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Short Communication

Enhancing oleanolic acid production in engineered *Saccharomyces cerevisiae*

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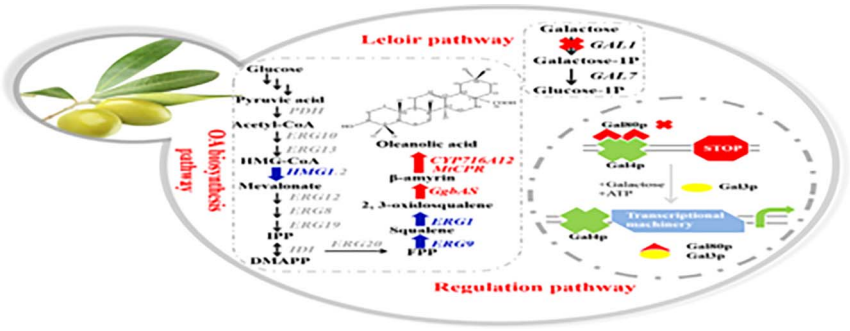
GRAPHICAL ABSTRACT



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ABSTRACT

Oleanolic acid is a plant-derived pentacyclic triterpenoid compound with various biological activities. Recently, biosynthesis of oleanolic acid in microbes has been demonstrated as a promising and green way, but the production is too low for industrialization. To improve oleanolic acid production, this study constructed a novel pathway for biosynthesis of oleanolic acid in *Saccharomyces cerevisiae* by improving the pairing efficiency between cytochrome P450 monooxygenase and reductase. Furthermore, to improve the transcriptional efficiency of heterologous genes, the cellular galactose regulatory network was reconstructed by knocking out galactose metabolic genes *GAL80* and *GAL1*. Finally, the 3-hydroxy-3-methylglutaryl-CoA reductase, squalene synthase and 2,3-oxidosqualene synthase were further overexpressed, increasing oleanolic acid production up to 186.1 ± 12.4 mg/L in flask shake. Combined with fermentation optimization, the final oleanolic acid production was 606.9 ± 9.1 mg/L with a yield of 16.0 ± 0.8 mg/g DCW which was 7.6-fold higher than the reported maximum production.

1. Introduction

Oleanolic acid (OA) is an important plant-derived triterpenoid with various biological activities, including anti-inflammation, anti-viral, anti-tumor and hepatoprotective effect (Pollier & Goossens, 2012). Thus, OA has been widely used in agriculture, food, cosmetics and pharmacy industry (Liu, 2005). Currently, OA is primarily manufactured by extraction from plants while the OA content in plants is low and generally needs a long period (Xia et al., 2011). Compared to plants, microbes exhibit several advantages in producing natural chemicals such as fast-growing, land-saving and controllable culture conditions. With the development of synthetic biology, various valuable

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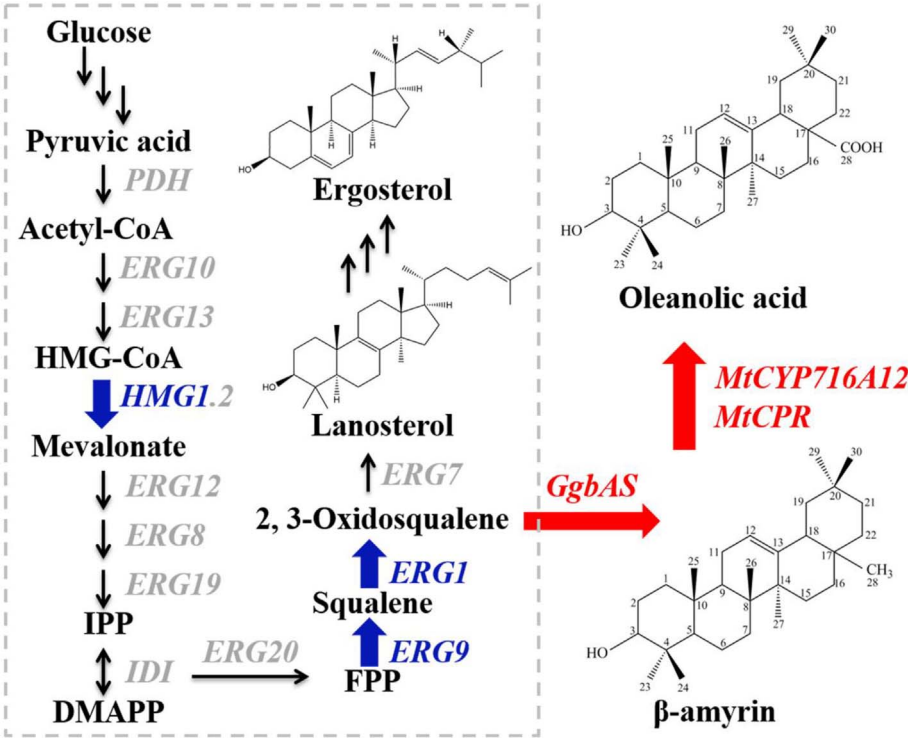

Fig. 1. Scheme for oleanolic acid (OA) biosynthesis pathway in engineered *S. cerevisiae*. Grey dotted box represent the native ergosterol pathway in *S. cerevisiae*. Single arrows represent one-step conversions, triple arrows represent multiple steps, blue arrows represent overexpressed endogenous genes and red arrows represent overexpressed heterogeneous genes. *GgbAS*: β-amyrin synthase gene from *Glycyrrhiza glabra*, *MtCYP716A12*: oleanolic acid synthase gene from *Medicago truncatula*, *MtCPR*: cytochrome reductase gene from *Medicago truncatula*, IPP: isopentenyl pyrophosphate, DMAPP: dimethylallyl pyrophosphate, FPP: farnesyl diphosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plant-derived terpenoids have been produced in microbial cell factories, such as artemisinic acid (Paddon et al., 2013), taxadiene (Zhou et al., 2015), β-amyrin (Liu et al., 2014) and glycyrrhetinic acid (Zhu et al., 2018).

Recently, the biosynthetic pathway of OA has been decoded (Fig. 1). It is generated by condensing six isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which can be generated through the mevalonate (MVA) pathway. Based on the endogenous MVA pathway, heterologous production of OA is accomplished by introducing three heterogenous genes (*bAS*, *CYP716A12* and *CPR*) into *Saccharomyces cerevisiae*, but the production was only 71.0 mg/L, which was too low for industrial production (Dai et al., 2014). Although many strategies have been tried to enhance its production, few of them are proven to be useful.

Recently, it has been reported that the electron transfer efficiency mediated by CPR may be a key issue of triterpenoids production in heterogeneous hosts (Zhu et al., 2018). The uncoupling of CYP450 and CPR would generate reactive oxygen, which could cause cell damage, thus decreasing the production of triterpenoids. Herein, we hypothesize that the low production of OA may be due to the mismatch of redox between selected CYP450 and CPR. Furthermore, the expression level of key genes may be not high enough either. Thus, we first optimized oxidation-reduction system to improve OA production by testing four CPRs to couple with CYP716A12. Then, the transcriptional level of key genes were improved by the overexpression under GAL promoters. Although GAL promoters are strong promoters in yeast, they are inhibited by GAL80 under glucose condition (Bhat & Iyer, 2009). In addition, galactose, the inducer of GAL promoters, can be metabolized through galactokinase (GAL1) (Sellick et al., 2008) that deeply reduces its utilization efficiency. To solve these issues, genes *GAL80* and *GAL1* were knocked out to improve the transcriptional level of key genes under glucose condition. Finally, the truncated HMG-CoA reductase, squalene synthase and squalene epoxidase were overexpressed to enhance the precursor supply. These strategies improved the production of OA up to 186.1 ± 12.4 mg/L in flask shake. Combined with fermentation optimization, the final oleanolic acid production was 606.9 ± 9.1 mg/L which was 7.6-fold higher than the reported maximum production. To our knowledge, this is the highest titer of OA in microbial cell factories, which dramatically promotes the industrialization process of OA production.

2. Material and methods

2.1. Strains, media and cell cultivation

S. cerevisiae strain JDY52 (*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0*) derived from S288C, was used as the parent strain. SD medium was used for selection of engineered strains. Engineered strains were fermented at 30 °C in YPDG medium. *Escherichia coli* TOP10 cells were used for transformation and plasmid DNA extraction. Strains were cultivated at 37 °C in LB medium with 100 mg/L ampicillin.

2.2. Plasmids and strains construction

The *Glycyrrhiza glabra* β-amyrin synthase gene (*GgbAS*) (GenBank: AB037203), *Medicago truncatula* oleanolic acid synthase gene (*CYP716A12*) (GenBank: FN995113) and *M. truncatula* cytochrome P450 reductase gene (*MtCPR*) (GenBank: KU878869.1) were synthesized after codon optimization by GENEVIZ (Beijing, China). Genes of *Arabidopsis thaliana* cytochrome P450 reductase gene (*AtCPR*) (GenBank: NP_194183.1), *Lotus japonicus* cytochrome P450 reductase gene (*LjCPR*) (GenBank: AB433810.1) and *Glycyrrhiza uralensis* cytochrome P450 reductase gene (*GuCPR*) (GenBank: KY798117.1) were stored in our lab. The truncated HMG-CoA reductase gene (t*HMGR1*), squalene synthase gene (*ERG9*) and squalene epoxidase gene (*ERG1*) were PCR-amplified from the genome of *S. cerevisiae*. All the plasmids and primers are summarized in Table S1 and Table S2, respectively. Transformation of *S. cerevisiae* strains was performed by the standard lithium acetate method (Gietz & Schiestl, 2007). The strains used in this study are summarized in Table S3. Primers used for strain construction are summarized in Table S4.

2.3. OA production analysis

Cells were harvested from fermentation culture via centrifugation, the pellet was resuspended in 1 mL distilled water and crushed by a Bead Beater (NEXT ADVANCE, Germany) 6 times (each time for 5 min) via 0.5 mm glass bead. The samples were then centrifuged at 12,000 rpm for 1 min. The supernatant were extracted three times with equal volume of ethyl acetate. The organic phase was evaporated, trimethylsilylated, and analyzed by GC-MS for analyzing oleanolic acid.

1 μL of sample was analyzed by GC-MS using an SHIMADZU GCMS-QP2010 equipped with a SHIMADZU SH-Rxi-5Sil MS (30 m, 0.25 mm × 0.25 μm) GC column. Compound separation was achieved with an injector temperature of 250 °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 28 min. Mass detection was achieved with electric ionization using SIM-scan mode with diagnostic ions monitored as followed: *m/z* 189, *m/z* 203, *m/z* 279, *m/z* 320, *m/z* 393, *m/z* 482, *m/z* 600. Furthermore, samples were analyzed by LC using an SHIMADZU HPLC system. For chromatographic separation, a waters symmetry C18 column (250 mm × 4.6 mm, 5 μm) was used. The mobile phase consisting of methanol (A) and water (B), and a program of A: B = 9:1 for 30 min was used. The flow rate was 1.0 mL/min and the column temperature was set at 30 °C.

3. Results and discussion

3.1. Engineering S. cerevisiae for the synthesis of OA

The biosynthesis pathway of OA in plants can be divided into three parts: (1) forming the core structure 2,3-oxidosqualene, (2) forming the pentacyclic triterpenoids backbone, β-amyrin, (3) CYP450 and CPR adding the functional groups at the C-28 position of β-amyrin. *S. cerevisiae* was chosen as the chassis host for producing OA based on the endogenous 2,3-oxidosqualene biosynthesis pathway. To construct the entire pathway of OA biosynthesis, *GgbAS*, *MtCYP716A12* and *AtCPR1* were introduced into the JDY52 strain using the multiple plasmids co-transformation method, generating the recombinant strain OA01. The fermentation products were identified and determined by GC-MS, and compared with the authentic standards. The results demonstrated that OA was successfully synthesized in strain OA01 (Fig. 2a). Furthermore, the production of OA was determined to be 2.5 ± 0.2 mg/L with yield of 0.6 mg/g DCW by LC analysis.

3.2. Introducing a novel reduction system to enhance OA production

To achieve efficient pairing between CYP450 and CPR, three CPRs (*MtCPR*, *LjCPR* and *GuCPR*) from different plants were introduced to the engineered strain which contained *GgbAS* and *MtCYP716A12*, generating three strains OA02 (*MtCPR*), OA03 (*LjCPR*) and OA04 (*GuCPR*). As expected, LC analysis showed that the production of OA was changed significantly with different CPRs. Specifically, all chosen CPRs could transfer electrons to CYP716A12, and the corresponding coupling efficiency of CPR with CYP716A12 were ranked as follows: *MtCPR* > *GuCPR* > *LjCPR* > *AtCPR* (Fig. 2d). The maximum OA production of 9.0 ± 0.7 mg/L was obtained with strain OA02, 2.6-fold higher than strain OA01. In addition, the production of OA in strain OA04 and OA03 was 7.1 ± 0.5 mg/L and 6.3 ± 0.3 mg/L, respectively. The above results demonstrated that CPRs showed diverse effects on the catalytic activity of CYP716A12 and further affected the production of OA. It has been proposed that CYP450 plays vital roles in triterpenoids biosynthesis and CPR is the most important redox partner of multiple P450s. The CYP450 and CPR from the same plant may possess higher pairing efficiency than that from different plants. Therefore, choosing CYP450 and CPR both from *M. truncatula* efficiently boosted OA production in *S. cerevisiae*.

3.3. Reconstructing the galactose regulatory network to enhance OA synthesis

Although strain OA02 could produce OA, the production was only 9.0 ± 0.7 mg/L. This might be due to the swinging growth burden caused by the introduced three plasmids, as evidenced by the result that the biomass of strains OA01-04 were no more than 5 g/L (Table 1). To solve this issue, *GgbAS*, *MtCYP716A12* and *MtCPR* were integrated into the genome of *S. cerevisiae*. Furthermore, *MtCYP716A12* and *MtCPR* were overexpressed under the promoter P_{GAL1} and P_{GAL10}, respectively. Although P_{GAL1} and P_{GAL10} are strong promoters in yeast, large portion of their inducer galactose were consumed through Leloir pathway that deeply reduced its utilization (Fig. S1). In addition, the price of galactose is approximately 20 times of glucose, so the dosage of galactose should be controlled to reduce the cost. To achieve this, galactokinase (*GAL1*) was knocked out to maintain the concentration of intracellular galactose, which can make galactose serves as an inducer rather than

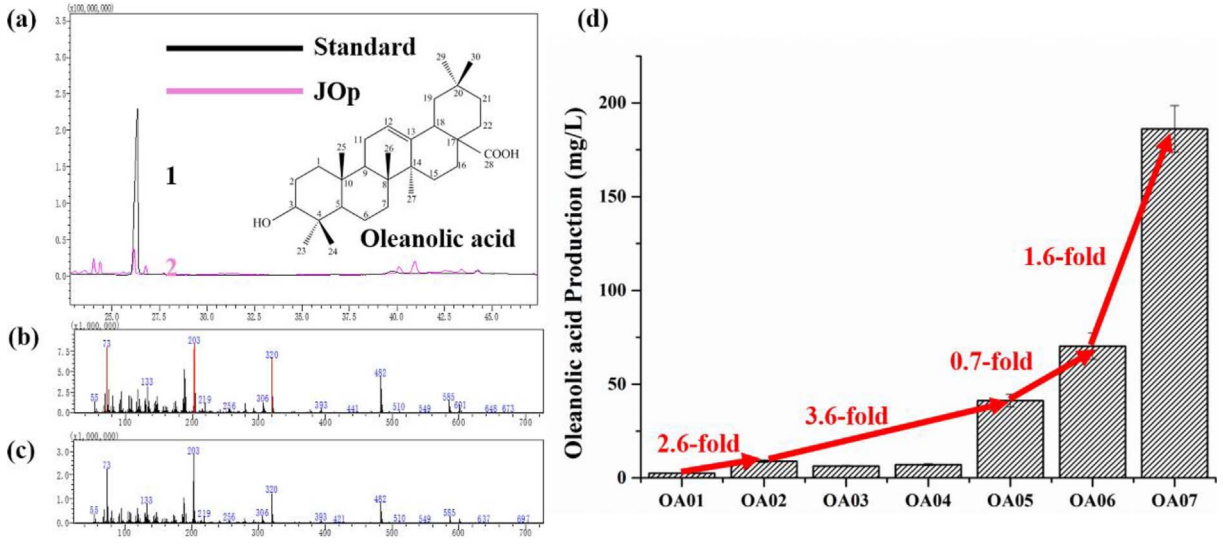

Fig. 2. GC-MS results and oleanolic acid (OA) production of engineered strains. (a) GC spectrogram of oleanolic acid. Peak-1 and peak-2 in the GC spectrogram corresponds to the authentic OA stranded and OA produced by OA01 strain; (b) MS spectrogram of the authentic OA; (c) Fragmentation patterns of OA produced by OA01 strain; (d) The OA production of strains OA01-07. The experiment was performed in triplicate measurements, and the errors stand for one standard deviation.

Table 1
Fermentation results of engineered strains.

Strains	Biomass (g/L)	Squalene (mg/L)	Ergosterol (mg/L)	Amyrin (mg/L)	Oleanolic acid (mg/L)
OA01	4.4 ± 0.4	1.0 ± 0.1	2.0 ± 0.3	0.6 ± 0.1	2.5 ± 0.2
OA02	4.4 ± 0.3	0.6 ± 0.0	2.2 ± 0.1	0.7 ± 0.1	9.0 ± 0.7
OA03	4.8 ± 0.2	0.7 ± 0.1	1.9 ± 0.1	0.6 ± 0.0	6.6 ± 0.6
OA04	4.5 ± 0.3	0.8 ± 0.0	2.8 ± 0.1	0.7 ± 0.0	7.1 ± 0.5
OA05	9.4 ± 0.8	ND	19.5 ± 2.4	2.1 ± 0.1	41.3 ± 3.4
OA06	9.0 ± 0.7	ND	25.3 ± 1.7	3.6 ± 0.5	70.3 ± 7.0
OA07	14.7 ± 0.5	ND	28.0 ± 1.9	11.6 ± 0.6	186.1 ± 12.4

ND, no detected. All the experiments were performed in triplicate measurements, and the errors stand for one standard deviation.

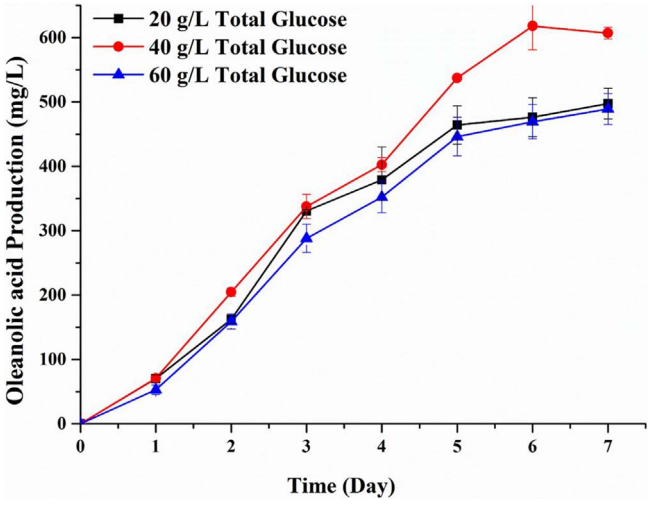

Fig. 3. Oleanolic acid production of engineered strain OA07 in 5 L bioreactor with different glucose concentration. The media used for fermentation was YPD composed of 20 g/L, 40 g/L and 60 g/L glucose, respectively. Fermentation was carried out at 30 °C with agitation speed of 400 rpm and airflow rate of 1–3 vvm. The pH was maintained at pH 5.0 by automatic addition of 5 M NH_4OH. The fermentation was performed in three measurements and the errors stand for one standard deviation.

the carbon source, thus improving OA production and reducing the costs.

Based on the above point, strain OA05 was constructed by inserting genes *GgbAS* (controlled by the *FBA1* promoter), *MtCYP716A12* (controlled by the *GAL1* promoter) and *MtCPR* (controlled by the *GAL10* promoter) into genome GAL1 site that not only relieved plasmid burden but also to reduce the industrial cost by knocking out *GAL1* gene. The biomass of strain OA05 was 9.38 ± 0.8 g/L with OA titer of 41.3 ± 3.4 mg/L which was 3.6-fold higher than OA02.

Furthermore, *GAL80* gene was knocked out to reconstruct galactose regulatory network. It has been reported the transcriptional level of GAL promoters is inhibited by glucose. GAL80 is a negative transcriptional regulator, which prevents the transcriptional activator Gal4 from binding to GAL promoters in response to galactose (Fig. S1). GAL80 has been proposed to switch the regulatory sugar from galactose to glucose. By knocking out gene *GAL80*, the genes under GAL promoters can express normally under glucose condition. To improve the utilization of GAL promoters, gene *GAL80* was knocked out from strain OA05 and the engineered strain without *GAL80* was named OA06, which produced OA up to 70.3 ± 7.0 mg/L, which was 0.7-fold higher than OA05.

3.4. Increasing precursors supply for efficient biosynthesis of OA

Precursors should be over supplied to further increase the production of OA. In *S. cerevisiae*, HMG-CoA reductase is the rate-limiting enzyme of MVA pathway, so the truncated *HMGR1* was overexpressed to increase the carbon flux through the MVA pathway. In addition, 2,3-oxidosqualene is a metabolic node of both endogenous ergosterol biosynthesis pathway and heterogeneous OA biosynthesis pathway. To improve OA production, squalene synthase (*ERG1* gene) which joins two farnesyl pyrophosphate moieties to form squalene and squalene epoxidase (*ERG9* gene) which catalyzes the epoxidation of squalene to 2,3-oxidosqualene were also overexpressed.

The genes *tHMGR1* (controlled by *TPI1* promoter), *ERG1* (controlled by *GAL1* promoter), *ERG9* (controlled by *GAL10* promoter) were integrated into the chromosome of OA06 at the *NTS* site to increase precursor supply for improving OA production. The resulting strain OA07 produced 186.1 ± 12.4 mg/L OA which was 1.6-fold higher than strain OA06.

3.5. Optimizing fermentation process for improving OA production

To explore the highest performance of strain OA07, batch fermentation was carried out in a 5 L fermentor containing 3 L medium. To find out the most appropriate initial glucose, different total glucose (20 g/L, 40 g/L and 60 g/L) in the medium have been tested. As shown in Fig. 3, the OA production was 606.9 ± 9.1 mg/L under 40 g/L glucose, which was approximately 2.3-fold higher than that in the flask shake, and 7.5-fold higher than the previously reported value.

4. Conclusion

In this work, plant-derived OA production in engineered *S. cerevisiae* has been effectively boosted via pairing the *MtCYP716A12* with *MtCPR* to optimize the oxidation-reduction system. In addition, reconstructing galactose regulatory network also improved the transcriptional level of key genes and enhanced OA production. Under these metabolic strategies, OA titer was increased up to 186.1 ± 12.4 mg/L in flask shake. Combined with fermentation optimization, the final OA production was 606.9 ± 9.1 mg/L with the yield of 16.0 ± 0.8 mg/g DCW which was increased over 7.5-fold compared to the maximum production reported.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.02.096.

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