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Identification of steroid C27 monooxygenase isoenzymes involved in sterol catabolism and stepwise pathway engineering of *Mycobacterium neoaurum* for improved androst-1,4-diene-3,17-dione production

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Abstract

Cholesterol oxidase, steroid C27 monooxygenase and 3-ketosteroid-Δ^1-dehydrogenase are key enzymes involved in microbial catabolism of sterols. Here, three isoenzymes of steroid C27 monooxygenase were firstly characterized from *Mycobacterium neoaurum* as the key enzyme in sterol C27-hydroxylation. Among these three isoenzymes, steroid C27 monooxygenase 2 exhibits the strongest function in sterol catabolism. To improve androst-1,4-diene-3,17-dione production, cholesterol oxidase, steroid C27 monooxygenase 2 and 3-ketosteroid-Δ^1-dehydrogenase were coexpressed to strengthen the metabolic flux to androst-1,4-diene-3,17-dione, and 3-ketosteroid 9α-hydroxylase, which catalyzes the androst-1,4-diene-3,17-dione catabolism, was disrupted to block the androst-1,4-diene-3,17-dione degradation pathway in *M. neoaurum* JC-12. Finally, the recombinant strain JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$ produced 20.1 g/L androst-1,4-diene-3,17-dione, which is the highest reported production with sterols as substrate. Therefore, this work is hopes to pave the way for efficient androst-1,4-diene-3,17-dione production through metabolic engineering.

Keywords *Mycobacterium neoaurum* · Steroid C27 monooxygenase · Metabolic engineering · Androst-1,4-diene-3,17-dione

Abbreviations

AD	4-Androstene-3,17-dione
ADD	Androst-1,4-diene-3,17-dione
9α-OH-AD	9α-Hydroxy-4-androstene-3,17-dione
9α-OH-ADD	9α-Hydroxy-androst-1,4-diene-3,17-dione
3β-HSD	3β-Hydroxysteroid dehydrogenase
CFU	Colony forming units
ChoM	Cholesterol oxidase
SMO	Steroid C27 monooxygenase
HP-β-CD	Hydroxymethyl-β-cyclodextrin
KSDD	3-Ketosteroid-Δ^1-dehydrogenase
KSH	3-Ketosteroid 9α-hydroxylase

Introduction

Androst-1,4-diene-3,17-dione (ADD), an important pharmaceutical androgen steroid, is widely used as an important precursor in the synthesis of steroid hormone medicines [25, 29]. Traditionally, in the pharmaceutical industry, ADD was synthesized by multistep chemical methods from natural steroid sapogenin, diosgenin [18]. One of the well-established route in its commercial usage is chemical modification of diosgenin to ADD followed by further synthesis to pharmaceutical steroids [14]. However, the chemical conversion of diosgenin to ADD and other valuable steroids has many shortcomings such as high-cost processes,

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relatively low yields, waste of land resources, and high pollution [35]. Therefore, with the constant improvement of the environmental protection consciousness and the further development of green technology, non-pollution and non-toxic technology have inevitably become the main direction of industrial development [1, 30].

Microbial conversion of the widely spread natural sterols (phytosterol, cholesterol, ergosterol, etc.) to produce valuable steroid precursors has been an important alternative way in the pharmaceutical industry [11]. Since the initial discovery of the gene cluster encoding sterol catabolism, many key enzymes involved in this process have been well identified and characterized in recent years [22, 36]. So far, the main key intermediates produced from sterol microbial metabolism were the C_{19} steroids such as 4-androstene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD) [11]. Since these products are widely used as important precursors in the synthesis of steroid hormone medicines, it has great significance and value to realize the biotransformation of the low-cost sterols into these high-value products. Generally, sterols were firstly bio-converted to AD [48], AD was then transformed to other intermediates that were catalyzed by the corresponding enzymes [7]. The biotransformation of sterols to AD involves two processes: the modification of 3β-ol-5-ene to 3-oxo-4-ene moiety on steroid nucleus catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD) or cholesterol oxidase [8, 9, 44], and the side-chain degradation of sterols [34]. Then AD is catalyzed to ADD by 3-ketosteroid-Δ^1-dehydrogenase (KSDD) [39]. Besides, AD and ADD are easily catalyzed to 9α-hydroxy-AD (9α-OH-AD) and 9α-hydroxy-ADD (9α-OH-ADD) by 3-ketosteroid-9α-hydroxylase (KSH), respectively [38]. Moreover, 9α-OH-ADD further degrades to carbon dioxide and water by spontaneous ring B destruction [41, 42], which results in the degradation of the key intermediates (Fig. 1).

In the biotransformation process of sterols' to AD, the initial step of sterols side-chain oxidation is hydroxylation catalyzed by steroid C27 monooxygenase at C27 [11]. CYP125, a well-studied steroid C27 monooxygenase belongs to cytochrome P450 family and catalyzes three successive oxidations of the sterol terminal carbon to an acid as shown for *Rhodococcus jostii* RHA1, *Mycobacterium bovis* BCG and *M. tuberculosis* H37Rv [5, 24, 40]. A crucial role of CYP125A1 in hydroxylating cholest-4-en-3-one at C27 and oxidizing to cholest-4-en-3-one-27-oic acid was demonstrated for *M. tuberculosis* H37Rv [21]. However, there are no literatures report about the effect of steroid C27 monooxygenase on AD/ADD production and any other steroid C27 monooxygenase isoenzymes involved in sterol catabolism in mycobacteria. Thus, it is necessary to characterize steroid C27 monooxygenase isoenzymes in *Mycobacterium* and identify their functions in AD/ADD production.

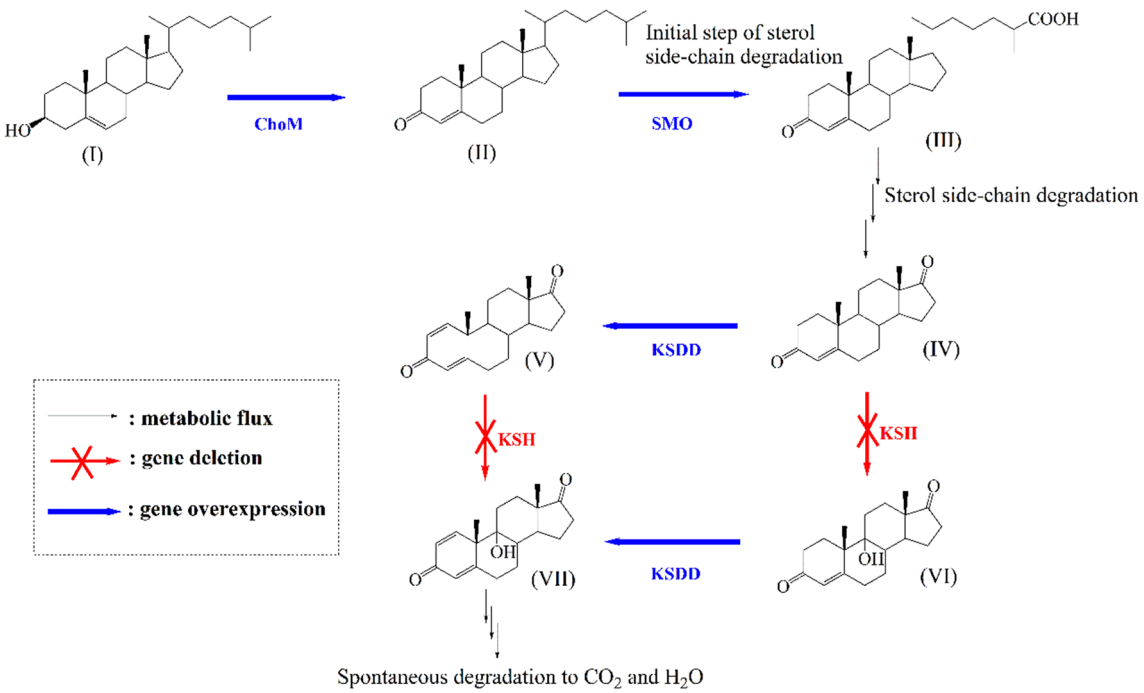

Fig. 1 Microbial metabolic pathway of sterols in *Mycobacterium neoaurum*. The depicted metabolites are: (I) cholesterol, (II) 4-cholesten-3-one, (III) cholest-4-en-3-one-27-oic acid, (IV) 4-androstene-3,17-dione (AD), (V) androst-1,4-diene-3,17-dione (ADD), (VI) 9α-hydroxy-4-androstene-3,17-dione (9α-OH-AD), (VII) 9α-hydroxy-androst-1,4-diene-3,17-dione (9α-OH-ADD). *ChoM* cholesterol oxidase, *SMO* steroid C27 monooxygenase, *KSDD* 3-ketosteroid-Δ^1-dehydrogenase, *KSH* 3-ketosteroid 9α-hydroxylase

It is well known that the genus *Mycobacterium* is the most efficient AD/ADD producer [11, 19, 33]. Many efforts have been devoted to increasing the AD/ADD yield by mutation breeding and genetic engineering. For instance, nitrosoguanidine mutagenesis and the combination of mitomycin C and UV treatments were used to achieve the high sterol transformation efficiency and AD/ADD yield [13, 15]. By disrupting $ksdd_M$ gene in *Mycobacterium*, AD production was improved from sterol bioconversion, while by overexpressing $ksdd_M$ gene, ADD production was improved [3, 39]. In a recent study, the cholesterol oxidases in *M. neoaurum* were identified and applied by increasing the AD and ADD production [45]. However, so far, there is still no report concerning the improvement of AD/ADD yield through metabolic engineering techniques. Thus, it is imperative to tune sterols' metabolic flux to improve the AD/ADD yield and block their degradation pathway.

In our previous studies, strain *M. neoaurum* JC-12, capable of transforming phytosterol into ADD as the main product, was obtained [28]. Enzymes cholesterol oxidase (ChoM) and 3-ketosteroid-Δ^1-dehydrogenase (KSDD) were identified as the key enzymes playing important roles in bioconverting sterols into AD/ADD in *M. neoaurum* [27, 48]. In this study, we firstly discovered three steroid C27 monooxygenase isoenzymes (SMO1, SMO2 and SMO3) involved in the C27-hydroxylation in *M. neoaurum*. By gene knockout and complementation experiment, SMO2 was confirmed to possess the strongest function in C27-hydroxylation. To enhance ADD production, metabolic engineering strategy was carried out by disrupting KSH to block the ADD degradation pathway and coexpressing ChoM, SMO2 and KSDD to strengthen the metabolic flux toward ADD in *M. neoaurum* JC-12. Finally, the recombinant strain JC-1 $2_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$ produced ADD of 20.1 g/L, which is the highest production ever reported. In this work, we firstly reported the optional regulation of the sterol metabolism to drive increased metabolic flux toward the freewheeling products by metabolic engineering strategy, which supplies a new insight into the redesigned metabolic pathway for improving the steroid intermediates production from sterols.

Experimental

Strains, plasmids, primers and culture conditions

All strains, plasmids and primers used in this work are listed in Table 1 and Table S1. *E. coli* strains JM109 and BL21 (DE3), cultured in Luria–Bertain (LB) medium, were used for plasmid construction and heterologous expression, respectively. Strain *M. neoaurum* JC-12 was stored in our laboratory and used for constructing engineering strains. For sterol biotransformation, *M. neoaurum* strains were inoculated in 10 ml seed medium (10 g/L glucose, 10 g/L peptone, 6 g/L beef extract, 10 g/L NaCl, pH 7.5) and cultivated at 30 °C and 160 rpm for 48 h. Then, 5 mL culture was transferred into 100 mL fermentation medium containing 20 g/L glucose, 10 g/L peptone, 6 g/L beef extract, 3 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$ and 5×10^{-4} g/L $MnCl_2{\cdot}4H_2O$, pH 7.5. To improve the sterol biotransformation, hydroxymethyl-β-cyclodextrin (HP-β-CD) was used to enhance the solubility of sterols [32, 47]. Sterols and HP-β-CD with a ratio of 1:3 (w/w) were added into the fermentation medium to conduct the transformation. Without special conditions, the fermentation was carried out with 20 g/L phytosterol (added with 60 g/L HP-β-CD). A 5-L fermentor (Biotech Co., Shanghai, China) was used to scale up the flask cultures with agitation speed of 400 rpm and 1 volume of air per unit of medium per minute (vvm) at 30 °C and pH 7.5. In addition, to identify the function of SMO in *M. neoaurum*, 4-cholesten-3-one agar medium containing 5 g/L 4-cholesten-3-one based on the minimal medium (g per liter of distilled water, $(NH_4)_2HPO_4$ 1.5, $MgSO_4{\cdot}7H_2O$ 0.2, K_2HPO_4 0.4, $FeSO_4{\cdot}7H_2O$ 5×10^{-4}, $ZnSO_4{\cdot}7H_2O$ 2×10^{-4}) and added with 20 g/L agar was used in this study. The corresponding antibiotics and inducers were added when needed.

Heterologous expression and purification of SMO isoenzymes in *E. coli*

The genome of *M. neoaurum* JC-12 was sequenced and three putative steroid C27 monooxygenase genes designated as *Smo1* (gene ID: MH881437), *Smo2* (gene ID: MH881438) and *Smo3* (gene ID: MH881439) were selected for further research in this study. The genes *Smo1*, *Smo2* and *Smo3* were amplified by PCR techniques using primers listed in Table S1. The amplified fragments were inserted into the *Sac* I/*Hin*d III or *Bam*H I/*Eco*R I sites of pET28a vector to create pET28a-*Smo1*, pET28a-*Smo2* and pET28a-*Smo3*, respectively. Then these recombinant plasmids were transformed into *E. coli* BL21 to construct recombinant strains BL21/pET28a-*Smo1*, BL21/pET28a-*Smo2* and BL21/pET28a-*Smo3*, which were confirmed by DNA sequencing. These recombinant strains were cultured in 50 mL LB medium with 50 mg/L kanamycin at 37 °C. The protein expression was induced by 0.05 mM IPTG when the OD_{600} value of the culture reached 0.6–0.8. After continuous cultivation for 12 h at 16 °C, the cells were harvested by centrifugation at 10,000×g for 10 min and washed with 50 mM phosphate buffer (pH 7.0). Then the pellets were suspended in phosphate buffer for sonication. The cell-free extracts were obtained by centrifugation at 10,000×g for 40 min and used for further protein sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, protein purification and enzyme activity

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Sources
Strains		
Escherichia coli		
JM109	General host for gene cloning	Invitrogen
BL21 (DE3)	Host for recombinant protein expression	Promega
BL21/pET28a-*Smo*	*E. coli* BL21 (DE3) harboring pET28a-*Smo1*/*Smo2*/*Smo3*	This study
Mycobacterium neoaurum		
ATCC 25795	Type strain of *M. neoaurum*	ATCC
JC-12	Wild type strain, converting sterols to ADD with small amount of AD	[28]
Mut_{S1}/Mut_{S2}/ Mut_{S3}/$Mut_{(S1\&S2)}$/ $Mut_{(S2\&S3)}$/$Mut_{(S1\&S3)}$/ $Mut_{(S1\&S2\&S3)}$	*Smo*-deleted mutant of *M. neoaurum* ATCC 25795	This study
Com_{S1}/Com_{S2}/ Com_{S3}/$Com_{(S1\&S2)}$/ $Com_{(S2\&S3)}$/$Com_{(S1\&S3)}$/ $Com_{(A1\&A2\&A3)}$	*Smo*-complemented strains of the *Smo*-deleted mutants	This study
JC-12_{S1}/JC-12_{S2}/JC-12_{S3}	SMO over-expressed strain of *M. neoaurum* JC-12	This study
JC-$12_{\Delta kshA}$	*kshA*-deleted mutant of *M. neoaurum* JC-12	This study
JC-$12_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$	SMO2, ChoM and KSDD coexpressed strain of JC-$12_{\Delta kshA}$	This study
Plasmids		
pMD18-T	*E. coli* clone vector; Amp^R	Takara
pET28a(+)	*E. coli* expression vector; Kan^R	Novagen
pMV261	Shuttle vector of *E. coli* and *mycobacterium*, carrying the heat shock promoter *hsp*60; Kan^R	Dr. W.R. Jacobs Jr.
pMV306	Mycobacterial integrating vector, Kan^R	Dr. W.R. Jacobs Jr.
p2NIL	Recombination vector of *mycobacterium*; Kan^R	[12]
pGOAL19	*Hyg* Pag_{85}-*lacZ* P_{hsp60}-*sacB*, *Pac*I cassette vector; Amp^R	[12]
pET28a-*Smo*	pET28a carrying *Smo1* or *Smo2* or *Smo3* gene; Kan^R	This study
p2N-Δ*Smo*	p2NIL harboring a crossover PCR product which covers the flanking regions of *Smo1* or *Smo2* or *Smo3* gene and the selection cassette from pGOAL19; Kan^R and Hyg^R	This study
p2N-Δ*kshA*	p2NIL harboring a crossover PCR product which covers the flanking regions of *kshA* gene and the selection cassette from pGOAL19; Kan^R and Hyg^R	This study
p261-*Smo*	pMV261 carrying *Smo1* or *Smo2* or *Smo3* gene; Kan^R	This study
p261-*Smo1-Smo2*	p261-*Smo1* carrying *Smo2* gene with its SD sequence inserted after *Smo1*; Kan^R	This study
p261-*Smo1-Smo3*	p261-*Smo1* carrying *Smo3* gene with its SD sequence inserted after *Smo1*; Kan^R	This study
p261-*Smo2-Smo3*	p261-*Smo2* carrying *Smo3* gene with its SD sequence inserted after *Smo2*; Kan^R	This study
p261-*Smo1-Smo2-Smo3*	p261-*Smo1-Smo2* carrying *Smo3* gene with its SD sequence inserted after *Smo2*; Kan^R	This study
p261-*Smo2-choM-ksdd*	p261-*Smo2* carrying *choM* and *ksdd* gene with their SD sequences inserted after *Smo2*; Kan^R	This study
p306-*Smo*	pMV306 carrying *Smo1* or *Smo2* or *Smo3* gene under the *hsp*60 promoter; Kan^R	This study
p306-*Smo1-Smo2*	pMV306 carrying *Smo1* and *Smo2* gene under the *hsp*60 promoter; Kan^R	This study
p306-*Smo1-Smo3*	pMV306 carrying *Smo1* and *Smo3* gene under the *hsp*60 promoter; Kan^R	This study
p306-*Smo2-Smo3*	pMV306 carrying *Smo2* and *Smo3* gene under the *hsp*60 promoter; Kan^R	This study
p306-*Smo1-Smo2-Smo3*	pMV306 carrying *Smo1*, *Smo2* and *Smo3* gene under the *hsp*60 promoter; Kan^R	This study

M. neoaurum ATCC 25795 (accession number: ATCC 25795, American Type Culture Collection)

Amp^R ampicillin-resistant, Km^R kanamycin-resistant

assay. Purification was performed as the instructions of HisTrap™ HP column [43]. The method of Bradford was used to determine the protein concentration and bovine serum albumin was used as the standard [2].

Activity assay and protein properties of the SMO isoenzymes

The SMO activity was determined by monitoring substrate

concentrations using HPLC as described previously with minor modification [21]. One unit of SMO activity is defined as the amount of enzyme required to convert 1 μmol of 4-cholesten-3-one to 4-cholesten-3-one-27-oic acid at 30 °C and pH 7.0 per minute. The optimum pH for SMO activity was determined by SMO enzyme activity assay at 30 °C in several buffers (50 mM citrate–sodium citrate buffer, pH 3.0–6.0; 50 mM phosphate buffer, pH 6.0–8.0; 50 mM glycine–sodium hydroxide buffer, pH 8.0–10.0). The optimum temperature was examined in 50 mM phosphate buffer (pH 7.0) using the standard reaction mixture with temperatures ranging from 10 to 60 °C. The kinetic parameters (K_M, V_{max} and k_{cat}) of these three isoenzymes were determined by fitting a plot of rate versus substrate concentration to the Michaelis–Menten equation using nonlinear regression in the software GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA, USA).

Functional analysis of SMO1, SMO2 and SMO3 in sterols metabolism

To identify the function of SMO isoenzymes, the disruption and complementation of their corresponding genes were carried out and the engineered strains used were constructed as given in the Supplementary Methods. By measuring the viability of the *Smo*-deleted strains grown on 4-cholesten-3-one agar medium plates with 4-cholesten-3-one as the sole energy and carbon source, the roles of SMO1, SMO2 and SMO3 on the growth of *M. neoaurum* with sterol as the sole energy and carbon source were identified. The different *Smo*-deleted strains were firstly inoculated into 10 mL seed medium and cultivated on a rotary shaker at 30 °C and 160 rpm for 24 h. Then, 1 mL of these cultures were diluted appropriately with distilled physiological saline and then plated on the 4-cholesten-3-one agar medium plates. CFU (colony forming units) of these strains were calculated at 3 and 7 days, respectively. Besides, the SMO enzyme activity of these *Smo*-deleted strains in the seed medium was also detected at 3 and 7 days, respectively. The 4-cholesten-3-one degradation properties of the different *Smo*-deleted strains were analyzed by shake-flask fermentation. After cultivation on a rotary shaker at 30 °C and 160 rpm for 24 h, 2 mL of the seed medium cultures were inoculated into 50 mL fermentation medium added with 1 g/L 4-cholesten-3-one and 3 g/L HP-β-CD. Then, the difference in the behavior among the *Smo*-deleted mutants in 4-cholesten-3-one degradation was clarified with the cultivation period elongated to 250 h. The residue of substrate was analyzed by HPLC.

Overexpression of SMO isoenzymes in *M. neoaurum* JC-12

To analyze the effects of SMOs on sterol transformation, plasmid pMV261 was used as an overexpression vector to augment the expression of SMO1, SMO2 and SMO3 in *M. neoaurum* JC-12. Genes *Smo1*, *Smo2* and *Smo3* were amplified using primers *Smo1*-f & r, *Smo2*-f & r and *Smo3*-f & r (Table S1). The *Sac* I/*Hin*d III fragment of *Smo1*, *Bam*H I/*Eco*R I fragment of *Smo2*, and *Bam*H I/*Eco*R I fragment of *Smo3* genes were inserted into the corresponding sites of pMV261 to construct the recombinant plasmids p261-*Smo1*, p261-*Smo2* and p261-*Smo3*. Then these plasmids were transferred into *M. neoaurum* JC-12 to generate the recombinant strains JC-12$_{S1}$, JC-12$_{S2}$ and JC-12$_{S3}$ (Table 1).

Metabolic engineering of *M. neoaurum* JC-12 for the production of ADD

A metabolic engineering strategy was carried out by disrupting KSH and coexpressing ChoM, SMO2 and KSDD. To delete *kshA* gene in *M. neoaurum* JC-12, recombinant strain JC-12$_{\Delta kshA}$ with p2N-Δ*kshA* was constructed in accordance with Mut$_{S1}$ as described in the Supplementary Methods. To coexpress ChoM, SMO2 and KSDD in the *kshA*-deleted mutant JC-12$_{\Delta kshA}$, the primers *choM*-SD-f (containing an SD sequence for ribosome binding) and *choM*-r were used to amplify the *choM* gene. The *choM* fragment was solely digested by *Eco*R I and inserted into the *Eco*R I site of plasmid p261-*Smo2* to create the p261-*Smo2-choM* with SMO2 and ChoM. Then, the *ksdd* gene was amplified using primers *ksdd*-SD-f and *ksdd*-r, and the solely *Hin*d III digested fragment was inserted into the *Hin*d III site of plasmid p261-*Smo2-choM* to create p261-*Smo2-choM-ksdd* with SMO2, ChoM and KSDD. The plasmid p261-*Smo2-choM-ksdd* was transformed into JC-12$_{\Delta kshA}$ to construct the recombinant strain JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$, in which the *kshA* gene was knocked out and *Smo2*, *choM* and *ksdd* genes were augmented. The KSH, ChoM and KSDD enzyme activities were assayed according to previous studies [27, 28, 46].

Analytical methods

A 1 mL sample was taken from culture broth and extracted with 4 mL ethyl acetate. After centrifugation, 2 mL of the supernatant was analyzed by Shimadzu HPLC equipped with C18 column (Diamonsil ®C18, 5 μm particles, 250 mm × 4.6 mm) and UV/visible detector. 4-Cholesten-3-one was detected at 240 nm and the mobile phase composed of acetonitrile and isopropanol (85:15, v/v) [26]. ADD was detected at 254 nm and the mobile phase composed of methanol and water (70:30, v/v). The flow rate was 1 mL/min and the column temperature was 30 °C [48]. Biomass accumulation was determined as the number of CFU per mL of cultural liquid during fermentation [20]. Residual glucose detection was done by a biological sensing analyzer (SBA, China) [49].

Results and discussion

Characterization of SMO isoenzymes from *M. neoaurum*

Mycobacterium neoaurum JC-12 is a good producer of ADD with few by-products of AD using sterols as substrate [28]. By analyzing the genome sequence results of *M. neoaurum* JC-12, three putative steroid C27-monooxygenases encoded by the genes *Smo1* (gene ID: MH881437), *Smo2* (gene ID: MH881438) and *Smo3* (gene ID: MH881439) were selected for further research. The amino acid identity between SMO1 and SMO2 was about 54%, and the identity between SMO1 and SMO3 was 65.6%. The phylogenetic tree is shown in Fig. S1 and SMO1, SMO2 and SMO3 share high similarity with the cytochrome P450 of *Mycobacteriaceae* (WP_019510071.1, 99%), cytochrome P450 of *Mycobacteriaceae* (WP_023986299.1, 99%) and cytochrome P450 of *Mycobacteriaceae* (WP_019512517.1, 99%), respectively.

To characterize the C27-hydroxylation catalytic activity, these three SMO isoenzymes were purified (Fig. S2) and the enzyme activities were analyzed (Table S2). Besides, the steady-state kinetic constants (K_M and k_{cat}) for the oxidation of 4-cholesten-3-one by SMO1, SMO2 and SMO3 are shown in Table 2. The K_M of SMO2 was lower than that of SMO1 and SMO3, and the k_{cat} and k_{cat}/K_M of SMO2 was higher than that of SMO1 and SMO3. These results indicate that the catalytic activity of SMO2 is higher than SMO1 and SMO3. Since the enzymatic properties of SMOs from *M. neoaurum* ATCC 25795 and *M. neoaurum* JC-12 have no differences, we designated SMO1, SMO2 and SMO3 as the same.

SMO2 exhibits stronger functions in sterol catabolism than SMO1 and SMO3

Since sterol catabolic pathway in *M. neoaurum* ATCC 25795 is intact, and there are no intermediates (AD/ADD) accumulated to inhibit cell growth and respiration [10]. Thus, we selected it as an experimental strain rather than *M. neoaurum* JC-12 to determine the specific functions of the enzymes SMO1, SMO2 and SMO3 in sterol catabolism. On the bases of *M. neoaurum* ATCC 25795, seven *Smo*-deleted mutants (Mut_{S1}, Mut_{S2}, Mut_{S3}, $Mut_{(S1\&S2)}$, $Mut_{(S1\&S3)}$, $Mut_{(S2\&S3)}$ and $Mut_{(S1\&S2\&S3)}$) and their corresponding complemented strains were constructed as described in Supplemental Methods. As shown in Table S3, all *Smo*-deleted mutants exhibited low enzyme activities and *Smo2*-deleted mutant showed lower activity than *Smo1* or *Smo3*-deleted mutants. Compared with wild-type strain ATCC 25795, all *Smo*-deleted mutants grew poorly on 4-cholesten-3-one agar plates and *Smo2*-deleted mutant grew poorer than *Smo1* or *Smo3*-deleted mutants (Fig. S3). The cell growth of mutant strain $Mut_{(S1\&S2)}$ was inhibited the most among two-gene deleted mutants. When *Smo1*, *Smo2* and *Smo3* were all disrupted in $Mut_{(S1\&S2\&S3)}$, cell growth was severely affected, indicating that the catabolism of sterols was greatly blocked. However, the complemented strains of *Smo*-deletion mutants showed better growth state on 4-cholesten-3-one agar plates and their activities toward 4-cholesten-3-one were restored to some extent. In addition, *Smo2*-complemented mutant exhibited better cell-growth properties than *Smo1* and *Smo3*-complemented mutants. These results showed that a combination of SMO1, SMO2 and SMO3 was required for *M. neoaurum* cell growth and SMO2 was more important than SMO1 and SMO3 for cell growth and enzyme activity.

To further assess the effects of *Smo1*, *Smo2* and *Smo3* on the catabolism of sterols, a fermentation medium containing sufficient nutriment supplemented with 4-cholesten-3-one and HP-β-CD was used for the degradation of 4-cholesten-3-one. There was no obvious difference in the cell growth between the wild-type strain *M. neoaurum* ATCC 25795 and its mutants Mut_{S1}, Mut_{S2}, Mut_{S3}, $Mut_{(S1\&S2)}$, $Mut_{(S1\&S3)}$, $Mut_{(S2\&S3)}$ and $Mut_{(S1\&S2\&S3)}$ in the nutriment medium (data not shown). As shown in Fig. 2, the wild type could completely degrade 1 g/L 4-cholesten-3-one within 100 h, while the *Smo1*-deleted mutant Mut_{S1}, *Smo2*-deleted mutant Mut_{S2} and *Smo3*-deleted mutant Mut_{S3} degraded 79%, 66% and 87% 4-cholesten-3-one within 100 h. The mutants Mut_{S1},

Table 2 Steady-state kinetic constants (K_M and k_{cat}) of SMO1, SMO2 and SMO3 towards 4-cholesten-3-one

Strains	Enzymes	K_M (μM)	k_{cat} (min^{-1})	K_{cat}/K_M (μM^{-1} min^{-1})
M. neoaurum JC-12	SMO1	28.3 ± 1.6	167 ± 6	5.9
	SMO2	23.5 ± 1.5	180 ± 8	7.7
	SMO3	32.8 ± 2.3	156 ± 5	4.8
M. neoaurum ATCC 25795	SMO1	28.3 ± 1.3	167 ± 5	5.9
	SMO2	23.5 ± 1.8	180 ± 6	7.7
	SMO3	32.8 ± 2.5	156 ± 3	4.8

All assays were performed in triplicate with three independent measurements. Error bars represented standard deviations of the biological replicates

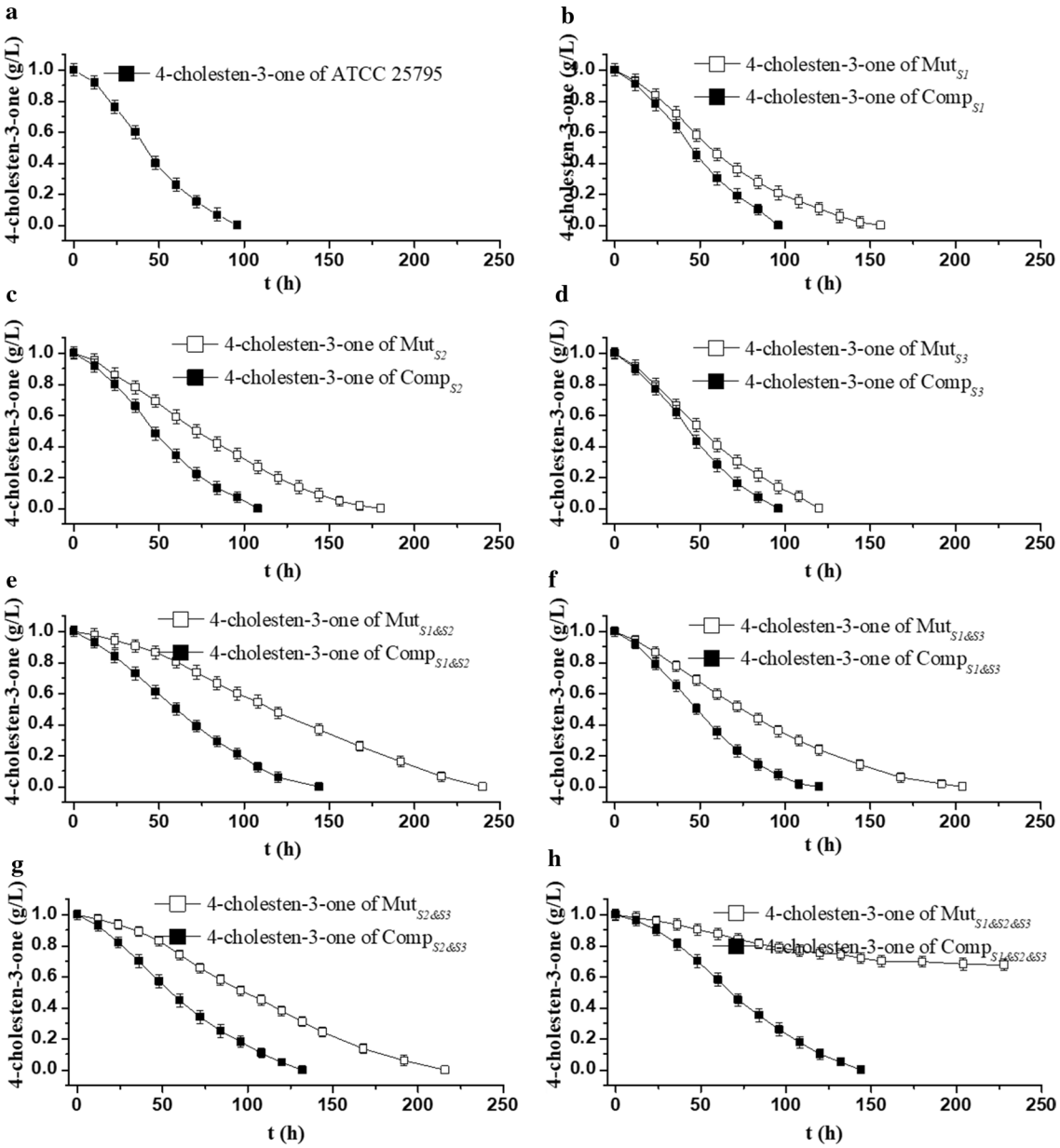

Fig. 2 The utilization of 4-cholesten-3-one by the *Smo*-deleted mutants and their corresponding complements. The strains were cultured in the fermentation medium added with 1 g/L 4-cholesten-3-one. The residue of 4-cholesten-3-one was detected by HPLC. All assays were performed in triplicate with three independent measurements. Error bars represented standard deviations of the biological replicates

Mut$_{S2}$ and Mut$_{S3}$ required 156 h, 180 h, and 120 h to completely degrade 1 g/L 4-cholesten-3-one. Among the mutants with the two *Smo* gene deletion (Mut$_{(S1\&S2)}$ Mut$_{(S2\&S3)}$ and Mut$_{(S1\&S3)}$), Mut$_{(S1\&S2)}$ retarded 4-cholesten-3-one degradation most seriously, while Mut$_{(S1\&S3)}$ retarded the degradation slightly. Besides, the results of Fig. 2 also provided an additional clue that there should be other enzymes in *M. neoaurum* which exhibit a similar function to SMOs, as Mut$_{(S1\&S2\&S3)}$ could still retain a partial capacity to degrade 4-cholesten-3-one. Recent studies have indicated that some other steroid C27 monooxygenases, such as CYP142A1 in *M. tuberculosis* and CYP142A2 in *M. smegmatis,* play a similar role to SMOs for the initial degradation of sterol side chain [5, 21]. All these results suggested that SMO was the key enzyme in the sterol catabolism pathway, and SMO2 showed stronger functions in sterol metabolism. Therefore, it would be helpful to enhance the sterol conversion efficiency to valuable steroids by improving the enzymatic activity through metabolic engineering method. Since there was no obvious difference in cell growth between ATCC25795

and *Smo*-deleted mutants (data not shown), the difference in the behavior in 4-cholesten-3-one degradation was mainly caused by *Smo* deletion. In this sense, these results were in accordance with that identified on the 4-cholesten-3-one agar plates.

Improved ADD production by SMO2 expression in *M. neoaurum*

Since SMO1, SMO2 and SMO3 play important roles in the sterol catabolism pathway, it is intriguing to determine whether the ADD yield could be significantly enhanced by improving the activity of SMO in *M. neoaurum* JC-12. Therefore, in this study, to detect the effects of SMO isoenzymes on ADD production, recombinant strains JC-12$_{S1}$ overexpressing SMO1, JC-12$_{S2}$ overexpressing SMO2 and JC-12$_{S3}$ overexpressing SMO3 were constructed, respectively.

As shown in Fig. 3, SMOs overexpression has no obvious effects on cell growth and glucose consumption, while the SMO-augmented strains could greatly increase the phytosterol conversion rate and ADD production. Compared with the parent strain *M. neoaurum* JC-12, the sterol conversion rate of the recombinant strains JC-12$_{S1}$, JC-12$_{S2}$ and JC-12$_{S3}$ was increased from 47.6% to 59.4%, 65.1% and 54.7%, respectively. Accordingly, the ADD yield of the recombinant strains JC-12$_{S1}$, JC-12$_{S2}$ and JC-12$_{S3}$ was enhanced from 5.2 g/L to 6.5 g/L, 7.3 g/L and 6.1 g/L with an increase of 25.0%, 40.4% and 17.3%. These results suggested that ADD production could be enhanced by SMO over expression, and SMO2-augmentation exhibited the highest level of ADD yield improvement, which further verified that SMO2 was more important than SMO1 and SMO3 in sterol catabolism. In a previous study, although ChoM2 is more important than ChoM1 in the sterol catabolism in *M. neoaurum*, the ChoM1-augmented strains and the ChoM2-augmented strains exhibited no obvious difference in their transformation capacity to accumulate ADD or AD [45]. Unlikely, in this study, the more important role of SMO2 than SMO1 and SMO3 in sterol catabolism lead to higher ADD yield of SMO2-augmented strain than that of SMO1 or SMO3-augmented strains. All these results confirmed that SMOs play an important role in the sterol catabolic pathway, and SMOs augmentation is beneficial to ADD accumulation in *M. neoaurum*. This is the first report about the application of SMOs in improving ADD production, and these results strongly suggested that the SMOs over expression in the sterols transforming mycobacteria might be a viable way to enhance sterol transformation to valuable steroid intermediates. However, the ADD concentration gradually decreased after 144 h, and this was mainly due to the ADD degradation catalyzed by the KSH enzyme [4]. Thus, it is imperative to block the ADD degradation pathway by *ksh* gene disruption to realize ADD accumulation.

Blocking the pathway of ADD degradation by *kshA* gene disruption

As is well known, KSH activity is represented by two components: terminal oxygenase (KshA) and ferredoxin

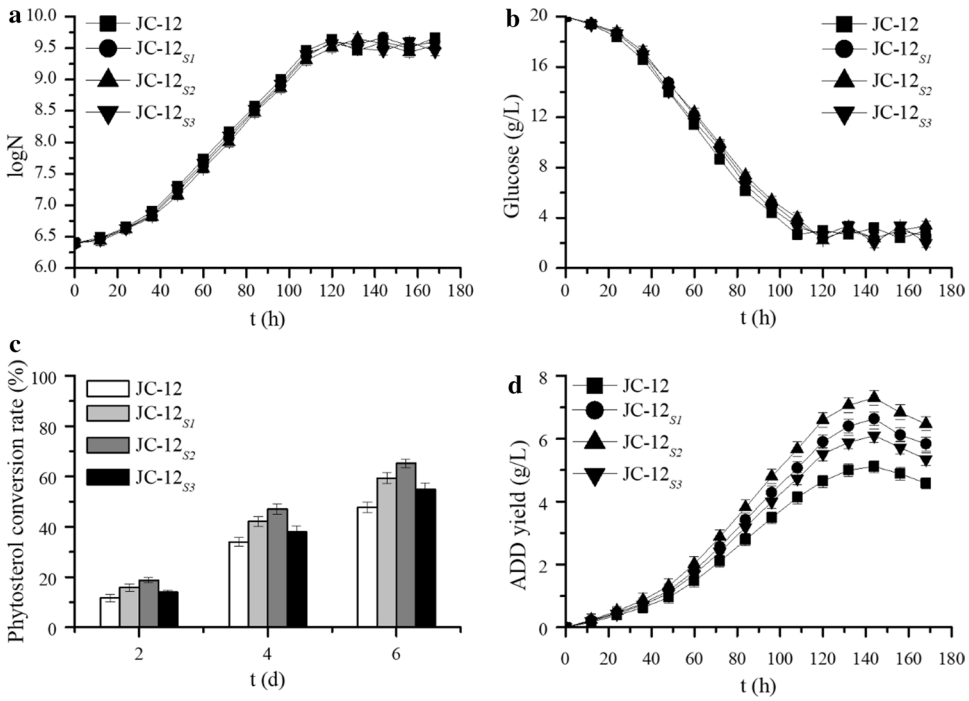

Fig. 3 Effects of the augmentation of SMOs on strain growth (**a**), glucose consumption (**b**), phytosterol conversion (**c**) and ADD production (**d**) in *M. neoaurum*. 20 g/L phytosterol (added with 60 g/L HP-β-CD) was supplemented as the substrate into the fermentation medium to conduct the transformation. *N* the number of CFU (colony forming units) per 1 mL of culture fluid. Glucose was fed when needed. All assays were performed in triplicate with three independent measurements. Error bars represented standard deviations of the biological replicates

reductase (KshB) in steroid 9α-hydroxylation [46]. A diversity of KshA activity can be usually observed in some strong sterol-using strains, indicating its significant role in the 9α-hydroxylation process of catalyzing the conversion of AD to 9α-OH-AD and degradation of ADD to 9α-OH-ADD in *M. neoaurum* [23, 38]. Therefore, we attempted to inactivate the KSH activity by disrupting the KshA component in *M. neoaurum* JC-12.

In this work, the *kshA* gene was knocked out to construct the mutant strain JC-12$_{\Delta kshA}$. Compared with *M. neoaurum* JC-12, KSH enzyme activity of JC-12$_{\Delta kshA}$ was hardly detected, which verified the successful disruption of KSH (Fig. S4). As shown in Fig. 4, fermentation curves showed that there were no difference in biomass and residual glucose between JC-12$_{\Delta kshA}$ and *M. neoaurum* JC-12, indicating that *kshA* gene disruption has no obvious effect on *M. neoaurum* cell growth. During sterol transformation by *M. neoaurum* JC-12, ADD reached the highest production of 5.2 g/L and decreased gradually after 144 h. This was because sterols could not be transformed into ADD, while ADD incessantly degraded into 9α-OH-ADD by catalysis of KSH. On the contrary, during the JC-12$_{\Delta kshA}$ sterol transformation process, ADD production was higher than that of *M. neoaurum* JC-12 and the highest yield reached 6.5 g/L at 144 h with no reduction later. This was because the KSH enzyme inactivation lead to the obstruction of the ADD 9α-hydroxylation pathway, which resulted in the higher ADD accumulation. Therefore, it is beneficial for ADD production through KSH disruption.

In the sterol catabolism process, ADD is converted to 9α-OH-ADD by KSH catalysis, while 9α-OH-ADD is an unstable compound followed by non-enzymatic ring B destruction with further full degradation [41, 42]. To protect the steroidal nucleus, previous efforts mainly focused on the chemical inhibition of KSH and the screening and improvement of microorganisms for higher AD/ADD yield [19]. In this work, we disrupted the *kshA* gene to block the KSH-catalyzed pathway from AD/ADD to 9α-OH-AD/9α-OH-ADD. The results showed that *kshA* deletion had no effect on cell growth, while ADD yield of JC-12$_{\Delta kshA}$ increased to some extent and ADD did not degrade. Therefore, inactivation of KSH activity was supposed to be a fundamental premise to develop a promising ADD biocatalyst.

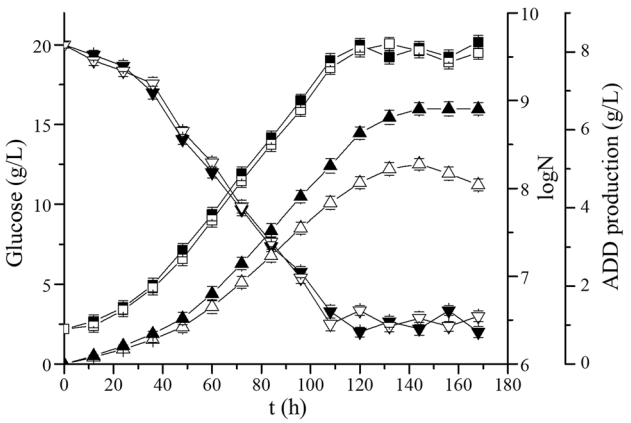

Fig. 4 Fermentation curves of *M. neoaurum* JC-12 (hollow symbols) and JC-12$_{\Delta kshA}$ (solid symbols). The fermentation was carried out with 20 g/L phytosterol (added with 60 g/L HP-β-CD). Glucose was fed when necessary. The parameters, such as glucose (inverted triangle), the biomass (square) and ADD concentration (triangle) were concurrently recorded during the fermentation. All assays were performed in triplicate with three independent measurements. Error bars represented standard deviations of the biological replicates

Significantly enhanced ADD production by coexpression of ChoM, SMO and KSDD in KSH-disrupted mutant JC-12$_{\Delta kshA}$

It has been reported that ChoM and KSDD were key enzymes in sterol transformation [26, 48], while SMO2 was also identified as a key enzyme in sterol catabolism and played a more important role than SMO1 and SMO3. Thus, we attempted to strengthen the sterol metabolic flux by coexpressing ChoM, SMO2 and KSDD in mutant JC-12$_{\Delta kshA}$ to realize a higher ADD production.

The successful construction of the recombinant strain JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$ was confirmed by DNA sequencing and SDS-PAGE analysis (data not shown), and the enzyme activity assay further certified the functional expression of these three enzymes (Table S4). As shown in Fig. 5a, the biomass of JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$ was slightly lower than that of JC-12$_{\Delta kshA}$. This was mainly because the over expression of three enzymes possibly increased the burden of cell growth, but this effect was not obvious. As shown in Fig. 5b, compared with JC-12$_{\Delta kshA}$, sterol conversion amount of JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$ enhanced from 49.6% to about 90%. Accordingly, the ADD yield increased from 6.5 to 11.6 g/L, almost a 78% increase (Fig. 5c). These results showed that the coexpression of ChoM, SMO2 and KSDD significantly improved the ADD production of *M. neoaurum* JC-12. In summary, it is a feasible metabolic engineering means to increase the transformation efficiency of sterols to the valuable steroid intermediate ADD by augmentation of ChoM, SMO2 and KSDD and disruption of KSH in *M. neoaurum*.

Some efforts have been made to identify the key enzymes involved in sterol transformation and improve the yield of the valuable steroid intermediates. Wei et al. reported the inactivation and augmentation of the primary KSDD in *M. neoaurum* NwIB-01 to increase the AD or ADD production from soybean phytosterol biotransformation [39]. Yao et al. identified two cholesterol oxidases involved in the initial step of sterol catabolism in *M. neoaurum*, and the augmentation of the ChoM2 activity achieved the increased AD and ADD production in *M. neoaurum* NwIB-R10 and in *M. neoaurum*

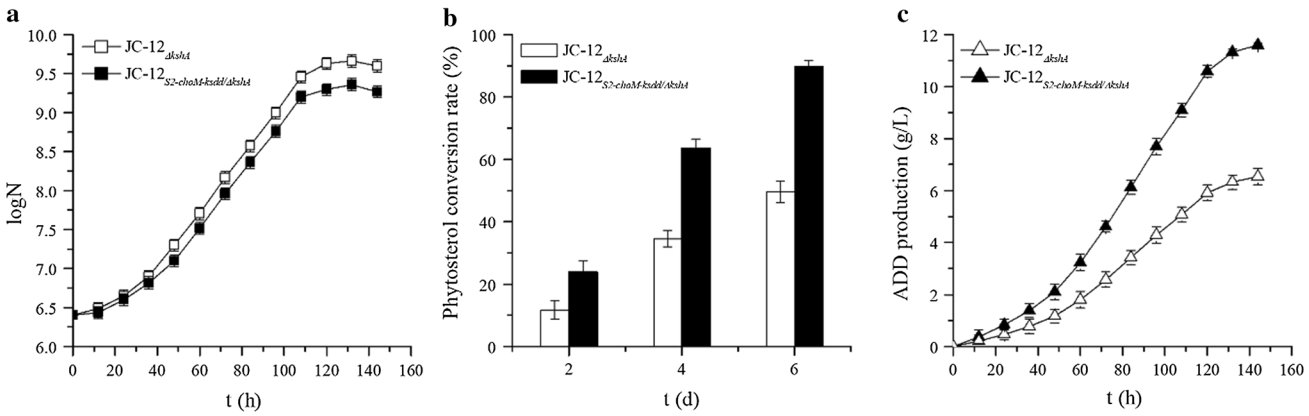

Fig. 5 Effects of the coexpression of ChoM, SMO and KSDD on ADD production in mutant JC-12$_{\Delta kshA}$. The fermentation was carried out with 20 g/L phytosterol (added with 60 g/L HP-β-CD). Glucose was fed when necessary. The parameters, such as the biomass, phytosterol conversion rate and ADD yield were concurrently recorded during the fermentation. All assays were performed in triplicate with three independent measurements. Error bars represented standard deviations of the biological replicates

NwIB-01MS, respectively [45]. However, this work only used genetic engineering by expressing or disrupting the gene in host strains, and no metabolic engineering strategy was used for enhancing ADD production. In the present work, we firstly identified the function of SMOs in sterols catabolism in *M. neoaurum* JC-12. Then, we coexpressed ChoM, SMO2 and KSDD in the KSH-disrupted strain to strengthen the metabolic flux. The final ADD production improved significantly. This is the first report for improving ADD yield through metabolic engineering strategy.

To evaluate the applicability of the strain JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$ in industry scale, the performance of this recombinant strain was carried out in a 5-L fermentor using 30 g/L phytosterol as substrate. As shown in Fig. 6, compared with the ADD production (7.9 g/L) by *M. neoaurum* JC-12 at 144 h, the recombinant strain JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$ produced the maximum ADD yield of 20.1 g/L at 120 h, with the conversion rate of 0.168 g/L/h, molar yield of 91.6% and ADD/AD molar ratio of 20:1. To our knowledge, this is the highest ADD production ever reported (Table 3). These results indicated that metabolic engineering of sterol catabolic pathway could be an effective strategy to increase the production of valuable steroidal intermediates from low-cost sterols in pharmaceutical industry scale.

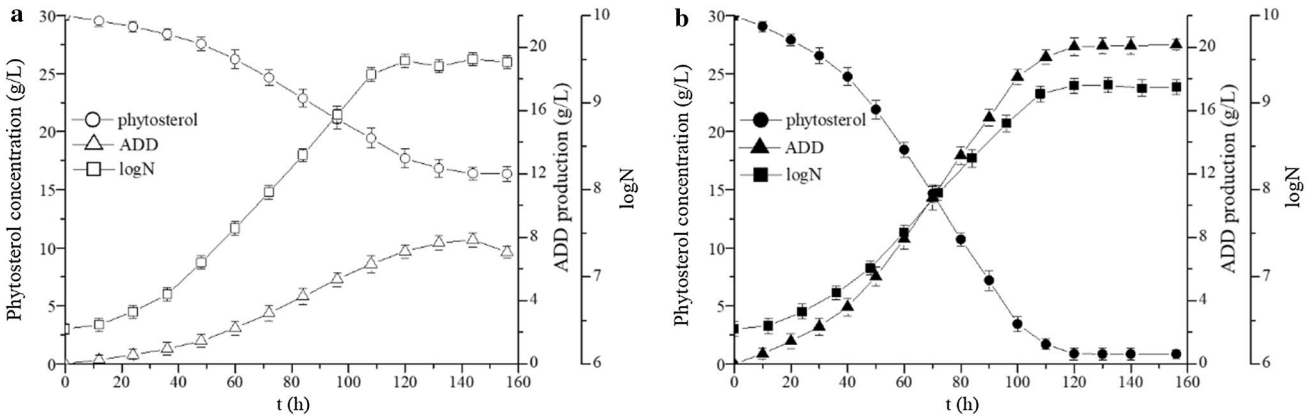

Fig. 6 Time profiles of ADD fermentation of parent strain *M. neoaurum* JC-12 (**a**) and the recombinant strain JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$ (**b**) in a 5-L fermentor. The fermentation was carried out with 30 g/L phytosterol (added with 90 g/L HP-β-CD). Glucose was fed when necessary. All assays were performed in triplicate with three independent measurements. Error bars represented standard deviations of the biological replicates

Table 3 Comparing of microbial production of ADD using different fermentative strains or biocatalysts

Strains and biotransformation mode	Substrates	Biotransformation duration (h)	Production (g/L)	Productivity (g/L/h)	References
Biocatalyst					
Mycobacterium sp. NRRL-B3683	Phytosterol	144	12.0	0.083	[37]
B. subtilis 168	AD	10	0.66	0.066	[48]
B. subtilis WB600	AD	48	0.45	0.009	[16]
E. coli BL21	AD	36	5.7	0.158	[25]
Fermentation					
M. neoaurum NwIB-04	Soybean phytosterols	96	4.94	0.051	[39]
M. neoaurum NwIB-01MS	Phytosterol	144	5.57	0.039	[45]
Chryseobacterium gleum	Cholesterol	24	0.08	0.003	[6]
Nocardia sp.	Cholesterol	96	0.11	0.001	[31]
Gordonia neofelifaecis	Cholesterol	96	0.44	0.005	[17]
M. neoaurum JC-12$_{S2\text{-}choM\text{-}ksdd/\Delta kshA}$	Phytosterol	120	20.1	0.168	This study

Fermentation: the transformation was carried out by using the fermentative strains; biocatalyst: the transformation was carried out by using the whole-cells [25]

Conclusion

Here, we identified three C27 monooxygenase isoenzymes as the key enzymes involved in sterol catabolism and determined SMO2 as the strongest one in *M. neoaurum* C27-hydroxylation. By blocking KSH to prevent the ADD degradation pathway and coexpressing three key enzymes, ChoM, SMO2 and KSDD, the final ADD yield reached 20.1 g/L, which is the highest ever reported. This work provides new insight into the redesigned microreactor, which efficiently produces ADD through metabolic engineering strategy. This strategy also paves the way for developing *M. neoaurum* as a microbial factory for the efficient production of other valuable steroid metabolites from sterols in the pharmaceutical industry.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest to declare.

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