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Research article

Over-expression of *Mycobacterium neoaurum* 3-ketosteroid-Δ1-dehydrogenase in *Corynebacterium crenatum* for efficient bioconversion of 4-androstene-3,17-dione to androst-1,4-diene-3,17-dione

Xian Zhang [a], Dan Wu [a], Taowei Yang [a], Meijuan Xu [a], Zhiming Rao [a,b,*]

[a] The Key Laboratory of Industrial Biotechnology of Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu 214122, PR China
[b] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, PR China

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ABSTRACT

Background: 3-Ketosteroid-Δ[1]-dehydrogenase (KSDD), a flavoprotein enzyme, catalyzes the bioconversion of 4-androstene-3,17-dione (AD) to androst-1,4-diene-3,17-dione (ADD). To date, there has been no report about characterization of KSDD from *Mycobacterium neoaurum* strains, which were usually employed to produce AD or ADD by fermentation.
Results: In this work, *Corynebacterium crenatum* was chosen as a new host for heterologous expression of KSDD from *M. neoaurum* JC-12 after codon optimization of the KSDD gene. SDS-PAGE and western blotting results indicated that the recombinant *C. crenatum* harboring the optimized *ksdd* (*ksdd*[II]) gene showed significantly improved ability to express KSDD. The expression level of KSDD was about 1.6-fold increased *C. crenatum* after codon optimization. After purification of the protein, we first characterized KSDD from *M. neoaurum* JC-12, and the results showed that the optimum temperature and pH for KSDD activity were 30°C and pH 7.0, respectively. The K_m and V_{max} values of purified KSDD were 8.91 μM and 6.43 mM/min. In this work, *C. crenatum* as a novel whole-cell catalyst was also employed and validated for bioconversion of AD to ADD. The highest transformation rate of AD to ADD by recombinant *C. crenatum* was about 83.87% after 10 h reaction time, which was more efficient than *M. neoaurum* JC-12 (only 3.56% at 10 h).
Conclusions: In this work, basing on the codon optimization, overexpression, purification and characterization of KSDD, we constructed a novel system, the recombinant *C. crenatum* SYPA 5-5 expressing KSDD, to accumulate ADD from AD efficiently. This work provided new insights into strengthening sterol catabolism by overexpressing the key enzyme KSDD, for efficient ADD production.

1. Introduction

Steroid drug intermediates are widely used for the commercial production of pharmaceutical steroid drugs. Compared with the chemical synthesis process, bioprocess of transforming sterols to steroid drug intermediates has its obvious advantage and has been widely used as a common and economical alternative method in the pharmaceutical industry [1]. The microbial transformation of steroids has long prevailed in the pharmaceutical industry since the 1950s [2,3]. Degradation of steroids can yield much valuable steroidal derivatives, such as 4-androstene-3,17-dione (AD), androst-1,4-diene-3,17-dione (ADD), 9α-OH-AD, and 9α-OH-ADD [4,5,6,7,8]. ADD has been acknowledged to be a worthwhile precursor in the synthesis of steroid pharmaceuticals such as oestrogens, contraceptive agents and progestogens [2]. Chemical synthesis has been the main method of ADD production in the pharmaceutical industry for a long time [2,6,7,9]. Nevertheless, the substantial consumption of organic chemicals and the production of chemical waste, make it an environmentally unfriendly approach. As an alternative and modest synthesis method, biocatalytic production of ADD has become a good alternative, mainly because it provides a superb combination of cost-effectiveness, sustainability and scalability [10,11,12,13].

3-Ketosteroid-Δ[1]-dehydrogenase (KSDD) [EC 1.3.99.4] catalyzes the insertion of a double bond between the C1 and C2 atoms of the chemically stable 3-ketosteroid A-ring (Fig. 1) [14]. Several steroid-degrading bacteria with KSDD activity have been reported, including *Mycobacterium*, *Rhodococcus*, *Comamonas*, and *Arthrobacter* [15,16,17]. The constructive N-terminal flavin adenine dinucleotide

* Corresponding author.
E-mail addresses: raozhm@jiangnan.edu.cn, zxshengwu@126.com (Z. Rao).
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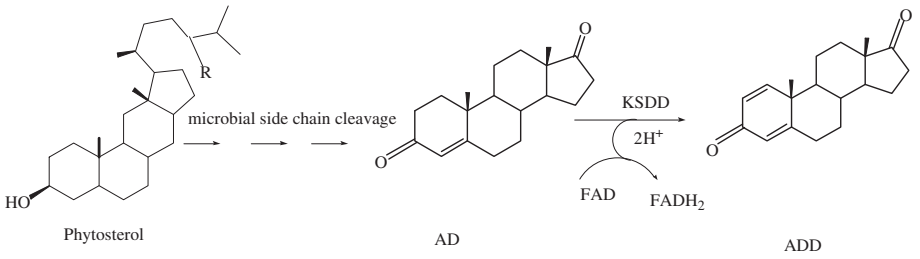

Fig. 1. Bioconversion of AD to ADD by 3-ketosteroid-Δ^1-dehydrogenase.

(FAD)-binding site was coincided with the sequence G-S-G-(A/G)-(A/G)-(A/G)-X_{17}-E [18,19]. According to the crystal structure of the KSDD from *Rhodococcus erythropolis*, the enzyme does not have any trans-membrane helices, and the protein behaves as a soluble protein [20].

Much work has been done on strain amelioration, enhancing or limiting KSDD expression by molecular or mutagenesis methods [21,22]. In our previous research, we isolated and identified *Mycobacterium neoaurum* JC-12 with the capacity of converting phytosterol to the product ADD in our laboratory. The *ksdd* gene was amplified and expressed in *Bacillus subtilis* 168 using plasmid pMA5. Regrettably, the expression level of KSDD was very low for purification and no high yields of ADD were obtained [18]. Nevertheless KSDD is a key enzyme for producing and accumulating ADD during steroid degradation. So far, there was no detail report that the KSDD from *M. neoaurum* has been successfully characterized. This lack of knowledge limits, both, the knowledge on the properties of KSDD and the optimum use of this enzyme for converting AD to ADD.

In this work, *Corynebacterium crenatum* was chosen as a new host for heterologous expression of KSDD. As the native gene *ksdd*^{I} showed a poor codon usage bias for *C. crenatum*, we suspected that it should be one of the limiting factors leading to the inefficient expression. In order to improve the expression of KSDD, we designed and constructed a full-length synthetic gene by optimizing the codon usage without changing its amino acid sequence. We also investigated the purification and characterization of the KSDD from *M. neoaurum* JC-12. Under optimal conditions, the efficient transformation of AD to ADD by the whole-cell of the recombinant *C. crenatum* SYPA 5-5 was achieved.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

C. crenatum strain SYPA 5-5 was screened and stored in our laboratory. It is an aerobic, Gram-positive, non-sporulating coryneform bacterium and a high-yield strain of amino acids in the industry [23]. *M. neoaurum* JC-12 and the vector pXMJ19 were preserved in our laboratory (Table 1). The strains *C. crenatum* SYPA 5-5/pXMJ19, *M. neoaurum* JC-12 and the recombinant *C. crenatum* SYPA 5-5 were cultivated at 30°C and 160 rpm in Luria–Bertani broth containing 0.5% glucose(LBG). Chloroamphenicol (50 mg/mL) was added to the growth medium if necessary. 4-Androstene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD) were supplied by the Sigma-Aldrich Chemical Co. Inc. (USA). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was purchased from Zhiyuan Bio-Technology Co., Ltd. (Binzhou, China). All other chemicals with the analytical grade could be purchased.

2.2. General cloning techniques

T4 DNA ligase, Mini Plasmid Rapid Isolation Kit and Mini DNA Rapid Purification kit were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Restriction enzymes were bought from TaKaRa Co. (Dalian, China).

2.3. Codon optimization of ksdd gene

According to the sequence of *ksdd* gene from *M. neoaurum* JC-12, the optimization of its codon was subjected to the Codon Adaptation Tool (http://www.jcat.de/) on the basis of codon preference of *Corynebacterium glutamicum* ATCC13032. The high GC content which did not favor for gene expressing was reduced. The modified gene was synthesized by Sangon Biotech Co., Ltd., (Shanghai, China).

2.4. Construction of recombinant plasmid pXMJ19-ksdd and transformation of C. crenatum SYPA 5-5

The modified gene *ksdd*^{II} and native *ksdd*^{I} were amplified with the primers *ksdd*^{II} R/*ksdd*^{II} F and *ksdd*^{I} R/*ksdd*^{I} F (Table 1). Both the modified and native genes were cloned into the plasmid pXMJ19 by designed primers with *Hind* III and *Bam*H I restriction sites (underline). Then the recombinant plasmids were transformed into

Table 1
Bacterial strains, plasmids, and primers.

Strains/plasmids/primers	Characteristics	Source
Strains		
C. crenatum SYPA 5-5	An industrial microorganism for production of amino acids	Laboratory stock
M. neoaurum JC-12	producing ADD from phytosterol	Laboratory stock
C. crenatum SYPA 5-5/pXMJ19	Cm^R	This study
C. crenatum SYPA 5-5/pXMJ19- *ksdd*^{I}	Cm^R	This study
C. crenatum SYPA 5-5/pXMJ19- *ksdd*^{II}	Cm^R	This study
Plasmid		
pXMJ19	Expression vector, p*tac*, *lacI*^{q}, Cm^R, Capable of replication in *Corynebacterium Glutamicum*	Laboratory stock
Primers	CGCAAGCTTAAAGGAGGGAAATCATGTTCTACATGACC GCTCAGG	
Optimal *ksdd*^{II} F	GACGGATCCTTA**GTGGTGGTGGTGGTGGTG**AGCCTTGCCAGCCAGGTG	
Optimal *ksdd*^{II} R	CGCAAGCTTAAAGGAGGGAAATCATGTTCTACATGACTGCCCAGG	
Native *ksdd*^{I} F	GACGGATCCTTA**GTGGTGGTGGTGGTGGTG**GGCCTTTCCAGCGAGATG	
Native *ksdd*^{I} R		

Cm^R Chloroamphenicol-resistant, the restriction enzyme sites were underlined, and the His-Taq coding region were bold typed.

C. crenatum SYPA 5-5 to obtain engineered *C. crenatum* strains by the electroporation methods described by Tauch et al. [24]. Chloramphenicol was chosen as the selectable marker to screen the recombinant *C. crenatum*, and then verified by DNA sequencing.

2.5. Expression of $ksdd^{II}$ and $ksdd^{I}$ in C. crenatum SYPA 5-5 with pXMJ19 and preparation of cell extracts

The recombinant plasmids pXMJ19-$ksdd^{II}$ and pXMJ19-$ksdd^{I}$ were introduced into *C. crenatum* SYPA 5–5. Transformants were obtained after growing 72 h on selective LBG agar plate added with chloroamphenicol. The recombinant cells were grown in 50 mL LBG medium for 14 h with 50 μL IPTG (200 mg/mL) to induce the enzyme expression. Cell pellets (8000 g; 10 min; 4°C) were washed with 10 mL 50 mM Tris–HCl buffer (pH 7.0). The supernatant of culture was used for KSDD enzyme activity assay. Pellets were suspended in 5 mL Tris–HCl buffer and sonicated for 15 min. 0.1 mM dithiothreitol (DTT) was added to protect the enzyme. In order to remove the cell fragments, cell extracts were centrifuged for 30 min at 10,000 × g in an SIGMA 3K-15 centrifuge. The supernatant of cell extraction was used for KSDD enzyme activity assay, SDS-PAGE (12% acrylamide) analysis or storage at -20°C with 10% glycerol.

2.6. SDS-PAGE analysis and determination of protein concentration

The samples used for SDS-PAGE were mixed with 2× SDS loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM DTT, 0.4 g/L SDS, 0.02 g/L bromophenol blue, and 20% (*v*/*v*) glycerol) with ratio 1:1 (*v*/*v*). The mixture was then boiled in water for 10 min, and centrifuged for 30 min at 10,000 × g. The samples were run on a SDS-PAGE as described by Laemmli [25]. Expression of KSDD was also detected by western blotting with a mouse monoclonal anti-His6 antibody. The samples were treated as described by Bao et al. [26], and the bands were detected by chemiluminescence with luminol and peroxide with the help of a Bio-Rad Chemidoc XRS. Bradford method was employed to determine the protein concentration by using BSA as standard protein [27].

2.7. KSDD enzyme activity assay

Enzyme activity of KSDD was determined spectrophotometrically at 30°C using 2,6-dichlorophenolindophenol (DCPIP) and phenazine methosulphate (PMS). 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 500 μM AD in methanol (2%). Cofactor (FAD) was added when necessary. Activity was expressed as units per milligram of protein; 1 U is defined as the reduction of 1 μmol/min DCPIP ($\varepsilon_{600\ nm} = 18.7 \times 10^3\ cm^{-1}\ M^{-1}$) [21,28]. No activity was found in reaction mixtures without 4-androstene-3,17-dione (AD).

2.8. Purification and characterization of the recombinant KSDD

The recombinant KSDD with histidine-tag was expressed in *C. crenatum* and purified by affinity chromatography on a Ni-NTA sepharose prepacked column His Trap HP (GE Healthcare Life Sciences, USA). Purification was performed according to the instructions of His Trap TM HP column [29]. The purified enzymes were subsequently assayed by SDS-PAGE. Bradford method was employed to determine the protein concentration. The eluted fractions containing the target protein were collected and assayed for KSDD activity. The purified enzyme can be stored at -20°C about half of month with 10% glycerol, 0.01 mM FAD and 0.1 mM DTT to maintain its stability.

To determine the optimal temperature for KSDD activity, the purified KSDD was assayed at pH 7.0 for 15 min at different temperatures (0°C–60°C). The thermal stability was assayed by incubating the purified enzyme at temperatures from 0°C to 60°C for 2 h. The residual enzyme activities were measured under standard assay conditions. The pH optimum of KSDD was examined at 30°C for 15 min at pH range from 3.0 to 10.0 (pH 3.0–pH 6.0, 0.05 M citrate-sodium citrate buffer; pH 6.0–pH 9.0, 0.05 M Tris–HCl buffer; pH 9.0–pH 10.0, 0.05 M borax-sodium hydroxide buffer). The pH stability was determined by incubating the enzyme in different buffers at 0°C for 2 h and the residual activity was measured at pH 7.0 and 30°C.

The influence of selected metal ions (K^+, Na^+, Ag^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}) and ethylenediaminetetraacetic acid (EDTA) at 1 mM final concentration on the activity of the purified KSDD was investigated. Most chemicals are chloric compounds except $AgNO_3$. Thus, we used $NaNO_3$ as a control experiment to investigate whether $NO3^-$ affect the results. Relative activity was assayed as a percentage of the activity without agents. Under different concentration of AD, Kinetic parameters were investigated with PMS as electron acceptor at a fixed concentration of 1.5 mM. The K_m and V_{max} values were determined from Lineweaver–Burk plots.

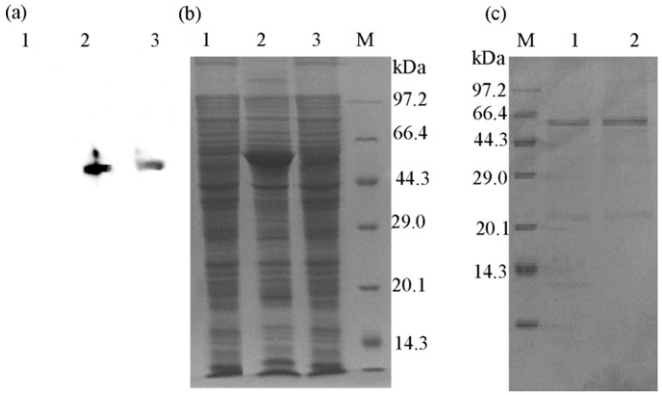

Fig. 2. SDS-PAGE analysis of cell-free extract and purified KSDD. (a) Western blot result of KSDD expressed in *C. crenatum*. Lane 1: *C. crenatum* SYPA 5-5/pXMJ19; lane 2: *C. crenatum* SYPA 5-5/pXMJ19-$ksdd^{II}$; lane 3: *C. crenatum* SYPA 5-5/pXMJ19-$ksdd^{I}$. (b) SDS-PAGE analysis of KSDD expressed in *C. crenatum*. Lane 1: cell-free extract of control *C. crenatum* SYPA 5-5/pXMJ19; lane 2: cell-free extract of *C. crenatum* SYPA 5-5/pXMJ19-$ksdd^{II}$; lane 3: cell-free extract of *C. crenatum* SYPA 5-5/pXMJ19-$ksdd^{I}$; lane M: protein marker (Takara Biotechnology Co., Ltd., Dalian, China). (c) SDS-PAGE analysis of purified KSDD. Lane 1, 2: 10 μL of purified mature His6-KSDD treated; lane M: protein marker (Takara Biotechnology Co., Ltd., Dalian, China).

2.9. Bioconversion of AD by recombinant strains C. crenatum/pXMJ19-ksdd

The bioconversion of AD was carried out in 250 Ml shake flasks with the recombinants *C. crenatum* and *M. neoaurum* JC-12. The cultural conditions of *C. crenatum* recombinants and *M. neoaurum* JC-12 were described previously. Cells were collected by an SIGMA 3K-15 centrifuge at late exponential phase (OD_{600} 4–6). Cell pellets were washed twice with 100 mL 50 mM Tris–HCl buffer (pH 7.0). After the pellets were resuspended in 50 mL Tris–HCl buffer (added with 1 mM K^+, Na^+ and Ca^{2+}), AD (1% (*w*/*v*)) and

Table 2
The enzyme activities of KSDD from *M. neoaurum* JC-12 and recombinant *C. crenatum*.

Strains	Enzyme activity (U/mg total protein)	
	Extracellular enzyme	Intracellular enzyme
M. neoaurum JC-12	NT	0.32 ± 0.06
C. crenatum SYPA 5-5/pXMJ19	NT	0.09 ± 0.03
C. crenatum SYPA 5-5/pXMJ19- $ksdd^{I}$	0.03 ± 0.01	1.56 ± 0.03
C. crenatum SYPA 5-5/pXMJ19- $ksdd^{II}$	0.04 ± 0.03	2.58 ± 0.05

NT, undetectable enzyme activity. All assays were performed with triplicate cultures.

Table 3
Purification of recombinant KSDD from *C. crenatum* SYPA 5-5/pXMJ19-*ksdd*^{II}.

Purification stage	Total protein (mg)	Total activity (U)	Specific activity (U/mg total protein)	Fold purification	Yield (%)
Crude cell extract	214.67 ± 0.06	553.84 ± 0.03	2.58 ± 0.05	1.00	100
Purified enzyme	3.15 ± 0.04	40.82 ± 0.06	12.96 ± 0.02	5.02	7.37

hydroxypropyl-β-cyclodextrin (HP-β-CD, 3% (*w*/*v*)) were added into the biotransformation system, which was carried out for 24 h. The extraction of steroids from the medium (1 mL) by ethyl acetate was analyzed by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Steroids were analyzed by HPLC (column: reversed phase Diamonsil C18, UV254 nm detection, liquid phase: methanol: water (7:3), column temperature: 30°C, flowrate: 1 mL min^{-1}) and TLC (F254 10 × 10 cm in petroleum ether/ethyl acetate (6:4), staining fluid: 20% sulfuric acid) [30].

3. Results

3.1. Construction of the recombinant C. crenatum SYPA 5-5

The native *ksdd*^{I} gene sequence was codon optimized for efficient translation. The synthesized gene *ksdd*^{II} and native gene *ksdd*^{I} were cloned onto an expression vector to generate two recombinant plasmids pXMJ19-*ksdd*^{II} and pXMJ19-*ksdd*^{I}. They were verified by digestion with *Hind* III and *Bam*H I into two DNA fragments. In order to generate the recombinant strain *C. crenatum* SYPA 5-5, the two recombinant plasmids were subsequently transformed into *C. crenatum* SYPA 5-5. The recombinant strains were selected with chloramphenicol and verified by DNA sequencing.

3.2. Overexpression and purification of KSDD

The possible expression of KSDD in *C. crenatum* SYPA 5-5 was investigated as follows. Crude cell extract was assayed by SDS-PAGE and western blotting; the molecular weight of the expressed protein was about 60 kDa (Fig. 2a, b). The intracellular and extracellular KSDD activity from *C. crenatum* SYPA 5-5 was assayed (Table 2). In the recombinant strains *C. crenatum* SYPA 5-5/pXMJ19-*ksdd*^{II}, the intracellular KSDD had much higher specific activity than *M. neoaurum* JC-12 and *C. crenatum* SYPA 5-5/pXMJ19-*ksdd*^{I}. From this we concluded that, compared with the wild type strain, the KSDD expression level of the mutant strain had been increased by about 1.6 fold (Table 2). It was also observed that there was no secretion of KSDD in *M. neoaurum* JC-12, *C. crenatum* SYPA 5-5/pXMJ19 and the recombinant strains. In conclusion, a high activity of KSDD has achieved in *C. crenatum* SYPA 5-5. The expression of KSDD has been significantly improved by codon optimization. Purification of the recombinant KSDD was carried out by using the Ni-NTA affinity chromatography. The result of SDS-PAGE analysis of the purified enzyme is given (Fig. 2c). The recombinant enzyme showed one band consistent with a molecular mass of about 60 kDa. The purification resulted in a yield of 7.37% and a purification of 5.02-fold (Table 3).

3.3. Characterization of KSDD

The purified KSDD was assayed at pH 7.0 for 15 min at different temperatures (0–60°C) to determine the optimal temperature for its reaction. It revealed that the optimal temperature of KSDD was 30°C (Fig. 3a). However, thermal stability profiles of the purified KSDD revealed that it was unstable at temperatures exceeding 30°C. The enzyme lost more than half of its activity after incubation at 30°C for 2 h (Fig. 3b), which corroborated previous report (KSDD from *Nocardia corallina*) [31]. The purified enzyme was also assayed at 30°C for 15 min at different pH (ranging from pH 3.0 to pH 10.0) to determine its pH optimum. The result showed that the optimal pH of KSDD was 7.0 (Fig. 4a). Furthermore, the profile of pH stability revealed that KSDD was fairly stable within a broad pH range for it retained more than 75% of its activity in pH ranging from 4.0–10.0 (Fig. 4b).

The effect of selected metal ions and EDTA on KSDD activity was also discussed in this study (Fig. 5). The results showed that KSDD activity was strongly stimulated by 1 mM K^+, Na^+ and Ca^{2+}, which had been revealed about other Δ^1-dehydrogenation reaction [32]. However, in the presence of 1 mM Ag^+, KSDD activity decreased to 28.05% compared to the control experiment. While subjected to the preferred reaction conditions, using AD as substrate, the purified KSDD exhibited typical Michaelis–Menten kinetics. The K_m and V_{max} values were 8.91 μM and 6.43 mM/min, respectively.

3.4. Efficient production of ADD by the recombinant C. crenatum

When using the whole-cell of the recombinant *C. crenatum* pXMJ19-*ksdd*^{I} and *C. crenatum* pXMJ19-*ksdd*^{II} as biocatalysts, the transformation from AD to ADD was assayed by HPLC (Fig. S1a) and

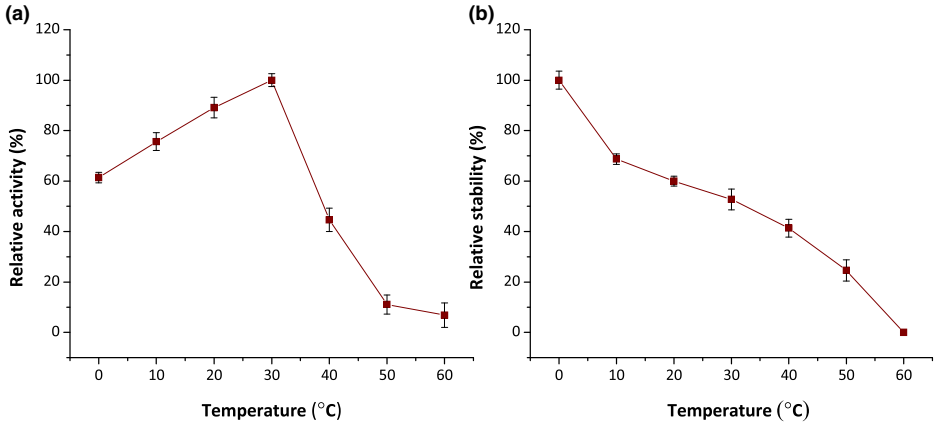

Fig. 3. Effect of temperature on KSDD activity (a) and stability (b).

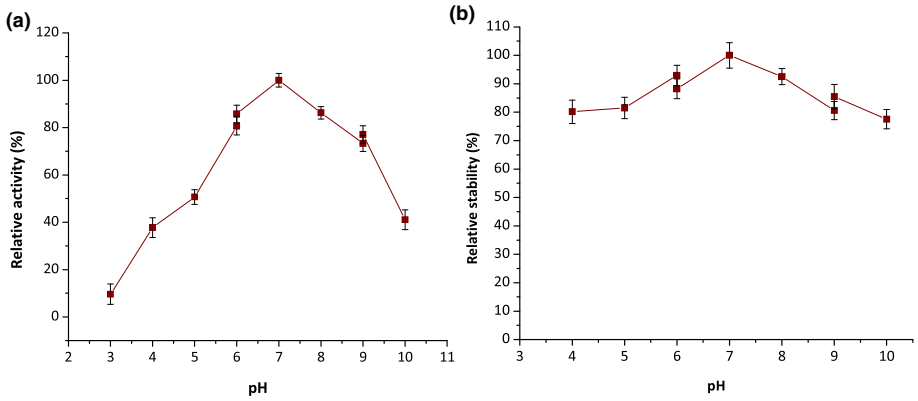

Fig. 4. Effect of pH on KSDD activity (a) and stability (b).

TLC (Fig. S1b). The results showed that the recombinant *C. crenatum* strains, which could over-express KSDD, were surely identified to have the capability to produce ADD when using AD as substrate. As a control, there was no ADD accumulated by the strain *C. crenatum* SYPA 5-5 harboring pXMJ19. By using recombinant *C. crenatum* pXMJ19-*ksdd*^{II} as biocatalyst, the production of ADD was improved to 8.39 g/L (Fig. 6). The overexpressed KSDD stimulated a shortened reaction duration about 13-fold, from 132 h to 10 h. As shown in Table 4, the recombinant strains *C. crenatum* pXMJ19-*ksdd*^{II} and *C. crenatum* pXMJ19-*ksdd*^{I} showed the maximum conversion rates from AD to ADD about 83.87% and 58.72% at 10 h. However, *M. neoaurum* JC-12 showed the conversion rate from AD to ADD only 3.56% at 10 h, and the maximum conversion rate of 23% at 132 h (Table 4 and Fig. 6). The results proved that the recombinant *C. crenatum* pXMJ19-*ksdd*^{II} cells could efficiently catalyze the transformation from AD to ADD.

4. Discussion

It has been reported that genes encoding KSDD1, KSDD2 and KSDD3 were found in the genome of *M. neoaurum*. However, KSDD3 performed the main function in steroid pathway and showed specific activities toward to the substrate AD [33]. Studies have tried to clone and heterologous express of KSDD from *M. neoaurum* in *R. erythropolis*, *Escherichia coli* and *Streptomyces lividans* [21,30,34,35,36]. Unfortunately, the low expression level of recombinant KSDD made it difficult using biocatalyst to transform AD to ADD. On the other hand, missing of the characterization of KSDD restricted the use of this enzyme, although it had been purified [21]. In this work, we successfully cloned and overexpressed KSDD from *M. neoaurum* in *C. crenatum* after codon adaption and optimization. The results of SDS-PAGE and western blotting indicated that a high expression level of recombinant KSDD (*C. crenatum* pXMJ19-*ksdd*^{II}) was achieved. The expression level of KSDD was improved approximately 1.65-fold in *C. crenatum* after codon optimization. After purification of KSDD, this work first characterized this enzyme and made the recombinant *C. crenatum* pXMJ19-*ksdd*^{II} as a biocatalyst for transforming AD to ADD. After characterization of KSDD, there was a huge loss of activity, with only 7.37% activity remaining for a 5-fold purification, because this enzyme was not very stable. It has been reported that this enzyme was not sensitive to carbonyl reagents and little sensitive to metal chelating agents [37]. Till now, no useful protectant has been reported. As reported that ions such as K^+, Na^+, Ca^{2+}, Fe^{2+} and Mg^{2+} could stimulate the Δ^1-dehydrogenation, we investigated the effect of mental ions on KSDD activity [32]. The results showed that KSDD activity was strongly stimulated by 1 mM K^+, Na^+ and Ca^{2+}. The redox-active ions such as Ag^+, Mn^{2+}, Cu^{2+} and Fe^{2+} might interfere with KSDD activity assay, and that was why they appeared inhibition effect on KSDD catalyzed reaction. On account of KSDD was very unstable above 30°C, in the following whole-cell biocatalysis, the strains were cultivated at 30°C and pH 7.0, which is favorable to maintain the integrity of cell and KSDD activity. Because of the low solubility of AD, HP-β-CD was added

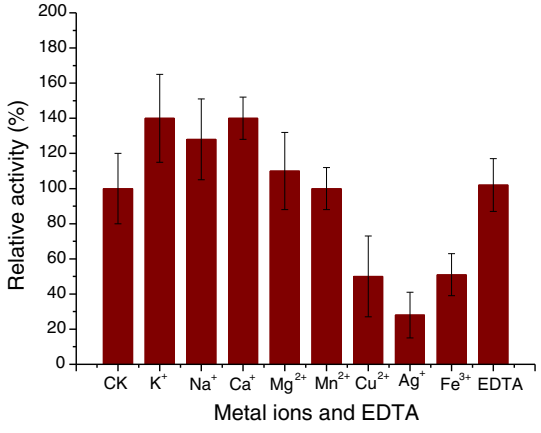

Fig. 5. Effects of metal ions and EDTA (1 mM) on KSDD activity. CK represents control experiment: no metal ion or EDTA added.

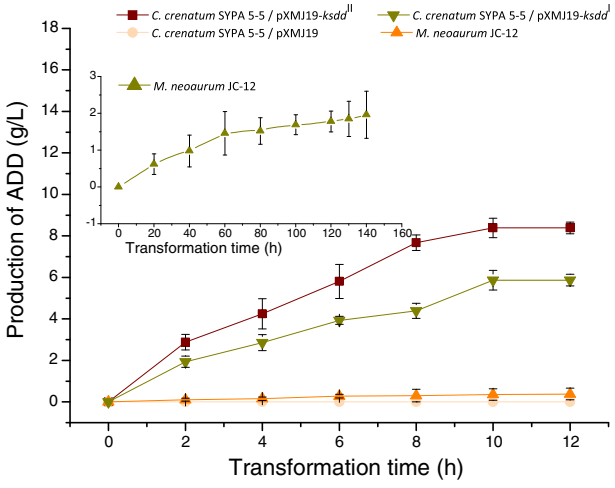

Fig. 6. Time course of ADD accumulation from AD by *C. crenatum* SYPA 5-5/pXMJ19-ksddII, *C. crenatum* SYPA 5-5/pXMJ19-ksddI, *C. crenatum* SYPA 5-5/pXMJ19 and M. neoaurum JC-12. (All assays were performed by three independent biological experiments, and the standard deviations of the biological replicates were represented by error bars).

Table 4
The whole-cell transformation from AD to ADD.

Strain	Time for culture (h)	Time for transformation (h)	The maximum transformation rate (%)
M. neoaurum JC-12	72	132	23.0
C. crenatum SYPA 5-5/pXMJ19	24	10	NT
C. crenatum SYPA 5-5/pXMJ19-*ksdd*^{I}	24	10	58.72
C. crenatum SYPA 5-5/pXMJ19-*ksdd*^{II}	24	10	83.87

NT, undetectable ADD. All assays were performed with triplicate cultures.

as an accessory solvent to increase the concentration of substrate. In order to further increase the conversion rate of AD to ADD, the stimulating metal ions for KSDD activity were also added.

In this work, *C. crenatum*, which has been widely employed as a safe microorganism in the industry, was employed as a novel whole-cell catalyst for bioconversion of AD to ADD. *E. coli* and *B. subtilis* have been employed for heterologous expression of KSDD [18]. Compared to *E. coli* and *B. subtilis* expression systems, *C. crenatum* produced more soluble protein after code optimization of *ksdd* gene, and this feature made it easy to purify and study the characterization of KSDD. The recombinant *C. crenatum* pXMJ19-*ksdd*^{II} showed a good performance of bioconversion from AD to ADD, and might be a promising strain in steroid industry. In the further work, site-specific mutagenesis of *ksdd* gene will be taken into consideration to improve the thermostability and the specific activity of recombinant KSDD. The protectants for maintaining KSDD activity will be selected and their mixture will be optimized on the ration. On the other hand, the optimization of the transformation system, including two-phase system, aqueous two-phase system and cloud point system will be applied to further improve ADD production.

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Conflict of interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2016.10.004.

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