, with an ionization voltage of 70 eV and an ion source temperature of 230 °C. And the parameter of scanning range was 50-700 m/z.

For analysis of sesquiterpenes, some parameters were adjusted as follows. For the GC system, the split ratio and the temperatures of injector and GC interface were adjusted to 50 and 250 °C, respectively. The carrier gas was high-purity helium with a constant flow of 2 mL/min. After 2 min at
40 °C, the column temperature increased to 210 °C at the speed of 20 °C/min, followed by increasing to 300 °C at the speed of 60 °C/min, holding for 2 min. For the MC system, the ion source temperature was set at 220 °C and the parameter of scanning range was 35-500 m/z.

**Extraction and analysis of total fatty acids**

For total fatty acids extraction, fresh samples of cell cultures were harvested after 120h fermentation (three duplicates). After being centrifugated and washed 3 times with distilled water, 1 mL of methanol solution containing 3 M hydrogen chloride, 0.1 mL of chloroform and 5 μL of heptadecanoic acids as internal standard (final concentration of 200 mg/L) were added into the precipitate, followed by incubating at 70 °C for 3 h with inverted and mixed every 40 min. After naturally cooling the samples to room temperature, 0.2 mL of sodium chloride particles were added and vortexed for 1 minute, followed by adding 0.5 mL of organic solvent n-hexane and vertexing for 3 min. After centrifugation at 12000 rpm for 5 min, the upper organic phase was filtered with a 0.22-μm organic film and stored at -80 °C, ready for the detection of total fatty acids concentration by GC-MS. The method of the analysis of total fatty acids by GC-MS was similar to the methods as reported previously [23]. For the GC system, 1 μL of the obtained sample was injected by an Agilent 7683 autosampler under the split ratio of 2, and the temperatures of injector and GC interface were both 280 °C. The carrier gas was high-purity helium with a constant pressure of 91 Kpa. After 2 min at 70 °C, the column temperature was increased with the speed of 8 °C/min to 290 °C, holding for 6 min. For the MC system, the ion source temperature was set at 250 °C and the parameter of scanning range was 50-800 m/z.

**RNA-seq analysis**

Culture samples were collected at logarithmic phase (take two parallel) after centrifugation at 5000rpm for 2 min, washed with PBS solution for 3 times and quickly froze with liquid nitrogen after centrifugation. The acquisition, analysis and sequencing of mRNA from the above samples were operated by Beijing Genomics Institute (BGI). The library construction was obtained from the cDNA, which was generated from mRNA after fragmented and reverse-transcribed by N6 primers. Then the library quality was determined using a Bioanalyzer 2100 (Agilent) analyzer and the libraries were
sequenced on the BGISEQ-500 sequencing platform. The sequencing results reads were filtered and stored in FASTQ format. The relative expression of genes was calculated using RSEM software, and its relative expression was calculated by FPKM method. Finally, the in-depth data analysis was performed on the BGI data analysis platform (http://report.bgi.com/ps/login/login.html).

**Morphological analysis by transmission electron microscopy**

Cells were harvested and the supernatant was discarded after centrifugation at 5000rpm for 2 min, followed by resuspending and fixing them with 2.5% glutaraldehyde solution overnight (4 °C). The mixture was rinsed three times with 0.1 M PBS buffer for 15 min, added 1% osmic acid to fix for 1 to 2 h and then rinsed for three times with 0.1 M PBS buffer. Then, the obtained sample was dehydrated by ethanol solutions with concentration of 30%, 50%, 70%, 80%, 90%, and 95%, successively, then treated as follows: 100% ethanol for 20 min; acetone solution for 20 min, acetone-embedding agent (V/V = 1/1) mixed solution for 1 h, acetone-embedding agent (V/V = 3/1) mixed solution for 3 h, and pure embedding agent for 12 h. After heating at 70 °C for 12 h, the embedded sample was sectioned with a Leica EM UC7 ultra-thin microtome (70 ~ 90 nm), stained with lead citrate and 50% uranyl acetate ethanol solution for 5 min, and then observed with a transmission electron microscope.

**Declarations**

**Ethics approval and Consent to participate**

Not applicable.

**Consent for publication**

All authors read and approved the final manuscript.

**Availability of supporting data and material**

Data will be made available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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Authors’ contributions

JLZ, QYB, YZP, JF and CCJ designed and performed the experiments. YXC and YJY conceived the project and guided writing the manuscript. All authors read and approved the final manuscript.

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Figures
Scheme diagram of engineering lipid metabolism to improve triterpenes production in Y. lipolytica. The strains without lipid manipulation possessed more saturated fatty acids and spherical cell (left). After regulating lipid metabolism, the content of unsaturated fatty acids enhanced twice and cell morphology elongated, facilitating lupeol efflux from cell to lipophilic solvent of isopropyl myristate, thereby improving cell activity and the capacity of single-cell triterpenes synthesis (right). Abbreviations: AcCoA, acetyl coenzyme A; UFAs, unsaturated fatty acids; SFAs, saturated fatty acids; IPM, isopropyl myristate.
Effect of metabolic engineering of MVA pathway and lipid metabolism on lupeol production.

(A) Relative modified genes involved in the MVA biosynthesis pathway and lipid manipulation, represented in red rectangles and blue rectangles, respectively. (B) Lupeol production under different modified conditions.
production of engineered strains. The strains selected for further investigations are marked with asterisks. Error bars represent ±SD of biological triplicates.

Figure 3

Effects of lipid manipulation on lipid content and composition. (A) Total fatty acids content. (B) Percentage of unsaturated fatty acids and saturated fatty acids. Error bars represent ±SD of technical triplicates.
Cell morphology of strain LU-9, LU-20, LU-21 and LU-22. (A) Morphological analysis by optical microscope (scale bars, 5 µm). (B) Morphological analysis by transmission electron microscopy (scale bars, 0.5 µm). The strain LU-20 with OLE1 overexpression maintained round morphology, which is similar to the control strain LU-9. However, disruption of PAH1-DGK1 in LU-21 and LU-22 led to distinctive elongated morphology.
Transcriptome analysis of genes related to membrane phospholipids synthesis. Color in each rectangle and circle represented the change ratio of FPKM. Abbreviations: Gro3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; CDP-DAG, cytosine diphosphate-diacylglycerol; PS, phosphatidyl serine; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline.
Figure 6

Effect of lipid modifications on the efflux of lupeol during two-phases fermentation. (A) Schematic of two-phase extractive fermentation. (B) The ratio of extra-/intra-cellular lupeol content after 120h of fermentations, which is calculated based on the lupeol titer as shown in Additional file 1: Fig. S4. (C) Effect of lipid modifications on cell growth. (D) The lupeol productivity after 120h fermentation. These results indicated that the efflux of lupeol was enhanced and ultimately improve the cell growth and the capacity of lupeol synthesis in engineered strains. Error bars represent ±SD of biological triplicates.
Figure 7

The production of other triterpenes and sesquiterpenes in the lipid-modified strain with OLE1 overexpression and PAH1-DGK1 knockout. (A) Schematic of α-amyrin and β-amyrin biosynthesis pathway in Y. lipolytica. (B) Effect of lipid manipulation on the production of α-amyrin and β-amyrin. (C) Schematic of longifolene class of sesquiterpenes biosynthesis pathway in Y. lipolytica, including longifolene, longipinene and longicyclene. (D) Effect of lipid manipulation on the production of longifolene class of sesquiterpenoids. Error bars represent ±SD of biological triplicates.

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