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Efficient biosynthesis of antitumor ganoderic acid HLDOA using a dual tunable system for optimizing the expression of CYP5150L8 and a *Ganoderma* P450 reductase

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Abstract

Ganoderic acid 3-hydroxy-lanosta-8,24-dien-26-oic acid (GA-HLDOA), an antitumor triterpenoid from the traditional Chinese medicinal higher fungus *Ganoderma lucidum*, is considered as a key precursor for biosynthesizing other ganoderic acids (GAs) with superior antitumor activities. Our previous study identified CYP5150L8 from *G. lucidum* as a lanosterol oxidase, and achieved heterologous biosynthesis of GA-HLDOA in *Saccharomyces cerevisiae*. However, low production of GA-HLDOA in either *G. lucidum* or heterologous host hindered its further investigation and application. In this study, we constructed a dual tunable system for balancing the expression of CYP5150L8 and a *Ganoderma* P450 reductase iGLCPR, and performed a comprehensive optimization of CYP5150L8 expression, iGLCPR expression, and glycerol usage. Then, we investigated the fermentation behavior of the best strain in optimized condition in flask and achieved 154.45 mg/L GA-HLDOA production, which was 10.7-fold higher compared with previous report. This study may facilitate the wide-spread application of GA-HLDOA and the discovery of unknown cytochrome P450s in downstream GAs biosynthesis.

KEYWORDS
cytochrome P450 reductase, cytochrome P450s, ganoderic acid HLDOA, metabolic engineering, *Saccharomyces cerevisiae*

1 | INTRODUCTION

Ganoderic acids (GAs), the unique secondary metabolites of medicinal mushroom *Ganoderma lucidum*, are a group of highly oxidized lanostane-triterpenoids (Baby, Johnson, & Govindan, 2015). Because of their

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important biological activities such as antitumor and antimetastasis, GAs' bioproduction has attracted wide attention (Tang, Liu, Zhao, Wei, & Zhong, 2006; Zhao, Fan, Wang, Feng, & Li, 2018). Although strategies focused on fermentation process optimization of *G. lucidum* greatly improved GAs content over the past decades, bioproduction of GAs still cannot meet the requirements for the wide range of application (Xu & Zhong, 2010). Further increasing GAs' production in *G. lucidum* fermentation is of particular importance but challenging due to (a) the slow growth of *G. lucidum*, (b) the notoriously immature genetic manipulations of medicinal mushroom (Xiao & Zhong, 2016), and (c) the cryptic biosynthetic pathway of GAs (Xiao et al., 2019).

GAs are biosynthesized via mevalonate (MVA) pathway using lanosterol as the direct precursor (Shiao, 1992). However, the processes of converting lanosterol to GAs are unclear, in which the cytochrome P450s (CYPs) are heavily involved (Chen et al., 2012; Xiao & Zhong, 2016). In our recent work, using a *Saccharomyces cerevisiae* strain capable of hyperproducing lanosterol as heterologous host, a systematic screening of 72 CYPs from *G. lucidum* revealed that CYP5150L8 is able to catalyze a three-step oxidation of lanosterol at C-26 to ganoderic acid 3-hydroxy-lanosta-8,24-dien-26-oic acid (GA-HLDOA) at a titer of 14.5 mg/L (Wang, Xiao, & Zhong, 2018). GA-HLDOA (called as ganoderic acid Z previously) was first discovered in 1983 and proved to be toxic to hepatoma cells (O. Toth, Luu, & Ourisson, 1983) without any follow-up preclinical studies. In another aspect, GA-HLDOA was proposed as the substrate to biosynthesize other GAs with promising biological activities (Chen et al., 2012; Xiao & Zhong, 2016). Most importantly, along with our paradigm for GAs related CYPs discovery using synthetic biology approaches, construction of a recombinant *S. cerevisiae* capable of hyper-producing GA-HLDOA would facilitate the identification of key CYPs responsible for downstream GAs biosynthesis (Wang et al., 2018). Taken together, hyperproduction of GA-HLDOA is urgently required.

As a monooxygenase, CYP is able to catalyze a variety of chemical reactions (e.g., hydroxylation, demethylation, epoxidation), and usually requires an extra partner—cytochrome P450 reductase (CPR) to transfer electrons from NAD(P)H (Nebert, Wikvall, & Miller, 2013). CPR plays a significant role in supporting the cellular function of CYP. If CPR mediated insufficient electron transfer occurs (also known as uncoupling event), CYP catalyzed reaction could generate more byproducts, leading to leakage of reducing equivalents and affecting CYP activity in the end (Shakunthala, 2010). Therefore the optimal balance between CYP and CPR expression is crucial to maximize CYP activity. To overcome this dilemma, one way is to create CYP-CPR fusion protein (Renault, Bassard, Hamberger, & Werck Reichhart, 2014). Another promising alternative is to select a suitable CPR with high electron transfer efficiency towards CYP, where a CPR with the same origin as CYP is often preferred (Durairaj, Jung, Park, Kim, & Yun, 2015). Our previous work indicated a predicted CPR (*gl20687*) was unlikely to support the function of CYP5150L8 in *S. cerevisiae* (Wang et al., 2018). In addition, a constant supply of electrons is critical to increase the catalytic efficiency of CYP (Roitel, Scrutton, & Munro, 2003). Many approaches, such as overexpression of the NAD(P)H-producing glucose/formate dehydrogenase or isocitrate reductase (Keasling, 2012; Y. Li et al., 2018; Paddon et al., 2013) or using photochemical and light-driven cofactor-free reduction system (Cirino & Arnold, 2003; Park et al., 2015; Sadeghi, Fantuzzi, & Gilardi, 2011; Schwaneberg, Appel, Schmitt, & Schmid, 2000), have been reported to increase the electron supply for CYP catalyzed reaction. However, their feasibility in industrial production remains to be confirmed. Adopting glycerol as carbon source in fermentation is considered as a simple but effective solution (Y. Li et al., 2018). Possible reasons include (a) NAD(P)H produced during glycerol metabolism (Xue, Chen, & Jiang, 2017), (b) improved stability of cell membrane proteins including CYPs mediated by glycerol (Bolen, 2001; Gekko & Timasheff, 1981), and (c) the correct folding of nascent polypeptides promoted by glycerol (Meng, Park, & Zhou, 2001).

In this study, we first attempted to improve GA-HLDOA production by testing activities of CYP5150L8 homologs, but failed to find a better substitute. Then we achieved 10.2-fold improvement of GA-HLDOA production by simultaneous overexpression of CYP5150L8 and a CPR from *G. lucidum* (iGLCPR) using plasmid with tunable copy number. To further increase the GA-HLDOA titer, we constructed a dual tunable system for balancing the expression of CYP5150L8 and iGLCPR, and performed a comprehensive optimization of CYP5150L8 expression, iGLCPR expression, and glycerol usage. A 17.8-fold improvement of GA-HLDOA production in 24-well plate was finally obtained. At last, we investigated the fermentation behavior of our best strain in the optimized condition in the flask and achieved 154.45 mg/L GA-HLDOA production.

2 | MATERIALS AND METHODS

2.1 | Yeast cultivation

The original strain *S. cerevisiae* YL-T3 (Wang et al., 2018) and its derivative strains were grown in SC-His, SC-His-Ura, SC-His-Ura-Leu (Dai, Liu, Huang, & Zhang, 2012), or YPD medium (containing 10 g/L of yeast extract, 20 g/L of beef peptone and 20 g/L glucose) with appropriate concentration of glycerol, G418 and/or hygromycin at 30°C.

2.2 | Construction of plasmids and strains

Strains and plasmids, and primers used in this study are listed in Tables 1 and S1, respectively. CYP5150L8 expression cassette was amplified from plasmid pRS426-HXT7p-CYP5150L8-FBA1t (Wang et al., 2018). The identified *G. lucidum* CPR-iGLCPR (*gl19526*) was amplified from complementary DNA (cDNA) of *G. lucidum* 260125 (Chen et al., 2012). The sequences of CYP5150L8-S1 (PIL33210.1) from *Ganoderma sinense* and CYP5150L8-S2 (XP_008037115.1) from *Trametes versicolor* were codon optimized for yeast expression. The G418-resistance gene (*KanMX*) and hygromycin-resistance gene (*hygromycin B*) were amplified from plasmid pUG6 (Güldener, Heck, Fielder, Beinhauer, & Hegemann, 1996) and pPS41H (Taxis & Knop, 2006), respectively. A truncated Ura3 promoter was adopted to drive the transcription of antibiotic genes (Bao et al., 2015). iGLCPR was amplified with primer pair CPR-F/CPR-R from cDNA of *G. lucidum* 260125. Plasmid pRS425-TEF1p-PGK1t (Wang et al., 2018) was linearized by *Pme*I (NEB, Beijing, China). The amplified iGLCPR fragment and linearized pRS425-TEF1p-PGK1t were ligated to produce plasmid pRS425-iGLCPR according to the procedure described in SoSoo

cloning kit (Tsingke, Beijing, China). C*yp5150l8-s1* and *cyp5150l8-s2* were synthesized and ligated to linearized plasmids pRS426-HXT7p-FBA1t to obtain plasmids pRS426-HXT7p-CYP5150L8-S1-FBA1t and pRS426-HXT7p-CYP5150L8-S2-FBA1t respectively by Ruimianbio Company (Shanghai, China). The B*amH*I linearized plasmid pRS426-HXT7p-FBA1t and *KanMX* expression cassette (amplified with primer pair tpKanMX-F and KanMX-R) were assembled to produce plasmid pRS426-HXT7p-FBA1t-G418r by DNA assembler (Shao, Zhao, & Zhao, 2009). The P*mel* linearized plasmid pRS426-HXT7p-FBA1t-G418r and *cyp5150l8* expression cassette (amplified with primer pair CYP5150L8-F and CYP5150L8-R) was ligated to produce plasmid pRS426-CYP5150L8-G418r according to the procedure described in SoSoo cloning kit. The *hygromycin B* sequence was first amplified with primer pair tpHyg-F/Hyg-R. The N*arI* linearized plasmid pRS425-iGLCPR and *hygromycin B* expression cassette, which was amplified with primer pair Hyg-F and Hyg-R using *hygromycin B* sequence as template, were further assembled to produce plasmid pRS425-iGLCPR-Hygr by DNA assembler. Similarly, the E*coR*I linearized plasmid pRS426-CYP5150L8-G418r and iGLCPR expression cassette, which was amplified with primer pair CPRp-F / CPRt-R using plasmid pRS425-iGLCPR as template, were assembled to produce plasmid pRS426-CYP5150L8-iGLCPR-G418r by DNA assembler. These plasmids were transformed into YL-T3 to generate corresponding engineered yeast strains (Table 1) using standard lithium acetate protocol (Gietz & Schiestl, 2007).

2.3 | Fermentation

For seed culture, strains were grown in SC-His-Ura or SC-His-Ura-Leu when appropriate at 30°C and 220 rpm to an OD_{600} of 1–2.5. For 24-well plate fermentation, the seeds were inoculated into 24-well plate with an initial OD_{600} of 0.05 and then grew at 30°C and 850 rpm with a humidity of 90% for 5 days. Each well contained 3 ml of YPD medium with appropriate concentration of glycerol, G418 and/or hygromycin. After 5 days of fermentation, samples were taken for

TABLE 1 Plasmids and strains used in this study

Plasmids and strains	Description	Source
Plasmids		
pRS41H	Plasmid containing *hygromycin B*	Taxis and Knop (2006)
pUG6	Plasmid containing *KanMX*	Güldener et al. (1996)
pRS426-HXT7p-CYP5150L8-FBA1t	Plasmid containing *cyp5150l8*	Wang et al. (2018)
pRS425-TEF1p-PGK1t	Helper plasmid	Wang et al. (2018)
pRS426-HXT7p-FBA1t	Helper plasmid	Wang et al. (2018)
pRS425-iGLCPR	Plasmid containing *iglcpr* expression cassette	This study
pRS426-HXT7p-CYP5150L8-S1-FBA1t	Plasmid containing *cyp5150l8-s1* expression cassette	This study
pRS426-HXT7p-CYP5150L8-S2-FBA1t	Plasmid containing *cyp5150l8-s2* expression cassette	This study
pRS426-HXT7p-FBA1t-G418r	A truncated *ura3* promoter was adopted for driving the expression of *KanMX*	This study
pRS426-CYP5150L8-G418r	Plasmid containing *cyp5150l8* and *KanMX* expression cassettes	This study
pRS425-iGLCPR-Hygr	Plasmid containing *iglcpr* expression cassette and a truncated *ura3* promoter was adopted for driving the expression of *hygromycin B*	This study
pRS426-CYP5150L8-iGLCPR-G418r	Plasmid containing *cyp5150l8*, *iglcpr* and *KanMX* expression cassettes	This study
Strains		
YL-T3	BY4742, *Δtrp1*, δDNA:: P_{PGK1}-tHMG1-T_{ADH1}-P_{TEF1}-LYS2-T_{CYC1}, TRP:: HIS-P_{PGK1}-ERG20-T_{ADH1}-P_{TEF1}-ERG9-T_{CYC1}-P_{TDH3}-ERG1-T_{TPL1}	Wang et al. (2018)
CYP5150L8	YL-T3 harboring plasmid pRS426-HXT7p-CYP5150L8-FBA1t	This study
CYP5150L8-S1	YL-T3 harboring plasmid pRS426-HXT7p-CYP5150L8-S1-FBA1t	This study
CYP5150L8-S2	YL-T3 harboring plasmid pRS426-HXT7p-CYP5150L8-S2-FBA1t	This study
CYP5150L8-iGLCPR	YL-T3 harboring plasmids pRS426-HXT7p-CYP5150L8-FBA1t and pRS425-iGLCPR	This study
CYP5150L8-S1-iGLCPR	YL-T3 harboring plasmids pRS426-HXT7p-CYP5150L8-S1-FBA1t and pRS425-iGLCPR	This study
CYP5150L8-S2-iGLCPR	YL-T3 harboring plasmids pRS426-HXT7p-CYP5150L8-S2-FBA1t and pRS425-iGLCPR	This study
CYP5150L8-r-iGLCPR	YL-T3 harboring plasmids pRS426-CYP5150L8-G418r and pRS425-iGLCPR	This study
CYP5150L8-iGLCPR-r	YL-T3 harboring plasmid pRS426-CYP5150L8-iGLCPR-G418r	This study
CYP5150L8-r-iGLCPR-r	YL-T3 harboring plasmids pRS426-CYP5150L8-G418r and pRS425-iGLCPR-Hygr	This study

analysis. The results represented the means ± standard deviation (*SD*) of three independent samples. For flask fermentation, the seeds were inoculated into 500 ml flasks containing 50 ml of YPD medium with appropriate concentration of glycerol, G418 and/or hygromycin with an initial OD_{600} of 0.05 and then grew at 30°C and 220 rpm. Samples were taken every day during 6 days of fermentation. The results represented the means ± *SD* of five independent samples.

2.4 | Determination of the plasmid copy number

Quantitative polymerase chain reaction (qPCR) was performed to determine the plasmid copy numbers using yeast total DNA extracts. To collect the yeast total DNA extracts, strain CYP5150L8-r-iGLCPR-r were grown in YPD medium supplemented with 4% glycerol and different concentrations of G418 and hygromycin. 0.5 ml of mid-log phase yeast cells in 24-well plate were harvested, washed by 1 ml of sterilized water, and resuspended in 250 μl of YP1 buffer from TIANprep Yeast Plasmid DNA Kit (TIANGEN, Beijing, China) containing 0.1 g of ziroconia/silica beads (0.5 mm diameter) (BioSpec Products, Bartlesville). Samples were vortexed for 40 min and extracted three times in 200 μl of hydroxybenzene-chloroform-isoamyl alcohol (25:24:1) at 13,500*g* for 10 min. The obtained supernatant was precipitated with equal volume of isopropanol. The tubes were placed at −20°C for 30 min and centrifuged at 4°C, 13,500*g* for 5 min. Then, the resultant precipitation was washed twice by 200 μl of 75% ethanol, kept at 60°C for 5 min, and redissolved in 100 μl of sterilized water for qPCR analysis. *Cyp5150l8* on the plasmid pRS426-CYP5150L8-G418r, *iglcpr* on the plasmid pRS425-iGLCPR-Hygr and *erg2* on the chromosome were selected as the target and reference genes, respectively. Primer pairs of qCYP5150L8-F and qCYP5150L8-R, qCPR-F and qCPR-R, qERG2-F and qERG2-R were designed for gene *cyp5150l8*, *iglcpr* and *erg2* in qPCR reactions, respectively (Table S1). Copy numbers were quantified as previously reported (Lian, Jin, & Zhao, 2016). SYBR® Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) on a Roche Light Cycler® 480 II (Roche, Rotkreuz, Switzerland) was used for qPCR analysis.

2.5 | Analyses of cell growth, glucose, glycerol, pH, and multiple metabolites

Yeast cell growth was determined by measuring absorbance at 600 nm (OD_{600}) using V-1000 spectrophotometer (AOE, Shanghai, China). The broth pH was determined with FiveEasy Plus FE20 pH meter (Mettler Toledo, Shanghai, China). After centrifugation and filtered through 0.22 μm PES syringe filter, the broth was used to determine residual glucose, glycerol, medium ethanol, and acetate using an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) equipped with a refractive index detector (RID) and an Aminex HPX-87H column (300 mm × 7.8 mm, BIO-RAD, California) at 55°C. These products were detected in 30 min by adopting 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. For precursors (squalene, 2,3-oxidosqualene, and lanosterol) and product GA-HLDOA extraction, 3 ml of fermentation broth was centrifuged and the cell pellet was mixed with an equivalent volume of methanol and incubated at 30°C and 250 rpm for 30 min. After centrifugation at 15,600*g* for 3 min, the supernatant was collected. After performing the above protocols for three times, the collections were combined and concentrated in vacuo using CentriVap concentrator (Labconco, Kansas City, MO) or RVC2-25CD plus concentrator (Marin Christ, Osterode, Germany). Then the dry products were redissolved in methanol and determined by Agilent 1260 Infinity II HPLC system (Agilent, Waldbronn, Germany) equipped with a Diode array detector (DAD) at 214 nm and Kinetex Biphenyl column (2.6 μm, 150 mm × 4.6 mm, Phenomenex, Torrance, CA). Mobile phase B contained methanol/acetic acid (100:0.1 vol/vol) and mobile phase A was 100% water. The column was eluted at a flow rate of 0.5 ml/min sequentially by a linear gradient of 80–100% B in 30 min, 100% B for 5 min, 100–80% B in 1 min and 80% B for 9 min.

3 | RESULTS AND DISCUSSION

3.1 | Overexpression of CYP5150L8 homologs or iGLCPR did not enhance GA-HLDOA production

In our previous work, CYP5150L8 was identified as a lanosterol oxidase responsible for the biosynthesis of GA-HLDOA (Wang et al., 2018; Figure 1). To obtain novel CYPs with higher catalytic efficiency for GA-HLDOA biosynthesis, an online blast search of Genbank was carried out using the amino acid sequence of CYP5150L8 as the query sequence. Two homologs of CYP5150L8, PIL33210.1 from *G. sinense* and XP_008037115.1 from *Trametes versicolor*, which exhibited 96% and 77% amino acid sequence similarity with CYP5150L8, were named as CYP5150L8-S1 and CYP5150L8-S2, respectively, and overexpressed in *S. cerevisiae* YL-T3 (Wang et al., 2018). After 120 hr fermentation, production of GA-HLDOA was hardly detected in the cell lysate of strain CYP5150L8-S1. Although GA-HLDOA (0.65 mg/L) was produced from strain CYP5150L8-S2, it was still much lower than that from strain CYP5150L8 (9.78 mg/L; Figure S1A). Meanwhile, the accumulation of lanosterol in CYP5150L8-S2 was much higher than that of CYP5150L8 (10.10 vs. 1.72 mg/L; Figure S1B). The results indicated that these CYP5150L8 homologs did not exhibit higher catalytic activity than CYP5150L8 regarding to biosynthesis of GA-HLDOA.

With high amino acid sequence similarity to CYP5150L8 (especially 96% similarity between CYP5150L8 and CYP5150L8-S1), none of the two novel CYPs could effectively convert lanosterol to GA-HLDOA (Figure S1), indicating the different amino acid residues among three CYPs may be critical to oxidase activity against lanosterol. CYP has a conserved core structure but variable regions associated with substrate recognition and binding (Ozols, Heinemann, & Johnson, 1985; Peterson & Graham, 1998), which include six substrate recognition sites (SRS1-6; Gotoh, 1992). A careful comparison of SRS sites in CYP5150L8, CYP5150L8-S1, and CYP5150L8-S2 reveals that Tyr_{106} and Asp_{107} in SRS1, Val_{214} in SRS2, Met_{404} in SRS5, Phe_{518} and Thr_{521} in SRS6 might have significant impact on lanosterol oxidase activity. Although we failed to discover a novel CYP with superior lanosterol oxidase activity than

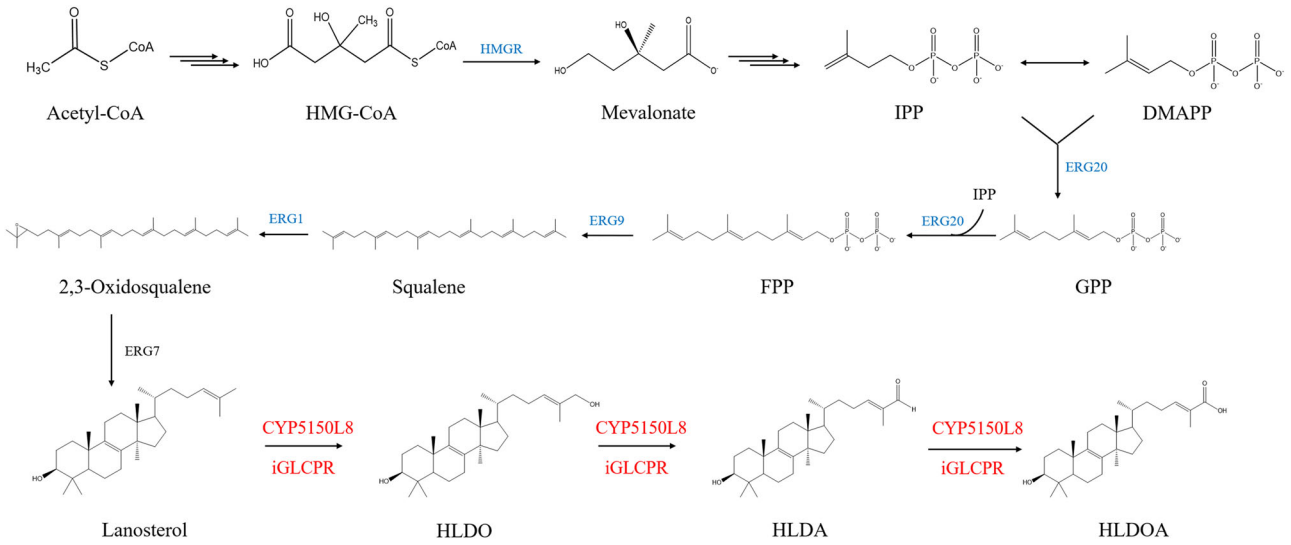


FIGURE 1 Biosynthetic pathway of ganoderic acid HLDOA (GA-HLDOA) in *Saccharomyces cerevisiae* . DMAPP, dimethylallyl diphosphate; ERG20, farnesyl diphosphate synthase; ERG9, squalene synthase; ERG1, squalene epoxidase; ERG7, lanosterol synthase; FPP, farnesyl diphosphate; HLDA, 3-hydroxy-lanosta-8,24-dien-26-al; HLDO, 3-hydroxy-lanosta-8,24-dien-26-ol; HLDOA, 3-hydroxy-lanosta-8,24-dien-26-oic acid; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenylisopentenyl diphosphate. Four genes overexpressed in the starting strain *S. cerevisiae* YL-T3 were highlighted in blue [Color figure can be viewed at wileyonlinelibrary.com]

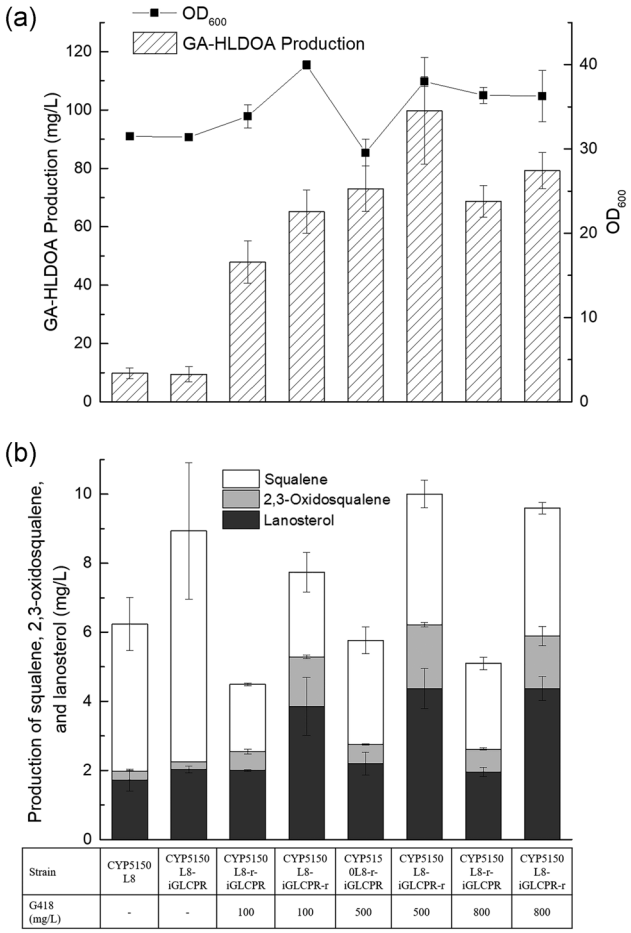
CYP5150L8, these amino acid residuals may be considered as potential targets for rational design or directed evolution of CYP5150L8 with improved lanosterol oxidase activity to synthesize GA-HLDOA in the future study (Figure S2).

In contrast, our previous work indicated that *gl20687* may not be a suitable partner of CYP5150L8 (Wang et al., 2018). To find a native CPR to better support the catalytic function of CYP5150L8, a careful reanalysis of the genomic and transcriptomic data of *G. lucidum* (Chen et al., 2012) was conducted and *gl19526* was identify as iGLCPR (Figure S3). It should be noted that another *G. lucidum* CPR, which harbored one mutation N32K as compared to iGLCPR, was biochemically characterized as a CYP reductase to support the activity of a *G. lucidum* CYP512U6 in a recent work (Yang et al., 2018). To test whether iGLCPR is a better partner of CYP5150L8, iGLCPR expression plasmid was cotransformed with CYP5150L8 expression plasmid into YL-T3 to generate strain CYP5150L8-iGLCPR. However, compared to strain CYP5150L8 with overexpression of CYP5150L8 alone, production of GA-HLDOA by CYP5150L8-iGLCPR was not improved (9.45 mg/L vs 9.78 mg/L; Figure 2a).

3.2 | Change the expression of CYP5150L8 and iGLCPR greatly affected GA-HLDOA production

To explore whether the production of GA-HLDOA can be improved via further increasing the expression of CYP5150L8, plasmid pRS426-CYP5150L8-G418r with tunable copy numbers was constructed. Previous studies showed that when *ura3* was poorly expressed driven by a truncated *ura3* promoter, cells had to maintain a higher copy number (~200 copies/cell) than that of our currently adopted 2μ–based plasmid (10–40 copies per cell) to survive when uracil was absent in the culture medium (Bao et al., 2015). To endow a plasmid with tunable copy numbers, the truncated *ura3* promoter, a 46 bp sequence upstream from the open reading frame of *ura3*, was adopted for driving the expression of *KanMX*, and stimulated expression of target protein was observed with increased G418 concentration (Lian et al., 2016). Hereby, we constructed plasmid pRS426-CYP5150L8-G418r for tunable expression of CYP5150L8. In addition, iGLCPR was cloned into pRS425 to minimize the potential impact of G418 concentration to the greatest extent possible. Plasmids pRS426-CYP5150L8-G418r and pRS425-iGLCPR were cotransformed into YL-T3 to yield strain CYP5150L8-r-iGLCPR. Alternatively, the expression cassettes of CYP5150L8 and iGLCPR were assembled into one plasmid pRS426-CYP5150L8-iGLCPR-G418r with tunable copy numbers and transformed to YL-T3 to yield strain CYP5150L8-iGLCPR-r, where the expression of CYP5150L8 and iGLCPR could be coregulated by changing the concentrations of G418 in the medium.

When 100 mg/L of G418 was added, 5-fold improvement of GA-HLDOA production was achieved by strain CYP5150L8-r-iGLCPR compared to strain CYP5150L8-iGLCPR in fermentation medium without the addition of G418 (47.90 vs. 9.45 mg/L). And another 1.5-fold improvement of GA-HLDOA production by strain CYP5150L8-r-iGLCPR was observed when G418 concentration was raised to 500 mg/L (73.01 vs. 47.90 mg/L; Figure 2a). However, when G418 was further increased to 800 mg/L, no obvious improvement of GA-HLDOA production was detected in CYP5150L8-r-iGLCPR compared with that in the presence of 500 mg/L (68.72 vs. 73.01 mg/L; Figure 2a), implying that only a suitable enhancement of CYP5150L8 expression would improve GA-HLDOA production. Additionally, a reduced accumulation of squalene but increased accumulation of 2,3-oxidosqualene in strain CYP5150L8-r-iGLCPR were detected no matter how much G418 was added, as compared to those precursors



Strain	CYP5150 L8	CYP5150 L8-iGLCPR	CYP5150 L8-r-iGLCPR	CYP5150 L8-iGLCPR-r	CYP515 0L8-r-iGLCPR	CYP5150 L8-iGLCPR-r	CYP5150 L8-r-iGLCPR	CYP5150 L8-iGLCPR-r
G418 (mg/L)	-	-	100	100	500	500	800	800

FIGURE 2 A suitable expression of CYP5150L8 and iGLCPR promoted GA-HLDOA production. (a) Cell growth, GA-HLDOA production and (b) the concentrations of precursors after 120 hr fermentation of different engineered yeasts. The error bars represent the standard deviation of three biological replicates

accumulated by strain CYP5150L8-iGLCPR in fermentation medium without G418 (Figure 2b). In contrast, among all the test conditions, accumulation of lanosterol in strain CYP5150L8-r-iGLCPR and CYP5150L8-iGLCPR were kept at the similar level (from 1.95 to 2.19 mg/L; Figure 2b).

When the expression of CYP5150L8 and iGLCPR were coregulated in the presence of 100 mg/L G418, GA-HLDOA production by strain CYP5150L8-iGLCPR-r was 1.4-fold higher than that of strain CYP5150L8-r-iGLCPR, in which only CYP5150L8 expression was elevated (65.16 vs. 47.90 mg/L; Figure 2a). However, when G418 concentration were further increased to 500 and 800 mg/L, no significant increase of GA-HLDOA production were observed by strain CYP5150L8-iGLCPR-r as compared with those produced by strain CYP5150L8-r-iGLCPR (99.68 ± 18.30 vs. 73.01 ± 7.78 mg/L, 79.20 ± 4.37 vs. 68.72 ± 5.36 mg/L; Figure 2a). These results indicated enhancing the expression of iGLCPR could improve GA-HLDOA production, but excessive iGLCPR expression may not benefit GA-HLDOA production. Interestingly, no matter how much G418 was adopted, increased accumulation of lanosterol, 2,3-oxidosqualaene and squalene were all observed in strain CYP5150L8-iGLCPR-r as compared with those produced by strain CYP5150L8-r-iGLCPR (Figure 2b).

Our previous study implied yeast endogenous CPR would only partly support the function of CYP5150L8 (Wang et al., 2018). It is reasonable that overexpression of iGLCPR stimulated GA-HLDOA production only when CYP5150L8 expression was increased, suggesting yeast endogenous CPR may be not enough to support CYP5150L8 to synthesize GA-HLDOA under such conditions (Figure 2). In the subsequent investigation, we also noticed that excess iGLCPR dramatically reduced GA-HLDOA no matter how much CYP5150L8 was overexpressed (Figure 3), suggesting iGLCPR expression may be the most important factor to the efficient production of GA-HLDOA. CPR and CYP are known to form 1:1 functional complex to fulfill enzyme function (Backes & Kelley, 2003). However, CPR was reported to produce diffusible reduced oxygen species (DROS) as well as deplete peroxide by itself, exhibiting a potential hazardous process and strongly affecting CYP kinetics (Manoj, Gade, & Mathew, 2010). In future, suppressing both DROS generation and equivalents consumption of CPR could be an interesting topic in terms of developing a novel CPR working specifically for target CYP.

3.3 | Construction of a dual tunable system for optimizing the expression of CYP5150L8 and iGLCPR to further strengthen GA-HLDOA production

Since the balance of CYP5150L8 and iGLCPR expression may play vital role to GA-HLDOA production (Figure 2b), a dual tunable system was constructed for individually regulating the expression of CYP5150L8 and iGLCPR. To construct another plasmid for separate regulation of iGLCPR expression, the truncated *ura3* promoter was adopted for driving the expression of hygromycin-resistance gene (*hygromycin B*), and assembled to yield plasmid pRS425-iGLCPR-Hygr. This plasmid, together with pRS426-CYP5150L8-G418r, were co-transformed into YL-T3 to generate strain CYP5150L8-r-iGLCPR-r, which allows regulating the expression of CYP5150L8 and iGLCPR by different concentrations of G418 and hygromycin, respectively. In addition, adding glycerol in fermentation is also considered to promote the expression of CYP and CPR, as well as to facilitate potential NADPH regeneration (Y. Li et al., 2018). Because it is very hard to predict the optimal CYP5150L8 and iGLCPR expression under different glycerol concentration, a comprehensive optimization among CYP5150L8 expression, iGLCPR expression, and glycerol concentration was accordingly performed.

By testing different combinations of G418 and hygromycin concentrations, we observed a dramatic drop in GA-HLDOA production with increased hygromycin concentrations (Figure 3a). When G418 concentration was kept at 300 mg/L and 2% (wt/vol) glycerol was added, GA-HLDOA production from strain CYP5150L8-r-iGLCPR-r was 107.93, 70.27, 19.43 and 14.98 mg/L when 300, 500, 800, and 1,000 mg/L of hygromycin was adopted. Similar trends of GA-HLDOA production were also observed when G418 concentration was kept at 500 and 800 mg/L with increased concentrations of hygromycin and at different glycerol

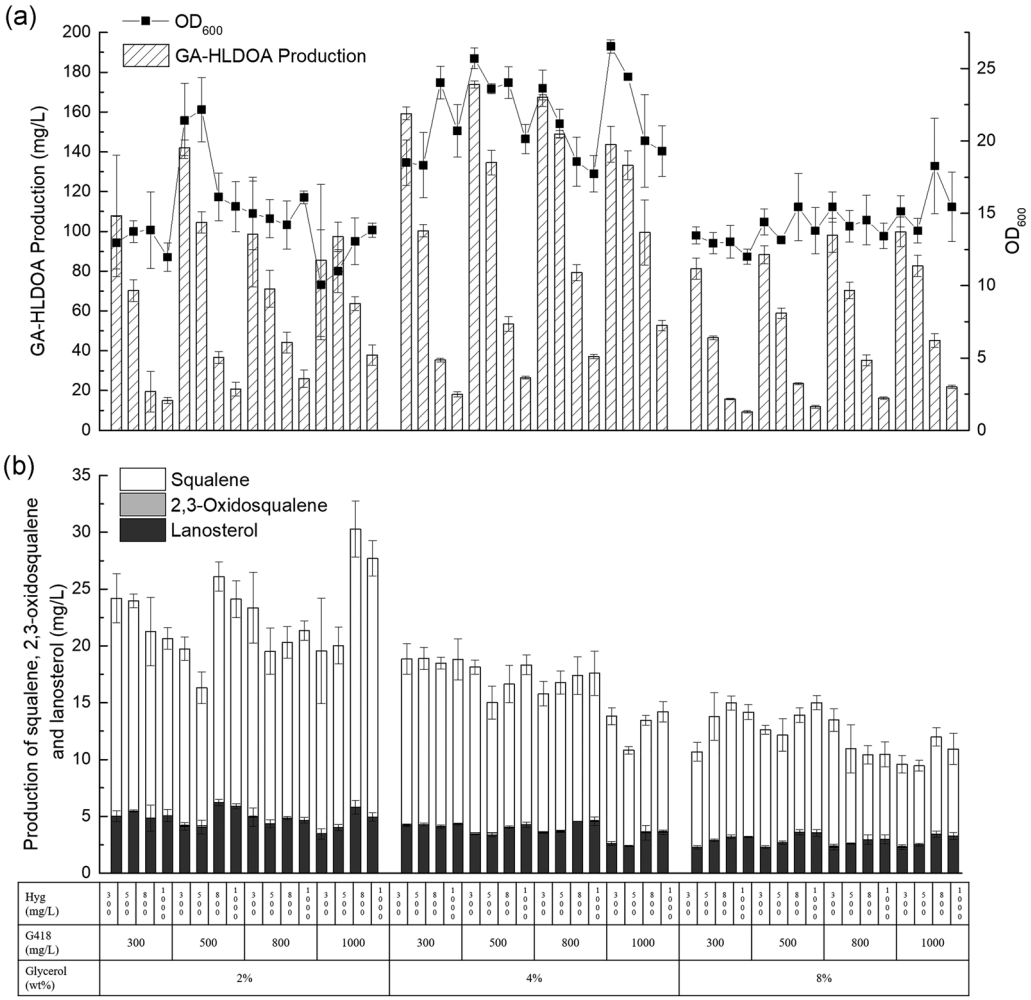


FIGURE 3 Modulation of CYP5150L8 and iGLCPR expression via plasmids with tunable copy numbers under different concentrations of glycerol, G418 and hygromycin. (a) Cell growth, GA-HLDOA production and (b) the concentrations of precursors after 120 hr fermentation of strain CYP5150L8-r-iGLCPR-r. The error bars represent the standard deviation of three biological replicates

concentrations (4% and 8% (wt/vol)), respectively (Figure 3a). In contrast, GA-HLDOA production have relatively small variation when CYP5150L8 expression was changed by using different amount of G418. For example, when hygromycin was kept at 300 mg/L and 2% glycerol was added, GA-HLDOA production from strain CYP5150L8-r-iGLCPR-r was 107.93, 141.96, 98.60, and 85.41 mg/L when 300, 500, 800, and 1,000 mg/L of G418 was adopted (Figure 3a). Furthermore, with a fixed concentration of G418 and hygromycin, addition of 4% glycerol was always the best condition for GA-HLDOA production as compared to those in the presence of 2% or 8% glycerol (Figure 3a). Concurrently with reduced GA-HLDOA production, reduced cell growth was also observed when 8% glycerol was added, as compared to those when 4% glycerol was added (Figure 3a), the reason of which remains unclear and requires further investigation. As a result, the optimal condition for GA-HLDOA production was 500 mg/L G418, 300 mg/L hygromycin and 4% glycerol, reaching 173.79 mg/L of GA-HLDOA after 120 hr fermentation (Figure 3a). In contrast to significant changes on GA-HLDOA production detected among all the tested conditions (from 9.31 to 173.79 mg/L), implementing the dual tunable system and glycerol addition did not obviously change the accumulation of precursors. The accumulation range of squalene, 2,3-oxidosqualene and lanosterol were 6.94–24.49, 0–0.18, 2.26–6.22 mg/L, respectively (Figure 3b).

Impaired cell growth was observed particularly when the dual tunable system was adopted (Figure 3). As for production of other triterpenoids via metabolic engineering strategies, cell growth retardation was also observed in many engineered *S. cerevisiae* strains (J. Li & Zhang, 2015; Madsen et al., 2011; Qiao et al., 2019; Zhang et al., 2015; Zhu et al., 2018). Possible explanations are (a) metabolic burden arising from expression of multiple copies of CYP5150L8 and iGLCPR, (b) GA-HLDOA toxicity, (c) the uncoupling events, or (d) the supply of the inadequate equivalent as mentioned above, which may need comprehensive host engineering. Harnessing the great potential of genome-scale engineering to develop a robust cell factory with an adaptive cell growth and GA-HLDOA biosynthesis is a promising alternative, where a specific high-throughput screening or selection method is usually required (Bao et al., 2018; Garst et al., 2017; Liang et al., 2017).

Next, the fold changes of copy numbers of CYP5150L8 and iGLCPR were determined in strain CYP5150L8-r-iGLCPR-r with different concentrations of G418 and hygromycin as compared with that without the presence of G418 and hygromycin. Under all the conditions tested, the copy numbers of CYP5150L8 and iGLCPR, as well as the GA-HLDOA production were remarkably increased once G418 and hygromycin were added (Figure 4). The fold changes of CYP5150L8 copy numbers were 3.70-, 4.00-, and 5.57-fold in 300, 500, and 800 mg/L of G418, and the corresponding fold changes of iGLCPR copy numbers were all at the similar level in 300 mg/L of hygromycin (Figure 4a,b). In contrast, although G418 concentrations were kept at 500 mg/L, the copy numbers of CYP5150L8 was much higher in 800 mg/L of hygromycin than those in 300 and 500 mg/L of hygromycin (Figure 4a). Surprisingly, when hygromycin concentration was increased from 500 to 800 mg/L, the fold change of iGLCPR copy numbers was decreased from 2.96- to 2.21-fold (Figure 4b). These results implied that G418 and hygromycin concentration mediated the changes of plasmid copy numbers in the dual tunable system may not follow the completely independent pattern, which was in line with a previous study (Lian et al., 2016). In addition, increased fold change of CYP5150L8 copy numbers (from 3.70-fold in 300 mg/L of G418 to 5.57-fold in 800 mg/L of G418) did not benefit GA-HLDOA production when 300 mg/L of hygromycin was adopted (Figure 4a–c). Further, significantly reduced GA-HLDOA production was observed with increased fold change of CYP5150L8 copy numbers (from 5.69- to 10.31-fold in 500 mg/L of G418) and decreased fold change of iGLCPR copy numbers (from 2.96-fold in 500 mg/L of hygromycin to 2.21-fold in 800 mg/L of hygromycin; Figure 4a–c), indicating the iGLCPR expression was not sufficient to support the catalytic function of CYP5150L8.

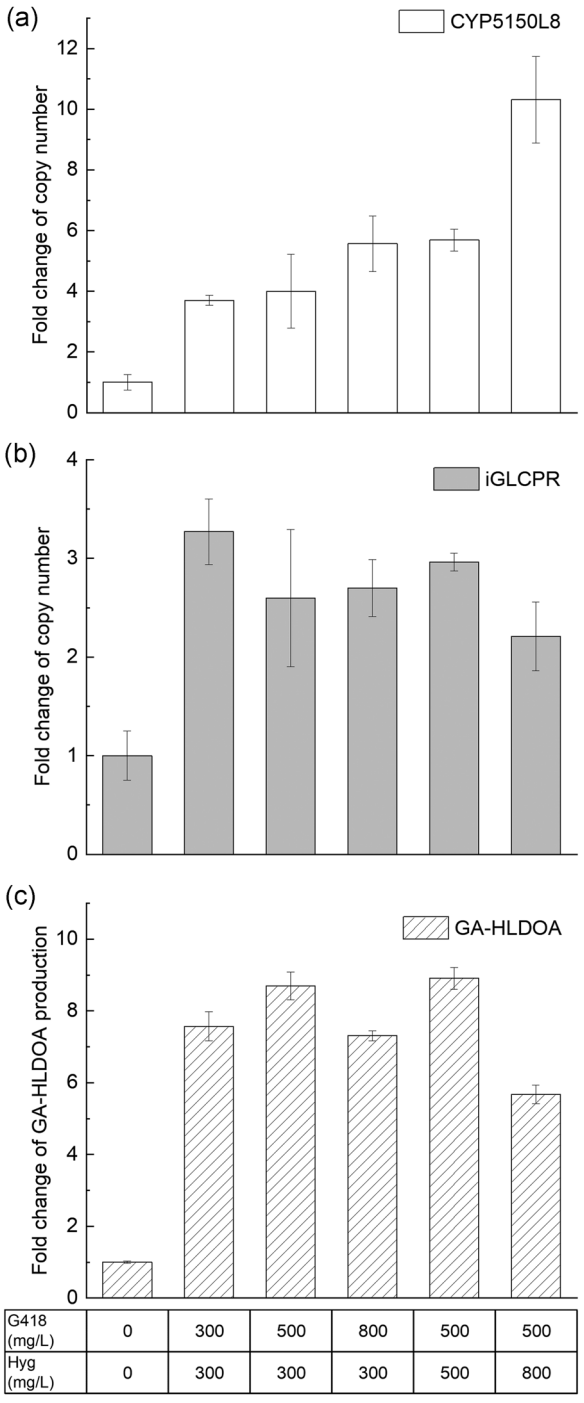


FIGURE 4 Fold changes of (a) CYP5150L8 copy number, (b) iGLCPR copy number, and (c) GA-HLDOA production of strain CYP5150L8-r-iGLCPR-r with different concentrations of G418 and hygromycin as compared to that without the presence of G418 and hygromycin. The error bars represent the standard deviation of three biological replicates

3.4 | Production of GA-HLDOA with the optimal condition in shake flask

After obtaining the optimal condition, we set out to investigate fermentation behavior of strain CYP5150L8-r-iGLCPR-r in shake flask in YPD medium containing 4% glycerol, 300 mg/L hygromycin, and 500 mg/L G418. Both cell growth and GA-HLDOA accumulation exhibited similar trends, increasing rapidly after 24 hr fermentation (Figure 5a). There was an obvious 24 hr lag phase when almost no cell growth and carbon source consumption was observed (Figure 5a,c). Then, 20 g/L glucose was rapidly consumed within the first 48 hr, while only small amount of glycerol (~4 g/L) was consumed after 144 hr (Figure 5c). Meanwhile, we also noticed the production and simultaneous consumption of acetic acid and ethanol between 24 hr and 96 hr (Figure 5d). The pH of the fermentation medium was around 6.1 before 96 hr and slightly increased to 6.5 after 96 hr (Figure 5c). Interestingly, lanosterol production was not detectable from 72 to 96 hr, and was accumulated from 96 to 144 hr, when only a small increase of GA-HLDOA was detected (Figure 5a,b). These results suggested a slow conversion of lanosterol to GA-HLDOA in late stage of fermentation. In addition, increasing accumulation of upstream intermediate squalene was observed during the whole fermentation process, reaching to 11.74 mg/L after 144 hr (Figure 5b). Finally, the production of 154.45 mg/L GA-HLDOA was achieved, which was 11% lower than that in 24-well plates but 10.65-fold higher than our previous report (Figures 3a and 5a; Wang et al., 2018).

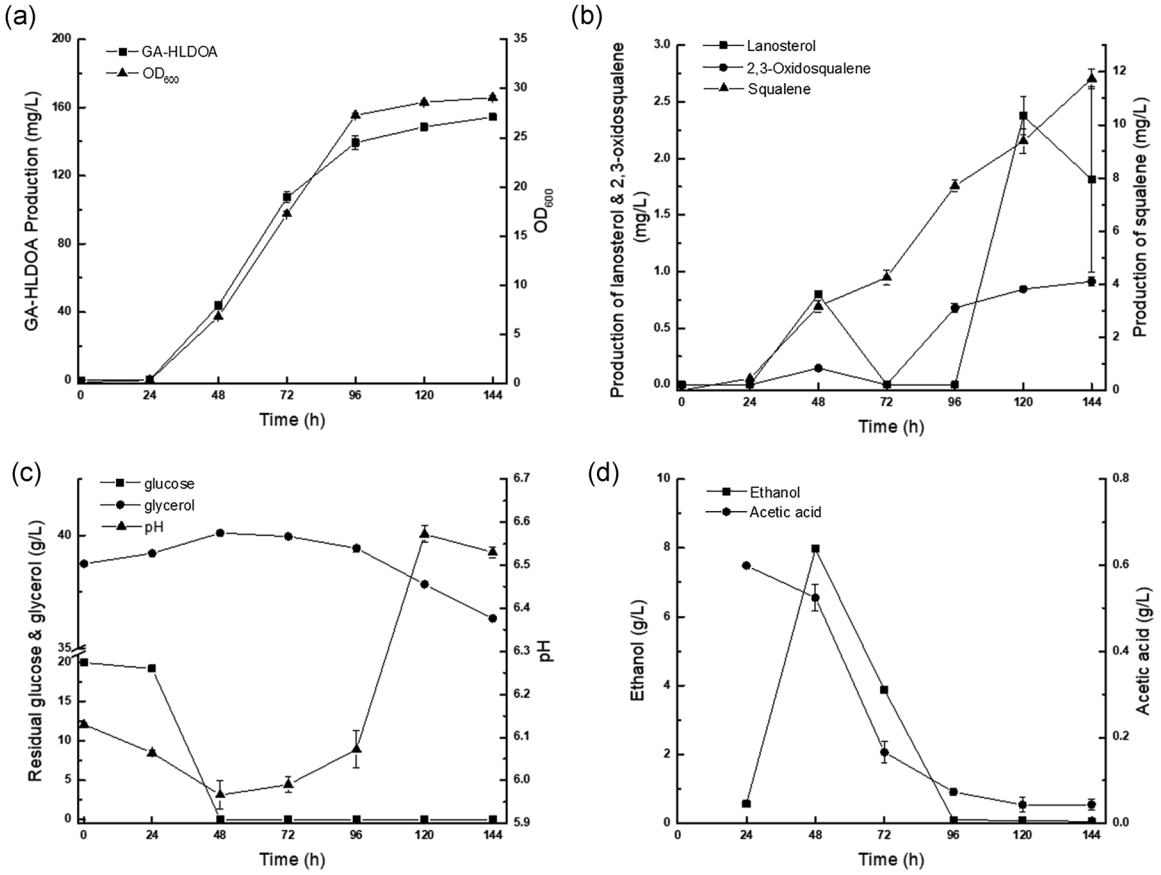


FIGURE 5 Time profile of (a) cell growth, GA-HLDOA production, (b) accumulation of lanosterol, 2,3-oxidosqualene and squalene, (c) residual glucose, glycerol, pH, and (d) ethanol and acetic acid in shake-flask fermentation of strain CYP5150L8-r-iGLCPR-r. The error bars represent the standard deviation of five biological replicates

A persistent and relatively high accumulation of squalene was detected in strain CYP5150L8-r-iGLCPR-r during fermentation in flask shakes (Figure 5b). Overexpression of squalene epoxide ERG1 on the tunable system was accordingly conducted, but decreased cell growth, increased squalene accumulation and reduced GA-HLODA production were surprisingly observed in the engineered strain, probably due to the metabolic burden imposed by ERG1 overexpression (data not shown). Fine-tuning the expression of ERG1 would be a solution to direct more metabolic flux to GA-HLDOA production.

4 | CONCLUSION

In this study, the production of GA-HLDOA, an important antitumor triterpenoid derived from mushroom *G. lucidum*, was comprehensively optimized in *S. cerevisiae* by adopting a dual tunable system for fine-tuning the expression of CYP5150L8 and iGLCPR. As a result, the engineered yeast was able to produce 154.45 mg/L GA-HLDOA, a 10.65-fold higher than that of the highest previously reported titer (14.5 mg/L; Wang et al., 2018). The engineered yeast in this study offers a unique versatile platform facilitating (a) the potential application of GA-HLDOA, (b) the discovery of cryptic genes involved in the biosynthesis of other important GAs and (c) the *de novo* biosynthesis of other important GAs and their derivatives.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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