Journal of Bioscience and Bioengineering
VOL. xxx No. xxx, xxx, xxxx

www.elsevier.com/locate/jbiosc

Cyclodextrins facilitate the efficient secretion of an anti-tumor triterpenoid ganoderic acid HLDOA by *Saccharomyces cerevisiae*

Xiaoting Lan[†] and Han Xiao[*,†]

State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, Laboratory of Molecular Biochemical Engineering, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 800 Dong-chuan Road, Shanghai 200240, China

Received 28 January 2020; accepted 28 March 2020
Available online xxx

As a large group of natural product with significant biological activities, triterpenoid secretion is of particular importance towards its bioproduction. Due to the lack of specific transporters, most triterpenoids are naturally accumulated inside the cells. In this study, by taking an antitumor triterpenoid ganoderic acid 3-hydroxy-lanosta-8,24-dien-26 oic acid (GA-HLDOA) as example, we discovered that addition of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) or 2,6-dimethyl-β-cyclodextrin (DM-β-CD) enable the fast and sufficient secretion of GA-HLDOA by the recombinant *Saccharomyces cerevisiae* strain as constructed in our previous study. In addition, these cyclodextrins (CDs) could not enter into cells, while no significant change of the cell membrane fluidity was observed after CDs treatment. This discovery provides a potential generally applicable method for triterpenoid secretion.

[**Key words:** Triterpenoid; Secretion; Ganoderic acid HLDOA; Cyclodextrin; *Saccharomyces cerevisiae*]

With tremendous applications in pharmaceutical, cosmetics and biomaterial industry, terpenoid bioproduction is now attracting widespread attention. To achieve efficient terpenoid bioproduction, there is a compelling need for terpenoid secretion. It could reduce the interference of terpenoid on the functionality of cell membrane (1), attenuate the feedback inhibition on the key enzymes of the terpenoid biosynthetic pathway (2), increase the efficiency of product recovery (3), and ultimately make contributions to commercial production of terpenoid. Successful secretion of monoterpenoids and sesquiterpenoids have been achieved by discovery, characterization and incorporation of corresponding transporters from their native producing strains (4,5). However, for triterpenoids secretion, such strategies are hard to generalize, because most of the triterpenoids are naturally accumulated inside the cells (6).

As a group of lanostane-type triterpenoids predominantly accumulated inside the cell, ganoderic acids (GAs) are the main bioactive secondary metabolites from traditional Chinese medicinal mushroom *Ganoderma lucidum* (7). Using *Saccharomyces cerevisiae* as a host, we found that overexpression of a cytochrome P450 monooxygenase CYP5150L8 from *G. lucidum* led to the production of an antitumor GA, 3-hydroxy-lanosta-8,24-dien-26 oic acid (GA-HLDOA) in *S. cerevisiae* (8). Further, we constructed a dual tunable system for controlling the expression of CYP5150L8 and a *G. lucidum* cytochrome P450 reductase iGLCPR, and significantly improved the production titer of GA-HLDOA in YPD medium containing 40 g/L of glycerol and appropriate antibiotics (9). Nonetheless, GA-HLDOA cannot be effectively secreted out in the engineered *S. cerevisiae* strain.

Thanks to the advances in synthetic biology, biosynthesis of many other triterpenoids has been achieved in *S. cerevisiae* in addition to GA-HLDOA. A few cases indicated that some *S. cerevisiae* strains can secret triterpenoid. For example, *S. cerevisiae* WAT11 and CEN-PK strains can naturally secret pentacyclic triterpenoids betulinic acid and botulin (10,11), and *S. cerevisiae* INVSc1 and CEN. PK2-1C can secret glycyrrhetinic acid (12). These results suggested that systematic screen of *S. cerevisiae* strains as GA-HLDOA producing strain would be a solution for its secretion.

Cyclodextrin (CD) is a class of cyclic oligosaccharides that are linked by a series of glucose subunits via α-1,4 glycosidic bonds. With a hydrophilic outer structure and a hydrophobic inner region, CD can form inclusions with a variety of non-polar molecules. Owning to this feature, CDs are extensively used as the agent to promote the water solubility of many compounds including terpenoids (13). In addition, CDs are applied as elicitors to stimulate the secondary metabolism (14). Thus, adoption of CDs as the culturing strategy may be general applicable regarding efficient secretion of triterpenoid.

In this study, the biosynthetic pathway of GA-HLDOA was introduced into different *S. cerevisiae* strains BJ5464, BY4741, CEN.PK2-1C, HZ848, INVSc1 and WAT11U. The corresponding engineered strains were not able to efficiently secret GA-HLDOA. Then, secretion of GA-HLDOA by previous engineered *S. cerevisiae* strain CYP5150L8-r-iGLCPR-r (9) was tested by adding different types of cyclodextrins. In particular, we found that only addition of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and 2,6-dimethyl-β-cyclodextrin (DM-β-CD) enable a fast and sufficient secretion of GA-HLDOA.

* Corresponding author.
E-mail address: smallhan@sjtu.edu.cn (H. Xiao).
† These authors contributed equally to this work.

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2020.03.014

Please cite this article as: Lan, X., and Xiao, H., Cyclodextrins facilitate the efficient secretion of an anti-tumor triterpenoid ganoderic acid HLDOA by *Saccharomyces cerevisiae*, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2020.03.014

MATERIALS AND METHODS

Strain construction and cultivation The plasmids pRS426-CYP5150L8-G418r and pRS425-iGLCPR-Hygr (9), harboring key enzymes responsible for GA-HLDOA biosynthesis, were simultaneously transferred into *S. cerevisiae* strains BJ5464, BY4741, CEN.PK2-1C, HZ848, INVSc1 and WAT11U using standard lithium acetate protocol to generate strains BJ5464-r, BY4741-r, CEN.PK2-1C-r, HZ848-r, INVSc1-r and WAT11U-r, respectively. *S. cerevisiae* strains were grown in SC-Ura-Leu medium or YPD medium (8) containing 40 g/L glycerol, 500 mg/L of G418 and 300 mg/L hygromycin at 30°C.

Fermentation Strains were grown in SC-Ura-Leu medium to an OD_{600} of 2.5. For flask fermentation, the seeds were inoculated into 250 mL flasks containing 50 mL of YPD medium, 40 g/L of glycerol, 500 mg/L of G418 and 300 mg/L of hygromycin with an initial OD_{600} of 0.05 and then grew at 30°C and 220 rpm. When needed, 5 mM of each type of cyclodextrin (α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), HP-β-CD or DM-β-CD) was added in the separate culture at the beginning of the fermentation. Alternatively, 385.38 mg of HP-β-CD or 325.83 mg of DM-β-CD was added into the fermentation medium after 72 h. For fermentation in T&J-B type 10 L stirred bioreactor (T&J Bioengineering, Shanghai, China), 200 mL seed culture of CYP5150L8-r-iGLCPR-r from shake flasks was inoculated into 6.5 L of YPD medium containing 40 g/L of glycerol, 5 mM of DM-β-CD, 500 mg/L of G418 and 300 mg/L of hygromycin at an initial OD_{600} of 0.1, and agitated by a standard six-blade turbine impeller at a speed of 300 rpm and an aeration rate of 1.2 vvm at 30°C.

Analyses of cell growth, glucose, glycerol, and multiple metabolites Yeast cell growth, residual glucose, glycerol, medium ethanol, and acetate were determined as previously reported (8). For extraction of ergosterol, squalene, lanosterol and GA-HLDOA, 20 mL of fermentation broth was centrifuged for separation of cells and the supernatant. Distilled water (20 mL) and 20 mL of ethyl acetate were added to the cell pellet, 20 mL of ethyl acetate was added to the supernatant, and incubated at 30°C and 220 rpm for 30 min. The organic phase was collected by centrifugation (4628 *g*, 20°C, 5 min) and evaporation, and the resulting residue was re-dissolved in methanol. The concentrations of multiple metabolites were determined by Agilent 1260 Infinity II HPLC system (Agilent, Waldbronn, Germany) equipped with a diode array detector (DAD) at 280 nm (for detection of ergosterol) and 210 nm (for detection of squalene, lanosterol and GA-HLDOA), and an Agilent SB-C18 column (5 μm, 4.6 mm × 250 mm). Mobile phase A was 100% water, and mobile phase B contained methanol/acetic acid (100: 0.1 v/v). A linear gradient for 80%–100% B in 30 min at 1 mL/min was adopted.

Detection of cyclodextrin The concentration of various cyclodextrins were determined by a colorimetric method using phenolphthalein with minor modification according to previous report (15). For preparation of CDs standard curve, 0.0325, 0.065, 0.1, 0.125 mM of DM-β-CD or HP-β-CD was added into a solution containing 200 μL of fermentation broth and 0.03 mM of phenolphthalein. After adjusting the pH to 11, the total volume of the solution was up to 10 mL. The absorbance of the solution was measured at 554 nm. The log absorbance value was plotted against log CDs concentration to obtain the plot. For detection of CDs concentration in samples, one mL of sample was centrifuged, and washed twice with 100 μL of distilled water for each time (2152 *g*, 20°C, 3 min). The supernatant was collected and diluted properly for CD detection. For the cell pellet obtained after the centrifugation, it was resuspended in 200 μL of distilled water containing 0.1 g of glass beads (Sigma–Aldrich, St. Louis, MO, USA) for cell disruption (5000 rpm, 20 min). After centrifugation (12,396 *g*, 20°C, 20 min), the supernatant was transferred to a new tube to detect the CDs concentration according to the aforementioned method.

Determination of cell membrane fluidity A total of 450 mL fermentation broth after 72 h fermentation of CYP5150L8-r-iGLCPR-r was collected and equally divided into 9 sterile test tubes containing 50 mL of fermentaion broth in each tube, which were further categorized into three groups. The first group was served as control group with no treatment. In the second and third group, 325.83 mg of DM-β-CD or 385.38 mg of HP-β-CD was added in each tube, respectively. Immediately after the addition of CDs, one OD of cells were taken from each group, centrifuged (1377 *g*, 20°C, 10 min) and washed with 1 mL of 0.1 M PBS (1377 *g*, 20°C, 5 min). The cell pellet was incubated with 1 mL of PBS containing 2 μM 1,6-diphenyl-1,3,5-hexatriene (DPH) for 8 min. This solution (200 μL) was added to a black 96-well plate (JingAn Biological, Shanghai, China) to detect the fluorescence using a multimode microplate reader (Tecan & Spark, Männedorf, Swiss) at 360 nm and 430 nm as excitation and emission wavelengths, respectively.

RESULTS AND DISCUSSION

Efficient secretion of GA-HLDOA cannot be achieved by multiple *S. cerevisiae* strains To investigate whether other *S. cerevisiae* strains can effectively secrete GA-HLDOA, we constructed various *S. cerevisiae* strains capable of producing GA-HLDOA (Fig. 1). When cell growth was stopped (Fig. 2A), their corresponding secretion efficiencies of GA-HLDOA for strains BJ5464-r, BY4741-r, CEN.PK2-1C-r, HZ848-r, INVSc1-r and WAT11U-r, as determined by the ratio between GA-HLDOA extracted from the medium, and GA-HLDOA extracted from both the cells and medium, were 41.19%, 32.14%, 12.27%, 10.97%, 21.64% and 38.19%, respectively (Fig. 2B). Only 6.03% of GA-HLDOA can be transported out by strain CYP5150L8-r-iGLCPR-r as we constructed previously (9), whereas it was the best producer of GA-HLDOA among all the strains (Fig. 2B). Accumulation of lanosterol could only be detected in the cells of CYP5150L8-r-iGLCPR-r, at the titer of 3.34 mg/L (Fig. 2C). For the rest of the engineered strains, lanosterol could be detected neither from the

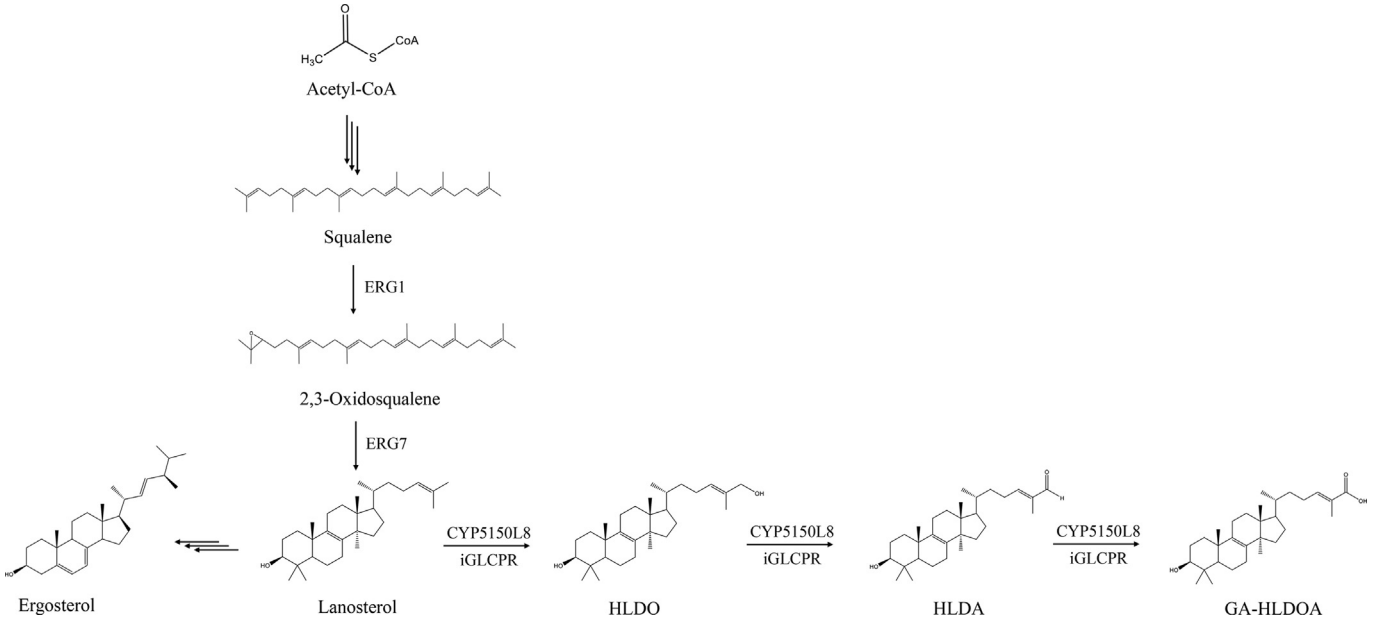

FIG. 1. Biosynthesis of GA-HLDOA in *S. cerevisiae*. ERG1, squalene epoxidase; ERG7, lanosterol synthase; CYP5150L8, a *G. lucidum* cytochrome P450; iGLCPR, a *G. lucidum* cytochrome P450 reductase. Multiple arrows represent multi-enzymatic reactions.

Please cite this article as: Lan, X., and Xiao, H., Cyclodextrins facilitate the efficient secretion of an anti-tumor triterpenoid ganoderic acid HLDOA by *Saccharomyces cerevisiae*, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2020.03.014

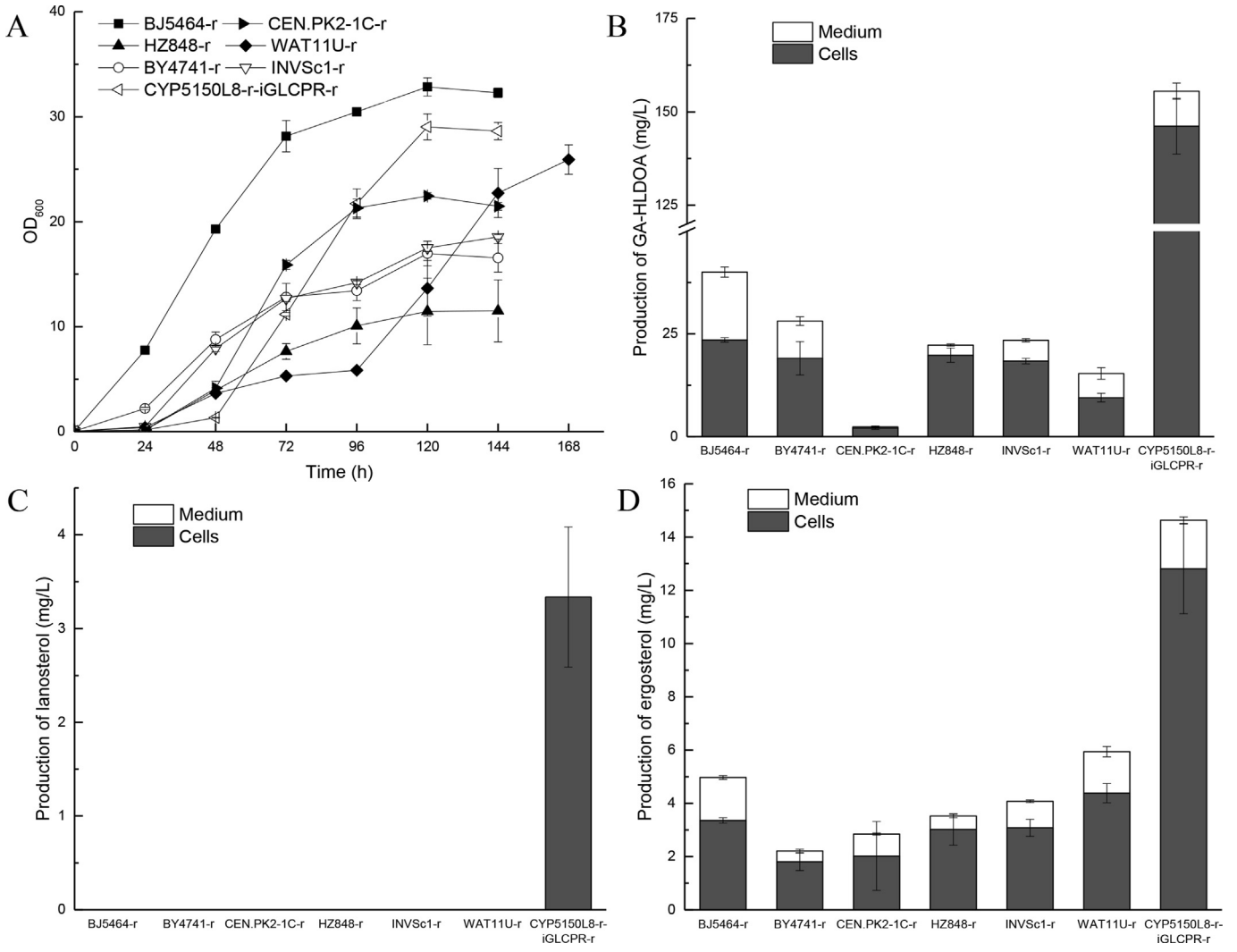

FIG. 2. Secretion of GA-HLDOA by different *S. cerevisiae* strains. (A) Time profile of cell growth. Production of (B) GA-HLDOA, (C) lanosterol and (D) ergosterol by strains BJ5464-r, BY4741-r, CEN.PK2-1C-r, HZ848-r, INVSc1-r, CYP5150L8-r-iGLCPR-r at 144 h, and by strain WAT11U-r at 168 h. The error bars represent the standard deviation of three biological replicates.

medium, nor from the cells, indicating their inadequate supply of precursor with regard to GA-HLDOA bioproduction (Fig. 2B, C). For ergosterol, the secretion efficiencies of strains BJ5464-r, BY4741-r, CEN.PK2-1C-r, HZ848-r, INVSc1-r, WAT11U-r and CYP5150L8-r-iGLCPR-r were 32.46%, 18.34%, 28.96%, 14.37%, 24.48%, 26.22% and 12.49%, respectively (Fig. 2D).

Despite the insufficient secretion of GA-HLDOA by all the strains, BJ5464-r exhibited the highest secretion efficiencies of both GA-HLDOA (41.19%) and ergosterol (32.46%) (Fig. 2B, D). Due to the double deletion of vacuolar aspartyl protease encoding gene *PEP4* and vacuolar proteinase B encoding gene *PRB1*, BJ5464 lacks the vacuolar degradation system and allows efficient production of various proteins (16), which may support the secretion of GA-HLDOA and ergosterol by facilitating expression of key proteins responsible for their bioproduction and secretion. Since the secretion capability of GA-HLDOA varied a lot in different strains (Fig. 2B), exploration of the determinants responsible for GA-HLDOA secretion by comparative transcriptomic and/or proteomics analysis would lay the foundation for reconstruction of an efficient secretion system for GA-HLDOA in future study.

Addition of DM-β-CD or HP-β-CD enables a fast and sufficient secretion of GA-HLDOA To find an alternative for efficient secretion of GA-HLDOA, we looked for CDs as the potential sequester of GA-HLDOA using the best producing strain CYP5150L8-r-iGLCPR-r. Five mM of three unsubstituted CDs, α-CD, β-CD, γ-CD or two β-CD derivatives, HP-β-CD and DM-β-CD, was individually added in medium at the beginning of fermentation. Compared with the untreated control, cells always exhibited higher OD_{600} after 72 h fermentation with γ-CD, while no significant differences on cell growth were observed at 144 h fermentation with other CDs (Fig. 3A). After 48 h, secretion of GA-HLDOA was not enhanced with α-CD, β-CD, or γ-CD treated cells as compared to the untreated cells, and the secretion efficiencies of GA-HLDOA were 1.43%, 1.67%, 4.60% and 12.13% with untreated, α-CD, β-CD, or γ-CD treated cells after 144 h, respectively (Fig. 3B). In contrast, 96.59% and 100% of produced GA-HLDOA were secreted in the medium after 48 h, while 71.13% and 95.15% of produced GA-HLDOA remained in the medium after 144 h treatment with HP-β-CD or DM-β-CD (Fig. 3B). It should be noted that production of GA-HLDOA by strain CYP5150L8-r-iGLCPR-r at 144 h was much

Please cite this article as: Lan, X., and Xiao, H., Cyclodextrins facilitate the efficient secretion of an anti-tumor triterpenoid ganoderic acid HLDOA by *Saccharomyces cerevisiae*, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2020.03.014

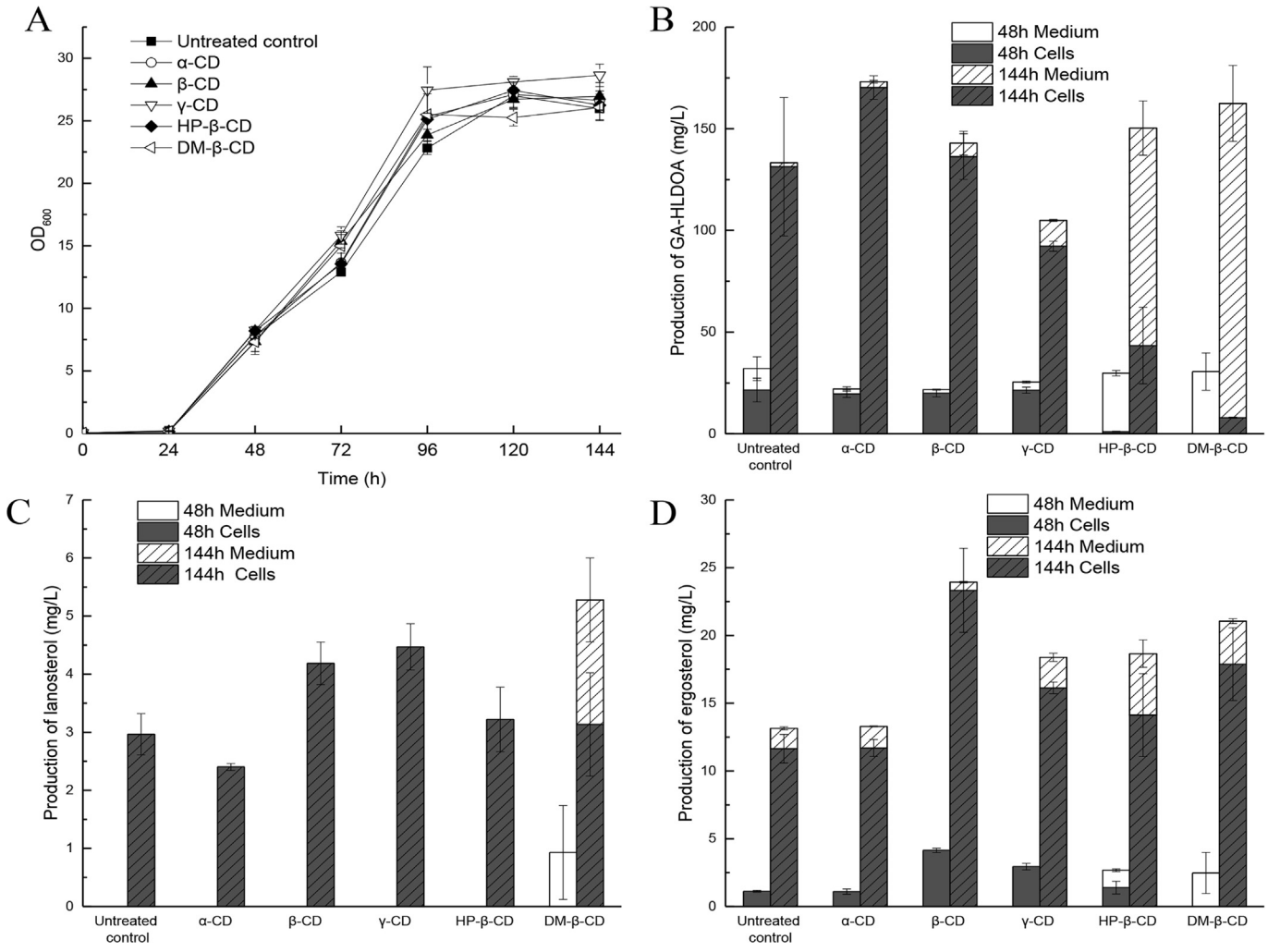

FIG. 3. Secretion of GA-HLDOA by application of CDs. (A) Time profile of cell growth. Production of (B) GA-HLDOA, (C) lanosterol and (D) ergosterol by CYP5150L8-r-iGLCPR-r at 48 h and 144 h with or without the treatment of CDs. The error bars represent the standard deviation of three biological replicates.

higher than that at 48 h (Fig. 3B). This information suggested that HP-β-CD may not be able to sequester GA-HLDOA with a comparable efficiency as DM-β-CD when GA-HLDOA titer was increased to a certain extent. For lanosterol production after 48 h, it could only be detected at the titer of 0.93 mg/L in the medium when DM-β-CD was added. After 144 h, 100% of produced lanosterol were from untreated, α-CD, β-CD, γ-CD or HP-β-CD treated cells, while 40.59% of produced lanosterol was from the medium of DM-β-CD treated cells (Fig. 3C). Secretion of ergosterol can only be detected in HP-β-CD and DM-β-CD treated cells after 48 h at the efficiencies of 47.99% and 100%, but even so, most of the ergosterol were still from the cells after 144 h treatment with various CDs. With time increasing, more ergosterol were produced but accumulated in cells treated with both HP-β-CD and DM-β-CD, suggesting that these cells compensated for the loss of ergosterol by enhancing its production (Fig. 3D).

To explore the capability of HP-β-CD and DM-β-CD to sequester GA-HLDOA, about 6 mM of HP-β-CD or DM-β-CD was added into the fermentation broth after 72 h. Within 5 min, 56.31% and 85.40% of GA-HLDOA were secreted out in HP-β-CD and DM-β-CD treated cells, while the secretion efficiencies further increased to 89.41% and 93.94% after 2 h treatment (Fig. 4A). Lanosterol can only be detected in the cells with HP-β-CD and DM-β-CD treatment, at the titers of less than 0.8 mg/L (Fig. 4B). By contrast, more than half of the ergosterol were in the medium after 2 h treatment with HP-β-CD and DM-β-CD (Fig. 4C). No significant reduce of CDs were observed in the medium during the 2 h treatment (Fig. 4D). Moreover, CDs from the cells were undetectable during the whole process, suggesting that they cannot enter into the cells. The cell wall structure of *S. cerevisiae* is open and porous. Previous study indicated that the yeast cell wall porosity has a hydrodynamic radius up to 5.8 nm (17). On the other hand, the methylated β-cyclodextrin is considered as a cylinder with 0.3 nm of radius and 0.8 nm of height (18). Although CDs could not enter the cell, we speculated that CDs could physically interacted with the yeast cell membrane by permeating through the pore of the cell wall. Meanwhile, these results also indicated that, to achieve the physical contact and subsequent sequester by extracellular CDs, the produced GA-HLDOA tends to be accumulated on the cell membrane.

The α-CD, β-CD and γ-CD are formed by six, seven, and eight α-D-glucopyranose units, respectively, which lead to different internal toroidal diameters. These differences could be the reason that both α-CD and γ-CD could not form stable inclusion complexes of GA-HLDOA for its secretion (Fig. 3). However, even for β-CD, with similar internal toroidal diameter as HP-β-CD and DM-β-CD, it still cannot sequester GA-HLDOA (Fig. 3). We speculate that the HP and DM modification on the β-CD could prevent the dissociation of the GA-HLDOA from the hydrophobic inner cavity of these CDs, and facilitate the stability of the complex in hydrophilic environment.

Please cite this article as: Lan, X., and Xiao, H., Cyclodextrins facilitate the efficient secretion of an anti-tumor triterpenoid ganoderic acid HLDOA by *Saccharomyces cerevisiae*, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2020.03.014

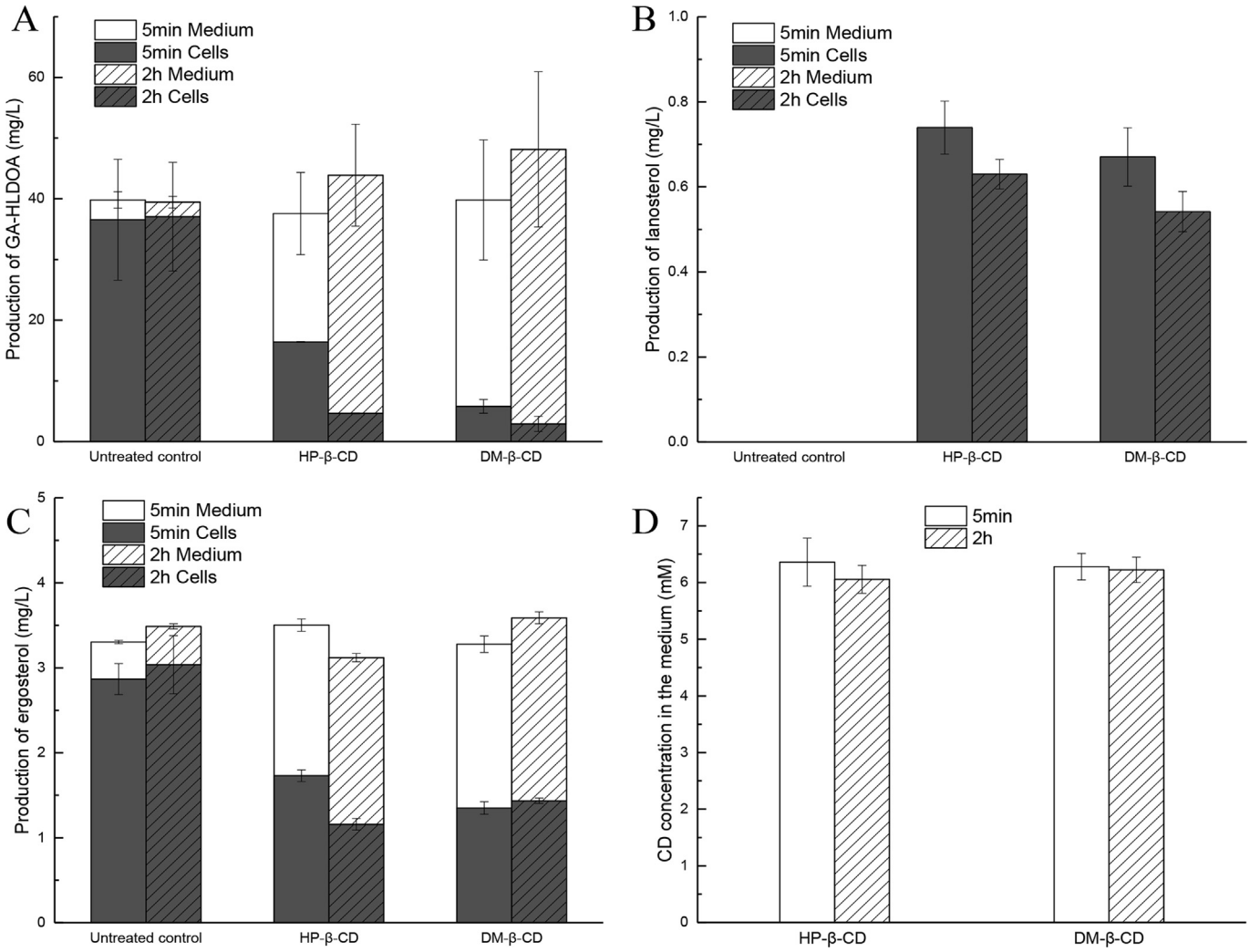

FIG. 4. Fast and sufficient secretion of GA-HLDOA using cells of CYP5150L8-r-iGLCPR-r with HP-β-CD or DM-β-CD treatment. Concentrations of (A) GA-HLDOA, (B) lanosterol, (C) ergosterol and (D) CDs after 5 min or 2 h incubation with CDs.

For both HP-β-CD and DM-β-CD treatment, secretion of GA-HLDOA was much higher than that of lanosterol (Figs. 3 and 4). The only chemical structure difference between lanosterol and GA-HLDOA is on the C-26, which are a methyl group for lanosterol, and a carboxyl group for GA-HLDOA, respectively (8). Possible reasons for the different secretion efficiencies of GA-HLDOA and lanosterol include (i) the inaccessibility between lanosterol and CDs, since lanosterol is not entirely accumulated on the cell membrane; (ii) a more stable binding by attachment of the substituents from the oxidized lanosterol (GA-HLDOA) and CDs than that from lanosterol and CDs (19). The second speculation was also supported by a previous study that M-β-CD was able to sequester more hydroxyl-β-amyrin than that of β-amyrin (an oleanane-type triterpenoid) (20).

In this study, production of GA-HLDOA was not further increased with HP-β-CD or DM-β-CD treatment (Figs. 3B and 4A), indicating two important clues for further improving the production of GA-HLDOA. First, since the enzyme and product are no longer in the same spatial location, the current titer of GA-HLDOA as produced by the engineered yeast may not exhibit feedback inhibition on biosynthesis of GA-HLDOA. Second, GA-HLDOA is spatially separated from the cell and it is unlikely to cause the problem of intracellular toxicity. Thus, for further improving the production of GA-HLDOA, as inspired by this study, genetic engineering strategy should not be focus on attenuating the feedback inhibition, nor reducing product toxicity.

However, it does not imply that the titer of GA-HLDOA would never cause feedback inhibition or product toxicity once it increases to a certain level. For the extraction view, although the compound which exists in intra of extracellular could be extracted completely, cell disruption is inevitable in this way. In contrast, cell disruption is not required once the product is secreted out. Moreover, since the potential inhibition and toxicity caused by the product is removed, cells could be collected for continuous production of GA-HLDOA for increasing the productivity.

No significant change on cell membrane fluidity after CDs treatment Methyl-β-cyclodextrin (M-β-CD) was able to deplete cholesterol or its analog ergosterol from plasma membrane in various eukaryotic cells including *S. cerevisiae*, which would increase the membrane fluidity in varied degrees (21). To test whether HP-β-CD or DM-β-CD can act on yeast cell membrane in a similar way, the cell membrane fluidity was monitored by DPH fluorescence polarization. The lower DPH anisotropy value reflects the higher cell membrane fluidity (22). The fluorescence anisotropy of HP-β-CD and DM-β-CD treated cells (0.18 ± 0.0035 and 0.18 ± 0.0035) were slightly lower than that of the untreated cells (0.19 ± 0.004), but no significant differences were observed

Please cite this article as: Lan, X., and Xiao, H., Cyclodextrins facilitate the efficient secretion of an anti-tumor triterpenoid ganoderic acid HLDOA by *Saccharomyces cerevisiae*, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2020.03.014

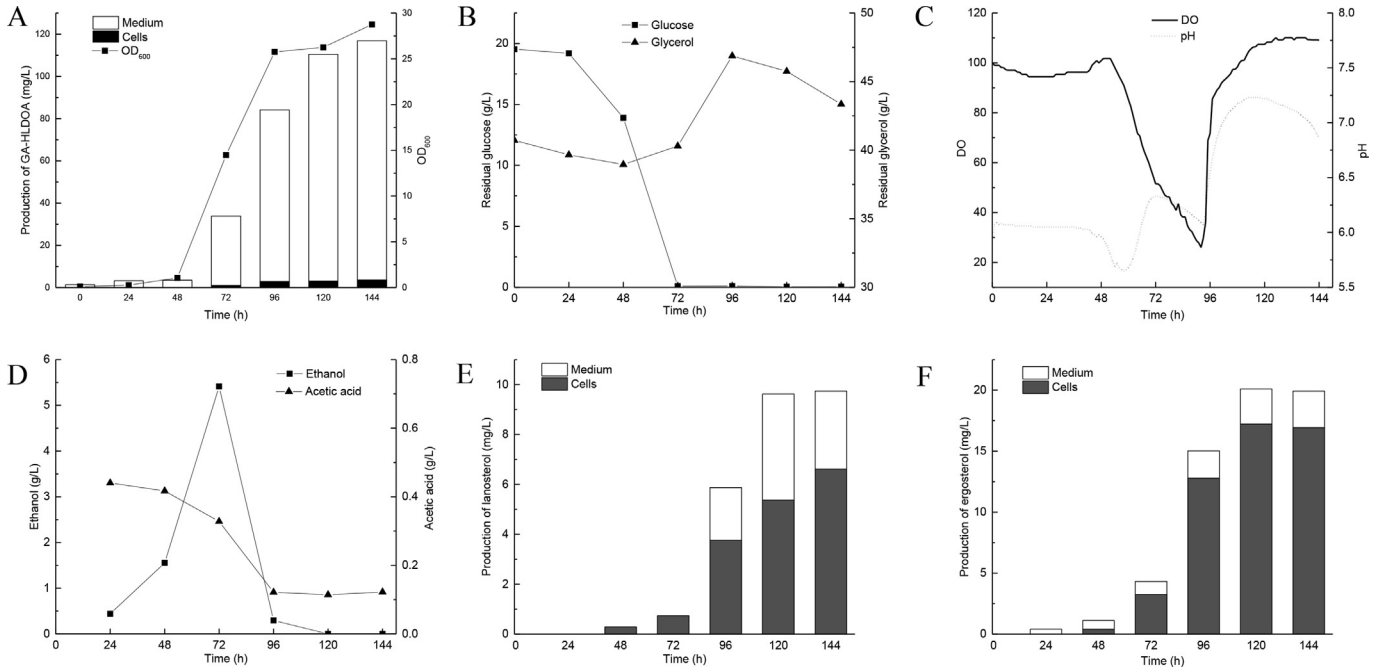

FIG. 5. Time profile of (A) cell growth and GA-HLDOA production, (B) residual glucose and glycerol, (C) pH and dissolved oxygen (DO), (D) ethanol and acetic acid, (E) lanosterol, (F) ergosterol in 10 L bioreactor fermentation of strain CYP5150L8-r-iGLCPR-r using 5 mM of DM-β-CD.

($p > 0.05$). These results indicated that no distinguishable change was detected on cell membrane fluidity after these CDs treatment.

Secretion of GA-HLDOA with DM-β-CD in 10 L bioreactor Since addition of DM-β-CD enables the faster and more sufficient secretion of GA-HLDOA than that of HP-β-CD (Figs. 3B and 4A), fermentation of CYP5150L8-r-iGLCPR-r in the presence of 5 mM DM-β-CD was performed in a 10 L bioreactor. Cells grew rapidly between 48 h and 96 h (Fig. 5A). In this period, the dissolved oxygen (DO) decreased from 100.9% at 48 h to the lowest level of 26% at 92 h, and quickly rebounded to 71.7% after 96 h (Fig. 5C). The glucose was completely consumed within 72 h, but the glycerol concentration in the medium changed slightly (Fig. 5B). The pH changed between 5.65 and 7.23 during the fermentation process (Fig. 5C). Production of ethanol and acetic acid, along with their consumption were all observed (Fig. 5D). During the whole fermentation process, over 96% of produced GA-HLDOA can always be extracted from the medium (Fig. 5A), demonstrating a stable and sufficient sequestration mediated by DM-β-CD. At the end of fermentation, 113 mg/L of GA-HLDOA was obtained from the medium, while 85% of ergosterol was still obtained from the cells (Fig. 5A, F). Interestingly, a continuous and stable secretion of lanosterol was observed after 96 h (Fig. 5E). Since a slow cell growth was simultaneously observed in this period, the reason that lanosterol obtained from the medium may be, at least partially be, attributed to the cell lysis (Fig. 5A, E).

Although the price of GA-HLDOA is not commercially available, the price of HP-β-CD and DM-β-CD are much less than other GAs. Using a naturally secreting strains to produce GA-HLDOA may save the cost of CDs. However, in our case, the best strain for secretion of GA-HLDOA could produce 40 mg/L of GA-HLDOA in total, while over half of the GA-HLDOA remained in the cells (Fig. 2B). The best producer of GA-HLDOA was able to produce over 100 mg/L of GA-HLDOA (Figs. 2B and 5A), although it requires CD for secretion of the product. Taken together, adopting CD as a culturing strategy may not cause a cost issue for bioproduction of GA-HLDOA. In future, we believe that after a systematic optimization of the biosynthetic pathway and cell regulation of GA-HLDOA, adopting these CDs as a culturing strategy would be a simple but effective method for enhancing the production of this triterpenoid.

In conclusion, we discovered that addition of HP-β-CD or DM-β-CD allows a fast and sufficient secretion of GA-HLDOA, an important antitumor triterpenoid, by *S. cerevisiae*. The work should be enlightening to develop a more general approach for triterpenoid secretion.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (no. 31971344) and Shanghai Municipal Natural Science Foundation (no. 17ZR1448900, no. 18ZR1420300). We thank Prof. Jian-Jiang Zhong (Shanghai Jiao Tong University) for his helpful suggestion on this study. Prof. Huimin Zhao (University of Illinois at Urbana-Champaign) kindly provided *S. cerevisiae* strains BJ5464, BY4741, CEN.PK2-1C, HZ848 and INVSc1, and Prof. Zongbao K. Zhao (Dalian Institute of Chemical Physics, Chinese Academy of Sciences) kindly sent us the strain WAT11U. Xiao H. thanks the DaSilva Award (The Society for Biotechnology, Japan) for financial support.

References

1. **Sun, W., Qin, L., Xue, H., Yu, Y., Ma, Y., Wang, Y., and Li, C.:** Novel trends for producing plant triterpenoids in yeast, Crit. Rev. Biotechnol., **39**, 618–632 (2019).
2. **Gavira, C., Höfer, R., Lesot, A., Lambert, F., Zucca, J., and Werck-Reichhart, D.:** Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by *Saccharomyces cerevisiae*, Metab. Eng., **18**, 25–35 (2013).
3. **Vermaas, J. V., Bentley, G. J., Beckham, G. T., and Crowley, M. F.:** Membrane permeability of terpenoids explored with molecular simulation, J. Phys. Chem. B, **122**, 10349–10361 (2018).
4. **Dunlop, M. J., Dossani, Z. Y., Szmidt, H. L., Chu, H. C., Lee, T. S., Keasling, J. D., Hadi, M. Z., and Mukhopadhyay, A.:** Engineering microbial biofuel tolerance and export using efflux pumps, Mol. Syst. Biol., **7**, 487 (2011).
5. **Wang, Y., Lim, L., DiGuistini, S., Robertson, G., Bohlmann, J., and Breuil, C.:** A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees, New Phytol., **197**, 886–898 (2013).

6. **Shitan, N.:** Secondary metabolites in plants: transport and self-tolerance mechanisms, Biosci. Biotechnol. Biochem., **80**, 1283–1293 (2016).
7. **Xiao, H. and Zhong, J.:** Production of useful terpenoids by higher-fungus cell factory and synthetic biology approaches, Trends Biotechnol., **34**, 242–255 (2016).
8. **Wang, W., Xiao, H., and Zhong, J.:** Biosynthesis of a ganoderic acid in *Saccharomyces cerevisiae* by expressing a cytochrome P450 gene from *Ganoderma lucidum*, Biotechnol. Bioeng., **115**, 1842–1854 (2018).
9. **Lan, X., Yuan, W., Wang, M., and Xiao, H.:** Efficient biosynthesis of antitumor ganoderic acid HLDOA using a dual tunable system for optimizing the expression of CYP5150L8 and a *Ganoderma* P450 reductase, Biotechnol. Bioeng., **116**, 3301–3311 (2019).
10. **Huang, L., Li, J., Ye, H., Li, C., Wang, H., Liu, B., and Zhang, Y.:** Molecular characterization of the pentacyclic triterpenoid biosynthetic pathway in *Catharanthus roseus*, Planta, **236**, 1571–1581 (2012).
11. **Zhou, C., Li, J., Li, C., and Zhang, Y.:** Improvement of betulinic acid biosynthesis in yeast employing multiple strategies, BMC Biotechnol., **16**, 59 (2016).
12. **Zhu, M., Wang, C., Sun, W., Zhou, A., Wang, Y., Zhang, G., Zhou, X., Huo, Y., and Li, C.:** Boosting 11-oxo-β-amyrin and glycyrrhetinic acid synthesis in *Saccharomyces cerevisiae* via pairing novel oxidation and reduction system from legume plants, Metab. Eng., **45**, 43–50 (2018).
13. **Carneiro, B. S., Costa Duarte, Í. F., Heimfarth, L., Siqueira Quintans, D. J., Quintans-Júnior, J. L., Veiga Júnior, F. V., and Neves de Lima, A.Á.:** Cyclodextrin–drug inclusion complexes: in vivo and in vitro approaches, Int. J. Mol. Sci., **20**, 642 (2019).
14. **Rizzello, F., De Paolis, A., Durante, M., Blando, F., Mita, G., and Caretto, S.:** Enhanced production of bioactive isoprenoid compounds from cell suspension cultures of *Artemisia annua* L. using β-cyclodextrins, Int. J. Mol. Sci., **15**, 19092–19105 (2014).
15. **Basappa, C., Rao, P., Rao, D. N., and Divakar:** A modified colorimetric method for the estimation of β-cyclodextrin using phenolphthalein, J. Food Sci. Technol., **33**, 517–520 (1998).
16. **Bond, C., Tang, Y., and Li, L.:** *Saccharomyces cerevisiae* as a tool for mining, studying and engineering fungal polyketide synthases, Fungal Genet. Biol., **89**, 52–61 (2016).
17. **Walker, L., Sood, P., Lenardon, M. D., Milne, G., Olson, J., Jensen, G., Wolf, J., Casadevall, A., Adler-Moore, J., and Gow, N. A. R.:** The viscoelastic properties of the fungal cell wall allow traffic of ambisome as intact liposome vesicles, mBio, **9**, e02383-17 (2018).
18. **Anderson, T. G., Tan, A., Ganz, P., and Seelig, J.:** Calorimetric measurement of phospholipid interaction with methyl-beta-cyclodextrin, Biochemistry, **43**, 2251–2261 (2004).
19. **Fourmentin, S., Ciobanu, A., Landy, D., and Wenz, G.:** Space filling of beta-cyclodextrin and beta-cyclodextrin derivatives by volatile hydrophobic guests, Beilstein J. Org. Chem., **9**, 1185–1191 (2013).
20. **Moses, T., Pollier, J., Almagro, L., Buyst, D., Van Montagu, M., Pedreño, M. A., Martins, J. C., Thevelein, J. M., and Goossens, A.:** Combinatorial biosynthesis of sapogenins and saponins in *Saccharomyces cerevisiae* using a C-16α hydroxylase from *Bupleurum falcatum*, Proc. Natl. Acad. Sci. USA, **111**, 1634–1639 (2014).
21. **Du, W. and Ayscough, K. R.:** Methyl beta-cyclodextrin reduces accumulation of reactive oxygen species and cell death in yeast, Free Radic. Biol. Med., **46**, 1478–1487 (2009).
22. **Companyo, M., Iborra, A., Villaverde, J., Martinez, P., and Morros, A.:** Membrane fluidity changes in goat sperm induced by cholesterol depletion using beta-cyclodextrin, Biochim. Biophys. Acta, **1768**, 2246–2255 (2007).

Please cite this article as: Lan, X., and Xiao, H., Cyclodextrins facilitate the efficient secretion of an anti-tumor triterpenoid ganoderic acid HLDOA by *Saccharomyces cerevisiae*, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2020.03.014