Efficient 9α-hydroxy-4-androsten-3,17-dione production by engineered Bacillus subtilis co-expressing Mycobacterium neoaurum 3-ketosteroid 9α-hydroxylase and B. subtilis glucose 1-dehydrogenase with NADH regeneration

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Abstract
3-Ketosteroid 9α-hydroxylase (KSH, consisting of KshA and KshB), a key enzyme in steroid metabolism, can catalyze the transformation of 4-androstene-3,17-dione (AD) to 9α-hydroxy-4-androstene-3,17-dione (9OHAD) with NADH as coenzyme. In this work, KSH from Mycobacterium neoaurum JC-12 was successfully cloned and overexpressed in Bacillus subtilis 168. The expression and purification of KSH was analyzed by SDS-PAGE and KSH activity assay. Preliminary characterization of KSH was performed using purified KshA and KshB. The results showed that KSH was very unstable, and its activity was inhibited by most metal ions, especially Zn²⁺. The whole-cells of recombinant B. subtilis, co-expression of KSH and glucose 1-dehydrogenase (GDH), were used as biocatalyst to convert AD to 9OHAD. The biocatalyst, in which the intracellular NADH was regenerated, efficiently catalyzed the bioconversion of AD to 9OHAD with a conversion rate of 90.4% and productivity of 0.45 g (L h)⁻¹, respectively. This work proposed a strategy for efficiently producing 9OHAD by using B. subtilis as a promising whole-cell biocatalyst host and co-expressing KSH and GDH to construct a NADH regeneration system.

Keywords: 3-Ketosteroid 9α-hydroxylase, Bacillus subtilis, Mycobacterium neoaurum, 9α-Hydroxy-4-androsten-3,17-dione

Background
9α-Hydroxylated steroids are important precursors in the synthesis of steroidal hormone pharmaceuticals, which have been attracted increasing attention (Donova and Egorova 2012; Donova 2007; Fernandes et al. 2003). Highly specific reactions are required to produce functionalized compounds with therapeutic use and commercial value. Due to the high region- and stereo-selectivity of the reactions, together with the mild conditions required, the high yield biological production process, which are more environmentally friendly than their chemical synthesis counterparts, has been developed (Fernandes et al. 2003). Microbial fermentation has been wildly used to accumulate some important steroids intermediates, such as 4-androstene-3,17-dione (AD), androst-1,4-diene-3,17-dione (ADD) and 9α-hydroxy-4-AD (9OHAD) (Zhang et al. 2013; Shao et al. 2015a; Yuan et al. 2015). The 3-ketosteroid 9α-hydroxylase (KSH) and 3-ketosteroid-Δ¹-dehydrogenase (KSDD) are key enzymes in the process of microbial steroids degradation. KSH catalyzes the 9α-hydroxylation reaction of
AD/ADD to 9OHAD/9α-hydroxy-4-ADD (9OHADD), whereas KSDD catalyzes the reaction of Δ1-dehydrogenation of AD/9OHAD to ADD/9OHADD. In this process, however, 9OHADD could subsequently form 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3HSA) by B-ring cleavage, spontaneously (Martin 1977; Kieslich 1985) (Fig. 1). Therefore, KSH combined with KSDD lead to the opening of the B-ring of steroid degradation.

The activity of KSH has been found in various bacterial genera, such as *Mycobacterium* (Wovcha et al. 1978; Brzoski 1982), *Rhodococcus* (Van der Geize et al. 2002; Petrusma et al. 2009; Datcheva et al. 1989) and *Arthrobacter* (Dutta et al. 1992). Heterologous expression of ksh and characterization of KSH have been reported, and the conserved sequences analysis demonstrated that KSH is a Rieske monoxygenase. It belongs to class IA monoxygenase, including a terminal oxygenase (KshA) and a ferredoxin reductase (KshB) (Petrusma et al. 2009; Capyk et al. 2009; Arnell et al. 2007). It has been certified that KshA and KshB are essential for KSH activity by gene deletion studies of kshA and kshB (Andor et al. 2006). There were some reports about microbial fermentation from phytosterols to 9OHAD. However, due to low enzyme activities of steroids degradation pathway, they took long fermentation durations (about 120–144 h). For example, it has been reported that the mutant *Mycobacterium* sp. 2–4 M can be used to produce 9OHAD as a major product from sitosterol, with a 50 % molar yield (Donova et al. 2005). By using the resting *Rhodococcus* sp. cells to transform AD to 9OHAD, the substrate conversion ratio reached to about 85 % (Angelova et al. 1996). Generally, it is difficult to accumulate 9OHAD using fermentation method despite bacteria that can degrade steroids, because 9OHAD could be Δ1-dehydrogenated to 9OHADD and then spontaneously form 3HSA in these strains. Since the present of KSDD isoenzymes prevented the accumulation of intermediates (Van der Geize et al. 2000), deletion of all ksd genes and overexpression of kshA resulted in accumulation of 9OHAD in *Mycobacterium neoaurum* (about 6.78–7.33 g L⁻¹). However, the fermentation duration was more than 150 h (Yao et al. 2014). Thus, the strains which could accumulate 9OHAD, might be lack of KSDD or deficiency in KSH (Seidel and Horhold 1992). Hence, double-stage fermentation method was developed to produce 9OHAD. The first step was the side-chain cleavage of sterols to form AD by one strain, and then the second step was 9α-hydroxylation of AD accomplished by another strain (Seidel and Horhold 1992).

Our laboratory has been devoted to using microorganisms to produce steroids intermediates with non-pollution and non-toxic biological technology (Shao et al. 2015a, 2016a). For example, we have co-expressed human 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) and *Saccharomyces cerevisiae* glucose 6-phosphate dehydrogenase (G6PDH) to construct the NADPH regeneration system for efficient testosterone (TS) production form AD (Shao et al. 2016b). The *M. neoaurum* JC-12 (CCTCC No. M208135), capable of producing AD and ADD from phytosterol or cholesterol by fermentation method, was isolated with phytosterol as the sole carbon source from soil (Zhang et al. 2013). Genes of steroids degradation pathway from *M. neoaurum* JC-12 had been heterologous over-expressed to construct bioconversion system for steroids intermediates production. For example, cholesterol oxidase gene (*choM*) had been over-expressed in *Bacillus subtilis* for bioconversion of cholesterol to 4-cholesten-3-one (Shao et al. 2015b). 3-ketosteroid-Δ1-dehydrogenase (*ksgd*) gene had been over-expressed in *B. subtilis* for bioconversion of AD to ADD (Zhang et al. 2013). The previous work indicated that *B. subtilis* might be a preferred host for bioconversion of steroids intermediates as compared with *M. neoaurum* strains. Hence, in this study, we cloned kshA and kshB gene from *M. neoaurum* JC-12 and first heterologously co-expressed them in *B. subtilis* 168. For efficiently bioconversion of AD to 9OHAD, glucose 1-dehydrogenase (GDH, EC 1.1.1.47, encoded by gdh gene) was co-expressed with KSH to construct a NADH regeneration system (Additional file 1: Fig. S1). The intracellular NADH concentration and the whole-cell bioconversion capability of recombinant *B. subtilis* were detected. This work provided a new reference for 9OHAD production.

**Methods**

**Bacterial strains, plasmids and culture conditions**

*Mycobacterium neoaurum* JC-12 (AD and ADD producing strain) was stored in our laboratory. *B. subtilis* 168 was used as a host for expression of KSH and KSD genes. The plasmids pET26b and pET28a (Novagen, Madison, WI, USA) were used to construct the expression vectors pET-26b-kshA (4·8 kb) and pET-28a-kshB (8·0 kb) with NdeI and BamHI sites. *M. neoaurum* JC-12 (CCTCC No. M208135), capable of producing AD and ADD from phytosterol or cholesterol by fermentation method, was isolated with phytosterol as the sole carbon source from soil (Zhang et al. 2013). Genes of steroids degradation pathway from *M. neoaurum* JC-12 had been heterologous over-expressed to construct bioconversion system for steroids intermediates production. For example, cholesterol oxidase gene (*choM*) had been over-expressed in *Bacillus subtilis* for bioconversion of cholesterol to 4-cholesten-3-one (Shao et al. 2015b). 3-ketosteroid-Δ1-dehydrogenase (*ksgd*) gene had been over-expressed in *B. subtilis* for bioconversion of AD to ADD (Zhang et al. 2013). The previous work indicated that *B. subtilis* might be a preferred host for bioconversion of steroids intermediates as compared with *M. neoaurum* strains. Hence, in this study, we cloned kshA and kshB gene from *M. neoaurum* JC-12 and first heterologously co-expressed them in *B. subtilis* 168. For efficiently bioconversion of AD to 9OHAD, glucose 1-dehydrogenase (GDH, EC 1.1.1.47, encoded by gdh gene) was co-expressed with KSH to construct a NADH regeneration system (Additional file 1: Fig. S1). The intracellular NADH concentration and the whole-cell bioconversion capability of recombinant *B. subtilis* were detected. This work provided a new reference for 9OHAD production.
168 was purchased from Bacillus Genetic Stock Center (BGSC). The E. Coli and B. Subtilis shuttle vector pMA5 (HpaII, ColE1, repB, Amp\(^R\), Km\(^R\)) was preserved in our lab (Zhang et al. 2013b). B. subtilis strains were cultivated at 37 °C and 160 rpm in LB medium (Luria–Bertani broth) with 0.5 % (w/v, weight/volume) glucose. Kanamycin (50 mg L\(^{-1}\)) was added to the growth medium for selecting the recombinants. M. neorarum JC-12 was grown at 30 °C and 160 rpm in liquid medium containing 0.5 % (w/v) glucose, 0.5 % (w/v) tryptone, 0.3 % (w/v) beef extract, 1.5 % (w/v) glycerol and 1.5 % (w/v) NaCl. 2 % (w/v) agar was added during cultivation on solid medium.

**Gene cloning and sequencing**

Restriction enzymes and T4 DNA ligase were purchased from TaKaRa Co. (Dalian, China). Extraction and purification of plasmids were carried out by Mini Plasmid Rapid Isolation Kit (Sangon Biotech Co., Ltd., Shanghai, China). Isolation of DNA restriction fragment from agarose gels was done using the Mini DNA Rapid Purification Kit (Sangon Biotech Co., Ltd., Shanghai, China). Nucleotide sequence of kshA and kshB were analyzed by Sangon Biotech Co., Ltd. Shanghai, China. Protein and nucleotide sequences alignment were performed using the function of the BLAST server at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Construction of recombinant B. subtilis 168/pMA5-ksh and B. subtilis 168/pMA5-ksh–gdh**

Primers used in this work are listed in Table 2. The construction steps of these plasmids are shown in Additional file 1: Fig. S2. The kshA was cloned from chromosomal DNA of *M. neoaurum* JC-12 with the forward primer KshA-F and reverse primer KshA-R. Primers were originally designed with NdeI and *MluI* restriction sites to clone kshA into the plasmid pMA5. Gene cloning of kshB was performed with forward primer KshB-F and reverse primer KshB-R1. The PCR product was ligated onto pMA5 after digested by BamHI. The identification of pMA5-kshB was performed by digestion of *MluI*. Then, we got HpaII-kshB from pMA5-kshB with the forward primer HpaII-F1 and reverse primer KshB-R2 (containing His-Tag). The construction of pMA5-ksh was performed by ligation of HpaII-kshB, digested by EcoRI and *SmaI*, onto pMA5-kshA. For co-expression of ksh and gdh to construct a NADH regeneration system, plasmid of pMA5-gdh (previously constructed by us, homologous over-expression of GDH from *B. subtilis* 168) was used as the template and primer pair HpaII-F2/Gdh-R was used to amplify HpaII-gdh. The construction of B. subtilis/pMA5-ksh–gdh was performed by ligation of HpaII-gdh onto pMA5-ksh, which were digested by *KpnI* and HindIII. The primer sequences were listed in Table 1. Transformation of *B. subtilis* cells were carried out according to the procedure described by Anagnostopoulos and Spizizen (1961). The recombinant *B. subtilis* with pMA5-ksh was selected by resistance to kanamycin and confirmed by DNA sequencing.

**Co-expression of KshA and KshB in B. subtilis 168 and protein purification**

The recombinant plasmid pMA5-ksh was introduced into *B. subtilis* 168. Transformants were obtained after growing overnight on selective LB medium supplemented with 50 mg L\(^{-1}\) of kanamycin. The recombinant cells were grown in LB medium (50 mL) for 24 h at 37 °C. Cell pellets (8000 rpm; 10 min; 4 °C) were washed with 100 mL of 50 mM Tris–HCl buffer (pH 7.0). Cell pellets were then resuspended in Tris–HCl buffer added with 5 mg lysozyme for 30 min and then sonicated for 10 min at 4 °C. Cell extracts were centrifuged for 30 min at 10,000 rpm in an SIGAMA 3K-15 centrifuge to remove cell debris. The purification of KSH-His was carried out by procedure described previously (Zhang et al. 2014). The final samples were verified via SDS-PAGE (12 % acrylamide).

### Table 1 Primers used in this work

| Primers | Sequences (5′–3′) | Restriction sites |
|---------|------------------|------------------|
| KshA-F  | ATC CATATG ACTACCGGAGACAGCCG | NdeI |
| KshA-R  | CGGACCCTTTAGTGGTTTGTTGTTGGTGTTGGCTGGCTGCCTGCCGGAC | |
| KshB-F  | CGCCGGATCC ATGACGGAGGAGCCGGCTCT | BamHI |
| KshB-R1 | CGGGGATCC TATTGGTCGGATGACTTC | BamHI |
| HpaII-F1| ACCGGGAATTC ATGACGGAGGAGCCGGCTCT | EcoRI |
| KshB-R2 | CCCCCTGGTTAGTGGTTTGTTGTTGGTGTTGGCTGGCTGACTTC | SmaI |
| HpaII-F2| ACCGGGAATTC ATGACGGAGGAGCCGGCTCT | KpnI |
| Gdh-R   | ACCGGGGATCTTTAACCACGGGGCCTGCCTGGAATGAAG | HindIII |

*The restriction enzyme sites were bold type*
**Enzyme activity assay**

The KSH activity was detected according to the procedure described previously (Petrusma et al. 2009). For KSH enzyme activity assay, the reaction mixture consists of 50 mmol L⁻¹ Tris–HCl buffer (pH 7.0), 105 µmol L⁻¹ NADH, 250 µmol L⁻¹ AD dissolved in 2 % methanol, and 20–25 µg co-expressed KSH (KshA and KshB). For KshB enzyme activity assay, the reaction mixture consists of 50 mmol L⁻¹ Tris–HCl buffer (pH 7.0), 0.1 mmol L⁻¹ 2,6-dichlorophenolindophenol, 0.25 mmol L⁻¹ NADH, and 1–2 µg KshB was added to the assay. Assays were performed at room temperature. GDH activity was detected as the procedure described before (Hilt et al. 1991).

**Determination of NADH and NAD⁺ concentrations**

The intracellular NADH and NAD⁺ concentrations of recombinant B. subtilis strains were determined according to the manufacturers’ instructions of Amplitite Fluorimetric NAD/NADH Ratio Assay Kit (15263) (Sunnyvale, USA). Sample preparation was followed by the procedure described previously (Bao et al. 2015).

**Bioconversion of AD by recombinant B. subtilis 168**

The bioconversion of AD was performed in shake flasks with the recombinant B. subtilis 168. The growth condition of B. subtilis strains was as previously described. After growing until late exponential phase (OD₆₀₀ = 4–6), cells were collected by an SIGMA 3 K-15 centrifuge. Cell pellets were washed with 200 mL of 50 mmol L⁻¹ Tris–HCl buffer (pH 7.0) for twice and then resuspended in 20 mL Tris–HCl buffer. AD and 0.2 % Tween-80 were then added into the bioconversion system. When using the NAD regeneration system as whole-cell biocatalyst, 1.5 % glucose was added as the substrate of GDH. Steroids extracted from the bioconversion solution (1 mL) by ethyl acetate were used for high-performance liquid chromatography (HPLC). For HPLC analysis, samples were diluted 5 times with ethyl acetate and filtered. Steroids were analyzed by HPLC with a reversed phase Diomonsil C18 at 35 °C using methanol–water (80: 20, v/v) solvent as mobile phase with a flow rate of 1 mL min⁻¹, and subsequent detected via determination of UV absorption at 254 nm.

**Results and discussion**

**kshA and kshB clone and sequence analysis**

The amplification and sequences alignment of kshA and kshB genes were conducted as described in Materials and methods. The kshA gene of M. neoaurum JC-12 encodes a protein of 395 amino acids. Protein sequence analysis showed that typical conserved sequences of class IA terminal oxygenase (Van der Geize et al. 2002; Petrusma et al. 2009), the Rieske Fe₂S₂ binding domain (CXHX₋₁₋₋₁₋₋CX₂₋₋₋₋₋₂H, residues 65–87) and the non-heme Fe²⁺ motif (DXₓ₋₁₋₋₁₋₋ₓₓₓₓ₋₋ₓₓₓₓ₋₋ₓₓₓₓ₋₋ₓₓₓₓ₋₋ₓₓₓₓ₋₋ₓₓₓₓ₋₋ₓₓₓₓ₋₋ₓₓₓₓ₋₋ₓₓₓₓ₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋щение


**Co-expression, purification and characterization of KshA and KshB**

The over-expressed KSH from M. neoaurum JC-12 in E. coli with the vector pET28a had been executed in our previous study, the recombinant KSH proteins mainly existed in the form of inclusion bodies and no KSH activity was detected. Thus, the recombinant plasmids of pMA5-kshA, pMA5-kshB and pMA5-ksh (i.e., pMA5-kshA–kshB), which allowed the gene kshA and kshB to be expressed under the control of HpaII promoter, were constructed. After transformation of the recombinant plasmids into B. subtilis 168, they were then selected by using kanamycin as the selectable marker.

In this study, the possible expression of KSH by recombinant B. subtilis was investigated by SDS-PAGE (Fig. 2), and the analysis of proteins showed that KshA and KshB were successfully expressed. The enzyme activity of KshB, the reductase component of KSH, accepts electrons from NAD(P)H and transfers it to oxygenase component KshA, had been successfully detected in B. subtilis 168/pMA5-kshB with B. subtilis 168 as control (data not shown). The previous study strongly suggested that the cooperation of KshA and KshB are critically important for maintaining KSH activity (Petrusma et al. 2009). Hence, KSH activity could be only detected when KshA and KshB were co-expressed (Table 2). The KSH activity of B. subtilis 168/pMA5-ksh was 0.57 U (mg total protein)⁻¹. However, KSH activity of M. neoaurum JC-12 were only about 0.02 U (mg total protein)⁻¹, suggesting co-expression of KshA and KshB in engineered B. subtilis successfully improved KSH activity.

KshA and KshB were purified with the C-terminal His-tag and showed KSH activity of 2.41 U mg⁻¹. The maximum KSH activity of co-purified KshA and KshB was observed at 33 °C and pH 7.0. Measurements showed that a rather narrow pH range was needed for KSH activity. However, the enzyme was not stable when stored at −20 and 0 °C, and the KSH activity was reduced by 28 and 67 % after 24 h, respectively. No metal ions were found enhanced the KSH activity conspicuously. On the
contrary, however, most metal ions were inhibitors of KSH, such as Fe$^{3+}$, Co$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$, in which Zn$^{2+}$ could inhibit KSH activity completely.

### Construction of NADH regeneration system for biocatalysis of AD to 9OHAD

GDH from B. subtilis 168 and KSH from M. neoaurum JC-12 were co-expressed to construct the NADH regeneration system (B. subtilis 168/pMA5-ksh–gdh). SDS-PAGE analysis proved GDH and KSH were successfully co-expressed in B. subtilis (Fig. 3). Compared to B. subtilis 168, of which GDH activity was 0.03 U (mg total protein)$^{-1}$, the KSH and GDH activities of B. subtilis 168/pMA5-ksh–gdh were 0.53 and 0.35 U (mg total protein)$^{-1}$, respectively, indicating the functional over-expression of GDH.

The significant role of cofactors in the biocatalysts was proved by comparing the intracellular concentrations of NAD$^+$ and NADH in the recombinant B. subtilis strains (Fig. 4). The results showed that the intracellular NADH pool in recombinant B. subtilis 168/pMA5-ksh–gdh was improved (17 %) as compared with B. subtilis 168/pMA5-ksh by over-expression of GDH, suggesting the NADH regeneration system was successfully constructed.

### Steroid transformation analysis of the recombinant B. subtilis 168/pMA5-ksh and B. subtilis 168/pMA5-ksh–gdh

The biosynthesized 9OHAD in M. neoaurum strains could be subsequently transformed to 9OHADD, which then undergoes a nonenzymatic ring cleavage to form 3HSA. However, the recombinant B. subtilis 168 could catalyze the transformation of 9OHAD from AD in one step without any degradation of 9OHAD. Moreover, B. subtilis has been widely used and regarded as a safe strain in industries (Schallmey et al. 2004).

While using whole-cells as biocatalyst, 1 g L$^{-1}$ AD was added as substrate to validate the bioconversion efficiency of different strains. The results showed that B. subtilis 168/pMA5-ksh and B. subtilis 168/pMA5-ksh–gdh successfully catalyzed the transformation of AD to 9OHAD (Fig. 5). However, as controls, no 9OHAD was detected during AD bioconversion by whole-cells of M. neoaurum JC-12, B. subtilis 168, B. subtilis 168/pMA5-ksha and B. subtilis 168/pMA5-kshB. Although both B. subtilis 168/pMA5-ksh and B. subtilis 168/pMA5-ksh–gdh showed significant improved KSH activity, NAD$^+$ was only continuously regenerated by GDH in B. subtilis 168/pMA5-ksh–gdh to keep a persistently 9OHAD productivity. The maximum conversion rate (g g$^{-1}$) of B. subtilis 168/pMA5-ksh was 70.1 % at 16 h, while B.

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**Table 2** Enzyme activity assay for KSH and GDH

| Strains           | KSH activity U (mg total protein)$^{-1}$ | GDH activity U (mg total protein)$^{-1}$ |
|-------------------|------------------------------------------|------------------------------------------|
| M. neoaurum JC-12 | 0.02 ± 0.01                              | –                                        |
| B. subtilis 168   | NT                                       | 0.03 ± 0.01                              |
| B. subtilis 168/pMA5-kshA | NT | 0.03 ± 0.01 |
| B. subtilis 168/pMA5-ksh   | 0.57 ± 0.02                              | 0.03 ± 0.01                              |
| B. subtilis 168/pMA5-ksh–gdh | 0.53 ± 0.02 | 0.35 ± 0.02 |
| Purified KshA     | NT                                       | –                                        |
| Co-purified KshA and KshB | 2.41 ± 0.05   | –                                        |

All assays were performed in triplicate
NT, enzyme activity was not detectable; –, not detectable

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![Fig. 2 SDS-PAGE analysis of cell-free extract and purified KshA and KshB. Lanes: M protein marker (Takara Biotechnology Co., Ltd., Dalian, China); 1 cell-free extract of B. subtilis 168; 2 cell-free extract of B. subtilis 168/pMA5-kshA; 3 cell-free extract of B. subtilis 168/pMA5-ksh; 4 cell-free extract of B. subtilis 168/pMA5-ksh–gdh; 5 purified KshA (45.1 kDa); 6 purified KshB (37.8 kDa); 7 copurified KshA and KshB](image2)

![Fig. 3 SDS-PAGE analysis of KSH and GDH co-expressed in B. subtilis. Lanes: M protein marker (Takara Biotechnology Co., Ltd., Dalian, China); 1 cell-free extract of B. subtilis 168; 2 cell-free extract of B. subtilis 168/pMA5-ksh–gdh](image3)
B. subtilis 168/pMA5-ksh–gdh showed a conversion rate (g g\(^{-1}\)) of 96.3 % at 2 h, indicating cofactor regeneration increased the conversion rate of the biocatalyst. After the batch conversion of B. subtilis 168/pMA5-ksh–gdh, the pH of the reaction mixture was decreased from 7.0 to about 6.3. Thus, the pH variation had little effect on this system.

From above experiments, B. subtilis 168/pMA5-ksh–gdh was expected a good candidate for transforming of AD to 9OHAD. However, due to the low solubility of the steroid substrates and products in aqueous conversion system, the repeated batch strategy of bioconversion from AD to 9OHAD was done in this work. While using 1 g L\(^{-1}\) AD as substrate for repeated batch bioconversion, the whole-cells of B. subtilis 168/pMA5-ksh–gdh could continuously transform total 8 g L\(^{-1}\) AD to about 7.23 g L\(^{-1}\) 9OHAD within 16 h with a conversion rate (g g\(^{-1}\)) of 90.4 % and productivity of 0.45 g (L h\(^{-1}\)) (Fig. 6). However, the enzyme activity of whole-cell biocatalyst decreased greatly (left about 38.9 % KSH activity and 30.6 % GDH activity) after 16 h. Thus the conversion duration was controlled within 16 h. In summary, the successful expression of M. neoaurum KSH in B. subtilis and the construction of NADH regeneration system made it possible to achieve one-step efficient transformation of AD to 9OHAD.
Conclusions

9OHAD, an important precursor in the synthesis of steroid pharmaceuticals, can be produced by microorganisms using fermentation method. However, due to the long fermentation duration and low substrate conversion rate, the productivity of biosynthesis of 9OHAD cannot meet the need of industrial production. This work cloned and over-expressed M. neoaurum KSH and B. subtilis GDH to construct a NADH regeneration system (B. subtilis pMA5-ksh–gdh), which could efficiently transform AD to 9OHAD. By using the NADH regeneration system as a biocatalyst integrated the repeated batch conversion strategy, 9OHAD production was improved to 7.23 g L⁻¹ with a conversion rate of 90.4 % and productivity of 0.45 g (L h)⁻¹. The results demonstrated that the NADH regeneration system of recombinant B. subtilis strain can be used in 9OHAD production. However, the low solubility of the steroid substrates in aqueous conversion system limits extremely the bioconversion rate. Thus, aqueous-organic two-phase systems and cloud point systems will be considered to be applied in the future research to improve the conversion rate of AD to 9OHAD (Wang et al. 2005).

Additional file

Additional file 1: Figure S1. The NADH regeneration system constructed by this work. Figure S2. Construction steps of the plasmids used in this work.

Authors’ contributions

ZX, ZL and RZ designed the study and performed the data analysis. ZX, ZL and XM carried out the laboratory work of the study. ZX, ZL and YT wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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