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Bioconversion of 4-androstene-3,17-dione to androst-1,4-diene-3,17-dione by recombinant *Bacillus subtilis* expressing *ksdd* gene encoding 3-ketosteroid-Δ^1-dehydrogenase from *Mycobacterium neoaurum* JC-12

Wenqing Zhang[a], Minglong Shao[a], Zhiming Rao[a,*], Meijuan Xu[a], Xian Zhang[a], Taowei Yang[a], Hui Li[b], Zhenghong Xu[b,*]

[a] *The Key Laboratory of Industrial Biotechnology, Ministry of Education, Laboratory of Applied Microbiology and Metabolic Engineering, School of Biotechnology, Jiangnan University, Jiangsu Province, Wuxi 214122, China*
[b] *School of Medicine and Pharmaceuticals, Jiangnan University, Jiangsu Province, Wuxi 214122, China*

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ABSTRACT

The enzyme 3-ketosteroid-Δ^1-dehydrogenase (KSDD), involved in steroid metabolism, catalyzes the transformation of 4-androstene-3,17-dione (AD) to androst-1,4-diene-3,17-dione (ADD) specifically. Its coding gene was obtained from *Mycobacterium neoaurum* JC-12 and expressed on the plasmid pMA5 in *Bacillus subtilis* 168. The successfully expressed KSDD was analyzed by native-PAGE. The activities of the recombinant enzyme in *B. subtilis* were 1.75 U/mg, which was about 5-fold that of the wild type in *M. neoaurum*. When using the whole-cells as catalysts, the products were analyzed by tin-layer chromatography and high-performance liquid chromatography. The recombinant *B. subtilis* catalyzed the biotransformation of AD to ADD in a percent conversion of 65.7% and showed about 18 folds higher than *M. neoaurum* JC-12. The time required for transformation of AD to ADD was about 10 h by the recombinant *B. subtilis*, much shorter than that of the wild-type strain and other reported strains. Thus, the efficiency of ADD production could be improved immensely. For industrial applications, the recombinant *B. subtilis* containing KSDD provides a new pathway of producing steroid medicines.

1. Introduction

Steroid drugs are important in the pharmaceutical industry and have been widely used in clinical applications. For example, the steroids, 4-androstene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD), are the most important steroidal derivatives in the process of steroid degradation by microbial strains [3]. AD can be transformed into multiple products by different microorganisms, such as ADD, 9α-OH-AD, and 9α-OH-ADD [5–9], which can be used as steroid medicines or the sterol drug intermediates. ADD, transformed from AD, is an important precursors in the synthesis of steroid pharmaceutical such as contraceptive agent, estrogen, and progestogen [2]. Although the chemical method has been used in the pharmaceutical industry [1,2,7,8], recent special attention is focused on preparation steroid intermediates by employing "green" and efficient enzymatic processes [10–13].

Recently, there have been some reports about microbial transformation of AD [4]. Several microorganisms including *Mycobacterium*, *Rhodococcus*, *Nocardia*, *Arthrobacter* were found to possess the transformation capability of AD [26–30]. It was reported that the most direct and efficient production of ADD was dehydrogenation of AD by microorganisms [15]. For industrial applications, the microbial enzymes with high rates and selectivity that can transform AD to ADD are extremely in demand.

The biocatalyst, 3-ketosteroid-Δ^1-dehydrogenase (KSDD), is the key enzyme for the production and accumulation of ADD in the process of steroid biotransformation [14], and it catalyzes the conversion of AD to ADD (Fig. 1). The introduction of C_1—C_2 double bond into ring A of steroid nucleus (biotransformation AD to ADD) can improve the biological activity and economical value of the original steroid substrate. For example, the 1-dehydro derivatives of cortisone and cortisol (i.e., prednisone and prednisolone) showed increased anti-allergic and anti-rheumatic activities with significantly reduced side affects [14]. In order to obtain the sole product of ADD or AD, many scholars have focused on the strain amelioration, enhancing KSDD expression, or making the target

Abbreviations: AD, 4-androstene-3,17-dione; ADD, androst-1,4-diene-3,17-dione; DCPIP, 2,6-dichlorophenolindophenol; FAD, flavin adenine dinucleotide; HPLC, high-performance liquid chromatography; KSDD, 3-ketosteroid-Δ^1-dehydrogenase; *ksdd*, the coding gene of 3-ketosteroid-Δ^1-dehydrogenase; PMS, phenazine methosulphate; TLC, tin-layer chromatography.

* Corresponding authors. Tel.: +86 510 85916881; fax: +86 510 85918206.
E-mail addresses: raozm@yahoo.com.cn (Z. Rao), zhenghxu@jiangnan.edu.cn (Z. Xu).

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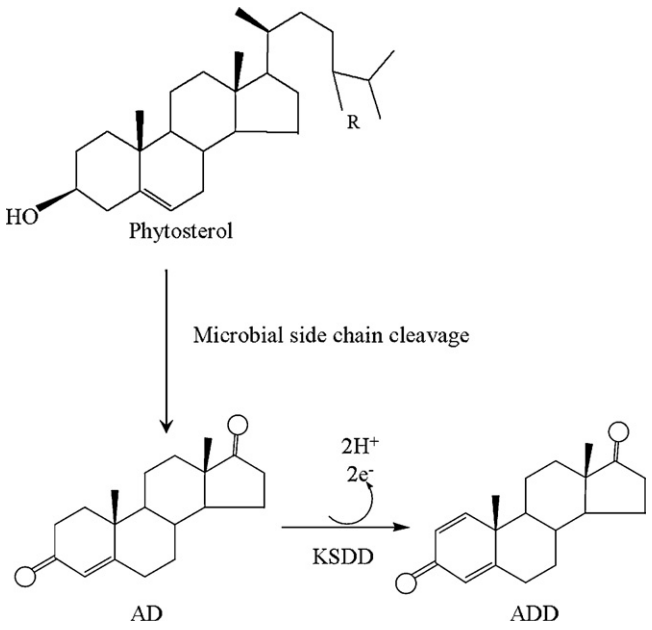

Fig. 1. Microbial transformation pathway of phytosterol to ADD.

gene *ksdd* inactivated by molecular or mutagenesis approaches [16,23]. Some labs succeeded in gene cloning of KSDD and its efficient heterologous expression in *Escherichia coli*, *Streptomyces lividans*, *Rhodococcus erythropolis*, etc. [17–19,21]. However, there is no report that the *ksdd* gene from *Mycobacterium neoaurum* has been successfully expressed in *Bacillus subtilis*.

Previously, we isolated *M. neoaurum* JC-12 that is capable of producing the sole product ADD from phytosterol in our laboratory. In this work, the *ksdd* gene was successfully amplified from *M. neoaurum* and expressed on the plasmid pMA5 in *B. subtilis* 168. The transformation of AD to ADD by the whole-cells of the recombinant *B. subtilis* was performed. This work provided a candidate for the industrial application of AD.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

B. subtilis 168 strain, *M. neoaurum* JC-12 strain and the vector pMA5 were preserved in our laboratory. *M. neoaurum* JC-12 that is capable of producing ADD was isolated from environment by our laboratory. The strains *B. subtilis* 168 and the recombinant *B. subtilis* 168 were cultivated at 37 °C and 160 rpm in liquid LB medium (Luria-Bertani broth). Kanamycin (100 mg/ml) was added to the growth medium when necessary. *M. neoaurum* JC-12 was grown at 30 °C and 160 rpm in liquid medium containing 0.5% (w/v) glucose (Sinopharm Chemical Reagent Co., Ltd., China), 0.5% (w/v) tryptone (Oxoid Ltd., Basingstoke Hampshire, England), 0.3% (w/v) beef extract, 1.5% (w/v) glycerol and 1.5% NaCl. For growth on solid medium, 2% (w/v) agar was added. 4-Androstene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD) were supplied by the Sigma–Aldrich Chemical Co. Inc. All other chemicals were of the analytical grade that could be obtained commercially.

2.2. General cloning techniques

Restriction enzymes were purchased from TaKaRa Co. (Dalian, China). T_4 DNA ligase was purchased from Fermentas or Sangon Biotech Co., Ltd. (Shanghai, China) and described by the manufacturer. Extraction and purification of plasmids were carried by Mini Plasmid Rapid Isolation Kit (Sangon Biotech Co., Ltd., Shanghai, China). Isolation of DNA restriction fragments from agarose gels was done using the Mini DNA Rapid Purification kit (Sangon Biotech Co., Ltd., Shanghai, China).

2.3. Nucleotide sequencing

Nucleotide sequence of *ksdd* was analyzed by Sangon Biotech Co., Ltd Shanghai, China. Protein and nucleotide sequence comparisons were performed using the function of the BLAST server at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Construction of recombinant plasmid pMA5-ksdd and transformation of B. subtilis

The *ksdd* gene was cloned from chromosomal DNA of *M. neoaurum* JC-12 with the forward primer [5′-ATCCATATGTTCTAC-ATGACTGCCCAGG-3′] and reverse primer [5′-GACGGATCCT-TAGTGGTGGTGGTGGTGGGCCTTTCCAGCGAGATG-3′]. Primers were originally designed with *Nde* I and *BamH* I restriction sites (underline) to clone *ksdd* into the plasmid pMA5. Transformation of the *B. subtilis* cells was carried out by the procedure described by Anagnostopoulos and Spizien [31]. The recombinant *B. subtilis* with pMA5-*ksdd* was selected by resistance to kanamycin and confirmed by DNA sequencing.

2.5. Expression of ksdd in B. subtilis 168 with pMA5 and preparation of cell extracts

The recombinant plasmid pMA5-*ksdd* was introduced into *B. subtilis* 168. Transformants were obtained after growing overnight on selective LB medium supplemented with 100 mg/ml kanamycin. The recombinant cells were grown in LB medium (50 ml) for 24 h. Cell pellets (8000 rpm; 10 min; 4 °C) were washed with 100 ml 50 mM Tris–HCl buffer (pH 7.0). The supernatant of culture was used for KSDD enzyme assays. Pellets were suspended in Tris–HCl buffer with 1:2 (w/w) ratio and sonicated 10 min. Cell extracts were centrifuged for 30 min at 10,000 rpm in an SIGAMA 3K-15 centrifuge to remove the cell debris. The supernatant of cell extraction was used for KSDD enzyme activity assays, analysis on native PAGE (12% acrylamide) or stored at −20 °C.

2.6. KSDD activity staining on native PAGE and KSDD enzyme activity assay

KSDD activity was visualized by incubating native PAGE [18] gels in 100 ml 50 mM Tris–HCl buffer containing 3.1 mg phenazine methosulphate, 2.9 mg steroid (AD in 500 μl ethanol) and 41 mg nitroblueterazolium (NBT) dissolved in 500 μl 70% dimethylformamide. Staining was allowed to proceed for several hours until clear activity bands were visible. The reaction was stopped by replacing the staining solution with 10% acetic acid. No KSDD activity staining was found in controls with ADD. Enzyme activities of the intracellular and extracellular parts were spectrophotometrically at 30 °C using phenazine methosulphate (PMS) and 2,6-dichlorophenolindophenol (DCPIP). The reaction mixture (1 ml) consisted of 50 mM Tris–HCl buffer (pH 7.0), 1.5 mM PMS, 40 μM DCPIP, appropriate concentration of the supernatant or cell extract, and 200 mmol/l AD in methanol (2%). Activities are expressed as units per milligram of protein; 1 U is defined as the reduction of 1 μmol min^{-1} DCPIP ($\xi_{600\,nm} = 18.7 \times 10^3\ cm^{-1}\ M^{-1}$) [16,22]. No activity was detected in reaction mixtures without 4-androstene-3,17-dione (AD).

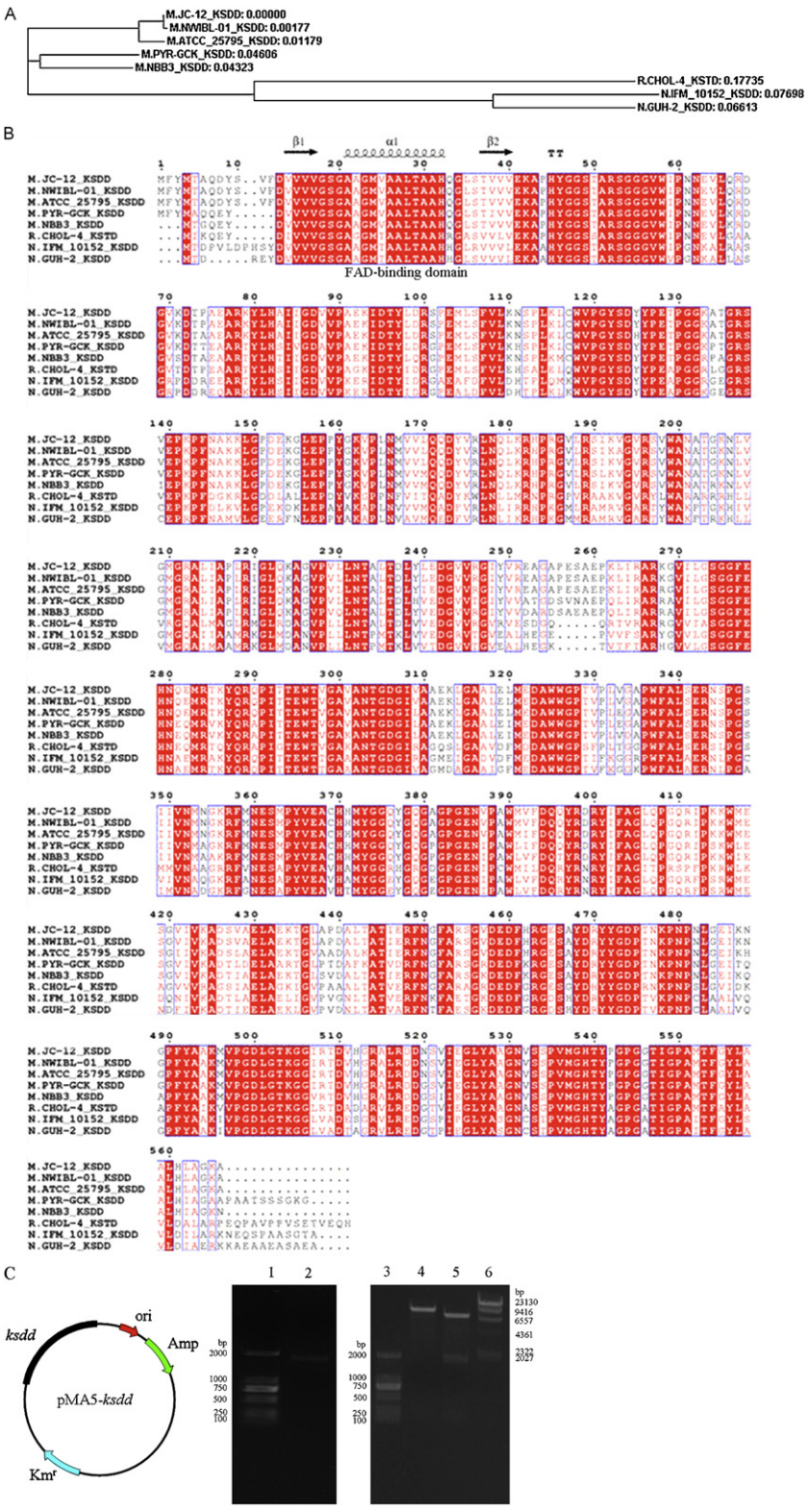

Fig. 2. Phylogenetic tree analysis and conesrved sequence. (A) Phylogenetic tree of KSDD enzymes from different species. The values are relative evolutionary distance. (B) The sequence alignment of known and putative KSDD. M.JC-12_KSDD, KSDD from *M. neoaurum* JC-12; M.NWIBL-01_KSDD, KSDD from *M. neoaurum* NwIBL-01; M.ATCC_25795_KSDD, KSDD from *M. neoaurum* ATCC25795; M.PYR-GCK_KSDD, KSDD from *M. gilvum* PYR-GCK; M.NBB3_KSDD, KSDD from *M. rhodesiae* NBB3; R.CHOL-4_KSTD, $KSTD_3$ from *R. rubber* Chol-4; N.IFM_10152_KSDD, KSDD from *N. farcinica* IFM 10152; N.GUH-2_KSDD, KSDD from *N. cyriacigeorgica* GUH-2. (C) Gene cloning and construction of the over expression plasmid pMA5-*ksdd* for KSDD production in *B. subtilis* 168, identification of recombinant plasmids. The length of *ksdd* is 1701 bp. The length of pMA5 is 7500 bp. Lane 1, DL2000 marker; lane 2, the amplified *ksdd* gene by PCR; lane 3, DL2000 marker; lane 4, the recombinant plasmid pMA5-*ksdd* digested by *BamH*I; lane 5, the recombinant plasmid pMA5-*ksdd* digested by *BamH*I/*Nde*I; and lane 6, λ DNA/*Hind* III marker.

2.7. Bioconversion of AD by recombinant strain B. subtilis 168 with pMA5-ksdd

The bioconversion of AD was done in shake flasks with the recombinant *B. subtilis* and *M. neoaurum* JC-12. The growth conditions of *B. subtilis* recombinants and *M. neoaurum* JC-12 were as previously described. After growing until late exponential phase (OD_{600} 4∼6), cells were collected by an SIGAMA 3K-15 centrifuge. Cell pellets were washed with 200 ml 50 mM Tris–HCl buffer (pH 7.0) twice. After that pellets were suspended in 20 ml Tris–HCl buffer. Then AD (0.1% (w/v)) and Tween 80 (0.1% (v/v)) were added into the biotransformation system. The bioconversion was performed for 24 h. Steroids extracted from the medium (0.5 ml) by ethyl acetate were used for HPLC (high-performance liquid chromatography) and TLC (tin-layer chromatography) analyses. For HPLC analysis, samples were diluted 10 times with ethyl acetate and filtered. Steroids were analyzed on HPLC (column: reversed phase Diamonsil C18, $UV_{254\,nm}$ detection, liquid phase: methanol:water (7:3), column temperature: 30 °C, flow rate: 1 ml min^{-1}) and TLC (F_{254} 10 × 10 cm in petroleum ether/ethyl acetate (6:4), staining fluid: 20% sulfuric acid) [19]. Retention times for HPLC were t_{ADD} = 7.3 min and t_{AD} = 9.9 min. Retention factors for TLC were $R_{f,AD}$ = 0.64 and $R_{f,ADD}$ = 0.5.

3. Results

3.1. Gene cloning and sequence analysis

The amplification of *ksdd* gene was achieved as described in Section 2. A phylogenetic tree was constructed by the service of EMBL-EBI [20]. The resulting protein sequence showed high similarity to that of several known KSDD including *M. neoaurum* NwIBL-01 (0.00177), *M. neoaurum* ATCC25795 (0.01179), *M. gilvum* PYR-GCK (0.04606), *M. rhodesiae* NBB3 (0.04323), *R. rubber* Chol-4 (0.17735), *N. cyriacigeorgica* GUH-2 (0.06613) and *N. farcinica* IFM 10152 (0.07698). The relative evolutionary distance of KSDD proteins of those listed bacteria was showed in their followed parentheses (Fig. 2A). These enzymes are flavoproteins, and the constructive N-terminal flavin adenine dinucleotide (FAD)-binding site was coincide with the sequence as previously described, G-S-G-(A/G)-(A/G)-(A/G)-X_{17}-E (Fig. 2B) [16,24,25].

3.2. Construction of the recombinant strain B. subtilis 168/pMA5-ksdd

The *ksdd* gene was amplified by PCR technique. It was cloned into the *Nde* I/*BamH* I sites of pMA5 carrying the control of the *Hpa*II promoter to construct a recombinant vector pMA5-*ksdd*. The recombinant plasmid pMA5-*ksdd* was divided by digesting with *Nde* I and *BamH* I into two major DNA fragments with sizes of 1701 and 7500 bp, respectively, which were consistent with the expected sizes. The recombinant plasmid pMA5-*ksdd* was verified (Fig. 2C).

The recombinant plasmid pMA5-*ksdd* was subsequently transformed into *B. subtilis* 168 to construct the recombinant strain *B. subtilis* 168/pMA5-*ksdd*, which allowed the gene *ksdd* to be expressed under the control of *Hpa*II promoter in *B. subtilis*. The recombinant *B. subtilis* was selected with kanamycin as the selectable marker and verified by DNA sequencing.

3.3. Expression and enzyme activity assay of KSDD

The possible presence of KSDD expression in *B. subtilis* 168 was subsequently investigated. Crude cell extract was prepared and assayed by native PAGE as described in Section 2. Staining for KSDD activity on native PAGE gels loaded with extracts of *M. neoaurum* JC-12 revealed a weak activity bands (Fig. 3 (lane 1)). The band with the highest electrophoretic mobility was expressed in the recombinant *B. subtilis* 168 (Fig. 3 (lane 3)). Meanwhile, there was no activity band on native PAGE gels loaded with extracts of *B. subtilis* 168 (Fig. 3 (lane 2)).

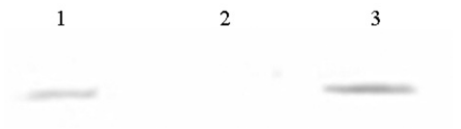

Fig. 3. KSDD activity staining on native PAGE, KSDD activity staining on native PAGE gels by using 4-androstene-3,17-dione as substrate, loaded with appropriate concentration of cell extracts of *M. neoaurum* JC-12 (lane 1), *B. subtilis* 168 (lane 2), the recombinant *B. subtilis* 168 (lane 3). No activity bands could be visualized in controls using AD as substrate for staining.

The activities of the intracellular and extracellular KSDD from the recombinant *B. subtilis* 168 were assayed by spectrophotometrical method. The enzyme activities of KSDD in the host cell *B. subtilis* 168 have not been detected. The activities of the intracellular and extracellular parts of KSDD were 1.75 ± 0.09 U/mg and 0.08 ± 0.01 U/mg, respectively. However, the activity of the intracellular parts of KSDD which was obtained from *M. neoaurum* JC-12 was merely 0.33 ± 0.02 U/mg. And no extracellular enzyme produced by *M. neoaurum* JC-12 was detected in the medium (Table 1).

3.4. Products analysis of the recombinant cells

When using the whole-cells of the recombinant *B. subtilis* as catalyst, AD was transformed, and the products were analyzed by tin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The results from TLC and HPLC indicated that the recombinant cells catalyzed the transformation of AD to ADD. The identity of the reaction product was conformed by TLC and HPLC (Fig. 4). As a control, no ADD formation was detected during AD bioconversion by whole-cell of strain *B. subtilis* 168/pMA5. From those above experiments, the ADD production capacity of the recombinant *B. subtilis* 168 has been surely confirmed when using AD as substrate.

3.5. Steroid transformation analysis

The whole-cells of *B. subtilis* 168/pMA5-*ksdd* were expected to accumulate ADD from AD. So the stoichiometrical bioconversion of AD into ADD by the strains was carried out. The results showed that no accumulation of ADD from AD was observed by the strain *B. subtilis* 168/pMA5. While in the recombinant strain *B. subtilis* 168/pMA5-*ksdd*, the percent conversion of AD reached the maximum 65.7% in 10 h after 0.1% (w/v) AD was added. However, in the strain *M. neoaurum* JC-12, the percent conversion of AD reached at 3.56% and the maximum 25% at 132 h (Fig. 5). These results demonstrated that the strain *B. subtilis* 168/pMA5-*ksdd* was a successful ADD-producing strain with AD as substrate (Table 1). Thus, it was expected that the recombinant *B. subtilis* 168 will be used in the steroid industry for the transformation of AD to ADD.

4. Discussion

Microbial strains that can transform AD to ADD are needed for industrial applications. In the previous experiment, the transformation of AD in *M. neoaurum* JC-12 had exhibited a low-efficiency owing to its low KSDD activity. Therefore, in order to improve the KSDD activity and the conversion efficiency of AD, *B. subtilis* was used to express KSDD in this work. We successfully obtained the KSDD gene from the chromosome of *M. neoaurum* JC-12 by PCR technique and constructed *ksdd* onto the plasmid to gain the recombinant pMA5-*ksdd*. Then the recombinant plasmid was

Table 1
The enzyme activities of KSDD and the whole-cell transformation of AD to ADD.

Strain	Enzyme activity		The whole-cell transformation of AD		
	Extracellular enzyme (U/mg)	Intracellular enzyme (U/mg)	Time for cell culture (h)	Time for transformation (h)	The maximum transformation rate (%)
M. neoaurum JC-12	NT	0.33 ± 0.02	72	132	25
B. subtilis 168	NT	NT	24	10	NT
B. subtilis 168/pMA5-*ksdd*	0.08 ± 0.01	1.75 ± 0.09	24	10	65.7

NT, no detectable enzyme activity. All assays were performed with either duplicate or triplicate cultures.

transformed to *B. subtilis* 168. The recombinant strain *B. subtilis* 168/pMA5-*ksdd* was acquired. The successfully expressed KSDD was demonstrated by the analysis of native-PAGE. The recombinant strain *B. subtilis* 168/pMA5-*ksdd* was used for whole-cell transformation of AD to ADD, and the products of the transformation of AD were analyzed with TLC and HPLC. In the recombinant *B. subtilis*168/pMA5-*ksdd*, KSDD activity was improved about 5-fold higher than that of *M. neoaurum* JC-12. And importantly, the

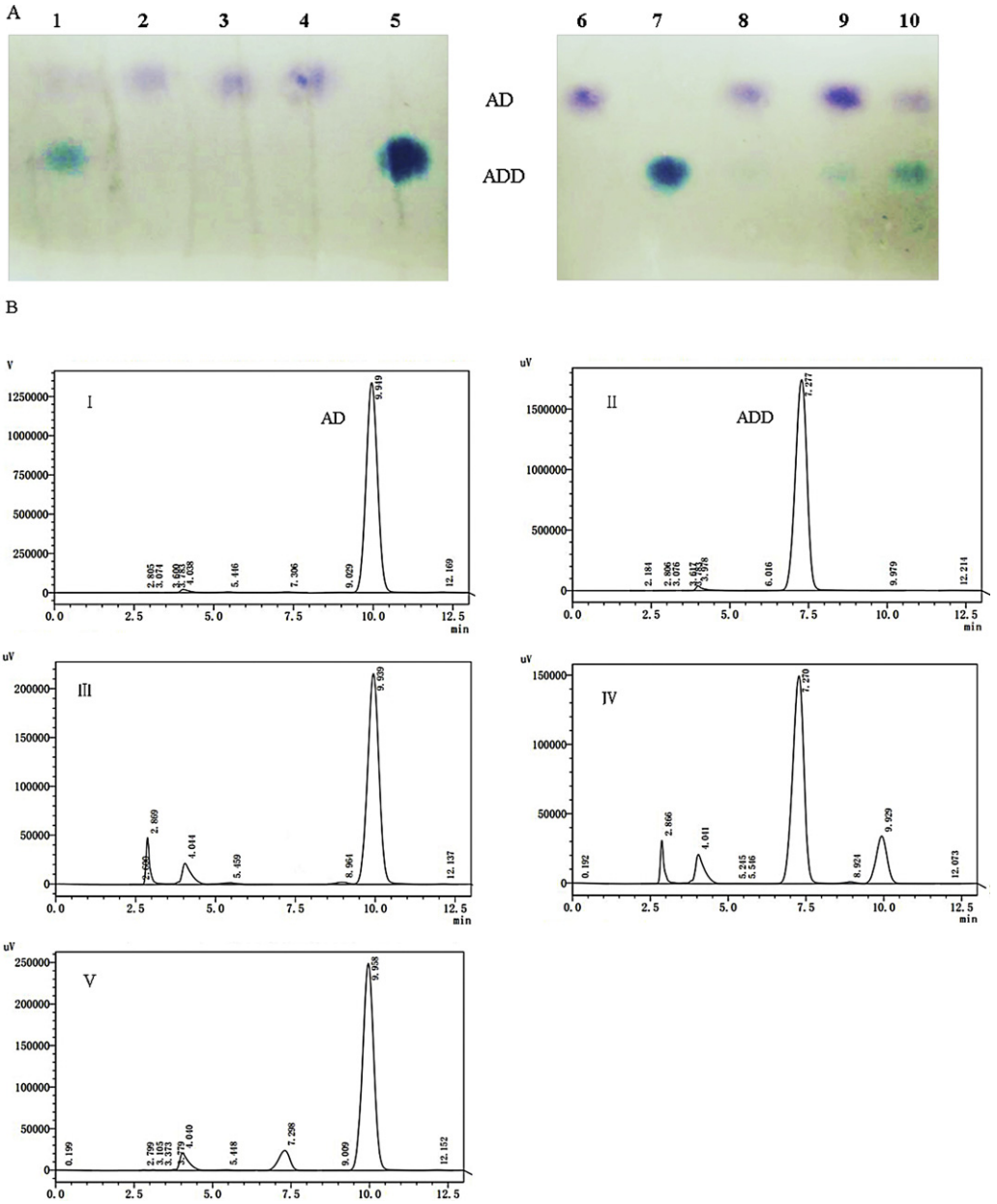

Fig. 4. TLC and HPLC analysis of transformation products. (A) TLC analysis of transformation products. Lane 1, the products by *B. subtilis* 168/pMA5-*ksdd*; lane 2, the products by *B. subtilis* 168/pMA5; lane 3, the products by *B. subtilis* 168; lane 4, the standard sample of AD; lane 5, the standard sample of ADD; lane 6, the standard sample of AD; lane 7, the standard sample of ADD; lane 8, the products by the 2 h cultures of *B. subtilis* 168/pMA5-*ksdd*; lane 9, the products by the 4 h cultures of *B. subtilis* 168/pMA5-*ksdd*; lane 10, the products by the 8 h cultures of *B. subtilis* 168/pMA5-*ksdd*. (B) HPLC analysis of transformation products. Peaks: I, the standard samples of AD; II, the standard samples of ADD; III, the product by *B.subtilis* 168/pMA5; IV, the product by *B.subtilis* 168/pMA5-*ksdd*; and V, the product by *M. neoaurum* JC-12.

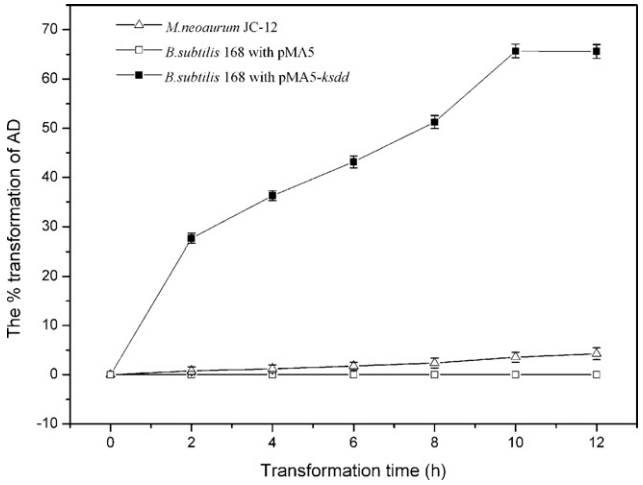

Fig. 5. ADD accumulations with AD as substrate. Time course of AD transformation to ADD by the *B. subtilis* 168/pMA5, *B.subtilis* 168/pMA5-*ksdd* and *M. neoaurum* JC-12 strains.

activity of KSDD expressed in *B. subtilis* could remain stable for a long period. The further research suggested that the KSDD activity mainly existed in intracellular, and it may ascribe to the transmembrane segments of this enzyme. It was previously reported that the expressed KSDD in *E. coli* was mainly located in the inner membrane in the form of inclusion bodies [16,17,22]. The overexpressed KSDD from *M. neoaurum* JC-12 in *E. coli* with the vectors pET28a and pET22b was executed in our original study, and the recombinant proteins mainly existed in the form of inclusion bodies. The enzyme activity of KSDD was not detected (on the vector pET28a) or very weak (on the vector pET22b) in *E. coli*, and the recombinant protein lost its activity in a very short time. From this point of view, the *B. subtilis* system for KSDD expression is much better than *E. coli*.

The percent conversion of AD by the recombinant *B. subtilis*168/pMA5-*ksdd* was improved significantly, which was about 18-fold higher than that of *M. neoaurum* JC-12. The percent conversion of AD reached maximum in 10 h by the recombinant *B. subtilis* 168, while it required 4–6 days by *M. neoaurum*. The biosynthesis ADD in *M. neoaurum* contains the two steps: first, from phytosterol to AD; then from AD to ADD. Compared to *M. neoaurum*, the *B. subtilis* 168 catalyzed the transformation of ADD from AD in one-step. Moreover, the recombinant *B. subtilis* was cultured much more easily than *M. neoaurum*. So the much higher conversion of AD by the recombinant *B. subtilis* 168 was due to its rapid growth and short steroids conversion periods. And most importantly, the *B. subtilis* was a very safe microorganism that is widely used in industry.

In conclusion, the enzyme KSDD from *M. neoaurum* JC-12 was successfully expressed with stabilized enzyme activity in *B. subtilis* 168. The recombinant *B. subtilis* showed obviously superiority on transformation of AD to ADD. Based on works above, the genes related to ADD synthesis from phytosterol are considered to be cloned in *B. subtilis* 168 to achieve one-step efficient transformation of low-cost substrate to ADD, making the recombinant *B. subtilis* 168 become a promising strain for the production of ADD in industry.

Acknowledgments

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