Improving the Production of 9,21-dihydroxy-20-methyl-pregna-4-en-3-one from Phytosterols in *Mycobacterium Neoaurum* by Modifying Multiple Genes and Improving the Intracellular Environment

Chen-Yang Yuan

SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Zhi-Guo Ma

SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Jing-Xian Zhang

SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Xiang-Cen Liu

SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Gui-Lin Du

SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Jun-Song Sun

SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute <https://orcid.org/0000-0002-7339-3250>

Ji-Ping Shi

SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Bao-Guo Zhang (✉ zhangbg@sari.ac.cn)

Shanghai Advanced Research Institute <https://orcid.org/0000-0002-0800-0342>

Research

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Abstract

Background

Steroid drugs are particularly important for disease prevention and clinical treatment. However, traditional chemical methods are rarely implemented during the whole synthetic process to generate steroid intermediates due to the intricate steroid structure. Novel steroid drug precursors and their ideal bacterial strains for industrial production have yet to be developed. Among these, 9-OH-4-HP is a potential steroid drug precursor for the synthesis of corticosteroids. In this study, a combined strategy of blocking Δ^1 -dehydrogenation and the C19 pathway as well as improving the intracellular environment was investigated to construct an effective 9-OH-4-HP-producing strain.

Results

A Δ^1 -dehydrogenation-deficient strain of wild-type *Mycobacterium neoaurum* DSM 44074 produces 9-OH-4-HP with a molar yield of 4.8%. *hsd4A*, encoding a β -hydroxyacyl-CoA dehydrogenase, and *fadA5* encoding an acyl-CoA thiolase, were separately knocked out to block the C19 pathway in the Δ^1 -dehydrogenation-deficient strain. The two engineered strains could accumulate 0.59 g L⁻¹ and 0.47 g L⁻¹ 9-OH-4-HP from 1 g L⁻¹ phytosterols. Furthermore, *hsd4A* and *fadA5* were knocked out simultaneously in the Δ^1 -dehydrogenation-deficient strain. The 9-OH-4-HP production from the Hsd4A and FadA5 double-deficient strain was 11.9% higher than that of the Hsd4A -deficient strain and 40.4% higher than that of the strain with FadA5 deficiency, and its selectivity reached 94.9%. Subsequently, the catalase *katE* from *Mycobacterium* and an NADH oxidase, *nox*, from *Bacillus subtilis* were overexpressed to improve the intracellular environment. Ultimately, 9-OH-4-HP production reached 3.58 g L⁻¹ from 5 g L⁻¹ phytosterols, and the selectivity of 9-OH-4-HP improved to 97%.

Conclusion

hsd4A and *fadA5* are key enzymes in the C19 pathway for phytosterol side chain degradation. Deletion of *hsd4A* and *fadA5* could almost entirely block the C19 pathway. Improving the intracellular environment of *Mycobacterium* during phytosterol bioconversion could accelerate the conversion process and enhance the productivity of target sterol derivatives.

Background

Steroid drugs, including mineralocorticoids, glucocorticoids, and sex hormones, are crucial in the prevention and clinical treatment of various diseases, including diabetes, asthma, neurodegenerative disorders, cancer, hormone metabolic syndrome, and many others [1, 2]. Steroid drugs represent one of the best-selling classes of drugs and are the second-largest pharmaceutical on the market. Global pharmaceutical industry predictions indicate that many drugs based on steroid compounds will retain market leadership in the future.

In industrial manufacturing, two major valuable intermediates of sterols, C19 steroids and C22 steroids, can be used to synthesize sex and adrenocortical hormones. However, traditional chemical methods are rarely implemented in the whole synthetic processes of modifying steroid intermediates due to the intricate steroid structure. Thus, the pursuit of novel steroid drug precursors has intrigued researchers. Certain C22 steroids, including 21-hydroxy-20-methyl-pregna-4-en-3-one (4-HP), 21-hydroxy-20-methyl-pregna-1,4-dien-3-one (1,4-HP), and 9,21-dihydroxy-20-methyl-pregna-4-en-3-one (9-OH-4-HP), are ideal precursors for steroid drug synthesis [3]. Among these steroids, 9-OH-4-HP is a highly valuable steroid derivative for the synthesis of corticosteroids because of its substituents at positions 9 and 21. Early studies identified 9-OH-4-HP as a by-product of sterol bioconversion to generate 9-hydroxy steroid derivatives in several *Mycobacterium* species, such as *Mycobacterium* sp. 2-4M, which produces a 1.5%-1.6% molar yield of 9-OH-4-HP [4]. *Mycobacterium* sp. VKM Ac-1815D, *Mycobacterium* sp. VKM Ac-1817D, and *Mycobacterium fortuitum* ATCC-6842 have also been reported to accumulate a small amount of 9-OH-4-HP during the 9-OH-AD production process [4–6]. However, ideal industrial strains have yet to be developed.

Actinomycetes, such as *Mycobacterium*, share similar sterol metabolic pathways. The initial step of sterol metabolism is the conversion of sterols to 4-en-3-sterone, followed by degradation of the sterol side chain and cleavage of the steroid skeleton [3, 7, 8]. The cleavage of the steroid B ring is performed by Δ^1 -dehydrogenation catalysed by 3-ketosteroid-1(2)-dehydrogenase (KstD) and C9-hydroxylation catalysed by 9 α -hydroxylase (KSH) [9, 10]. Consequently, blocking C9 hydroxylation resulted in various C19 steroids. 9 α -Hydroxy derivatives, such as 9-hydroxy-androst-4-ene-3,17-dione (9-OH-AD), are important precursors in the manufacture of several modern glucocorticoid drugs bearing a halogen at the 9 α position [11]. An engineered strain of *Mycobacterium neoaurum* ATCC 25795 in which *kstDs* are knocked out can accumulate 6.02 g L⁻¹ 9-OH-AD as the main product from 15 g L⁻¹ phytosterols [12]. *kstd1* and *kstd2* have also been reported to be effective for 9-OH-AD degradation in *Rhodococcus rhodochrous* DSM43269 [13]. By overexpression of certain related genes and knockout of *kstDs*, the production of 9-OH-AD increased by 45% in *M. neoaurum* MS136 [14]. A few wild-type strains of *Mycobacterium* have been reported to be able to produce 9-OH-AD from plant or animal sterols via a single-step microbial conversion [4]. Normally, 9 α -hydroxy derivatives can be obtained from industrial strains in which *kstDs* are deleted.

In contrast, side chain degradation is rather complicated. Some key enzymes involved in the side chain metabolic pathway remain uncertain, limiting the comprehensive understanding of the process. Dual competing pathways, the overwhelming C19 steroid pathway and the C22 steroid pathway, are involved in phytosterol side chain degradation. Recently, the 17-hydroxysteroid/22-OH-BNC-CoA dehydrogenase Hsd4A was found to be relevant during C22 steroid formation [3]. Inactivation of Hsd4A enabled the production of C22 steroids from sterols. For example, *M. neoaurum* *NwIB-XII* accumulates androst-4-ene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD), which are both C19 steroids, as the main products after culture with cholesterol. The *hsd4A* knockout strain accumulates 4-HP and 1,4-HP as the main products. Nevertheless, C19 steroids still accumulated in the *hsd4A* knockout strain from

phytosterols, indicating incomplete blockage of the C19 steroid pathway. An Hsd4A- and KstDs-deficient strain of *M. neoaurum* ATCC 25795 gives a 32% molar yield of 9-OH-4-HP and a 15% molar yield of 9-OH-AD from 40 g L⁻¹ phytosterols [3]. FadA5, a thiolase, was reported to be essential for the production of AD/ADD from cholesterol by *M. tuberculosis* H37Rv [15]. A FadA5-deficient strain of *M. neoaurum* NwIB-XII also accumulates 4-HP and 1,4-HP as the main products. Thus, the deletion of *fadA5* may contribute to further blockage of the C19 pathway. Therefore, *hsd4A* and *fadA5* are important targets for modification by genetic engineering to develop microorganisms that can transform sterols into the valuable steroidal intermediate 9-OH-4-HP.

On the other hand, phytosterols and their metabolites are toxic to cells, as they could inhibit cell growth and biocatalytic activity [16]. A steady-state intracellular environment could be beneficial for phytosterol degradation by *Mycobacterium*. Toxic steroid intermediates cause cells to produce reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), during aerobic metabolism. A high level of H₂O₂ might harm cell growth, hence slowing the rate of phytosterol degradation and decreasing the yield of metabolites [17], and vice versa. For example, elimination of H₂O₂ from *M. neoaurum* JC-12 increased the 4-HP yield by 24%. In addition, during the phytosterol degradation process, intracellular nicotinamide adenine dinucleotides (NAD⁺ and NADH) are consumed, which participate in multistep reactions during phytosterol degradation, such as when dehydrogenation occurs. NAD⁺/NADH regeneration and maintenance of the redox balance are considered the rate-limiting factors in the steroid degradation pathway [17, 18]. Manipulation of NAD⁺/NADH contents could enhance the production of AD and ADD to various degrees [17–19]. Overexpression of NADH oxidase in *M. neoaurum* JC-12 increased ADD production by 43% [17], and an increase in the amounts of the ratio of NAD⁺/NADH in *M. neoaurum* TCCC 11978 enhanced the productivity of ADD by 93%. Thus, the elimination of H₂O₂ and regeneration of NAD⁺ could contribute to higher concentrations of phytosterol metabolites.

Herein, an engineered strain of *M. neoaurum* DSM 44074, which is a sterol consumer, was constructed for the bioconversion of phytosterols to 9-OH-4-HP. A *kstDs* knockout strain was constructed based on *M. neoaurum* DSM 44074, and the C19 steroid pathway was further blocked by knocking out both *hsd4A* and *fadA5*. By improving the intracellular environment, an efficient 9-OH-4-HP-producing strain was generated. This strain may contribute to the development of steroid drug precursors.

Results

Accumulation of 9 α -hydroxy derivatives

To eliminate Δ^1 -dehydrogenation and accumulate 9 α -hydroxy derivatives from phytosterols, *kstDs* were identified and knocked out from the genome of the wild-type strain *M. neoaurum* DSM 44074, a steroid-degrading *Mycobacterium* that can completely degrade phytosterols to produce CO₂ and H₂O [7]. The genome of *M. neoaurum* DSM 44074 was sequenced as described in the Methods section. Three putative *kstD* genes (gene 5102 for *kstd1*, gene 5236 for *kstd2*, and gene 5233 for *kstd3*) were identified in

M. neoaurum DSM 44704. *kstDs* was successfully knocked out from the genome of *M. neoaurum* DSM 44704 as described in the Methods section, and the mutant strain $\Delta KstD$ was obtained. The cell growth of the $\Delta kstD$ strain showed no significant difference from that of the wild-type strain (Fig. S1). The wild-type *M. neoaurum* DSM 44074 strain and the genetically modified strain $\Delta kstD$ were incubated with phytosterols for 168 h. The resulting metabolites were extracted from the culture supernatants and analysed by HPLC. Compared with the wild-type strain *M. neoaurum* DSM 44074, which showed no detectable product by HPLC analysis (Fig. 2a), the $\Delta kstD$ strain produced 9-OH-AD as the main product with a retention time of 4.2 min (Fig. 2a, peak A), along with 9-OH-4-HP as a by-product with a retention time of 7.1 min (Fig. 2a, peak B). No ADD was detected during phytosterol bioconversion by $\Delta kstD$, which indicated that the phytosterol degradation pathway was interrupted because of the elimination of Δ^1 -dehydrogenation by *kstDs* knockout. When MP01 medium plus 1 g L⁻¹ phytosterols was used for incubation of the $\Delta kstD$ strain, 0.62 g L⁻¹ 9-OH-AD and 0.04 g L⁻¹ 9-OH-4-HP were produced within 60 h (Fig. 3a and 3b). The molar yield of 9-OH-AD reached 84.9%. 9-OH-4-HP was the major by-product during phytosterol bioconversion by $\Delta kstD$, but the molar yield of 9-OH-4-HP was only 4.8%, and the selectivity of 9-OH-4-HP was 5.6% (Table 2). Thus, 9 α -hydroxy derivatives successfully accumulated during phytosterol bioconversion by *Mycobacterium*, but the purity and yield of 9-OH-4-HP remained unsatisfactory.

Construction of a 9-OH-4-HP-producing strain

Dual pathways, the C19 steroid pathway and the C22 steroid pathway, competes during phytosterol side chain degradation. The C19 steroid pathway is the dominant pathway in *M. neoaurum* DSM 44074, which could be confirmed that $\Delta kstD$ produces 9-OH-AD as the main product along with 9-OH-4-HP as a by-product after culture with phytosterols. The two pathways diverge at 22-hydroxy-3,24-dioxo-4-ene-cholest-COA (22-OH-24-CDOE-COA), which could be Δ^{22} -dehydrogenated by the β -hydroxyacyl-CoA dehydrogenase Hsd4A to generate 3,22,24-trioxo-4-ene-cholest-COA (24-CTOE-COA). 24-CTOE-COA could subsequently be catalysed by the thiolase FadA5, leading the phytosterol degradation pathway to the C19 pathway.

Thus, to construct a 9-OH-4-HP-producing strain, *hsd4A* and *fadA5* were identified in the genome of *M. neoaurum* DSM 44074 and separately knocked out in $\Delta kstD$, resulting in the strains $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$.

The cell growth of $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$ showed no significant difference from that of the wild-type strain *M. neoaurum* DSM 44074 (Fig. S1). The strains $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$ were cultured with phytosterols for 168 h, and the metabolites were analysed by HPLC (Fig. 2b). As shown in Fig. 3b, 9-OH-4-HP successfully accumulated in both strains $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$. The selectivity of 9-OH-4-HP from the $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$ strains were 88.6% and 86.0%, respectively. Nevertheless, both strains still showed only a small amount of 9-OH-AD accumulation (Fig. 3a). The selectivity of 9-OH-AD from strains $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$ were 7.2% and 8.0%,

respectively. After culture with 1 g L⁻¹ phytosterols in MP01 medium, the strain $\Delta kstD\Delta hsd4A$ accumulated 0.59 g L⁻¹ 9-OH-4-HP and 0.13 g L⁻¹ 9-OH-AD, while 0.47 g L⁻¹ 9-OH-4-HP and 0.08 g L⁻¹ 9-OH-AD were obtained from strain $\Delta kstD\Delta fadA5$. The molar yields of 9-OH-4-HP and 9-OH-AD from strain $\Delta kstD\Delta fadA5$ were both lower than those from strain $\Delta kstD\Delta hsd4A$. The molar yield of 9-OH-4-HP from strain $\Delta kstD\Delta fadA5$ was 20.3% lower than that from strain $\Delta kstD\Delta hsd4A$, and the molar yield of 9-OH-AD from strain $\Delta kstD\Delta fadA5$ which was 38.5% lower than that from strain $\Delta kstD\Delta hsd4A$.

Considering that 9-OH-AD still accumulated in both the $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$ strains, the C19 steroid pathway of the phytosterol degradation pathway was not completely blocked in either strain. Thus, to enhance the purity and productivity of 9-OH-4-HP and obstruct the yield of 9-OH-AD, *hsd4A* and *fadA5* were both knocked out in strain $\Delta kstD$, and strain $\Delta kstD\Delta hsd4A\Delta fadA5$ was obtained. The cell growth of the $\Delta kstD\Delta hsd4A\Delta fadA5$ strain showed a trend similar to that of the wild-type strain *M. neoaurum* DSM 44074 (Fig. S1). As shown in Fig. 2b, after culture with phytosterols and metabolites being analysed by HPLC, the strain $\Delta kstD\Delta hsd4A\Delta fadA5$ accumulated 9-OH-4-HP as the main product, while the accumulation of 9-OH-AD was significantly decreased compared with the strains $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$. The selectivity of 9-OH-4-HP and 9-OH-AD from strain $\Delta kstD\Delta hsd4A\Delta fadA5$ were 94.9% and 2.0%, respectively. The purity of 9-OH-4-HP from strain $\Delta kstD\Delta hsd4A\Delta fadA5$ was higher than those from strains $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$. After culture with 1 g L⁻¹ phytosterols, 0.66 g L⁻¹ 9-OH-4-HP was obtained from strain $\Delta kstD\Delta hsd4A\Delta fadA5$, which is 11.9% more than that from strain $\Delta kstD\Delta hsd4A$ and 40.4% more than that from strain $\Delta kstD\Delta fadA5$ (Fig. 3b). The selectivity and production of 9-OH-4-HP from strain $\Delta kstD\Delta hsd4A\Delta fadA5$ were both higher than those from strains $\Delta kstD\Delta hsd4A$ and $\Delta kstD\Delta fadA5$, indicating that double knockout of *hsd4A* and *fadA5* could effectively block the accumulation of AD homologues.

Moreover, to verify the functions of *hsd4A* and *fadA5* during phytosterol degradation, $\Delta kstD\Delta hsd4A$ -*hsd4A*, the *hsd4A* complementation strain, and $\Delta kstD\Delta fadA5$ -*fadA5*, the *fadA5* complementation strain, were also constructed. As shown in Figure 2c, when the two complementation strains were cultured with phytosterols and metabolites being analysed by HPLC, the accumulation of 9-OH-AD was recovered. The purities of 9-OH-AD from $\Delta kstD\Delta hsd4A$ -*hsd4A* and $\Delta kstD\Delta fadA5$ -*fadA5* were 90.0% and 88.5%, respectively, which is nearly consistent with those of strain $\Delta kstD$. These results indicated that Hsd4A and FadA5 were key enzymes in the C19 steroid pathway during phytosterol degradation. Phylogenetic trees of Hsd4A and FadA5 were constructed to elucidate evolutionary relationship of the two enzymes (Fig. S2).

Evaluation of the 9-OH-4-HP producer

After culture with 1 g L⁻¹ phytosterols, the molar yield of 9-OH-4-HP from $\Delta kstD\Delta hsd4A\Delta fadA5$ was 78.9%. To evaluate the ability of $\Delta kstD\Delta hsd4A\Delta fadA5$ to transform phytosterols into 9-OH-4-HP, different concentrations of phytosterols were incubated with $\Delta kstD\Delta hsd4A\Delta fadA5$.

As shown in Fig. 4b, the yields of 9-OH-4-HP from the bioconversion of 2 g L⁻¹, 5 g L⁻¹, 8 g L⁻¹, and 10 g L⁻¹ phytosterols by $\Delta kstD\Delta hsd4A\Delta fadA5$ were 1.43 g L⁻¹, 2.78 g L⁻¹, 1.98 g L⁻¹, and 1.73 g L⁻¹, respectively. 9-OH-AD was also obtained during the incubation, showing yields of 0.06 g L⁻¹, 0.10 g L⁻¹, 0.03 g L⁻¹, and 0.04 g L⁻¹, respectively (Fig. 4a). The productivity of 9-OH-4-HP was enhanced as the concentration of phytosterols increased up to 5 g L⁻¹. However, at phytosterol concentrations of 8 g L⁻¹ and 10 g L⁻¹, the productivity of 9-OH-4-HP decreased, showing results only slightly higher than that with 2 g L⁻¹ phytosterols, and was obviously lower than that with 5 g L⁻¹ phytosterols. The molar yields of 9-OH-4-HP from different concentrations of phytosterols are listed in Table 3. A downward trend in the molar yield of 9-OH-4-HP appeared as the concentration of phytosterols increased from 2 g L⁻¹ to 10 g L⁻¹. However, the purity of 9-OH-4-HP remained stable. Previous research has reported that phytosterols and their metabolites could be noxious to cells during bioconversion [16, 20-22], which might account for the poor performance of $\Delta kstD\Delta hsd4A\Delta fadA5$ during phytosterol bioconversion as the concentration of phytosterols increased.

Intracellular environmental balance contributes to higher 9-OH-4-HP production

The 9-OH-4-HP-producing strain $\Delta kstD\Delta hsd4A\Delta fadA5$ did not perform well during the bioconversion of phytosterols when the concentration of phytosterols was higher than 2 g L⁻¹. This might be due to multiple factors that influence the bioconversion of phytosterols.

A series of redox reactions occur during phytosterol degradation, which use oxygen as an electron acceptor, and cholesterol dehydrogenases/isomerases require intracellular nicotinamide adenine dinucleotides (NAD⁺ and NADH) as cofactors [23, 24]. NAD⁺ and NADH play important roles during phytosterol transformation. They act in many oxidation-reduction reactions and regulate various enzymatic activities and genetic processes. The intracellular NAD⁺ concentration decreased due to its consumption. Therefore, NAD⁺ and NADH have critical effects on the maintenance of the intracellular redox balance. Regeneration of NAD⁺ and enhancement of the NAD⁺/NADH ratio may be of great assistance during phytosterol transformation.

In addition, hydrogen peroxide (H₂O₂) is produced due to incomplete oxidation during aerobic metabolism and the regeneration of flavin adenine dinucleotide (FAD) during the phytosterol transformation process [17]. A high level of H₂O₂ can damage proteins, DNA, and lipids in cells, resulting in inhibition of cell growth and metabolite yield [25].

To enhance the ability of strain $\Delta kstD\Delta hsd4A\Delta fadA5$ to transform phytosterols into 9-OH-4-HP, the catalase *katE* from DSM 44074 and the NADH oxidase *nox* from *Bacillus subtilis* [17] were co-expressed in strain $\Delta kstD\Delta hsd4A\Delta fadA5$ to construct strain $\Delta kstD\Delta hsd4A\Delta fadA5$ -NK.

The extracellular H₂O₂ concentrations of the two strains $\Delta kstD\Delta hsd4A\Delta fadA5$ and $\Delta kstD\Delta hsd4A\Delta fadA5$ -NK were measured when they were cultured with 5 g L⁻¹ phytosterols for 168 h. As shown in Figure 5b, the extracellular H₂O₂ concentration of strain $\Delta kstD\Delta hsd4A\Delta fadA5$ showed an upward trend during the bioconversion process. The extracellular H₂O₂ concentration increased from 0.59 $\mu\text{mol L}^{-1}$ at the beginning to 1.05 $\mu\text{mol L}^{-1}$ after 168 h and reached a peak of 1.10 $\mu\text{mol L}^{-1}$ at 120 h. In contrast, the extracellular H₂O₂ concentration of strain $\Delta kstD\Delta hsd4A\Delta fadA5$ -NK remained nearly stable during the bioconversion process at approximately 0.51 $\mu\text{mol L}^{-1}$. Therefore, the overexpression of *katE* eliminated excessive extracellular H₂O₂. Moreover, to verify the toxicity of H₂O₂, cell growth of the strains $\Delta kstD\Delta hsd4A\Delta fadA5$ and $\Delta kstD\Delta hsd4A\Delta fadA5$ -NK was also measured. As shown in Figure 5a, the biomass of strain $\Delta kstD\Delta hsd4A\Delta fadA5$ -NK was higher than that of strain $\Delta kstD\Delta hsd4A\Delta fadA5$, indicating that the elimination of extracellular H₂O₂ could help with cell growth.

Likewise, the NAD⁺/NADH ratios of the strains $\Delta kstD\Delta hsd4A\Delta fadA$ and $\Delta kstD\Delta hsd4A\Delta fadA$ -NK were also measured after they were cultured with 5 g L⁻¹ phytosterols for 168 h. As shown in Figure 5c, the NAD⁺/NADH ratio of strain $\Delta kstD\Delta hsd4A\Delta fadA$ -NK was consistently higher than that of strain $\Delta kstD\Delta hsd4A\Delta fadA$ by at least 10.9%. At 96 h, the NAD⁺/NADH ratio was enhanced by 25.4% in strain $\Delta kstD\Delta hsd4A\Delta fadA$ -NK compared with strain $\Delta kstD\Delta hsd4A\Delta fadA$. The overexpression of *nox* could significantly influence the NAD⁺/NADH ratio during phytosterol bioconversion.

9-OH-4-HP productivity was also measured to test whether overexpression of *katE* and *nox* could enhance the ability of $\Delta kstD\Delta hsd4A\Delta fadA$ to transform phytosterols into 9-OH-4-HP. The recombinant strain $\Delta kstD\Delta hsd4A\Delta fadA$ -NK was cultured with 1 g L⁻¹, 2 g L⁻¹, 5 g L⁻¹, 8 g L⁻¹, and 10 g L⁻¹ phytosterols for 168 h, and the productivity of 9-OH-4-HP was measured every 24 h. As shown in Figure 5d, the final productivities of 9-OH-4-HP from 1 g L⁻¹, 2 g L⁻¹, 5 g L⁻¹, 8 g L⁻¹, and 10 g L⁻¹ phytosterols were 0.68 g L⁻¹, 1.53 g L⁻¹, 3.58 g L⁻¹, 2.51 g L⁻¹, and 2.73 g L⁻¹, respectively. Compared with $\Delta kstD\Delta hsd4A\Delta fadA$ cultured with the same concentrations of phytosterols, the productivities of 9-OH-4-HP were enhanced by 3.03%, 6.99%, 28.7%, 26.8%, and 57.8%. The highest yield of 9-OH-4-HP was obtained when strain $\Delta kstD\Delta hsd4A\Delta fadA$ -NK was cultured with 5 g L⁻¹ phytosterols, with a molar yield that reached 85.5%, which was 28.8% higher than that of $\Delta kstD\Delta hsd4A\Delta fadA$. Moreover, no significant difference in the productivity of 9-OH-AD was observed between the two strains $\Delta kstD\Delta hsd4A\Delta fadA5$ and $\Delta kstD\Delta hsd4A\Delta fadA5$ -NK (Fig. 5e), indicating that the purity of 9-OH-4-HP was also enhanced during phytosterol bioconversion by the strain $\Delta kstD\Delta hsd4A\Delta fadA5$ -NK. All of the results above confirm that regulation of the intracellular NAD⁺/NADH ratio and H₂O₂ levels could be an effective way to improve sterol transformation efficiency and the production of steroid intermediates.

Discussion

By genome sequencing, three *kstDs* were found in *M. neoaurum* DSM 44074. *kstD2* and *kstD3* in *M. neoaurum* DSM 44074 showed 100% similarity with those in *M. neoaurum* ATCC 25795, a strain that was

deemed to be the same strain as *M. neoaurum* DSM 44074. However, *kstD1* in *M. neoaurum* DSM 44074 showed 5 mismatches with that in *M. neoaurum* DSM 44074, causing 3 amino acid changes. When the *kstds* knockout strain of *M. neoaurum* DSM 44074 was cultured with phytosterols, AD and 4-HP were nearly undetectable in the final products. The *kstd* knockout strain accumulated 9-OH-AD as the main product and 9-OH-4-HP as a by-product. Certain other strains have also been reported to accumulate 9-OH-AD from phytosterols. *Mycobacterium* sp. 2-4M [26] showed a 50% molar yield of 9-OH-AD, a 22% molar yield of AD and a 2% molar yield of 4-HP from 5 g L⁻¹ sitosterol [27]. In a *kstds* knockout strain of *M. neoaurum* ATCC 25795, a 55% molar yield of 9-OH-AD and a 15% molar yield of AD were obtained from 15 g L⁻¹ phytosterols. Compared with other 9-OH-AD-producing strains, the purity and molar yield of 9-OH-AD from $\Delta kstD$ after culture with phytosterols were notably higher. The accumulation of AD from *kstds* knockout strains might be due to residual Δ^1 -dehydrogenation activity. The genome of *R. ruber* contains at least two other possible ORFs other than *kstD1*, *kstD2*, and *kstD3* with certain identity to *kstds* (approximately 38%) [28]. The existence of more than 3 KstDs has also been reported for other *Rhodococcus* species, such as *R. jostii* Rha1 [29]. Thus, inactivation of all KstD activities ought to be the fundamental premise to develop promising 9 α -hydroxy derivatives.

Due to dual competing pathways, the dominant C19 steroid pathway and the C22 steroid pathway exist in the phytosterol degradation pathway. 9-OH-4-HP is usually produced as a by-product in 9-OH-AD-producing strains. M. V. Donova reported a wild-type strain *Mycobacterium* sp. 2-4M, which produces 9-OH-AD as the major product with a 1.5%-1.6% molar yield of 9-OH-4-HP [26]. *Mycobacterium* sp. VKM Ac-1815D, *Mycobacterium* sp. VKM Ac-1817D, and *Mycobacterium fortuitum* ATCC-6842 have also been reported to accumulate a small amount of 9-OH-4-HP during the 9-OH-AD production process [4–6]. The enzymes that catalyse 22-hydroxy-3,24-dioxo-4-ene-cholest-COA into 4-HP homologues remain unidentified. Thus, Hsd4A is normally chosen to manipulate the metabolic flux to generate AD homologues or 4-HP homologues. Xu reported the characterization of Hsd4A *in vivo* and *in vitro*, testifying that deletion of *hsd4A* resulted in blockage of the C19 steroid pathway and enhanced the accumulation of 4-HP homologues. During the Hsd4A investigation, Xu constructed a 9-OH-4-HP-producing strain by knocking out *hsd4A* in the *kstD*-deficient strain of *M. neoaurum* ATCC 25795. This mutant strain displayed 32% molar yield of 9-OH-4-HP and 15% molar yield of 9-OH-AD from 40 g L⁻¹ phytosterols [3]. Here, in this research, it was confirmed that double knockout of *hsd4A* and *fadA5* could further block the C19 steroid pathway. The purity and molar yield of 9-OH-4-HP of strain $\Delta kstDs\Delta hsd4A\Delta fadA5$ were notably higher than those of Xu's strain. Although strain $\Delta kstDs\Delta hsd4A\Delta fadA5$ did not perform well when cultured with higher concentrations of phytosterols, the purity of 9-OH-4-HP was not influenced, indicating its potency as a promising 9-OH-4-HP producer.

AD homologues accumulated in the 9-OH-4-HP producer strains $\Delta kstDs\Delta hsd4A$, $\Delta kstDs\Delta fadA5$, and $\Delta kstDs\Delta hsd4A\Delta fadA5$, indicating incomplete blockage of the C19 steroid pathway. Similar results have been previously reported. Analysis of the *M. neoaurum* CCTCC AB2019054 genome revealed that there were 6 proteins with >38% identity and 10 proteins with 31–38% identity to Hsd4A, which may compensate for its function [30]. The most identical gene, *hsd4A2* (45% identity to *hsd4A*), was deleted,

and fermentation analysis revealed that it can indeed produce 4-HBC at a significantly increased ratio. A similar result was found in *M. neoaurum* DSM 44074. Eight proteins showed certain identities to Hsd4A, indicating the presence of isoenzymes of Hsd4A in the genome of *M. neoaurum* DSM 44074.

Some attempts have been made to enhance the ability of microorganisms to transform phytosterols. Considering the toxicity of phytosterols and their derivatives to cells, the balance of the intracellular environment could improve the ability of microorganisms to transform phytosterols. Intracellular factors such as NAD⁺ and NADH have drawn increasing attention in recent years. NAD⁺ and NADH participate in multiple steps during steroid bioconversion, and the intracellular NAD⁺ concentration decreases as it is consumed. Regeneration of NAD⁺ and enhancement of the NAD⁺/NADH ratio have been proven to be able to enhance the ability of microorganisms to transform phytosterols. Overexpression of NADH oxidase in *M. neoaurum* JC-12 increased ADD production by 43% [17]. Overexpression of flavin oxidoreductase and NADH oxidase from *Lactobacillus brevis* in *M. neoaurum* TCCC 11978 increased the NAD⁺/NADH ratio by 113% and 192%, respectively, and significantly enhanced the conversion ratio of AD(D) [18]. The type II NADH dehydrogenases *ndhN* and *ndhF* were overexpressed in *M. neoaurum* MNR, resulting in an increase in the NAD⁺/NADH ratio from 3.93 to 5.91 and 10.96, respectively. The highest molar biotransformation rates of AD with 5 g L⁻¹ phytosterol feed were 5.32% and 12.38% higher than those of the original strain, respectively.

In addition, reactive oxygen species are generated during the conversion process of sterols, which impair cell viability and hinder the conversion of sterols to steroid synthons. Elimination of ROS and H₂O₂ have been reported to be an effective method to improve cell growth under phytosterol feed and enhance phytosterol bioconversion. Combinatorial augmentation with catalase, mycothiol, and ergothioneine increased 4-HP productivity by 47.5% in *M. neoaurum* Will-*egt&msh&cat* [31]. Elimination of H₂O₂ in *M. neoaurum* JC-12 increased the 4-HP yield by 24%. In this study, when combining the abilities of NAD⁺ regeneration and H₂O₂ elimination, the performance of the new mutant strain $\Delta kstDs\Delta hsd4A\Delta fadA5$ -NK to transform phytosterols into 9-OH-4-HP improved. The extracellular H₂O₂ concentration of $\Delta kstDs\Delta hsd4A\Delta fadA5$ -NK after culture with 5 g L⁻¹ phytosterols remained at a low level, which was only 49.4% that of the extracellular H₂O₂ concentration of $\Delta kstDs\Delta hsd4A\Delta fadA5$ culture under the same conditions. The NAD⁺/NADH ratio was also enhanced by 25.4% after 96 h. Although the molar yield of 9-OH-4-HP decreased as the phytosterol concentration increased, the molar yield of 9-OH-4-HP from strain $\Delta kstDs\Delta hsd4A\Delta fadA5$ -NK was significantly higher than that from strain $\Delta kstDs\Delta hsd4A\Delta fadA5$ at the same phytosterol concentration. The highest yield of 9-OH-4-HP was 3.58 g L⁻¹, which was achieved when $\Delta kstDs\Delta hsd4A\Delta fadA5$ -NK was fed 5 g L⁻¹ phytosterols. The molar yield of 9-OH-4-HP from strain $\Delta kstDs\Delta hsd4A\Delta fadA5$ -NK was 28.7% higher than that from strain $\Delta kstDs\Delta hsd4A\Delta fadA5$ with 5 g L⁻¹ phytosterol feeding. All of these results proved that the elimination of H₂O₂ and regeneration of NAD⁺ could be an effective method to improve phytosterol bioconversion.

When 10 g L⁻¹ phytosterols were cultured with $\Delta kstDs\Delta hsd4A\Delta fadA5$ -NK, the molar yield of 9-OH-4-HP was 32.6%, indicating further manipulation to enhance its molar yield. A number of different aqueous-organic two-phase systems have been studied, such as water-vegetable oils and water-ionic liquids [32]. The use of cyclodextrins could enhance the uptake of phytosterols by microorganisms [16, 33]. Recently, some novel methods have been developed to improve phytosterol bioconversion. Increasing cell permeability could improve the production of phytosterol metabolites [34, 35]. Deletion of the transmembrane transporter trehalose monomycolate *mmpL3* in a 4-HP-producing strain derived from *M. neoaurum* ATCC 25795 increased 4-HP production by 24.7%. A “resting cell-cyclodextrin” system has also been widely used in industry and research to improve bioconversion ability.

Methods

Bacterial strains, plasmids, medium, and reagents

The stains and plasmids used in this study are described in Table 1. *E. coli* DH5 α stored in the laboratory was used for plasmid amplification. Wild-type *M. neoaurum* DSM 44074 (DSM 44704) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, GERMANY). All other strains were derived from *M. neoaurum* DSM 44704. Common plasmids and primers (Table S1) were used to construct the mutants. *E. coli* DH5 α was cultured at 37°C and 200 rpm in 50 mL of Luria-Bertani (LB) medium (10 g L⁻¹ tryptone, 10 g L⁻¹ NaCl, 5 g L⁻¹ yeast extracts, pH 7.0). *Mycobacterium* cells were cultured in MYD medium (0.6 g L⁻¹ K₂HPO₄·3H₂O, 5.4 g L⁻¹ NaNO₃, 6 g L⁻¹ glucose, 15 g L⁻¹ yeast extract and an initial pH value 7.5) and fermented with MP01 medium (10 g L⁻¹ corn steep powder, 20 g L⁻¹ glucose, 2 g L⁻¹ K₂HPO₄·3H₂O, 1.0 g L⁻¹ MgSO₄·7H₂O, 2.0 g L⁻¹ NaNO₃, 2‰ Tween 80 (v/v), and an initial pH value 7.5) at 30°C and 200 rpm.

The phytosterols consisted of 45% β -sitosterol, 37% campesterol, and 18% stigmasterol, which were purchased from Yunnan Biological Products Co., Ltd. (Yunnan, China). AD and 9-OH-AD were obtained from Shanghai Macklin Biochemical Co., Ltd. (China). (2-Hydroxypropyl)- β -cyclodextrin (HP- β -CD) was purchased from Zhiyuan Biotechnology Co., Ltd. (Shandong, China). The ClonExpress One Step Cloning Kit was purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). The Hydrogen Peroxide (H₂O₂) Content Assay Kit and Nicotinamide Adenine Dinucleotide NAD(H) Content Assay Kit were obtained from Sangon Biotech Co., Ltd. (Shanghai, China).

Bioinformatic analysis

The genome of *M. neoaurum* DSM 44074 was sequenced by Shanghai Majorbio Co., Ltd. The DNA sample was extracted and sheared into 400-500 bp fragments using a Covaris M220 Focused Acoustic Shearer (Covaris, USA). Illumina sequencing libraries were prepared from the sheared fragments using a NEXTflex™ Rapid DNS-Seq Kit (Bioo Scientific, USA). The sequencing data were assembled using SOAPdenovo2([GitHub - aquaskyline/SOAPdenovo2: Next generation sequencing reads de novo](https://github.com/aquaskyline/SOAPdenovo2)

assembler). Further prediction and annotation were produced by Glimmer ([Glimmer \(jhu.edu\)](http://Glimmer.jhu.edu)) and BLAST (blast.ncbi.nlm.nih.gov). The putative genes for *kstD*, *hsd4A*, and *fadA5* were identified by comparison with known gene sequences taken from the NCBI database. MEGA-X software ([Home \(megasoftware.net\)](http://Home.megasoftware.net)) was used to construct a phylogenetic tree of *hsd4A* and *fadA5* with the known amino acid sequences taken from the NCBI.

Mutant strain construction

A CRISPR-assisted nonhomologous end-joining strategy was used to delete the target gene in *M. neoaurum* DSM 44074 based on previous reports. The PSBY1 plasmid harbouring *cpf1* was obtained from Jiang [36], and the PCR-Hyg plasmid harbouring sgRNA was obtained from Sun [37]. ClonExpress[®] One Step Cloning Kit mutated spacers were used to construct different plasmids harbouring target sgRNA. The plasmid harbouring target sgRNA was transfected into *M. neoaurum*, and the PSBY1 plasmid was transfected beforehand by electroporation. The recombinant clones were sequenced using specific primers to determine the deletion.

The vector P38Mu (pMV306 with the Psmc promoter) with kanamycin resistance was used to overexpress the target gene. The genes *hsd4A*, *fadA5*, *katE* from *M. neoaurum* DSM 44074, and *nox* from *Bacillus subtilis* were recombined on P38Mu. Specific primers were used to amplify the corresponding gene, and the PCR product was inserted into the Nde⁺ site (and Hind⁺ site, if two genes were inserted) of P38Mu using the ClonExpress[®] One Step Cloning Kit.

Bioconversion and analysis

The transformation capability of the mutant strains was identified in MP01 medium with an initial phytosterol concentration of 1 g L⁻¹. A concentration gradient was later tested to further determine the capability of phytosterol bioconversion. Phytosterols were prepared in (2-hydroxypropyl)- β -cyclodextrin (HP- β -CD) at a ratio of 1:1.5. The recombinant cells were inoculated into 30 mL of MYD medium in a 250 mL shaker flask and cultured at 30°C and 200 rpm. Three millilitres of seed medium was transferred to 30 mL of MP01 medium in a 250 mL shaker flask with a baffle when the optical density reached the mid-log exponential phase. The fermentation of *M. neoaurum* DSM 44074 and recombinant strains was sampled every 12 or 24 h, and three replicates were used to measure the steroids. The bioconversion mixture was extracted with 3 volumes of ethyl acetate, and the solvent was removed to give a residue that was redissolved in methanol. The resulting solution was used for HPLC analysis. HPLC was performed on a Shimadzu Separations module connected to a Shimadzu SPD-M20A detector equipped with a C18 column (250 mm $\times$ 4.6 mm, 5 μ m) and detected at a wavelength of 254 nm. A mixture of methanol and water (80:20, v/v) was used as the mobile phase at a flow rate of 0.8 mL min⁻¹.

Extracellular H₂O₂ concentrations were measured according to the operating manual of the Hydrogen Peroxide (H₂O₂) Content Assay Kit. NADH and NAD⁺ intracellular concentrations were measured

according to the operating manual of the Nicotinamide Adenine Dinucleotide, NAD(H) Content Assay Kit.

The accumulation of 9-OH-4-HP was achieved by blocking the C19 steroid pathway and 3-ketosteroid- Δ^1 -dehydrogenation. Compared with single deletion of *hsd4A* and single deletion of *fadA5*, double deletion of *hsd4A* and *fadA5* could further block the C19 steroid pathway. By eliminating H_2O_2 and regenerating NAD^+ in the triple *hsd4A*, *fadA5*, and *kstDs* knockout strain, the highest 9-OH-4-HP productivity was 3.58 g L^{-1} with 5 g L^{-1} phytosterol feed.

Abbreviations

AD: androst-4-ene-3,17-dione; ADD: androst-1,4-diene-3,17-dione; 9-OH-AD: 9-hydroxy-androst-4-ene-3,17-dione; 4-HP: 21-hydroxy-20-methyl-pregna-4-en-3-one; 1,4-HP: 21-hydroxy-20-methyl-pregna-1,4-dien-3-one; 9-OH-4-HP: 9,21-dihydroxy-20-methyl-pregna-4-en-3-one; KstDs: 3-ketosteroid- Δ^1 -dehydrogenases; KSHs: 9 α -hydroxylase; HPs: C22 steroids; Hsd4A: β -hydroxyacyl-CoA dehydrogenase; FadA5: acyl-CoA thiolase; ROS: Reactive oxygen species; H_2O_2 : hydrogen peroxide; NAD^+ / NADH: nicotinamide adenine dinucleotides; NOX: NADH oxidase; KatE: catalase; HPLC: high performance liquid chromatography

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated and analyzed during this study are included in this published article and its additional files.

Author's contributions

BGZ and YCY designed the study. YCY carried out the gene knockout and overexpression. YCY and ZGM performed the phytosterol bioconversion. YCY, XCL, GLD and JXZ analyzed the data. YCY wrote the manuscript. BGZ, JSS and JPS reviewed the manuscript.

All authors read and approved the final manuscript.

Competing interests

The authors declare no conflict of interest

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Tables

Table 1

Strains and plasmids used in this study

Name	Description	Source
Strains		
<i>M. neoaurum</i> DSM 44704	Wild type strain, Sterol consumer with no detectable intermediates	
$\Delta kstD$	<i>kstD1</i> , <i>kstD2</i> , and <i>kstD3</i> deleted in <i>M. neoaurum</i> DSM 44704	This study
$\Delta kstD\Delta hsd4A$	<i>hsd4A</i> deleted in $\Delta kstD$ strain	This study
$\Delta kstD\Delta fadA5$	<i>fadA5</i> deleted in $\Delta KstD$ strain	This study
$\Delta kstD\Delta hsd4A$ - <i>hsd4A</i>	$\Delta kstD\Delta hsd4A$ strain harboring P38Mu-Hsd4A	This study
$\Delta kstD\Delta fadA5$ - <i>fadA5</i>	$\Delta kstD\Delta fadA5$ strain harboring P38Mu-FadA5	This study
$\Delta kstD\Delta hsd4A\Delta fadA5$	<i>hsd4A</i> and <i>fadA5</i> deleted in $\Delta kstD$ strain	This study
$\Delta kstD\Delta hsd4A\Delta fadA5$ -NK	$\Delta kstD\Delta hsd4A\Delta fadA5$ strain harboring P38Mu-NK	This study
Plasmids		
PSBY1	Derived from pMV261 and contains FnCpf1 <i>C. glutamicum</i> codon-optimized; Kan ^R	[36]
PCR-Hyg	Plasmid for sgRNA production	[37]
Pam-KstD1	PCR-Hyg containing <i>kstD1</i> spacer	This study
Pam-KstD2	PCR-Hyg containing <i>kstD2</i> spacer	This study
Pam-KstD3	PCR-Hyg containing <i>kstD3</i> spacer	This study
Pam-Hsd4A	PCR-Hyg containing <i>hsd4A</i> spacer	This study
Pam-FadA5	PCR-Hyg containing <i>fadA5</i> spacer	This study
pMV306	<i>Mycobacterium</i> integrative vector without promoter, kan ^R	[38]
P38Mu	pMV306 with Psmyc promoter, Kan ^R	[39]
P38Mu-Hsd4A	Recombinant P38Mu for expression of <i>hsd4A</i>	This study
P38Mu-FadA5	Recombinant P38Mu for expression of <i>fadA5</i>	This

		study
P38Mu-NK	Recombinant P38Mu for expression of <i>nox</i> and <i>katE</i>	This study

Table. 2

Relative production purity of *M.neoaurum* DSM 44074 and its derivative strains

strain	Relative Purity (%)			
	9-OH-AD	9-OH-4-HP	AD	Others
DSM 44704	0	0	0	0
$\Delta kstD$	90.4 $\pm$ 3.1	5.6 $\pm$ 2.5	1.9 $\pm$ 0.2	2.1 $\pm$ 0.8
$\Delta kstD\Delta hsd4A$	7.2 $\pm$ 2.2	88.6 $\pm$ 1.3	2.4 $\pm$ 0.5	1.8 $\pm$ 1.3
$\Delta kstD\Delta fadA5$	8.0 $\pm$ 1.9	86.0 $\pm$ 3.5	3.3 $\pm$ 0.4	2.7 $\pm$ 0.5
$\Delta kstD\Delta hsd4A-hsd4A$	90.0 $\pm$ 2.2	5.2 $\pm$ 1.7	1.8 $\pm$ 0.2	3.0 $\pm$ 0.3
$\Delta kstD\Delta fadA5-fadA5$	88.5 $\pm$ 3.6	4.4 $\pm$ 2.3	1.3 $\pm$ 0.1	5.8 $\pm$ 1.2
$\Delta kstD\Delta hsd4A\Delta fadA5$	2.0 $\pm$ 1.1	94.9 $\pm$ 1.2	0.3 $\pm$ 0.1	2.8 $\pm$ 0.3
$\Delta kstD\Delta hsd4A\Delta fadA5$ -NK	2.0 $\pm$ 1.1	97.0 $\pm$ 1.1	0.3 $\pm$ 0.1	0.7 $\pm$ 0.3

Table. 3

Maximum yield and molar yield of 9-OH-4-HP from $\Delta kstD\Delta hsd4A\Delta fadA5$ and $\Delta kstD\Delta hsd4A\Delta fadA5$ -NK

phytosterols concentration (g L ⁻¹)	$\Delta kstD\Delta hsd4A\Delta fadA5$		$\Delta kstD\Delta hsd4A\Delta fadA5$ -NK	
	Maximum yield (g L ⁻¹)	Maximum molar yield (%)	Maximum yield (g L ⁻¹)	Maximum molar yield (%)
1	0.66 $\pm$ 0.02	78.9 $\pm$ 2.4	0.68 $\pm$ 0.04	81.2 $\pm$ 4.8
2	1.43 $\pm$ 0.06	84.8 $\pm$ 3.6	1.53 $\pm$ 0.06	90.8 $\pm$ 3.6
5	2.78 $\pm$ 0.11	66.4 $\pm$ 2.6	3.58 $\pm$ 0.15	85.5 $\pm$ 3.6
8	1.98 $\pm$ 0.08	31.5 $\pm$ 1.2	2.51 $\pm$ 0.14	38.5 $\pm$ 2.1
10	1.73 $\pm$ 0.09	20.7 $\pm$ 1.1	2.73 $\pm$ 0.19	32.6 $\pm$ 1.1

Figures

Figure 2

Phenotypic analyses of the metabolites of phytosterol by *M. neoaurum* DSM 44074 and its derivative strains. Peak A, 9-OH-AD; Peak B, 9-OH-4-HP. (a) HPLC chromatogram comparison of the products of *M. neoaurum* DSM 44074 and $\Delta kstD$ with 1 g L⁻¹ phytosterols feed. (b) HPLC chromatogram comparison of the products of $\Delta kstD$, $\Delta kstD\Delta hsd4A$, $\Delta kstD\Delta fadA5$ and $\Delta kstD\Delta hsd4A\Delta fadA5$ with 1 g L⁻¹ phytosterols feed. (c) HPLC chromatogram comparison of the products of $\Delta kstD$, hsd4A complement strain $\Delta kstD\Delta hsd4A-hsd4A$ and fadA5 complement strain $\Delta kstD\Delta fadA5-fadA5$, with 1 g L⁻¹ phytosterols feed. (d) structure of peak A, 9-OH-AD, and peak B, 9-OH-4-HP.

Figure 3

9-hydroxy steroids accumulation from 1 g L⁻¹ phytosterols. (a) Time course of 9-OH-AD accumulation; (b) Time course of 9-OH-4-HP accumulation; single deletion of *hsd4A* or *fadA5* caused increasement of 9-OH-4-HP, and double deletion of *hsd4A* and *fadA5* could obviously increase the productivity and selectivity of 9-OH-4-HP.

Figure 4

9-hydroxy steroids accumulation of $\Delta kstD\Delta hsd4A\Delta fadA5$ from different concentrations of phytosterols. (a) Time course of 9-OH-AD accumulation; (b) Time course of 9-OH-4-HP accumulation; As the phytosterols concentration increased, the ability of the strain $\Delta kstD\Delta hsd4A\Delta fadA5$ to transform phytosterols was inhibited.

Figure 5

Time profiles of 9-hydroxy steroids accumulation, extracellular H₂O₂ concentration, and the NAD⁺/NADH ratio of the strain $\Delta kstD\Delta hsd4A\Delta fadA5$ and the strain $\Delta kstD\Delta hsd4A\Delta fadA5-NK$. (a) the cell growth; (b) intracellular NAD⁺/NADH ratio; (c) extracellular H₂O₂ concentration; (d) time course of 9-OH-4-HP accumulation of $\Delta kstD\Delta hsd4A\Delta fadA5-NK$ from different concentrations of phytosterols; (e) time course of 9-OH-AD accumulation of $\Delta kstD\Delta hsd4A\Delta fadA5-NK$ from different concentrations of phytosterols.

Expression of Nox and KatE is beneficial for cell growth, increases the ratio of intracellular NAD⁺/NADH, decreases the extracellular H₂O₂ concentration, and enhance the yield of 9-OH-4-HP under a high concentration of phytosterol.

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